CATCH ME IF YOU CAN: DETECTING IMMUNE-CANCER CELL INTERACTIONS FOR BETTER IMMUNOTHERAPY

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ABSTRACT

Kaitlyn Maffuid Quebbemann: Catch Me if You Can: Detecting Immune-Cancer Cell Interactions for Better Immunotherapy
(Under the direction of Yanguang Cao)

The tumor and tumor microenvironment (TME) consists of multiple cells in communication, including immune, fibroblasts, vascular cells., and cancer cells. Cellular communication between immune cells and cancer cells significantly influences patient response to immunotherapy and accounts for treatment resistance and interpatient response variability. Thus, it is important to study the complex interactions occurring in the TME, through proximity dependent cell labeling methods. In this dissertation, we used the enzyme-mediated intercellular proximity labeling strategy (EXCELL) to 1) detect and visualize immune cell labeling in a time- and concentration-dependent manner in vitro and 2) detect immune cell infiltrate in vivo.

Using flow cytometry and confocal microscopy, we showed EXCELLs ability to label and detect immune cells with biotin. We showed that this process is both time- and concentration- dependent with an increase in immune cell labeling over time. Furthermore, we translated EXCELL from in vitro to in vivo showcasing EXCELL's ability to label primary murine B and T cells with biotin post interacting with cancer cells.

Overall, our findings suggest that the EXCELL method has great potential for improving our understanding of immune cell dynamics within the TME, ultimately leading to more potent pharmacological effects and cancer immunotherapy strategies.

In loving memory of my Grandma, Janet Maffuid Leavy. I love and miss you.

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LIST OF ABBREVIATIONS

αβ T cell	Alpha beta T cell
λβ T cell	Gamma delta T cell
2CT-CRISPR	Two-cell type CRISPR
2D	Two dimensional
3D	Three dimensional
ABM	Agent based model
ACT	Adoptive cell therapy
ADCC	Antibody dependent cellular cytotoxicity
APC	Antigen presenting cell
BCR	B cell receptor
BiTE	Bispecific T cell engager
CAF	Cancer associated fibroblast
CAR	Chimeric antigen receptor
CCCExplorer	Cell-cell communication explorer
CCI	Cell-cell interaction
cDC	Common DC
CellPhoneDB	Cell phone database
CellTalkDB	Cell talk database
CLIP	Cre-induce intercellular labeling protein
CODEX	Co-detection by indexing
DC	Dendritic cell
DMEM	Dulbecco's modified eagle medium

ECM	Extracellular matrix
EXCELL	Enzyme-mediated intercellular proximity labeling
FAP	Fibroblast activation protein
FBS	Fetal bovine serum
G-BaToN	GFP-based touching nexus
GEMM	Genetically engineered mouse model
ICI	Immune checkpoint inhibitor
IFC	Imaging flow cytometry
IHC	Immunohistochemical staining
IMC	Imaging mass cytometry
LIPSTIC	Labelling immune partnerships by SorTagging intercellular contacts
M-MDSC	Monocyte MDSC
MAB	Monoclonal antibody
MC	Mast Cell
MDSC	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex
Mo-DC	Monocyte-derived DC
MRI	Magnetic resonance imaging
M_t	Mucosal mast cell
M_{tc}	Connective tissue mast cells
NET	Neutrophil extracellular traps
NHP	Non-human primate
NK	Natural Killer

PBS	Phosphate buffered saline
PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
pDC	Plasmacytoid DC
PDX	Patient derived xenografts
PenStrep	Penicillin-Streptomycin
PMN-MDSC	Polymorphonuclear MDSC
PUP-IT	Pupylation-based interaction tagging
PyMINEr	Python maximal information network exploration resource
QSP	Quantitative systems pharmacology
ROS	Reactive oxygen species
RPMI	Roswell park memorial institute 1640 medium
scRNA-seq	Single-cell RNA sequencing
scTensor	Single cell tensor
sLRPA	Ligand-receptor pair analysis
SoptSC	Similarity matrix-based optimization for single-cell data analysis
SpaOTsc	Spatially optimal transporting the single cells
SrtA	Sortase a
STORM	Stochastic optical reconstruction microscopy
TAM	Tumor associated macrophage
TAN	Tumor associated neutrophils
TCR	T cell receptor
T_{fh}	Follicular helper T cell

TIL	Tumor infiltrating lymphocytes
TME	Tumor microenvironment
TRACC	Transcriptional recorder for detecting cell-cell contacts
T_{reg}	Regulatory T cell
TSA	Tumor specific antigen
WT	Wild type

CHAPTER 1: DECODING THE COMPLEXITY OF IMMUNE-CANCER CELL INTERACTIONS: EMPOWERING THE FUTURE OF CANCER IMMUNOTHERAPY[1]

Summary

The tumor and tumor microenvironment (TME) consist of a complex network of cells, including malignant, immune, fibroblasts, and vascular cells, which communicate with each other. Disruptions in cell-cell communication within the TME, caused by a multitude of extrinsic and intrinsic factors, can contribute to tumorigenesis, hinder the host immune system, and enable tumor evasion. Understanding and addressing intercellular miscommunications in the TME are vital for combating these processes. The effectiveness of immunotherapy and the heterogeneous response observed among patients can be attributed to the intricate cellular communication between immune cells and cancer cells. To unravel these interactions, various experimental, statistical, and computational techniques have been developed. These include ligand-receptor analysis, intercellular proximity labeling approaches, and imaging-based methods, which provide insights into the distorted cell-cell interactions within the TME. By characterizing these interactions, we can enhance the design of cancer immunotherapy strategies. In this review, we present recent advancements in the field of mapping intercellular communication, with a particular focus on immune-tumor cellular interactions. By modeling these interactions, we can identify critical factors and develop strategies to improve immunotherapy response and overcome treatment resistance.

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Introduction

The direct (i.e., physical proximity) and indirect (i.e., paracrine signaling) interactions between cells play crucial roles in an organism's function and development. These interactions are the basic building blocks of physiological communication and are essential for tissue formation, immune response, homeostasis, and regeneration.[1] In direct cellular communications, contact between cell surfaces can occur via gap junctions, cell adhesion, tunnel nanotubes, and ligand receptor signaling. When cells interact indirectly, cellular information is shared through signaling from extracellular vesicles, cytokines, chemokines, growth factors, metabolites, and exosomes. These intercellular interacting mechanisms contribute to tissue development and physiological functions.[1]

A healthy immune system can precisely identify and eliminate precancerous cells before they can cause harm, a process referred to as tumor immune surveillance. Numerous extrinsic and intrinsic factors impair immune–precancerous cell interactions, contributing to tumorigenesis. Once developed, tumor cells evade and disrupt the host immune system, leading to an immune suppressive TME. The TME is a complex ecosystem comprised of tumor cells, immune cells, fibroblasts, extracellular matrix, and signaling molecules. The interaction between the immune cells and cancer cells within the TME evolves and can result in either pro- or anti-tumorigeneses.[2] Restoring the immune function and the network of healthy cell-cell communication within the TME has become a significant component of cancer immunotherapy.

The advent and implement of cancer immunotherapy began in the 1980s with an interferon-alpha 2 inhibitor as the first immunotherapeutic agent approved by the FDA in 1986.[3] Since then, numerous other immunotherapies have been implemented, including immune checkpoint inhibitors, oncolytic viruses, bispecific T cell engagers, cytokine therapies, and

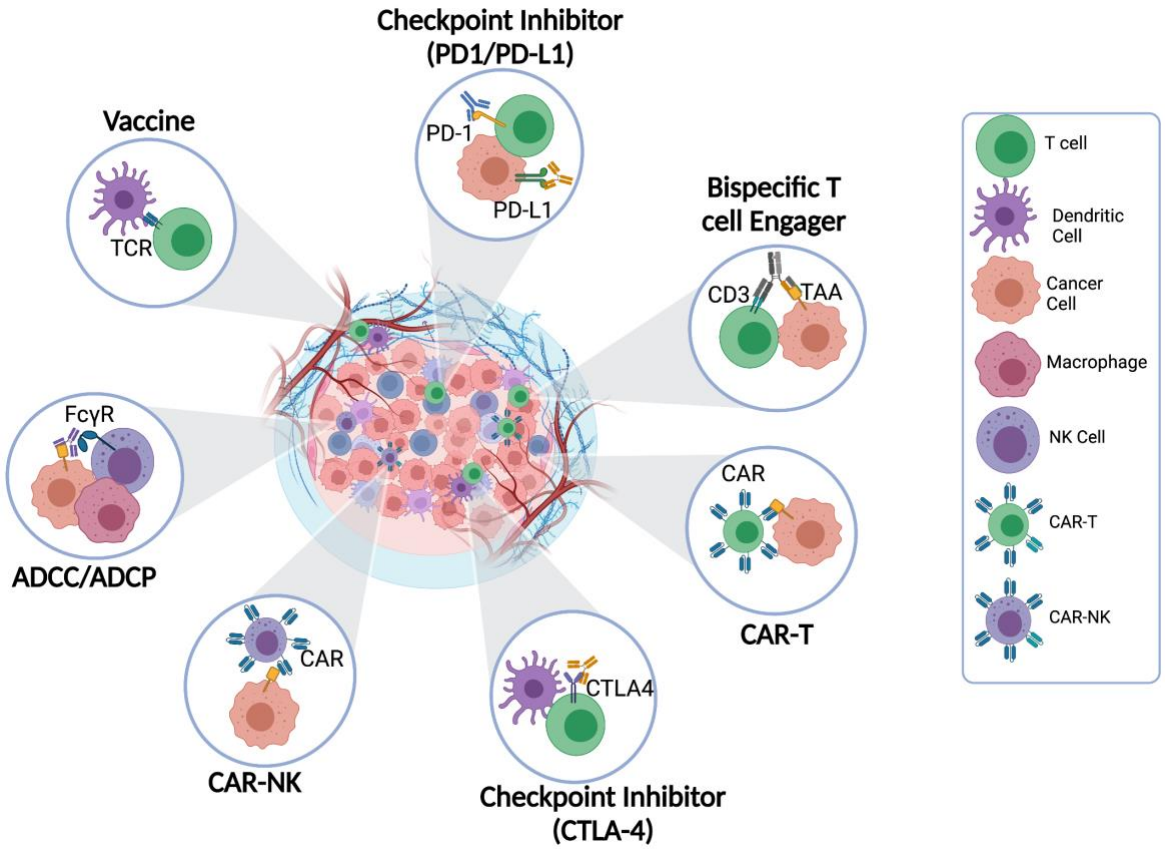
adoptive cell therapies. The central concept of immunotherapy is to restore or reactivate the host anti-tumor immune system.[3] As promising as these immunotherapies are, clinical responses vary significantly from patient to patient, primarily because of distinct patterns of immunosuppressive TMEs and the disruption in cell-cell interactions. Approximately 30-40% of patients respond to immunotherapy, with fewer achieving a durable response.[4] Variability in the TME is primarily due to the different degrees of tumor infiltrated lymphocytes (TILs) and their functions. Some patients have "hot" tumors with higher TILs, which typically confer positive responses to immunotherapy.[5] On the other hand, some patients have "cold" tumors with little to no TILs, and these tumors often develop resistance to immunotherapy.[5] Elucidating the mechanism of resistance and characterizing the distorted patterns of intercellular interactions between tumor and immune cells within TMEs has become critical for the development of more potent immunotherapies.

Studies over the years have showcased cell types present in the TME associated with positive and negative outcomes of immunotherapy. High $CD8^+$ T cell abundance has historically been associated with favorable overall survival, whereas increased regulatory T cells are associated with poor overall survival.[6,7] However, recent pan-cancer analysis shows that this is not always true. This pan-cancer analysis showed that high CD8+ abundance is not always associated with a better prognosis, as the spatial cellular assemblies are also crucial.[8] This suggests that different immune cells will have various prognostic factors depending on the location and type of cancer. The current prognostic markers such as programmed death- ligand 1 (PD-L1) expression and tumor mutation burdens, and microsatellite instability are approved for some cancers; however, there are still no robust prognostic biomarkers across cancers. Methods that can characterize the deformed intercellular interactions and identify cell (sub-)population

that are involved in the interactions are extremely critical for prognosis. Understanding which cell population in each cellular state is associated positive or negative outcome to immunotherapy is extremely valuable to patient stratification, prognostic biomarkers, and resistance mechanisms.

This chapter will delve into the various forms of cell-cell interaction in cancer, how we can measure cell-cell interaction, and how we can harness the power of cell-cell interactions for better cancer immunotherapies.

Figure 1.1: Cell-Cell interactions are the pharmacological basis of immunotherapy



The primary pharmacological mechanism of immunotherapy entails the activation or engagement of diverse immune cell populations to identify and eradicate cancer cells. This crucial process heavily relies on the dynamic interplay between immune cells and cancer cells within the TME. Within the TME, immune cells, including T cells, natural killer (NK) cells, dendritic cells (DCs), and macrophages, establish various types of interactions with cancer cells. These interactions encompass intricate molecular signaling pathways, direct cell-to-cell contact, and the exchange of soluble factors. The ultimate objective of immunotherapeutic approaches is to either alleviate immune suppressive interactions within the TME or activate immune effector functions, thereby unleashing the anti-cancer pharmacological effects of immunotherapy.

Cell-Cell Interactions During Immunotherapy

Almost all types of immunotherapies involve direct cell-cell interactions for anti-tumor effect. One of the key cell-cell interactions during immunotherapy is the interaction between T cells and tumor cells. T cells can recognize and target tumor cells through the recognition of specific antigens presented by the tumor cells. However, tumors can evade T cell recognition by downregulating antigen presentation or by producing immune-suppressive molecules. Cell-Cell interactions are the pharmacological basis of immunotherapy (Figure 1.1). The most successful immunotherapy − immune checkpoint inhibitors – have been approved for over 19 types of cancer treatment.[9] Blocking the immune checkpoint, CTLA-4 or PD-1/PD-L1, can restore the function of TILs for cytotoxic effects, which entail direct physical and functional contact between TILs and tumor cells. TILs, once engaged with target cells, can secrete perforin and granzyme B for cytotoxic effects.[10]

Another mechanism in which immune cells can have a cytotoxic effect on cancer cells is the process of antibody-dependent cellular cytotoxicity (ADCC).[10,11] In ADCC, tumor specific monoclonal antibodies (mAbs) recognize tumor-selective antigens on the surface of cancer cells. The Fc receptor expressed by the effector immune cell binds the Fc portion of the antibody attached to the cancer cells. Upon binding, the immune cell secretes proteins and enzymes, inducing cancer cell lysis. Many IgG-based targeted therapies, such as rituximab and trastuzumab, can trigger antibody-dependent cellular cytotoxicity (ADCC) through interactions between Fc and Fcγ receptors expressed on effector cells, initiating direct cell-cell interactions and cytotoxicity.[11]

Another therapeutic approach that utilizes antibodies is bispecific T cell engagers (BiTEs).[12] BiTEs are characterized by having two different antigen-binding sites in a single

molecule, with one site binding to T cell receptors to activate cytotoxic T lymphocytes, and the other site binding to tumor-specific antigens (TSAs). The engagement between cytotoxic T lymphocytes and tumor cells triggered by BiTEs leads to the elimination of the tumor cells. Examples of BiTEs include CD3 and 4-1BB, which activate cytotoxic T lymphocytes, and target tumor-associated antigens (TAA) such as CD19 and CD20. BiTEs redirect cytotoxic T lymphocytes to specifically recognize and engage tumor cells, initiating cell-cell contact known as the immunological synapse, and inducing cytotoxicity.[12,13]

Chimeric antigen receptor (CAR) cell therapies represent a novel immunotherapeutic approach that signifies a significant advancement in personalized cancer treatment.[14] This approach involves genetically modifying T cells or natural killer (NK) cells to express synthetic receptors (CARs) that can bind to tumor antigens.[14] This genetic modification enables the redirected T or NK cells to specifically recognize cancer cells and initiate immune responses against them.

Oncolytic virus therapy holds promise as an immunotherapy approach that involves T cell activation and cell-cell interactions. This therapy utilizes either genetically engineered or naturally occurring viruses that can selectively replicate within and kill cancer cells while sparing non-cancerous cells.[15,16] Upon administration, the oncolytic virus activates the immune system, leading to the recruitment of natural killer (NK) cells and CD8+ T cells to the tumor site. This process results in the reduction of regulatory T cells (T_{regs}) and facilitates an effective immune response against the cancer cells.[15,16]

In summary, intercellular interactions between effector cells (such as cytotoxic T lymphocytes and NK cells) and tumor cells have emerged as crucial steps for the efficacy of immunotherapies. Therapeutic approaches such as BiTEs, CAR cell therapies, and oncolytic

virus therapy exploit these interactions to enhance the immune response against cancer cells and hold promise for improving cancer treatment outcomes.

Cell-Cell Interactions During Tumorigenesis

The immune system consists of two compartments: innate cells (such as macrophages, neutrophils, dendritic cells, and natural killer cells) and adaptive cells (B cells and T cells). Innate cells rapidly respond to foreign pathogens, presenting antigens to adaptive cells to initiate specific immunological responses. In the context of cancer, antigen-presenting cells detect tumor antigens and present them to naïve lymphocytes. This communication primes and activates lymphocytes, which then migrate to the tumor site. The activated T cells recognize and eliminate tumor cells. The innate and adaptive immune cells collaborate in a process called cancer immunoediting to eliminate tumors. However, if this process is unsuccessful or suppressed, the tumor microenvironment forms.

The progression of the TME towards malignancy can be understood within the framework of cancer immunoediting. Cancer immunoediting refers to the dynamic interplay between the host immune system and tumor cells, whereby immune mechanisms either restrain or promote tumor development. This process can be divided into three distinct phases: elimination, equilibrium, and escape.[17]

During the elimination phase, innate and adaptive immune responses collaborate to recognize and eliminate malignant cells. The innate immune system, including dendritic cells and antigen-presenting cells, primes and activates T cells by presenting tumor antigens. These activated T cells are then mobilized to directly interact with cancer cells, leading to their destruction.[17] However, tumors can evade elimination by exploiting immune checkpoints, such as PD-1/PD-L1 and CTLA-4, which act as brakes on T cell activity. When T cells engage with

cancer cells bearing PD-L1 or CTLA-4, inhibitory signals are transmitted, causing T cell exhaustion and inactivation.[17]

Tumors that successfully evade elimination enter the equilibrium phase, during which they remain dormant but develop resistance mechanisms against immune surveillance.[17] This resistance is mediated by immunosuppressive cell types, including tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), cancer-associated fibroblast (CAF), and regulatory T cells (Tregs).[18] These cells actively communicate with other components of the TME, dampening T cell activation, modulating effector cell function, and promoting tumor progression. Of special significance, CAFs and TAMs play pivotal roles in carcinogenesis and the maturation of TMEs.[18] CAFs can promote tumor growth, angiogenesis, invasion of tumor cells into surrounding tissues, and modulating tumor response to immunotherapy.[19–21] They also secrete various signaling molecules and cytokines that can modulate immune responses and create an environment favorable for tumor growth. The equilibrium phase sets the stage for the subsequent escape phase, characterized by clinically detectable tumor growth and the need for therapeutic intervention.[17,18]

Effective immunotherapy can revert tumors to the elimination phase, where the suppressive mechanisms are counteracted, leading to the elimination of the tumor.[17] However, a partial response to immunotherapy may shift the tumor back into the equilibrium phase, enabling the emergence of resistant clones and eventually leading to the escape phase, signifying acquired resistance to immunotherapy.[17] In cases where immunotherapy fails to induce a response, the tumor demonstrates innate resistance to treatment.[17]

Understanding the intricate dynamics of cancer immunoediting and intercellular interactions between tumor and immune cells is crucial for the development of effective therapeutic

approaches aimed at restoring immune control over tumors and achieving durable clinical responses.

In the following sections, the communication of immune cell subtypes in the TME will be summarized to highlight the importance of immune cell and cancer cell communication.

CD8+ T Cells

CD8+ T cells express T-cell receptor (TCR) which recognize peptide antigens that are presented in MHC-I cells. MHC molecules bind to protein pieces from pathogens and display them on the surface of cells to be recognized by their specific T cell. CD8+ T cells are effectors in anti-cancer immune response and defense against other intracellular pathogens. While CD8+ T cell TCRs bind to the MHC-1 complex on an interacting cell, a co-stimulatory signal must be activated from the interaction between CD28 on the T cells to CD80 or CD86 on antigen presenting cells (APC) for the CD8+ T cell to exert its killing mechanisms. CD28 interaction also determines the level of effect exerted by the CD8+ T cell.[22] CD8+ T cells have major mechanisms for killing a foreign cell including the secretion of cytokines such as TNF-α and INF-γ, release of cytotoxic granules like perforin and granzymes that causes apoptosis in the target cells.

Within the TME, the location and amount of CD8+ T cells present within a tumor and invasive tumor margins has been correlated with a mostly positive prognosis in patient response. Data summarized by Fridman et al. showed that the correlation between patients with high CD8+ T cell and overall survival resulted in a positive patient prognosis in colorectal, breast, ovarian, hepatocellular, NSCLC, bladder, pancreatic, gastric, and melanoma.[23] Interestingly, CD8+ T cell infiltration has conflicting prognosis for some cancer with both positive and negative prognosis in head and neck, prostate cancers, and negative prognosis in renal cell carcinoma.[23,24]

Furthermore, many patients can have an initial response or partial response but fail to have a durable response to immunotherapy. While these mechanisms are still being studied, one mechanism for ineffective patient response is T cell exhaustion where the T cells lose their effector functions via prolonged antigen stimulation from the tumor cell.[25]

CD8+ T cells also have a regulatory subset, regulatory CD8+ lymphocytes. These cells are able to directly kill their target cell, release immunosuppressive cytokines, and are able to inhibit proliferation of effectors CD4+ and CD8+ effector T cells.[26] In colorectal cancer patients, regulatory CD8+ T cells were isolated from patient tumors and exhibited immunosuppressive properties.[27] The location and function of CD8+ T cells and how they are being communicated to are important factors to keep considering when looking to use these cells as a biomarker for TME susceptibility to ICI.

CD8+ T cells are the backbone for current successful immunotherapies such as CAR-T, and ICI. CD8+ T cells communicate directly with cells through checkpoints that are either stimulatory or inhibitory. Some of the most common inhibitory molecules include PD-1, CTLA-4, and LAG-3. Tumor cells like to take advantage of this direct communication pathway by not allowing regulatory cells and mechanisms to turn off the inhibition effect leading to T cell exhaustion. T cell infiltrates within the TME have been shown to determine the probability of patient response to immunotherapy.

CD4+ T Cells

CD4+ T lymphocytes are the other branch of T cells expressing TCRs that recognize the MHC-II molecules on APCs. CD4+ T cells work to help mediate immune response through both direct and indirect communication via cytokine release. CD4+ T cells play a variety of roles and help conduct adaptive immune responses with their ability to recruit and assist effector cells and

regulate cells after immune response. CD4+ T cells have both pro and anti-tumorigenic properties and have many subsets including Th1, Th2, Th9, Th17, follicular helper (Tfh), and T_{reg}.[28,29,30] The subsets of CD4+ T cells each have pro- and anti- tumor functions ranging from supporting the cytotoxic effects of effector cells to promoting tumor metastasis and angiogenesis.[28,29,30]

CD4+ T cells are recruited into the TME through indirect communication of chemokines CXCL9, CXCL10, and CCL5 that are released from the tumor cells. CD4+ T cells indirectly communicate with effector cells via cytokines. CD4+ T cells also directly communicate with other immune cells in the TME by binding to dendritic cell (DC) via CD40/CD40L interaction and Tfh cells communicate with B cells via CD40/CD40L. Tfh cells also secrete IL-21 to modify antigen presentation of B cells and DC to CD8+ T cells to encourage pro-tumorigenic functions.[31,28,32]

In response to immunotherapy, CD4+ T cells present in the TME can be indicators for patients having either poor or beneficial prognosis. Most commonly, CD4+ cells present within the TME are associated with poor prognosis, especially when studying T_{reg} cells.[33,34] However, some CD4+ T cells have been positively correlated with patient outcomes in colorectal, non-small cell lung cancer and melanoma patients.[29,34] In non-small cell lung cancer patients, the amount of CD4+ T cells present had a positive correlation to patient response before anti-PD-1 treatment, and in melanoma patients after anti-CTLA-4 treatment. The positive correlation could be due to the subset of Th1 CD4+ T cells present in the TME points to a more favorable prognosis in patients, but more studies need to be done on the influence on the different subsets.[28,29,31]

Gamma Delta T cells

Gamma delta (γδ) T cells are denoted by their TCR being composed of γ and δ chains and are a small subset of T cells only accounting for <5% of cells in the blood.[35,36] This small set of T cells are different from their alpha beta (αβ) T cells counterparts, CD4+ and CD8+ T cells. γδ T cells are generally found in epithelial and mucosal tissues and contribute to the first line of defense against pathogens.[35,36] γδ T cells can recognize markers from infection, tumorigenesis, and other cellular stress markers without the requirement of antigen presentation from MHC molecules. Most γδ T cells are activated in an MHC-independent manner a major difference compared to αβ T cells.[37,36] This makes γδ T cells an interesting cells subtype when looking at MHC-I deficient tumors and using them for allogeneic cell therapies.[36] Most T cell research and clinical approaches look at αβ T cells with current cancer immunotherapies based on αβ T cells. γδ T cells share similar qualities with αβ T cells such as cytotoxic effector functions and pro-inflammatory cytokine production.[36,37]

Within the TME, this subtype has been reported to be a favorable prognostic indicator among 22 different immune cell populations in 39 cancer types.[38] γδ T cells are a favorable prognostic biomarker in colorectal[39], triple negative breast cancer[40], hepatocellular carcinoma[41], gastric cancer[42], head and neck[43], bladder[44], Merkel cell carcinoma[45], and non-small cell lung cancer.[46] γδ T cells possess anti-tumorigenic functions through the activation of the γδ T cell receptor, communication with natural killer receptors or CD16 which can induce ADCC.[37] γδ T cells directly kill the tumor through release of cytolytic granules and can activate the cytotoxic mechanisms of αβ T cells via IFNγ and TNF. γδ T cells also communicate with DC and NK cells via secretion factors to recruit them to the TME to exert anti-tumor effects.

Due to γδ T cells having a unique mechanism of action compared to their αβ T cells counterpart as well as having mainly anti-tumor activities they are an interesting cell type target for immunotherapies. Ongoing clinical trials in various solid and hematological cancers are investigating different γδ T cell-based therapies, including γδ T cell engagers, expanding γδ T cell subsets using adoptive cell therapy (ACT), CAR-induced γδ T cells using ACT, and ACT with γδ TCR-engineered T cells.[37,36] The combination of using γδ T cells as a possible indicator for patient outcome as well as development of new therapies makes this subtype an exciting subtype to study further.

B Cells

B lymphocytes are branch of the adaptive immune cell types that mature in secondary lymphoid organs such as lymph nodes and the spleen and are responsible for creating antibodies and can also recruit other cells to destroy a pathogen or infected cell, and are activated through their B cell receptor (BCR).[47] B cells are drawn to the TME through indirect communication mechanisms of chemokines CXCL13 and CCL21. B cells traffic into the TME and are localized to tertiary lymphoid structures that are next to tumors. Within the TME, B cells are heterogenous, interact with both APCs and T cells, and play a large role in T cells response.[47] Like T cells, B cells also possess pro- and anti-tumorigenic processes. The protumor roles of B cells include inhibition of cytotoxic T cells via IL-10, production of CD1D+/CD5+ and TIM-1+ B_{regs}, and PD-L1+ B cells.[48] These factors promote a TME that favors the production or immunosuppressive Tregs and production of pro-inflammatory cytokines that influence tumor growth.[47] B cells also secrete specific antibodies specific for tumor cells causing decreased tumor growth and ADCC from NK and myeloid cells. B cells also engage in direct communication with other cells by acting as APCs and priming T cells for activation to carry out anti-tumor effects. B cells can also

directly interact with the tumor cells via the BCR binding to tumor antigens producing granzyme B from the B cells leading to cytolytic activity.[47]

High presence of B cells in the TME has been shown to have a positive correlation with patient prognosis and response in many cancers such as melanoma[49], sarcoma[50], breast cancer[51], non-small cell lung cancer[52], colorectal[53], bladder[54], and pancreatic cancer.[55] They are not however, a favorable prognosis in clear cell renal cell carcinoma.[56] It is very interesting that the presence of B cells within the TME is associated with positive prognosis in an array of cancers and are an interesting TME cell maker to look at moving forward.

B cells also express checkpoint molecules liked PD-1, PD-L1, and CTLA-4. The presence of B cells in the TME have been shown to be positive prognosis markers of patient response to ICBs. In melanoma patients that responded to ICI, B cells helped to predict patient response through activation of PD-1 T cells. Similarly, in sarcoma and non-small cell lung cancer patients, B cells were the highest cell type biomarker for patients who responded to ICI. Interestingly, circulating B cells that produce TNF or IL-6 have been shown to be associated with patients not responding to anti-CTLA-4 therapy.[48,57] These cases of B cell response in ICI highlight the ability of B cells as a predicator or response to ICI therapy.

Natural Killer Cells

NK cells are a member of the innate immune system and play an integral role in the communication between the innate and adaptive immune responses. NK cells consist of 5-15% of systemic circulation cells representing a small but important population.[58,59] NK cell functions include quick response to foreign pathogens, killing of virally infected cells, detect and control tumor cell growth, and aid in protecting against disease. NK cells are referred to as immunosurveillers and patrol the body being constantly in communication with other cells.[59]

NK cells can kill tumor cells without any of the priming and activation that is required for T or B cells. Similarly, to the cancer-immune cycle of T cells, NK cells can also directly interact in their own NK-cancer cycle. NK cells are recruited into the TME where they recognize tumor cells and are activated, leading them to kill tumor cells systemically helping alert adaptive immunity. NK killing of tumor cells causes the release of tumor antigen to increase, which can be presented to T cells and influence cytotoxic CD8+ T cell response showcasing the innate immune system helping to activate an adaptive immune response. Within the TME NK cells are found alongside the other cells, they are just present at a lower cell count, but still can be just as potent.[58,59]

Overall, high presence of NK cells has been shown to be correlated with higher survival probability in melanoma[60,61], non-small cell lung cancer, and renal cell carcinoma.[62] Interestingly, a small population of immature NK cells has been shown to promote progressions of triple negative breast cancer.[63] Due to NK cell's unique ability to mediate adaptive immune response, as well as exert cytotoxic effects on tumor cells, makes NK cells are an intriguing target for immunotherapies such as CAR-NK. CAR-NK has numerous advantages over CAR-T including better safety, less cytokine release syndrome, more cytotoxic activating mechanisms, and the ability to use manufactured cells since there is less risk of graft versus host disease using NK cells compared to T cells.[64] CAR-NK efficacy is currently being evaluated in several clinical trials of various hematological and solid malignancies with the most common one being B-cell leukemia/lymphoma targeting the CD19 antigen.[65] CAR-NK has promise but still needs to overcome some challenges including CAR-NK cell expansion and lifetime of CAR-NK cells.[64]

NK cells also play roles in ICB therapy. Studies have shown that PD-1 and PD-L1 blockade has an impact on NK cells and that NK cells contribute to the success of ICBs. Interestingly, T cells and NK cells share similar immune checkpoints and targeting these

receptors on NK cells could provide an interesting therapeutic angle.[66,67] An *in vivo* study showed that PD-L1 expressing tumors were able to escape NK mediated cytotoxicity via PD-1/PD-L1 interaction.[66] However, in PD-L1 negative tumors NK cells were able to exhibit cytotoxic effect on tumors cells.[66] This highlights this importance of NK cells as a bridge between the innate and adaptive immune systems.

Dendritic Cells

DCs are part of the innate immune cell classification that bridges the gap between innate and adaptive immune cells. DCs are tree shaped cells that have a large surface area, which allows them to have direct communication with a lot of surrounding cells like T and NK cells. DCs are able to activate antigen-specific T cells in secondary lymphoid organs and one mature DC can stimulate 100-3000 T cells.[68] DCs are a plastic cell population that can shape their phenotypes to the microenvironment in both lymphoid and non-lymphoid tissues, and have a variety of different subtypes including: common DC (cDC): cDC1, cDC2, plasmacytoid DC (pDC), and monocyte-derived DC (Mo-DC).[68]

During cancer, DCs will successfully present (TSA) to naïve T cells to activate them and exert effector and cytotoxic function of the tumor cells.[69,70] DCs help to assist in anti-tumor response via stimulation of CD8+ T cells, and are immunosuppressive in their communication with tumor cells by impairing antigen presenting and also help enable tumor escape.[69,71] Even though DCs play a large role in anti-cancer immunity, once a tumor is formed DCs become inactive and can no longer alert the adaptive immune systems. The different subtypes of DCs communicate with the tumor cells in the TME in different ways. cDC1 cells communicate directly with CD8+ T cells via cross-presentation to illicit a cytotoxic response. They also communicated with NK cells to promote anti-tumor immunity.[71,72] In the TME, cDC1 cells

transport tumor antigens to tumor draining lymph node for T cell priming and produce chemokines that recruit T cells into the TME.[71] Mo-DCs have been shown communicate with CD8+ T cells through cross-talk with Th1 and Th17 cells for anti-tumor effects and communicate with Tregs for pro-tumor effects.[71] pDCs inhibit tumor proliferation and exert cytotoxic effects directly on the tumor via IFN-α, and granzyme B. pDCs can also be pro-tumor by interacting with T_{regs} to promote their expansion and inhibit CD8+ T cells.[71]

High presence of cDC1 cells have shown positive outcomes and a higher overall survival in patients with cutaneous melanoma, breast cancer, and lung adenocarcinoma.[73] Similarly, studies in melanoma patients have shown high presence of cDC1 cells in the TME resulted in better overall survival and response to ICI.[74] Increased presence of cDC2 cells infiltrating the TME of melanoma patients were correlated with poor survival in these patients; however, cDC2 cells that were within the TME expressed an IL-12 resulting in patients have a better overall survival.[75] A similar phenomenon with cDC2 cells was shown in breast and lung cancer patients showing that the TME and location of cells in the TME is shaping how the cells respond.[75] pDCs have been found in multiple cancers such as melanoma, breast, ovarian, and colon.[76] In ovarian cancer, patients with pDCs in the primary tumor were correlated with reduced progression free survival, but not in patients with pDCs in other locations.[76] Mo-DCs enter the TME from inflammatory signals such as those from the CCL2/CCR2 axis. In melanoma patients, Mo-DCs were associated with CD8+ T cell proliferation via cross-antigen presentation. Mo-DCs in breast and ovarian cancer patients correlated with an immunosuppressive TME.[70,77,78] Similar to other immune infiltrate, there is correlation between DC subtype, cancer, and TME location with patient response.[70,78]

DCs are also a very interesting cell type for immunotherapy opportunities, and their effect on immunotherapy. Mo-DCs were positively correlated with effector TILs in the TME of mouse models responding to therapy. In melanoma patients who responded to anti-PD1 therapy, there was a significant level of Mo-DC cells present in the TME of responding patients compared to non-responders.[79] Tumor infiltrating DCs have a high level of PD-L1 expression which hinders anti-tumor response via cross-presentation to CD8+ T cells.[72] Normally, PD-L1 is upregulated onto DCs to protect them against the cytotoxic effects of CD8+ T cells, but this communication strategy works in favor of tumor progression within the TME.[72] Alternatively, DC-based vaccines have been studied with more than 200 complete clinical trials.[80] DCs are isolated from a patient or generated *in vitro,* then manipulated *ex vivo* and re-infused into patients with clinical trials in ovarian cancer have shown success with DC-based vaccines.[80] Upon administration, the DCs cause a T cell response targeting the existing malignant cells increasing therapeutic potency and reducing immune evasion. These examples provide rationale that DC cells and their subtypes should continue to be studied for immunotherapy opportunities.

Myeloid-Derived Suppressor Cells

MDSCs are generated in the bone marrow and are pathologically activated immature myeloid cells neutrophils and monocytes that have immunosuppressive activity. MDSCs balance out excessive inflammatory responses that happen during high inflammation occurrences such as pregnancy, sepsis, trauma, and surgery.[81] MDSCs are one of the most prominent cell types present in the TME.[81,82] There are two different subtypes of MDSCs, polymorphonuclear MDSC (PMN-MDSC), which are morphologically and phenotypically similar to neutrophils, and monocyte MDSC (M-MDSC) which are similar to monocytes.[83] PMN-MDSCs are found in peripheral lymphoid organs and play a role as a regulatory to tumor-specific immune response

such as T cell tolerance and have suppressive activity. As MDSC cells become more suppressive within the TME, the M-MDSC subtype is more prevalent then the PMN-MDSCs.[82,83]

MDSCs directly communicate with immune cells in the TME to inhibit them through different mechanisms. MDSCs communicate directly in the TME with T cells by inhibiting CD8+ T cells activation and promote T_{regs}.[81] MDSCs also communicate directly with macrophages and DCs through crosstalk, which allows them to amplify their immunosuppressive effects.[82,81] Furthermore, MDSCs communicate indirectly to suppressive pro-tumorigenic mechanisms such as IL-10, arginase-1, reactive oxygen species (ROS), and other inhibitory factors. These signals recruit Tregs into the TME as well as promote epithelial to mesenchymal transition.[82,83] An increased presence of MDSCs in the TME has been poorly correlated in numerous cancers including melanoma[84], non-small cell lung cancer[85], colorectal[86], pancreatic[87], breast[88], hepatocellular[89], and cervical.[90] Thus, targeting the MDSC cells has the potential to turn a cold tumor hot and reactivate the protumorgenic responses.[81,82]

MDSCs are not only immunosuppressive within the TME, but are also linked to patients' resistance to immunotherapy, chemotherapy, and radiotherapy. In a study done using a murine colorectal cancer model, mice treated with anti-CTLA-4 or anti-PD-1 therapy showed that high level of MDCS cells were interfering with the therapeutic efficacy.[91] Once MDSC presence was reduced, the tumor size was also reduced even in advanced or metastatic tumors. MDSCs can be a therapeutic target to alleviate immunosuppressive mechanisms and help make ICIs more effective. Some potential target mechanisms include depleting MDSC populations using chemotherapy or tyrosine kinase inhibitors.[92] Another way is to induce differentiation of MDSCs into mature monocytes that do not have immunosuppressive mechanisms through chemotherapy and casein kinase inhibitors.[91]

Tumor Associated Macrophages

Macrophages are a heterogenous population of innate immune cells whose roles include maintaining homeostasis, tissue repair, and development.[93] Macrophages are very plastic and respond to microenvironment signals around them causing them to differentiate into phenotypes with specific functions.[94] Interestingly, macrophages with multiple phenotypes can be present within the same microenvironment. Macrophages use the communication method of phagocytosis to ingest other cells, infectious agents, and other foreign pathogens. Upon ingestion of foreign particles, macrophages can then present the antigens to adaptive immune cells such as lymphocytes for them to exert an effector response.[93] Macrophages are classified based on their response to a polarizing agent and are divided into two main phenotypes: M1 or classical, and M2 or non-classical. M1 macrophages are proinflammatory, anti-tumoral, microbicidal, immunostimulating, and can cause tissue damage formation. M2 macrophages are anti-inflammatory, immunosuppressors, pro-tumor, and support angiogenesis and tissue repair.[93,95]

Within the TME, TAMs and are one of the main infiltrating innate immune cell types. The TAM population in the TME is heterogenous with both M1 and M2 subtypes present.[93,95] M1 TAMs generally exhibit anti-tumor activity while M2 TAMs are more pro-tumorigenic. The ratio between M1 and M2 TAMs has been noted as an important prognostic marker in the TME.[95] Low M1/M2 ratio is correlated with poor patient outcomes and tumor growth, while a high M1/M2 ratio has been associated with positive patient outcomes in ovarian, gastric, colorectal, lung, osteosarcoma, and others.[95] TAMs communicate with other cells in the TME to exert immunosuppressive effects. TAMs present PD-L1 and PD-L2 on their surface and can interact with PD-1 expressed on effector cells suppressing their cytotoxic effects.[96] TAMs also

communicate indirectly through cytokine secretion such as immunosuppressive cytokines IL-10 and TGF-β and can present CD4+ Th1 cell polarization.[97]

TAMs can directly communicate with effector T cells through checkpoint molecules such as PD-L1, PD-L2, B7-S1, and Galectin-9 causing the effector T cells to become immunologically exhausted.[96,98] Like DCs, TAMs can crosstalk with T_{regs} and recruit them into the TME through chemokines and cytokines contributing to ICI resistances and immune evasion. TAMs have been shown to play a role in ICI resistance via the FcγR receptor expressed on macrophages. For example, a study showed that anti-PD-1 antibodies with certain Fc domains were taken up by macrophages leading to ICI resistance.[97,98] Ongoing clinical trials are interested in looking at the synergistic ability of targeting TAMs with immunotherapy. The mechanisms of action targeting TAMs including inhibiting TAM recruitment into the TME and reprogramming them to an immunopromoting phenotype. This combination targeted TAM therapy is being combined with immunotherapy in clinical trials in patients with multiple cancers including lung, kidney, melanoma, colorectal, non-small cell lung cancer, pancreatic, and other solid tumors.[99]

Neutrophils

Neutrophils are part of the effector arm of the immune system and patrol the system looking for signs of foreign pathogens.[100] Neutrophils are equipped with a large amount of pathogen recognition receptors and opsonin mediated receptors.[100,101] Upon discovery of a foreign pathogen through one of their many recognition receptors, they exert cytotoxic effects by phagocytosis, reactive oxygen species (ROS), and the release of neutrophil extracellular traps (NET).[100,101] NETs released from neutrophils indirectly communicate with macrophages to promote cytokine production and enhance DC recruitment and antigen presentation. Neutrophils directly communicate with B cells, inducing B cell activation and antibody production and with

T cells to increase T cell recruitment and differentiation.[100,101] Neutrophils are key components in the activation and regulation of the adaptive immune response and have both immunosuppressive and immunostimulatory molecules that interact with lymphoid cells and macrophages.[100]

Within the TME, tumor associated neutrophils (TANs) possess both protumor and antitumor responses due to TAN diversity and plasticity.[102] TANs can aid in pro-tumorigenic functions by promoting angiogenesis, remodeling of the extracellular matrix, recruiting of cells to the pre-metastatic sites, and immunosuppression of effector cell types.[102] TANs also aid in antitumorigenic responses by directly killing the tumor cells through release of various cytokines and activation of CD8+ T cells and CD4+ T cells for effector functions. TANs can be divided into two subtypes, N1-TAN, and N2-TAN. N1-TANs are the antitumor phenotype and N2-TANs are the pro-tumor phenotype,[101,102] with signals from the TME causing TANs to polarize into N1 or N2 subtypes.[102,103] As the TME progresses, the antitumor mechanisms of TANs are no longer effective giving rise to the pro-tumor functions mentioned above.[103] The presence of high levels of TANs is correlated with negative patient prognosis in many cancers.[101,103] A study done in head and neck cancers looked at TANs in both early (non-metastatic) and late (metastatic) TMEs since neutrophils accumulate in the lymph nodes during cancer progression. During early non-metastatic TME stages, TANs in the lymph nodes influence anti-tumor T cell responses and positively impact patient prognosis.[103] However, in late metastatic TME stages, T cells are suppressed causing a negative impact on patient prognosis.[103] A study done in melanoma tumors showcased that neutrophils are necessary for eradication of antigen escape variants after adoptive T cell transfer therapy combined with anti-CTLA-4 treatment.[104] This combination treatment showed activation of anti-tumorigenic neutrophil signature and allowed neutrophil mediated tumor antigen killing. Moreover, another study showed that presence of neutrophils in the TME

is associated with successful immunotherapy treatment in murine lung cancer models.[105] The ongoing studies investigating neutrophils showcases that neutrophils can be important for effective anti-tumor response post immunotherapy treatment, and targeting neutrophils can lead to better tumor control.

Mast Cells

Mast cells (MC) are part of the innate immune system and have two major subtypes, mucosal mast cells (MC_t) and connective tissue mast cells (MC_{tc}). MC_t are primarily located in the external mucosa of the gastrointestinal and respiratory locations.[106] MC_{tc} cells are primarily located in the submucosa and connective tissues near blood and lymphatic vessels.[107] MCs are important components of the inflammatory response and are activated by allergen and pathogen antigens to release cytokines and mediators. Activated MCs have different responses based on the tissue and organ they are in with the most common sites being mucosa of respiratory tract, gastrointestinal tract, blood wounds, and connective tissues. Mast cells also contribute to vasodilation, angiogenesis, and elimination of bacteria.[106,107]

Mast cells also communicate with other immune cells such as T cells, B cells, fibroblasts, and eosinophils.[107] MCs can recognize pathogenic antigens by direct communication through PAMPs on mast cell surface. When a MC receptor binds to an antigen on a foreign cell the MC releases PAMP dependent inflammatory mediators to exert cytotoxic functions on the foreign cells. The release of these inflammatory mediators can recruit immune cells like NK cells and neutrophils to eliminate bacteria. MCs can also ingest and then present antigens through MHC-I and MHC-II to provide direct communication with T cells.[106,108]

Within the TME, MC are found at the periphery and within the TME and like other immune cells exhibits pro- and anti- tumorigenic activity depending on the location, amount, and

TME environment.[108] MCs are able to communicate with a variety of cells within the TME through cytokine secretion and degranulation.[109] MC are usually associated with poor patient prognosis in a variety of cancers including Hodgkin's lymphoma[110], pancreatic adenocarcinoma[111], and hepatocellular carcinoma.[112] Localization of MC in the TME is a large component in patient prognosis. For example, in colorectal cancer MC are an indicator of favorable prognostic factor except for when MC are localized to peritumoral part of the TME.[113] Specifically, MCs play an important role in the development of polyps and have been shown to be enriched in polyps in colorectal patients.[113]

MCs have been shown to be a factor in immunotherapy resistance and eradicating MCs from the TME improves immunotherapy effect.[114] PD-1 is expressed on activated MCs and is associated pro-tumor functions in the TME via releasing of more histamine and cytokines.[109] A study done looking at bioinformatics analysis of patients with melanoma to determine prognostic impact of MCs with anti-PD-1 immunotherapy. MCs were correlated with a poor prognostic factor and resistance to anti-PD1 therapy.[109] The inhibition of MCs and/or removal of them from the TME could provide a solution to patient response to immunotherapy.

Eosinophils

Eosinophils are effector cells that play important roles in host defense against parasitic infections and allergic diseases. Eosinophils regulate immune function and response through indirect communication via cytokines and direct communication and regulate immune responses in both innate and adaptive cell types.[115]

Eosinophils traffic into the TME from chemotactic signals from different cell types such as endothelial cell expressed adhesion molecules, CCL24 from tumors, macrophage derived CCL11, and CCR3 signaling.[116] Eosinophils have been shown to be present in various cancers

including, lymphoma[117], breast[118], ovarian[119], bladder[120,121], gastric[122], melanoma[123], and colorectal[124] exhibiting pro- and anti-tumorigenic functions depending on the cancer. Eosinophils exert anti-tumorigenic actions through both indirect and direct communications within the TME. Eosinophils can exert cytotoxic capabilities directly on the tumor itself through the secretion of cytotoxic proteins such as major basic protein and eosinophil-derived neurotoxin as well as granzymes.[116] Eosinophils can also directly communicate with tumor cells through ligand receptor interaction with NK cells, with CD8+ T cells indirectly, and recruit CD8+ T cells into the TME[115,116,125]. Like other immune cells in the TME, eosinophils also have pro tumorigenic functions. Eosinophils can indirectly communicate with T_{regs} via CCL22 to recruit them into the TME. Eosinophils also indirectly communicate with effector T cells to inhibit them, and release growth factors that support angiogenesis and tumor cell proliferation and survival.[115,125]

In immunotherapy, the effect of eosinophils on clinical response have been studied in breast, Hodgkin lymphoma, head and neck squamous cell carcinoma, melanoma, non-small-cell lung cancer, renal cell carcinoma, and urothelial carcinoma.[125] Overall, in all these cancers, eosinophils correlated with a better overall survival and decreased risk for progression showing that the presence of this cell type can have a positive influence on immunotherapy.[125] Interestingly, in cancer patients treated with immunotherapy can develop eosinophilia.[126] A retrospective study on melanoma patients revealed that patients who developed eosinophilia during immunotherapy treatment correlated with prolonged survival in these patients.[126] This finding showcases that eosinophilia has the potential to be a biomarker for patient response to immunotherapy.

Cancer-Associated Fibroblasts

Fibroblasts are a diverse and plastic group of cells that contribute to tissue architecture, produce the extracellular matrix, and promote wound healing, inflammation and scaring after injury.[127] Fibroblasts can survive in severe stress which could contribute to the ability of fibroblasts to survive damage that is induced by chemotherapy or radiotherapy.[128] In cancer, fibroblasts are referred to as cancer-associated fibroblasts (CAFs) are a significant component that make up the tumor stroma. There are multiple mechanisms that contribute to the activation of a CAF from a fibroblast.[129] These include DNA damage, TGF-β, physiological stress, inflammatory signals, contact signals, and receptor tyrosine kinase ligands.[129] In relation to CAFs, tumors have been referred to as wounds that do not heal[130] leading the fibroblasts to have a continuous repair response to the tumor.[128]

In the TME, fibroblasts are recruited into the TME by growth factors from the tumor cells mainly TGFβ and fibroblast growth factor 2.[128] CAFs secrete a multitude of factors that are either pro- or anti- tumor formation. CAFs encourage tumor angiogenesis that inhibits effector immune cell recruitment into the TME, immune cell activation, and promotes cancer cell migration and invasion. Within the TME, CAFs influence the extracellular matrix (ECM) of the TME by remodeling and reprogramming it. CAFs can work with the ECM of the TME to remodel it to not allow immune infiltrate into the TME serving as a barrier for the TME. However, this mechanism can reverse and CAFs can remodel the ECM to allow for effector cell infiltration and reveal binding sites within the TME for immune cell adhesion.[128] A pan-cancer analysis that analyzed data from the cancer genome atlas showed that high presence of CAFs correlated with negative overall survival in 21 tumor types.[131]

Due to the generally immunosuppressive nature of CAFs, they are of interest as a cellular target. There are ongoing clinical trials targeting specific cytokines and chemokines including TGF-β, hedgehog, CXCR4 and others.[21,129] Most of the drugs being studied in these clinical trials are small molecules inhibitors and blocking antibodies with the main mechanism being preventing CAF activation and function. Regarding immunotherapy, CAFs secrete numerous chemokines that causes an increase in the expression of checkpoint inhibitor molecules. CAFs have the ability to indirectly communicate with tumor cells through cytokines and vesicles inducing expression of PD-L1 on the tumor cells.[21] Furthermore, CAFs play a role in the effects of PD-1/PD-L1 immunotherapy in solid tumors including, non-small cell lung cancer, esophageal squamous cell carcinoma, breast, and colorectal cancer.[21] In both breast and colorectal cancer, CAFs increase expression of PD-L1 on tumor cells.[21] In non-small-cell lung cancer, CAFs release TGF-β creating a barrier at the edge of the TME limiting T cell infiltration to the tumor cells.[21] Taken together, the general immunosuppressives of CAFs in the TME coupled with the remodeling done by CAFs in the TME makes them an exciting candidate to target for therapeutic options.

Extracellular Matrix

ECM provides physical support for cells as well as regulates development, function, and homeostasis of the cells.[132,133] ECM provides structural support for the cells and sets the characteristics of the tissues.[132,133] The ECM assists in cell attachment, communication to nearby cells, cell growth and movement. The ECM is considered an active and dynamic environment that is continuously changing composition and structure based on the actions of the surrounding cells.[134] All of the tissues within our bodies have their own distinct ECM that has composition

and topographical differences due to the various cells present in each tissue microenvironment,[132] and is used as an anchorage site for cells and can facilitate or block the migration of cells.[132–134]

The ECM is composed of two main components the base membrane and the interstitial matrix.[134] The interstitial matrix interconnects cells in the stroma to the basement membrane, ensuring the structural integrity of tissues and subsequently organs, and alters cell differentiation and migration processes.[135] The interstitial matrix can change and remodel due to force stress and trauma.[134,135] The remodeling that occurs in the interstitial ECM can influence cell signaling, migration and tumor cell progression.[135] In opposition to the interstitial matrix, the basement membrane is a denser, more stable structure that surrounds muscle cells, lines basal surface of endothelial cells, and organizes tissues into compartments. Cells interact with the basement membrane by binding to it to maintain tissue homeostasis and to develop. Similar to the interstitial matrix the basement membrane needs to undergo remodeling for tumor cells to infiltrate.[133,135]

During tumor formation, the remodeling process of the ECM is altered to accommodate the tumor formation. The three major components of ECM remodeling in the primary tumor are deposition, modification, and degradation corresponding with increasing stages of the TME.[132,135] Like the immune cells that the ECM houses, the ECM itself has both anti- and pro-tumor formation properties with the majority being pro-tumor formation. During tumor formation, the most frequent pro-tumor composition change in the ECM is an increased amount of fibrillar collagen. This along with other increases in ECM components causes desmoplasia, a fibrotic phenotype that is a major characteristic of breast cancer.[135,136] ECM remodeling also plays a significant role in cancer metastasis. Circulating tumor cells leave the primary tumor site via angiogenesis and other factors and contain ECM that can protect the tumor cells from

immunosurveillance.[135,137] Endothelial cells promote attachment of circulating tumor cells to the endothelial wall of the metastasized organ remodeling the tissue at this location. Furthermore, primary tumor cells secrete various growth factors to remodel the ECM at the metastasized organ site priming it for the circulating T cells that will arrive.[132,135,138] In turn this leads to the formation of a metastatic tumor site.

The ECM is a major player in the structure and shaping of the TME as well as a large proponent of drug resistance. One of the major immunosuppressive mechanisms the ECM contributes to is creating a barrier that inhibits the infiltration of T cells into the TME. A major obstacle in immunotherapy success is the low presence of T cells within the core of the TME that are unable to get to the core due to the ECM barrier.[135,137] A higher density ECM can influence and determine the distribution of drugs into the TME while also determining effector cell distribution into the TME. Both T and NK cells are unable to penetrate the dense ECM of a TME. This along with the inability for distribution of immunotherapies into the TME, makes breaking down the ECM a great therapeutic option to improve immunotherapy effectiveness and response.[137] Preclinical models utilized collagenase treatment on the ECM to break down the barrier and allow for drug uptake.[139] Under this treatment, the degrading of the ECM improved drug uptake and response.[139] While this a viable option that has the potential to turn a cold tumor hot the ECM is under constant remodeling making it a difficult target to treat.

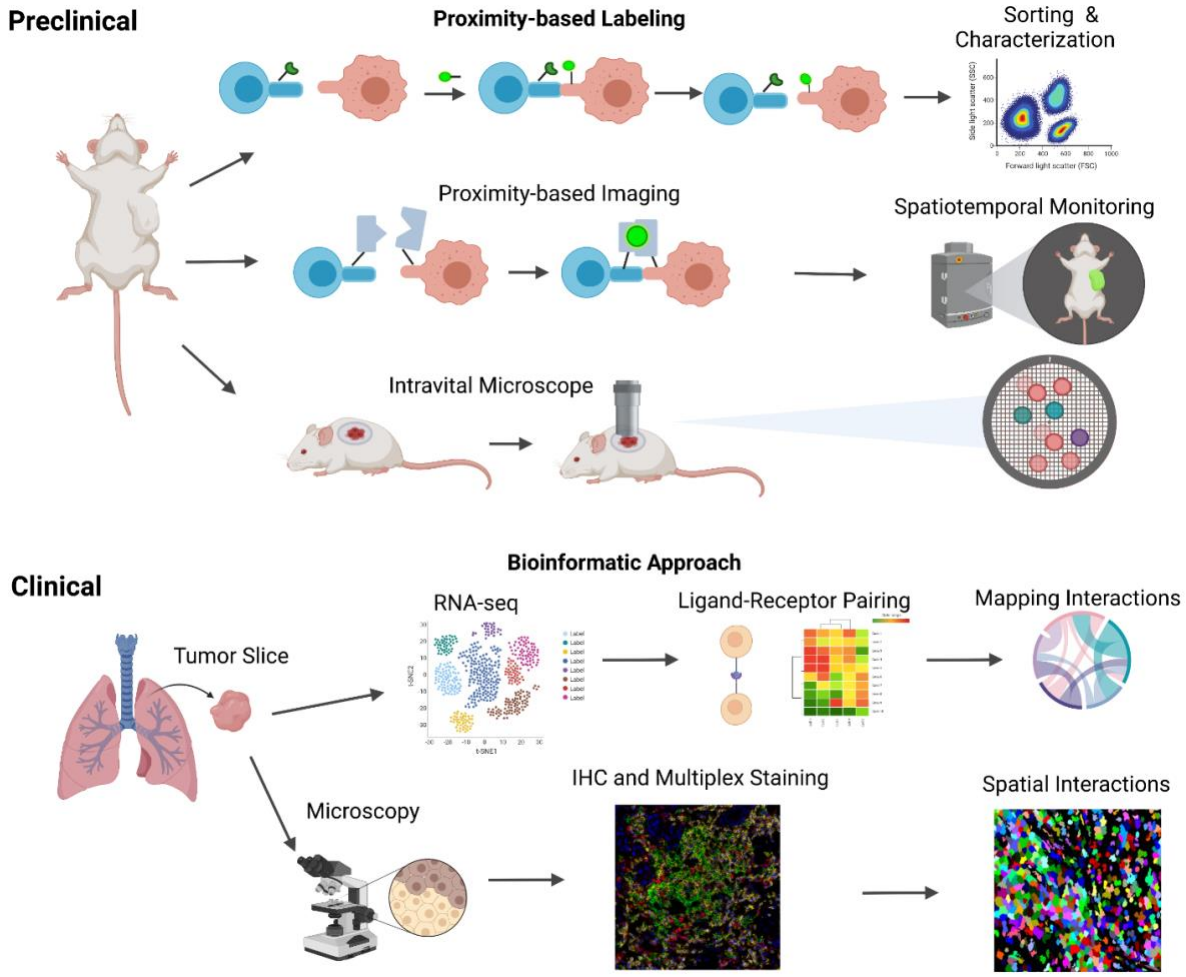
Experimental Techniques and Modeling Systems to Study Cellular Communication

The intricate interplay between immune cells and structural components within the TME significantly influences patient outcomes, therapeutic response, and disease progression.[140] While existing data shed light on the communicative relationships among immune cells and tumor cells within the TME, there remains a substantial knowledge gap regarding the intra-

patient and intra-cancer type communication. Consequently, the TME and its diverse cellular components have emerged as an enticing landscape for research, aiming to discover novel therapeutic strategies and optimize patient management.[140]

To investigate and elucidate the intricate cellular interactions within the TME, numerous methodologies and systems have been developed (Figure 1.2). These approaches encompass in vitro and in vivo models, employing molecular analysis techniques, proximity labeling methods, and bioinformatic approaches.[140,141] Understanding the composition of distinct cell types within different TMEs and patient contexts is pivotal for advancing therapeutic interventions and identifying prognostic biomarkers. In this section, we delve into various experimental techniques and modeling systems for the comprehensive study of cell-cell interactions, encompassing experimental modeling systems, microscopy/imaging, proximity labeling, and bioinformatic approaches.

Figure 1.2: Human and Murine Methods to Study Cell-Cell Interactions



Schematic overview of the current human and murine methods in studying intercellular interactions. Human samples, obtained primarily from tissue biopsies and blood samples, serve as valuable resources for investigating these interactions. Two key techniques employed in human models are single-cell RNA sequencing (scRNA-seq) and immunohistochemical staining (IHC). scRNA-seq enables the analysis of ligand-receptor interactions, facilitating the mapping of diverse cell-cell interactions. On the other hand, IHC provides spatial information, allowing for the identification of cell locations and their physical proximity to one another. In addition to the techniques utilized in human models, other innovative methods have been developed to study intercellular interactions. These include proximity-based intercellular labeling approaches such as LIPSTIC and EXCELL, which enable the identification of neighboring cells and the assessment of their interactions. Proximity-based intercellular imaging approaches such as confocal microscopy and CODEX offer further insights into intercellular communication by visualizing the spatial relationships between cells. Furthermore, intravital microscopy has emerged as a powerful tool for real-time monitoring of lymphocyte localization and movement within tumor microenvironments.

Table 1.1: Experimental Modeling Systems for Intercellular Interactions

Model System	Sample	Method Description	Reference
2D cell culture		Cells grow in a monolayer if adherent or suspended in a culture flask. These cultures are straightforward, cost-effective, and low-maintenance approach. Within the controlled environment, it is possible to investigate the interactions between different cell lines and observe their behavior and responses to treatments.	142
3D cell culture	Cells	Cell growth and interactions occur in 3D space, where cells interact with their surrounding environment and neighboring cells. Two approaches: scaffold-based methods using hydrogels or structural scaffolds and scaffold-free techniques (spheroids).	142
Spheroids	Cells	Organoids, also known as multicellular spheroids, are self-assembled structures that mimic the physiological environment and interactions found in vivo. It provides a more physiologically relevant context, allowing to investigate intercellular interactions and responses within a 3D microenvironment resembling in vivo conditions.	143
Organoids	Patient-derived cells and tissues	Primary patient-derived microtissues grown in a 3D extracellular matrix that represent in vivo physiology and genetic diversity, allowing to investigate intercellular interactions and responses in a patient specific manner.	144
Tissue Slices	Tumor Tissue	Tumor biopsy taken from patients or xenograft models, stained to assess tumor morphology and spatial location of cells.	145
Animal models	Tumor Tissue	Compatible with intravital and intercellular imaging/labeling techniques, as well as other genetic systems designed to detect cell-cell interactions upon contact or external stimulation, including UV or fluorescent light.	146

The study of cell-cell interactions employs various in vitro experimental systems, including two-dimensional (2D) cell culture, three-dimensional (3D) methods such as spheroids and organoids, as well as tissue samples (Table 1.1). Traditional 2D culture using primary cells and cell lines has long been considered a gold standard in cell culture due to its cost-effectiveness, long-term culture viability, low maintenance requirements, and user-friendly nature.[142] However, 2D culture falls short in mimicking the natural tissue structure and lacks biologically relevant cell-environment interactions when investigating complex environments

like TME or normal tissue structures. To address this limitation, 3D cell culture techniques have revolutionized in vitro methodologies by providing more physiologically relevant options.[142] Organoids and spheroids have gained popularity as they enable the comparison of in vivo organs in vitro. Organoids, derived from stem cells or patient tumor cells, are three-dimensional tissue cultures that replicate the morphological and genetic features of the original tumor, allowing for patient-specific models and in vitro representations of the TME.[144] However, organoids have limitations such as high patient variability, absence of specific essential cellular components, challenging culture maintenance, and higher costs.[144] On the other hand, spheroids are simpler three-dimensional clusters of cells derived from various cell types, including tumor tissues and hepatocytes.[143] They do not require scaffolding to form 3D cultures but rely on cell adhesion. However, spheroids lack the ability to self-assemble or regenerate, making them less desirable compared to organoids.[143] Both models enable three-dimensional assessment of tumors in vitro, providing improved translational models for clinical applications. Tissue biopsy slices are also valuable for identifying the spatial distribution and location of cells within the TME or normal tissues. However, the slicing process introduces variability in cell distribution due to the method employed.[145]

Nevertheless, in vitro systems lack the host tissue contexture and immune system, which are critical components for studying cell-cell interactions. Cell-cell interactions heavily rely on the tissue contexture, and techniques that support in vivo investigations have the potential to unveil novel modes of cell-cell interactions and their impact on tumor response to therapies. In vivo systems with an intact host immune system, such as syngeneic mouse models, are useful for studying the TME since the host immune system can interact with the TME.[147,148] However, a drawback of in vivo models lies in the fact that the interacting immune system being studied is

often the host mouse immune system, which differs significantly from the human immune system, especially concerning cellular interactions.[147]

Furthermore, tumor xenograft models using cell lines often undergo substantial genetic changes, fail to recapitulate the natural tumor structure, and may lead to mouse-specific tumor evolution.[146] While mice are the preferred experimental model for immunologists, there are significant differences between mice and humans, particularly in innate and adaptive immunity, leading to challenges in translating findings to humans.[149] The low success rate of clinical trials, which is less than 15%, can be attributed, in part, to the inadequate modeling of human diseases in animals and the limited predictability of animal models.[149] Future advancements in ex vivo models and platforms, such as microfluidics, hold promise in using patient-derived human samples to study cell-cell interactions, leading to better clinical translation.

Proximity-Based Labeling Approaches for Studying Cell-Cell Interactions

Intercellular proximity labeling approaches have revolutionized the study of cell-cell interactions by providing spatially resolved information. These methods involve the tagging or labeling of proteins or other molecules that are in proximity to a specific cell type or surface marker [150] (Table 1.2). By identifying and analyzing the labeled molecules, researchers can gain valuable insights into the neighboring cell types and their interactions. In the context of studying the TME, proximity labeling approaches play a crucial role. Understanding the interactions between cancer cells and immune cells within the TME is essential for developing effective immunotherapies. By labeling and tracking immune cells that have come in proximity or contact with tumor cells, we can investigate the molecular features of immune cells and assess how these cells influence the composition and function of the TME.

Table 1.2: Proximity-Based Labeling Approaches for Studying Cell-Cell Interactions

System	Scale	Application	Method	Reference
EXCELL	In vitro	Labeling Imaging	EXCELL (Enzyme-mediated intercellular proximity labeling) is a method that utilizes a variant of SrtA, mgSrtA, to enable the non-specific labeling of cell surface proteins containing a monoglycine residue at the N-terminus. Unlike other methods, EXCELL does not require pre-engineering of acceptor cells and was applied for in vitro studies.	[151]
G-BaToN	In vitro In vivo Ex vivo	Labeling Imaging	G-BaToN is a versatile system for physical contact labeling between cells. Sender cells express surface bound GFP, while receiver cells carry a synthetic element that selectively binds to GFP. Upon cell contact, GFP is transferred from sender to receiver cells, leading to fluorescence labeling of the receiver cells. This method requires pre-engineering of both sender and receiver cells and can be used for in vitro and ex vivo studies.	[152]
LIPSTIC	In vitro	Labeling	LIPSTIC (labelling immune partnerships by SorTagging intercellular contacts) is a proximity-dependent labeling method that employs bacterial sortase (SrtA) to detect receptor-ligand interactions between cells. It involves the attachment of biotin to cell surface proteins, which can be detected using flow cytometry. LIPSTIC can be used in both in vitro and in vivo settings by pre-engineering the cells on both sides of the interaction.	[153]
FucoID	In vitro Ex vivo	Labeling	FucoID is a method for identifying antigen-specific T cells using interaction-dependent fucosyl biotinylation. This technique enables the isolation of endogenous tumor antigen T cells from tumor digests without prior knowledge of the tumor-specific antigens and has been used for ex vivo studies.	[154,155]
PUP-IT	In vitro	Labeling	PUP-IT (Pupylation-based interaction tagging) is a method used to identify membrane protein interactions. In this approach, a small protein tag, Pup, is applied to proteins that interact with a PafA-fused bait, enabling transient and weak interactions to be enriched and detected by mass spectrometry. PUP-IT enables the identification and analysis of protein-protein interactions occurring at the membrane level.	[156]
2CT-CRISPR	In vitro Ex vivo	Genetic influence	Two-cell type CRISPR assay. This assay can genetically manipulate T cells to interact with cancer cells ex vivo to determine the genes that influence T cell effector function on cancer cells.	[157]
TRACC	In vitro	Labeling Imaging	TRACC (Transcriptional Readout Activated by Cell-Cell Contacts) is a system that utilizes light gating to detect cell-cell contacts based on transcriptional activity (TF). Cells are engineered to express a light-responsive TF that regulates the expression of a reporter gene. When two cells come into contact, a light signal is applied to activate the TF, resulting in the activation of the reporter gene and subsequent detection	[158]

System	Scale	Application	Method	Reference
			of the cell-cell contact, monitoring cell-cell interactions in a controlled and dynamic manner.	
Cherry-niche	In vivo	Labeling Imaging	Cherry-Niche is an innovative method that allows cells expressing a fluorescent protein to selectively label their surrounding cells in the tumor niche. This technique involves generating cancer cells capable of transferring a liposoluble fluorescent protein to their neighboring cells within the tumor microenvironment.	[159]
Caged luciferins	In vitro In vivo	Imaging	Caged luciferins are utilized for bioluminescent activity-based sensing. Activator cells expressing β-galactosidase catalyze the cleavage of caged luciferin, known as Lugal, resulting in the release of D-luciferin. The liberated D-luciferin can then enter nearby reporter cells, where it serves as a substrate for luciferase enzyme, leading to the production of light and allowing for the identification and visualization of cells that are in close proximity to the sender cells	[160]
SynNotch activated MRI	In vivo	Imaging	SynNotch system is utilized to induce the expression of an MRI contrast agent in recipient cells when they interact with sender cells expressing the corresponding synthetic notch receptor, enabling the detection and visualization of cell-cell communication events in real-time.	[161]
CLIP	In vivo	Labeling Imaging	CLIP (Cre-induced intercellular labeling protein) secretes a membrane-permeable fluorescent protein (mCherry) from a donor cell that can mark neighboring receptor cells. This method can label both direct cell contact receptor cells and receptor cells at a close-range distance.	[162]

Different from the IHC or fluorescent staining approaches, proximity labeling techniques can go beyond a time frozen snapshot, helping to identify and track cells giving a more dynamic approach to cell-cell interactions. This becomes important when studying where immune cells go after interacting with cancer cells, and how that cellular movement affects the composition of the TME. To ensure the applicability of proximity labeling approaches in both *in vivo* and *in vitro* environments, it is essential that these methods are non-disruptive and non-toxic to cells. This consideration ensures that the labeled cells maintain their physiological properties and behave naturally during the experimental process. By utilizing labeling techniques that are minimally

invasive and compatible with live cell imaging, researchers can gain a comprehensive understanding of cell-cell interactions in the TME.

Two prominent intercellular proximity labeling methods, EXCELL and LIPSTIC, employ the Staphylococcus aureus enzyme Sortase A (SrtA) to measure cell-cell interactions. LIPSTIC (Labeling Immune Partnerships by SorTagging Intercellular Contacts) enables the identification of ligand-receptor interactions between immune cells and their target cells.[151,153] Cells expressing SrtA on their surface covalently attach biotin molecules to neighboring surface proteins upon cell-cell contact.[163] Interacting cells are then exposed to a streptavidin-conjugated fluorescent dye, allowing for quantification of the interaction. LIPSTIC offers an unbiased approach for identifying ligand-receptor interactions and allows for the study of interaction dynamics over time. However, it relies on the genetic modification and expression of both donor and receiver cells, limiting its application to specific cell types or tissues.[153] EXCELL (Enzyme Mediated intercellular proximity labeling) represents a recent development using a SrtA variant mgSrtA, enabling promiscuous labeling of various cell surface proteins containing a monoglycine residue at the N-terminus.[151] Unlike LIPSTIC, EXCELL does not require genetic modification of the receiver cells and supports the identification of novel cellular interactions, including subtype identification of TILs interacting with tumor cells. For both approaches, the biotin-labeled proteins can then be isolated and identified using streptavidin-based purification methods such as flow cytometry and these labeled cells could be subject to further molecular characterization.

GFP-based-Touching Nexus or G-baToN harness the trogocytosis communication of cells to transfer GFP from a donor cell to an acceptor cell.[152] However, G-baToN approach requires the donor and receiver cells both to be transfected, which does not support identification of unknown or novel CCIs.[152] FucoID has several advantages over other proximity labeling

approaches. It enables the labeling of glycoproteins, which are an important class of proteins involved in many biological processes.[154,155] Additionally, the fucose tag is relatively small and minimally interferes with the function of the labeled proteins, which can reduce the likelihood of introducing artifacts into downstream analyses. FucoID can also be combined with other techniques such as single-cell RNA sequencing to gain a deeper understanding of the molecular mechanisms underlying intercellular communication. However, FucoID also has some limitations. It is dependent on the expression level and accessibility of the cell surface marker of interest, which may limit its application to certain cell types or tissues. Additionally, the labeling efficiency of FucoID may be affected by the density of glycoproteins on the cell surface and the availability of fucose residues. Careful experimental design and validation are necessary to ensure the accuracy and specificity of the results obtained using FucoID.[154,155]

A subsequent method that is able to detect membrane proteins through proximity labeling is population-based interaction tagging (PUP-IT).[156] In this system, the small protein tag Pup is weakly attached to proteins or prey interacting with the gene *PafA* or bait.[156] PUP-IT was utilized to label the interaction between CD28 expressing Jurkat T cells with CD80/86 expressing Raji B Lymphocytes. PUP-IT CD28 extracellular Jurkat T cells were able to label Raji B cell *in vitro.*[156] However, for this interaction to be observed both cells needed to be modified with the prey or bait genes. PUP-IT is also classified as a "weak" interaction and may not be suitable for long-term tracking.[156] This highlights that PUP-ID needs some knowledge of ligand-receptor interactions before using.

2CT-CRISPR assay is a novel and interesting approach for identifying genes that are essential for effector T cell function in tumors. In the 2CT-CRISPR assay, human T cells were represented as effectors and melanoma cells were represented as targets.[157] The purpose of this

assay was to determine if genetically manipulating the immune cell would influence the tumor cell during ligand-receptor interactions.[157] A recombinant TCR-engineered CD8+ T cell was used to target a specific antigen (NY-ESO-1) that can mediate tumor size in melanoma patients. The 2CT method was used to control the selection pressure and killing effects shown by the T cell as well as modulating the effector to target ratio. Furthermore, the 2CT method was used in combination with a CRISPR-Cas9 library that held over 100,000 single guide RNAs which impaired effector function in T cells. The 2CT method allowed for the analysis of genes necessary for immunotherapy specifically those that target effector T cell function. The 2CT method has exciting translation and clinical opportunities to uncover genes in immunotherapy-resistant patients.[157]

Other proximity-based methods for studying cell-cell interactions include TRACC (Transcriptional Readout Activated by Cell-Cell Contacts) and SynNotch activated MRI (magnetic resonance imaging), both of which exploit specific receptor-ligand interactions between two interacting cells to facilitate labeling and detection.[158,161] TRACC utilizes a g-protein-coupled receptor with a light-sensitive domain to detect cell-cell interactions using transcriptional readout.[158] This approach allows for the visualization and identification of cell populations involved in the interaction of interest. SynNotch activated MRI combines synthetic biology and imaging techniques to detect cell-cell interactions.[161] It involves the engineering of cells expressing a synthetic Notch receptor that can be activated upon interaction with a specific ligand presented by neighboring cells. Upon activation, the engineered cells produce a contrast agent detectable by MRI, enabling the visualization and tracking of the interacting cell populations.[161]

Most proximity-based methods are dependent on cell-cell contact and interaction. Cherry niche, caged luciferins, and CLIP (Cre-induced intercellular labeling protein) are three approaches that do not solely rely on direct cell-cell contact for labeling.[159,160,162] In the Cherry niche method, cells are engineered to express the enzyme Cherry-tagged ligase, which can attach a fluorophore to nearby cells expressing a complementary Cherry-tagged receptor.[159] This proximity labeling occurs within a specific microenvironment or niche defined by the presence of the ligase and receptor.[159] Caged luciferins, on the other hand, involve the use of caged luciferin molecules that can be activated by specific enzymes or stimuli produced by engineered cells.[160] Upon activation, the caged luciferins produce luminescent signals that can be detected and used to identify neighboring cells in the vicinity.[160] CLIP has an interesting methodology in that it can label cells that are in direct contact as well as those that are not in direct contact but in proximity.[162] This method involves the engineering of both the donor and receiver cells where the donor cell secretes a lipid-soluble tag containing mCherry that labels the recipient cells.[162] These approaches provide additional tools for studying cell-cell interactions, offering different mechanisms for labeling and detection beyond direct cell-cell contact.

In summary, intercellular proximity labeling or imaging approaches offer significant potential for elucidating the intricate cellular interactions and communication networks within the TME. These methods have been successfully employed in various research areas, including the investigation of tumor metastasis[159,164], T cell priming [153], cell migration [153], tumor-immune cell interactions [164], cellular therapy [164], and the examination of interactions between neurons and glioma cells.[158] As these techniques continue to advance, it is anticipated that their application will expand further, enabling a broader understanding of the factors and mechanisms that impede the pharmacological effects of immunotherapies. Overall, intercellular proximity labeling

approaches can provide valuable insights into the complex cellular interactions and communication networks in the TME, which can inform the development of more effective cancer immunotherapies.

Bioinformatic Techniques for Inferring Cell-Cell Interactions

Enzyme-based intercellular proximity labeling approaches are predominantly employed in experimental systems. However, with the growing availability of large clinical datasets, bioinformatic methods have gained significance in studying cell-cell interactions and identifying novel interactions. In clinical settings, bioinformatics methods play a crucial role in inferring intercellular interactions or communication by examining the coordinated expression patterns of ligand-receptor pairs' cognate genes. Ligand-receptor analysis has emerged as a valuable approach for investigating intercellular communication, particularly in the context of cancer immunotherapy. This approach enables the identification of specific ligand-receptor pairs involved in immune cell interactions with cancer cells or the TME (Table 1.3).

This approach proves especially valuable in deducing intercellular interactions that aren't solely reliant on cell-to-cell contact. This is evident when immune cells and cancer cells release diverse cytokines, chemokines, and growth factors that govern immune reactions and inflammation. These signaling molecules and their corresponding receptors may be modulated based on environmental cues. Through these bioinformatics methods, we can identify the likelihood of intercellular interactions based on their ligand-receptor profiles, which is potentially pivotal in forecasting patient prognosis and treatment outcomes.

Table 1.3: Bioinformatic Techniques for Inferring Cell-Cell Interactions

Platform	Data Source	Method	Reference
CellTalkDB	scRNA-seq	Manually curated database of ligand-receptor pairs from both human and mouse samples.	[165]
iTalk	scRNA-seq	Identifying and illustrating alterations in intercellular signaling network. R package made to analyze and visualize ligand-receptor	[166]
PyMINeR	scRNA-seq	Python maximal information network exploration resource. Fully automates cell type specific identification, and pathways as well as in silico detection of autocrine and paracrine signaling networks	[167]
CellChat	scRNA-seq	Open-source R package that is able to visualize, analyze and deduce intercellular communications from a data input. Uses mass action models and differential expression analysis to deduce cell-state specific signaling communications. Also provides visualization outputs to compare intercellular communication methods.	[168]
CellPhoneDB	scRNA-seq	Identifies biologically relevant interacting ligand-receptor pairs. Cells with the same cluster are pooled together as one cell state. Ligand-receptor interactions are derived based on the expression of a receptor of one state and a ligand of the other state.	[169]
Giotto	scRNA-seq	Open-source spatial analysis platform that contains two modules Giotto analyzer and Giotto viewer both independent and fully integrated. Analyzer provides instructions about steps in analyzing single cell expression data, and the viewer provides an interactive view of the data.	[170,171]
ICellNET	RNA-seq, scRNA-seq, and microarray	Transcriptomic based framework that integrates a database of ligand-receptor interactions, communication scores, connection of cell populations of interest with 31 human reference cell types, and three visualization methods.	[172]
SingleCellSignalR	scRNA-seq	Open-source R platform. Relies on a database of known ligand-receptor interactions called LR*db*.	[173]
CCC Explorer	Transcriptome profiles	Java based software. Uses a computational model to look at cell-cell communications ranging from ligand receptor interactions to transcription factors and target genes.	[174]
NicheNet	Gene expression data	Open-source R platform. Uses a database of ligand-receptor interactions to identify ligand-receptor interactions that could drive gene expression changes	[175]
SoptSC	RNA-seq	Similarity matrix-based optimization for single-cell data analysis. Uses a cell-to-cell similarity matrix via gene marker identification, lineage reference, clustering, and psuedotemporal ordering. From this information, predicts cell-cell communication networks.	[176]

Platform	Data Source	Method	Reference
SpaoTSC	scRNA-seq	Spatially optimal transporting the single cells. Method has two major components: 1. Constructing spatial metric for cells from scRNA-seq data and 2. Reconstructing the cell-cell communication networks from the data and identifying relationships between genes from intercellular relationships. Uses python.	[177]
scTensor	scRNA-seq	Open-source R-package. Instead of looking at one to one cell-cell interactions, this software focuses on many to many cell-cell interactions. scTensor looks at a three-way relationship (hypergraph) between ligand expression, receptor expression, and ligand-receptor pairs.	[178]

The analysis of ligand-receptor interactions primarily relies on single-cell RNA sequencing (scRNA-seq) or bulk RNA-seq data. The procedure typically involves the following steps:

- Data preprocessing: This step involves normalizing, quality controlling, and filtering gene expression data to ensure data integrity and reliability.
- Gene set selection: A specific set of ligand and receptor genes is chosen based on prior knowledge or by utilizing databases such as CellPhoneDB or Interactome INSIDER.
- Calculation of ligand-receptor expression: The expression levels of ligand and receptor genes are calculated for each cell or cell type within the dataset, whether it's scRNA-seq or bulk RNA-seq data.
- Ligand-receptor interaction analysis: Interactions between ligands and receptors are predicted by assessing the co-expression patterns of ligand and receptor genes across different cells or cell types. Several methods, including CellPhoneDB, scRNA-seq-based Ligand-Receptor Pair Analysis (sLRPA), and LigandNet, are available for performing this analysis.
- Visualization and interpretation: The results of the analysis are visualized using heatmaps, networks, or other visualization techniques. These results can be interpreted to identify specific ligand-receptor pairs that may be involved in intercellular communication and to gain insights into the underlying biological processes.

Bioinformatic methods offer valuable tools for inferring intercellular interactions and communication based on transcriptome data. These approaches provide valuable insights into the intricate network of cell-cell interactions in the context of cancer immunotherapy. Notably,

ligand-receptor analysis holds promise for identifying predictive biomarkers for immunotherapy response and monitoring treatment efficacy.

Nevertheless, it is important to acknowledge the limitations of these bioinformatic methods in inferring intercellular interactions. Firstly, their predictions solely rely on gene expression data and overlook additional factors like post-translational modifications or protein localization that can influence interactions. Secondly, these methods hinge upon existing knowledge of ligand-receptor pairs, which may be incomplete or imprecise. Thirdly, the biological relevance of the predictions is not guaranteed, necessitating experimental validation. Lastly, technical artifacts such as batch effects, sequencing depth, and normalization methods can influence the accuracy and reproducibility of the results. Considering these limitations, it is crucial to exercise caution and combine bioinformatic predictions with experimental validation to ensure the reliability and significance of the findings. Continued advancements in bioinformatic techniques and complementary experimental approaches will enhance our understanding of intercellular interactions and their role in cancer immunotherapy.

Scientific Questions That Can Be Answered from These Tools and Approaches

A wide array of scientific questions concerning cancer immunotherapy could be answered utilizing these CCI techniques. These questions include:

1. What are the subpopulations of immune cells interacting with the tumor cell during immunotherapy?
2. What are the molecular features of these interacting immune cells, and how are the molecular features related in response to immunotherapy?
3. Whether the effector cells that have interacted with tumor cells migrate across tumor metastatic lesions?

Ultimately, the answers to these questions can uncover the pharmacological actions of cancer immunotherapy and reveal the underlying molecular mechanism of resistance.

Conclusion

The TME represents an intricate network comprising diverse cell types engaged in communication, which plays a pivotal role in shaping the tumor landscape. Effective communication between immune cells and cancer cells holds great significance in determining patients' response to immunotherapy, as well as contributing to treatment resistance and interpatient variability in responses. Investigation of cell-cell communication in immune cells under normal and pathological conditions provides crucial insights into the mechanisms of cancer immunotherapy, patient responses, disease progression, and TME status. Various experimental and computational approaches exist for elucidating pathological intercellular interactions, both directly and indirectly, with the aim of identifying the communicating cell populations. Comprehensive understanding, modeling, and discovery of cell-cell interactions within the TME hold immense potential in identifying critical factors and strategies influencing immunotherapy response, treatment resistance, and TME status.

In recent times, advances in imaging, microscopy, cellular engineering, and bioinformatics have emerged as powerful tools in unraveling novel mechanisms and cellular relationships, thereby paving the way for improved immunotherapy options for patients. By leveraging these methodologies synergistically, it becomes possible to bridge existing knowledge gaps and gain a comprehensive understanding of treatment resistance and design more potent cancer immunotherapies.

SPECIFIC AIMS

AIM 1: Develop a method to label immune cells *in vitro* (Chapter 2)

1a. Generate stable cell lines expressing EXCELL machinery through lentiviral infection.

1b. Quantify time- and concentration- dependent labeling of an immune cell line through flow cytometry and confocal microscopy.

AIM 2: Measure the immune cells that are labeled in a pilot *in vivo* study (Chapter 3)

2a. Determine EXCELLs ability to label primary immune cells through flow cytometry.

2b. Quantify murine immune cell subtypes that interacted with a tumor bearing EXCELL using a flow cytometry panel for B and T cells.

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CHAPTER 2: UTILIZING A PROXIMITY DEPENDENT STRATEGY TO STUDY IMMUNECANCER CELL INTERACTIONS IN VITRO[1]

Summary

Immune cells play a critical role in surveilling and defending against cancer, emphasizing the importance of understanding how they interact and communicate with cancer cells to determine cancer status, treatment response, and the formation of the tumor microenvironment (TME). To this end, we conducted a study demonstrating the effectiveness of an enzyme-mediated intercellular proximity labeling (EXCELL) method, which utilizes a modified version of the sortase A enzyme known as mgSrtA, in detecting and characterizing immune-tumor cell interactions. The mgSrtA enzyme is expressed on the membrane of tumor cells, and labels immune cells that interact with tumor cells in a proximity-dependent manner. Our research indicates that the EXCELL technique can detect and characterize immune-tumor cell interactions in a time- and concentration-dependent manner, without requiring pre-engineering of the immune cells. Overall, our findings suggest that the EXCELL method has great potential for improving our understanding of immune cell dynamics within the TME, ultimately leading to more potent pharmacological effects and cancer immunotherapy strategies.

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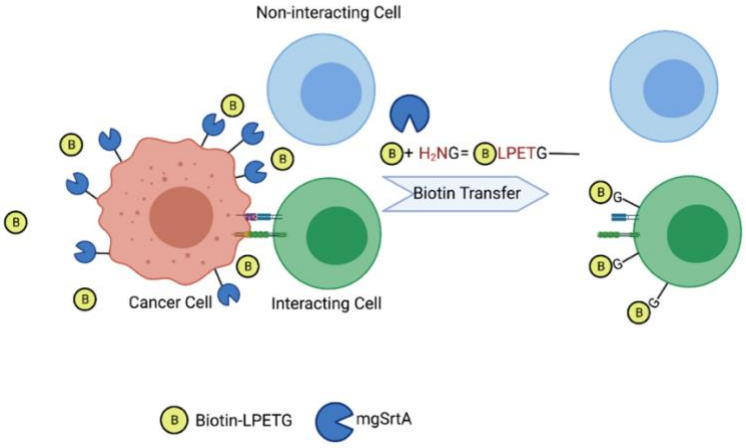
Figure 2.1: EXCELL Cell-Cell Interaction Labeling Approach



The plasmid-expressed mgSrtA enzyme is present on the cell surface. During cell-cell interactions in the presence of the Biotin-LPETG probe, the interacting cell's surface is labeled with biotin through transpeptidation, mediated by mgSrtA.

Introduction

Cell-cell interactions play a critical role in organ system function, cell development, tissue homeostasis, and the interaction of the immune system with disease cells.[1] Cell-cell interactions are crucial within the immune system, which is composed of a variety of cell types that communicate with each other in complex networks to ensure protections against pathogens and maintain self-tolerance.[1] In particular, immune cells play a key role in immunosurveillance against cancer, and when this process fails, it can increase the risk of cancer development and progression.[2,3] Therefore, the intercellular interaction and communication between immune cells and cancer cells are crucial in determining cancer status, progression, treatment response, and the formation of tumor microenvironment (TME).

The TME is a complex ecosystem composed of immune cells, blood cells, fibroblasts, blood vessels, and lymphocytes.[4] Tumors are classified based on their TME landscape, ranging from "hot" tumors that are infiltrated by tumor infiltrating lymphocytes (TIL) to "cold" tumors

that are devoid of immune infiltrate.[5] The intricate communication between immune and cancer cells is critical in shaping the landscape and status of TME and can significantly influence a patient's response to therapies, especially immunotherapy.[6] It remains challenging to investigate which types of cells are near tumor cells and are in direct contact with tumor cells in dynamic TMEs.

Tumors can evade the immune system through different mechanisms. These mechanisms include direct immunosuppression, which can be suppressed by tumor cells through immune-checkpoint pathways via PD-1/PD-L1 interaction.[2] The identification of immune-checkpoint inhibitor pathways has led to the development of immune checkpoint inhibitors (ICI), which target molecules such as CTLA-4 or PD-1/PD-L1[7]. ICIs have the ability to block inhibitory interactions and reactivate effector immune cells.[7,8] ICIs have shown efficacy in many cancers including melanoma, non-small cell lung cancer, and many others.[9,10] However, only a small percentage of patient respond to ICIs and there is high inter-patient and intra-cancer variability in response to ICI treatment.[11,12] Efforts to predict responders to ICI treatment have been suboptimal, emphasizing the need for a deeper understanding of the TME and the dynamic cell-cell interactions that occur within it.

There are several methods available for studying cell-cell interactions, including proximity-dependent techniques such as synNotch [13], FucoID [14], LIPSTIC [15] and G-BaToN [16], microscopy and imaging techniques[17], and bioinformatic techniques for molecular profiling[18] such as ligand-receptor pair analysis.[15,16] The choice of method will depend on the specific research question, the type of cells being studied, and the available resources. In this study, we utilized EXCELL technique to study cancer cell-immune cell interactions.[19] The EXCELL technique takes advantage of a transpeptidase enzyme called sortase A (SrtA) derived from

Staphylococcus aureus, which is naturally used to anchor proteins to bacterial cell walls.[20] SrtA recognizes substrate proteins with a specific motif called LPTXG.[21] An evolved version of SrtA called mgSrtA is used in the EXCELL method, which can promiscuously label various cell surface proteins containing a monoglycine residue at the N-terminus.[19] This increases the labeling potential on the interacting cell.[21] The objective of this study is to test the application of EXCELL technique *in vitro* to assess time and concentration dependency and specificity of EXCELL before moving into an *in vivo* model.

Methods

Peptides and Plasmids

Peptide probe Biotin-AALPETG*G (Cat # LT25866) was custom synthesized and purchased from LifeTein with a purity of 95.35%**.** Plasmid pcDNA3.1-CD40L-mgSrtA was a gift from P. Chen (Addgene plasmid # 125795; http://n2t.net/addgene:125795; RRID: Addgene_125795)

Cell Lines

HEK293T and MC38 Cells lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin (PenStrep) (%v/v). Raji B lymphocytes cell line was maintained in Roswell Park Memorial Institute (RPMI) 1640 Medium supplemented with 10% FBS and 1% PenStrep (%v/v).

Lentivirus Transduction

Plasmid pcDNA3.1-CD40L-mgSrtA was packaged into Lentivirus via GeneScript Custom Lentivirus Packaging in 1000 µl at a concentration of $1x10^8$ IFU/mL (GeneScript, USA). Specifically, the EXCELL components of the original plasmid were PCR'd out from the existing vector and into GeneScript's lenti-compatible transfer plasmid vector. Next, the transfer

plasmid was co-incubated with the packaging plasmids for co-infection resulting in third generation lentivirus packaging. Lentivirus was harvested, aliquoted, and titered to measure below 10^8 IFU/ml as determined by p24 ELISA. Of note, the plasmid comes with a FLAG tag to allow sorting of positive transfection. MC38 and HEK293T cells were plated at 100,000 cells/well in a 12-well plate and adhered to plate overnight. After 24 hrs 30 µl of Lentivirus was added dropwise to each well with cells in the 12-well plate except for control well. The 12 well plate was spinfected at 1,000 x g for 20 min at room temperature and placed into incubator. The morning after infection, media in the 12-well plate was changed. 48 hrs post infection. Antibiotic selection using Puromycin was added at 1ug/mL to kill the cells that do not contain the desired construct.

Stable Cell Line Generation

Cells were kept under selection for 3 days to ensure only the living cells contained the desired constructs. Next, cells were sorted by flow cytometry to obtain a purified cell population with the highest viral expressing cells. Cells were harvested and washed with 2% FBS in PBS. Cell pellets were resuspended in 100 µl of 2% FBS in PBS solution and 1ul of PE anti-FLAG antibody was added. Cells incubated for 30 minutes. Cells were washed 3 times with 2% FBS in PBS solution. Cells were then filtered with 70µm cell strainer into appropriately labeled 50ml tubes. Cell suspension was pipetted in polypropylene tubes for flow cytometry. Cells were sorted for the top 15% highest expressing FLAG signal. After sorting cells were placed into a 12-well plate with 15% Geneticin 10% FBS DMEM medium.

EXCELL Approach

The enzyme mgSrtA is present on the surface of cancer cells. As shown in Figure 1, when an immune cell encounters a cancer cell in the presence of Biotin-LPETG probe, which has a

sortase recognition motif LPETG, a ligand-receptor interaction is triggered, leading to the labeling of the interacting cell with biotin via mgSrtA-mediated transpeptidation. In the process, the enzyme cleaves the threonine-glycine bond in the motif and forms an acyl intermediate with the threonine in the peptide. Consequently, the interacting immune cell is labeled with biotin, while non-interacting cells remain unlabeled. The glycine residues present on N-terminus proteins on the cell surfaces conjugate the biotin peptide probe to the cell surface proteins, resulting in biotin labeling of the cell for downstream detection.

RT-qPCR Identifications of mgSrtA in Cell Lines

Custom TaqMan Probe was generated from ThermoFisher to identify the mgSrtA expression levels. RNA was extracted using Qiagen Rneasy Mini Kit (Cat # 74104). cDNA synthesis was performed using Verso cDNA synthesis kit (Cat # AB1453A). Gene expression was quantified using RT-qPCR performed on a thermocycler using TaqMan Universal PCR Master Mix (Cat # 4304437) and GAPDH as a housekeeping gene. Percent of mgSrtA gene expression versus control was analyzed using the double delta Ct method.

Flow Cytometry

HEK293T and/or MC38 cell lines were trypsonized using non enzymatic cell dissociation media and centrifuged cells at 400 x g for 10 min to pellet cells. Raji B cells were placed into 14ml falcon tube and centrifuged at 400 x g for 10 min to pellet cells. Cells were washed three times with PBS and counted on hemocytometer to determine cell viability and concentration. HEK293T and MC38 cells were stained with anti-DYDDDPK antibody and Raji B was stained with FITC anti-CD20 antibody. All cells were incubated with antibody for 30 minutes at 4 C in the dark. All cells were washed with cold PBS. HEK293T and Raji B or MC38 and Raji B cells were co-cultured together. 1 µl of biotin LPETG*G was added to 100 µl of combined cell culture

for a final concentration of 100µM biotin-LPETG*G. Cell combinations were left to incubate for 30 minutes to allow for biotin attachment. Cell combinations were washed 3x with PBS to remove any excess biotin. Cell combinations was resuspended in 100 µl of PBS and Streptavidin Alexa Fluor 647 conjugated antibody was added was added to the cell combination to stain for biotin. Cell combinations were incubated for 30 min at 4°C. Cell combinations were washed three times with PBS to remove any excess antibody. Cells were resuspended in a final volume of 400 µl and processed on a Attune NxT Flow Cytometer at UNC Chapel Hill Flow Cytometry Core. Data was analyzed using FlowJo.

Confocal Microscopy

HEK293T flag and HEK293T WT cell line were trypsonized using non enzymatic cell dissociation solution. Raji B cells were removed from cell culture dish. All cells were centrifuged at 400 x g for 10 min. Cells were counted on hemocytometer to assess viability and concentration. Cell concentration was adjusted to 10,000 cells for each cell type. HEK293T WT or FLAG were co-incubated with Raji B cells in 100 µl of PBS in the presence of 100 µM biotin-LPETG*G for 30 minutes. Cell combinations were washed 3 times with PBS. Cell combinations were stained with Streptavidin-Alexa 647 Conjugate antibody for biotin detection and incubated for 30 minutes at 4°C. Cell combinations were washed with PBS to remove excess antibody. Cell combinations were resuspended in 100 µl 0.5% BSA in PBS and placed into appropriately labeled well of Ibidi µ-Slide 18 Well Glass Bottom plate (Cat # 81817). Microscopy was performed on the Leica STELLARIS 8 FALCON STED confocal microscope at the UNC Hooker Imaging Core Facility. Gamma was set to 0.45 to adjust for contrast in images.

Statistical Analysis

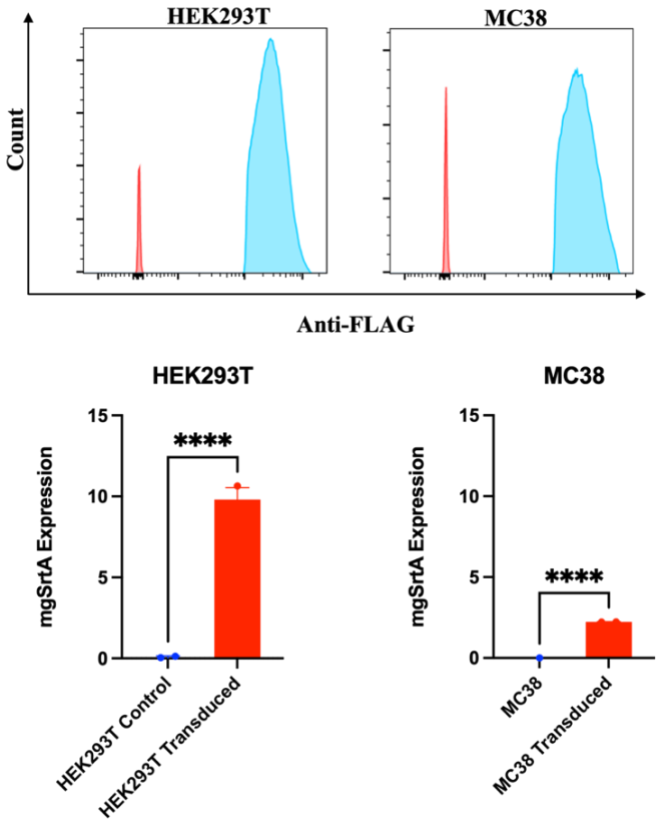
Comparisons between control and transduced HEK293T and MC38 mgSrtA expression were performed using students T Test. Similarly, the comparison of labeled Raji B cells compared to self-labeled HEK293T cells as well as total splenocytes compared to biotin positive splenocytes were performed using students T Test. Data were analyzed using GraphPad Prism v10 with a significance level of $P<0.05$.

Results

Generation of Stable Cell Lines

To ensure optimal mgSrtA expression in HEK293T and MC38 cell lines, the plasmid pcDNA3.1-CD40L-mgSrtA was packaged into lentivirus vector and used to generate stable expressing cell lines. Cells were infected as described above and sorted for FLAG tag (DYKDDDK) expression using anti-FLAG antibody (Figure 2a). The top 15% of cells expressing FLAG tag were sorted and cultured to ensure cells expressing the highest amount of plasmid were cultured for stable cell line generation. A custom TaqMan probe was created to measure mgSrtA gene expression via RT-qPCR. RT-qPCR data shows that mgSrtA gene expression was present in both cell lines, HEK293T and MC38, compared to their respective control (Figure 2.2). Both HEK293T and MC38 cells expressed a higher expression of mgSrtA compared to their respective controls.

Figure 2.2: Generation of Stable HEK293T- and MC38-mgSrtA Expressing Cell Lines



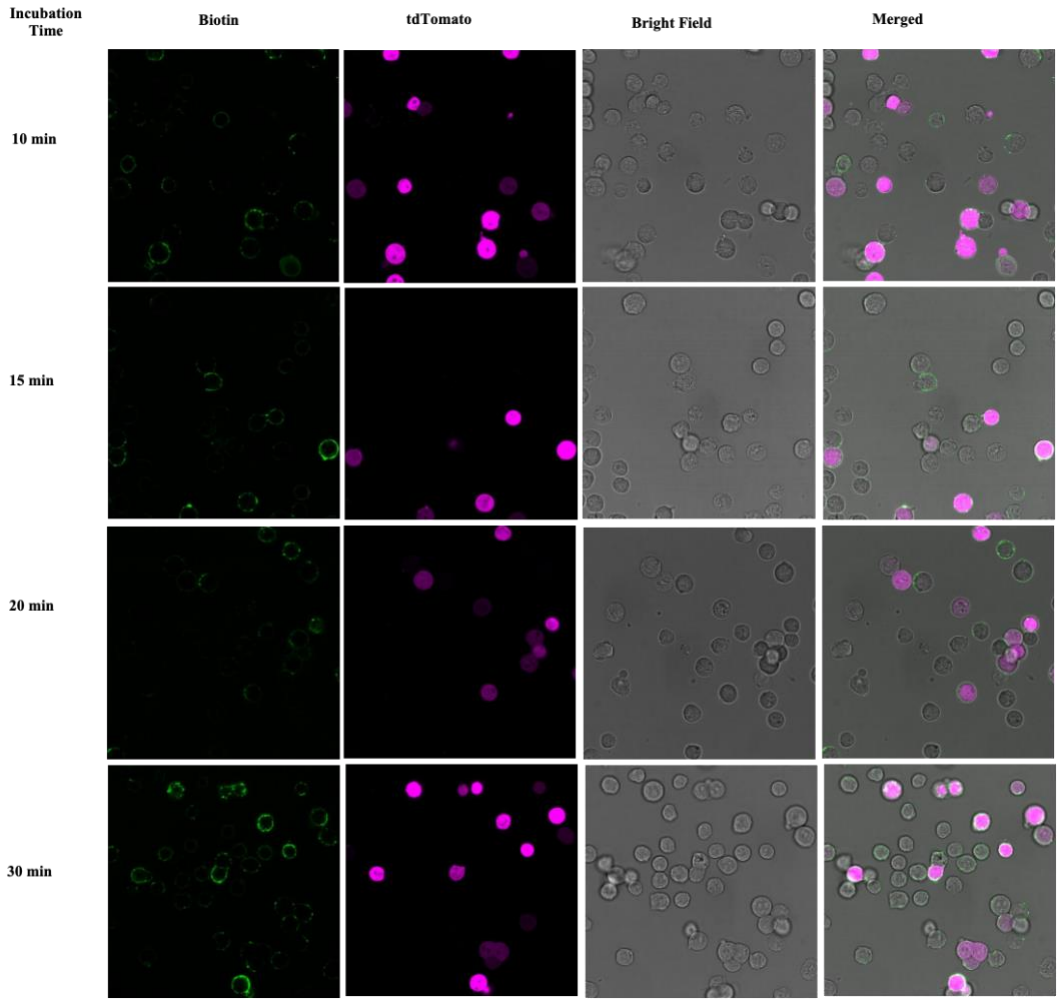
22. The image displays anti-FLAG staining, with control cells represented in red and plasmid construct expressing cells in blue. b. The RT-qPCR results exhibit the expression of mgSrtA in HEK293T and MC38 infected cell lines (shown in blue) compared to control cell lines (depicted in red). Student T-Test was run to determine stastical significance with **** referring to P<0.0001 and a n=3 for each group.

Imaging Cell-Cell intercellular Interaction In Vitro

After generating stable cell lines expressing mgSrtA in HEK293T and MC38 cells, the next step was to establish cell-cell interactions in a co-culture model *in vitro*. Raji B were used as an immune cell model, while HEK293T and MC38 served as cancer cell line models. To visualize these interactions, confocal microscopy was employed at different time points (10, 15, 20, and 30 minutes) post-co-culture. For clearer (non-overlapping) visualization in the confocal microscopy images, a low cell density was chosen, preventing robust statistical analysis of labeling efficiency. Biotin labeling was used to detect Raji B cells, which was outlined in green

on the cell membrane, and internal marker tdTomato was used to visualize HEK293T cells shown in magenta. In Figure 3, there was some level of self-labeling, i.e., HER293T cells label biotin on their own membrane, which was also observed in previous studies [19]. It can be observed that as the incubation time increased from 10 minutes to 30 minutes, there is an increase in the Raji B cells that are labeled.

Figure 2.3: Imaging Immune-Cancer Intercellular Interactions.



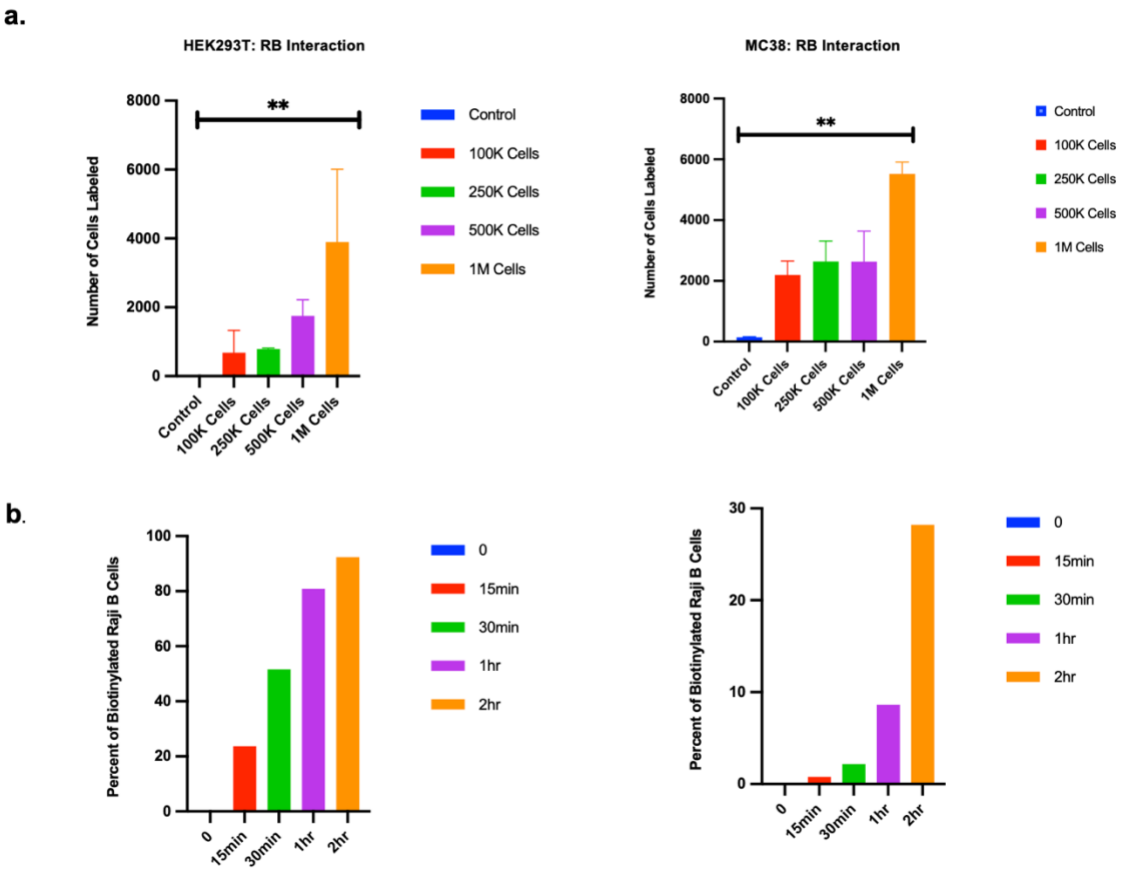
Confocal microscopy images were captured to observe the cell-cell interactions between Raji B and HEK293T cells over a period of 10 to 30 minutes. Biotin labeling, which is localized to the cell membrane, is represented in green. The mgSrtA expressing HEK293T cells are labeled with tdTomato, appearing in magenta. Bright field imaging was used to visualize the localization of both cell types in the frame. The merged channels display the biotin labeling of interacting cells facilitated by mgSrtA. To adjust for channel contrast, a gamma value of 0.45 was applied.

Cell density- and Time-Dependent Intercellular Interaction in Vitro

To further investigate the dynamics of cell-cell interactions using EXCELL-mediated proximity labeling, we tested its cell density- and time-dependency. First, we investigated the labeling of Raji B cells after directly contact with HEK293T and MC38 cells at various cell concentrations. Raji B cells were co-cultured with HEK293T and MC38 cells at different ratios, ranging from 100,000 cells to 1 million Raji B cells, while keeping a constant number of 1 million HEK293T or MC38 cells. The results showed that the number of Raji B cells that were biotinylated increased with the increasing concentration of Raji B cells in the cell-cell interaction (Figure 4a) (n=3/group), indicating a concentration-dependent labeling of Raji B cells upon direct ligand-receptor interaction.

Next, time-dependent proximity labeling between Raji B and HEK293T or MC38 cells was examined (Figure 4b). Consistent with the confocal microscopy images shown in Figure 3, there was an increased percentage of Raji B cells that were biotinylated after interaction with both cell lines over time in both cell lines. The gradual accumulation of biotinylated cells over the time frame indicated the stability of the biotinylated product, consistent with the previous observation that biotinylation involves covalent and stable attachment of biotin to glycine moiety of the protein on cell membrane. This time-dependent increase in Raji B cell labeling further confirmed the dynamic nature of the cell-cell interactions. Overall, the *in vitro* experiments presented in this study provided evidence for the ability of EXCELL to label immune cells in a time- and cell density-dependent manner.

Figure 2.4: Cell Density- (a) and Time- (b) Dependent Labeling of Raji B Cells



The graph presents the number of Raji B cells that were biotinylated after interacting with HEK293T or MC38 mgSrtA expressing cells, with different amounts indicated in the legend (n=3/group). b. The graph illustrates the percentage of biotinylated Raji B cells labeled over time, ranging from zero to two hours, following interaction with either HEK293T or MC38 cells. One way ANOVA was run showing statistical significance ** representing P<0.01 on the cell concentration dependent assay. There were no biological replication rather technical replications in the time-dependent assay.

Discussion

The EXCELL method has demonstrated its applicability in detecting and characterizing immune-tumor cell interactions through *in vitro* data. Using flow cytometry and confocal microscopy techniques, EXCELL was able to show that upon direct interaction with either HEK293T or MC38 cell lines, Raji B cells were labeled with biotin. Furthermore, the confocal images showcased that as time increased from 10 min to 30 min there was an increase in the intensity of the biotin labeling the Raji B cells as well as an increase in the number of cells that were labeled. Furthermore, there were some cells present especially in the earlier time points that had not been biotinylated yet. This gave us promise that the cells are in fact being labeled through proximity dependent transfer and there is no off-target labeling or antibody sticking occurring. By labeling immune cells in a concentration- and time-dependent manner, EXCELL can help identify and characterize immune cells interacting with cancer cells *in vitro* and provides rationale that EXCELL can be utilized *in vivo*.

While these experiments showcase EXCELLs ability to label an immune cell line *in vitro* without any engineered signaling from the tumor cell, there are some limitations. One of the major advantages also happens to be a limitation. As mentioned above EXCELL utilizes an evolved version of the enzyme SrtA, mgSrtA, to facilitate the biotin attachment onto the interacting cell. The difference between SrtA and mgSrtA is that SrtA recognizes the N-terminal glycine residue with -GGG and mgSrtA recognizes those with -G which occupy 96% of membrane in comparison to 1.10%.[22,19] This significantly improves the labeling ability of EXCELL on the cell membrane, but also leads to a lot of self-labeling on the cancer cell and non-specificity due to the promiscuous nature of the mgSrtA enzyme.

Overall, EXCELL showed promise *in vitro* to label interacting cells and has promise as a powerful tool for investigating immune-tumor cell interactions *in vivo* to determine the immune cells that are trafficked into the tumor on their own accord without any engineered signaling from the tumor cell. Future studies involving primary murine cells and a pilot *in vivo* study will be interesting for detecting EXCELLs ability to label primary cells.

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CHAPTER 3: UTILIZNG A PROXIMITY LABELING STRATEGY TO STUDYING IMMUNE-CANCER INTERACTION IN VIVO[1]

Summary

Previously we showed that EXCELL can be utilized *in vitro* to label Raji B cells with biotin in a time and concentration dependent manner through flow cytometry and confocal microscopy. Next, we were interested in developing the EXCELL method further to be able to label primary murine cells *ex vivo* and in a pilot *in vivo* study. Our research indicates that the EXCELL method can detect and characterizing immune-cancer cell interactions *in vivo.* Using EXCELL we were able to detect various types of immune cell subpopulations *in vivo* that have migrated out of the tumor into the spleen, providing insights into the role of peripheral T cell recruitment in tumor progression. The EXCELL method holds promise for detecting immune cell interaction *in vivo* as well as studying immune tumor cell dynamics and potentially uncover novel subtypes of immune cells within the TME.

[1]Portions of Chapter 3 were published September 28, 2023 Citation: "Maffuid, K., Cao, Y. Utilizing a Proximity Dependent Labeling Strategy to Study Cancer-Immune Intercellular Interactions *In Vitro* and *In Vivo.* JPET doi: 10.1124/jpet.123.001761"

Introduction

Understanding the presence and characteristics of TILs in the TME has the potential to provide valuable insights into tumor progression and patient response to ICIs. For example, studies have shown that a high presence of CD8+ T cells in the TME of colorectal cancer patients is associated with better overall survival.[1] Similarly, in melanoma patients, a high presence of CD8+ T cells pre-ICI treatment has been correlated with a positive patient response to ICI treatment.[2] Other immune cell types such as regulatory CD4+ T cells (T_{regs}), B cells, dendritic cells (DC), and natural killer (NK) cells, can also have both positive and negative effects on patient response to the treatment.[3] It has been observed that the prognostic impact of TILs can vary depending on factors such as tumor type, cell location in the TME, and state of cell activation.[4,5] Therefore, there is a need to further understand the complex and dynamic interactions between tumor cells and immune cells in the TME and identify novel phenotypes or sub-phenotypes of TILs that can provide insights into the status of the TME. Understanding the cell populations present in the TME can aid in predicting tumor aggressiveness and patient response to treatment.

Currently, there are no *in vivo* techniques in place to study dynamic labeling of immune cells in the TME without pre-engineering the immune cells. There are several methods available for studying cell-cell interactions, including proximity-dependent techniques such as synNotch [6], FucoID [7], LIPSTIC [8] and G-BaToN [9], microscopy and imaging techniques [10], and bioinformatic techniques for molecular profiling[11] such as ligand-receptor pair analysis.[8,9] The choice of method will depend on the specific research question, the type of cells being studied, and the available resources. In this study, we optimized the EXCELL technique to study cancer cell-immune cell interactions in a mouse *in vivo* model. The objective of this aim was to test the

application of EXCELL technique in tumor systems to gain a better understanding of and characterize the TILs of an *in vivo* TME.

Methods

Splenocyte and Lymphocyte Isolation and Flow Cytometry

Spleen and Lymph Nodes were isolated from healthy C57B/6 mice, counted on a hemocytometer, and resuspend at 1 million cells/mL. MC38-wt and MC38-mgSrtA cell lines were trypsonized using non enzymatic cell dissociation media and centrifuged cells at 400 x g for 10 min to pellet cells. Cells were washed three times with PBS and counted on hemocytometer to determine cell viability and concentration. HEK293T and MC38 cells were stained with anti-DYDDDPK antibody and splenocytes and lymphocytes were stained with CD45 antibody. All cells were incubated with antibody for 30 minutes at 4°C in the dark. All cells were washed with cold PBS. MC38-mgSrtA and either splenocytes or lymphocytes were co-cultured together. 1 µl of biotin LPETG*G was added to 100 µl of combined cell culture. Cell combinations were left to incubate for 30 minutes to allow for biotin attachment. Cell combinations were washed 3x with PBS to remove any excess biotin. Cell combinations was resuspended in 100 µl of PBS and Streptavidin Alexa Fluor 647 conjugated antibody was added was added to the cell combination to stain for biotin. Cell combinations were incubated for 30 min at 4°C. Cell combinations were washed three times with PBS to remove any excess antibody. Cells were resuspended in a final volume of 400 µl and processed on a Attune NxT Flow Cytometer at UNC Chapel Hill Flow Cytometry Core. Data was analyzed using FlowJo.

In Vivo Pilot Study

Male and female C57BL/6 mice were maintained at UNC vivarium in accordance with the Guide of the Care and Use of Laboratory Animals. A total of six mice were used in this pilot

study with five mice being xenografted with the MC38-mgSrtA expressing cells and one mouse with wild-type (WT) MC38 cell line as the control. On day zero, mice were bilaterally xenografted with 100 µl of 1 million MC38-mgSrtA or MC38 cells. On day five post-inoculation, all mice were injected with 300nM total of Biotin-LPETG*G penta peptide. 150 nM of peptide was administered and incubated for 30 min then the remaining 150nM was administered and incubated for 40min. Mice were then euthanized, and the spleen was harvested. Splenocytes from each mouse and were counted on a hemocytometer. Next, splenocytes were run through StemCell Technologies biotin positive selection kit II (Cat #17683) to isolate biotin labeled cells. Biotin positive cells were counted on hemocytometer to determine percentage of biotin labeled cells and cell viability. Biotin positive labeled cells were stained with flow panel including CD19, CD40, TCRβ, and CD69 markers and analyzed on Attune NxT flow cytometer. Flow data was analyzed using FlowJo software.

Statistical Analysis

The comparison between total splenocytes and biotin positive splenocytes isolated from the mice were compared using a Student's T-Test. The comparison of the cell types of isolated form the mouse samples were analyzed using one-way ANOVA with multiple comparisons. Data were analyzed using GraphPad Prism v10 with a significance level of $P<0.05$.

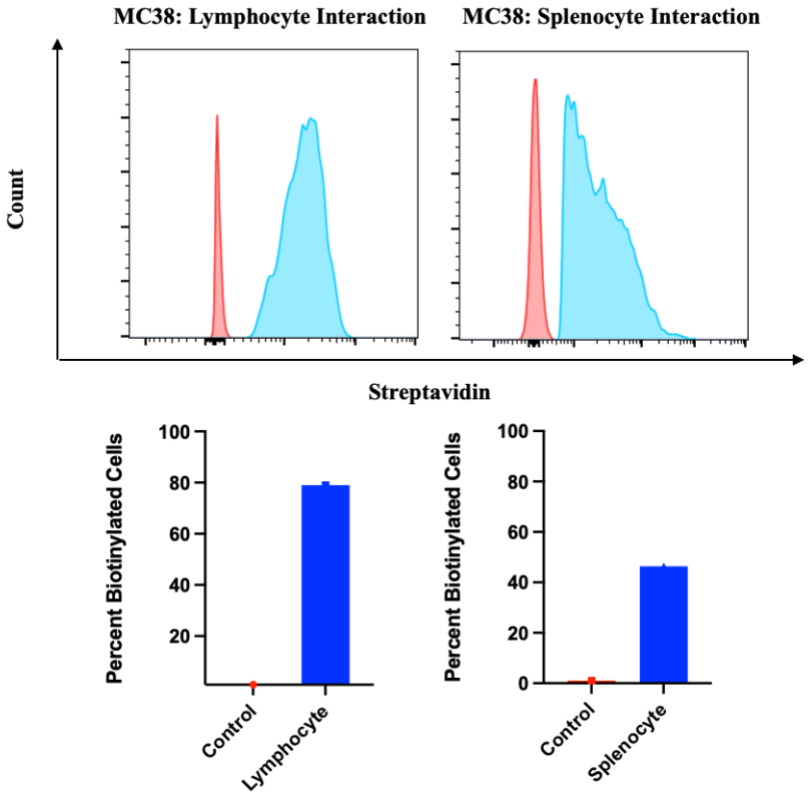
Results

Determining proximity dependent labeling in vivo

Prior to the xenograft study, we examined whether the MC38-mgSrtA cell line could label primary immune cells originating from lymph nodes or spleen. By co-culturing MC38 cells with murine lymphocytes and splenocytes in the presence of biotin-LPETG*G, we observed successful labeling of the respective immune cells, as shown in Figure 3.1. This result indicates

that EXCELL has the potential to label primary immune cells from lymphoid tissues, suggesting its applicability in detecting intercellular interactions between immune and tumor cells in an *in vivo* setting.

Figure 3.1 Biotinylation of Lymphocytes and Splenocytes



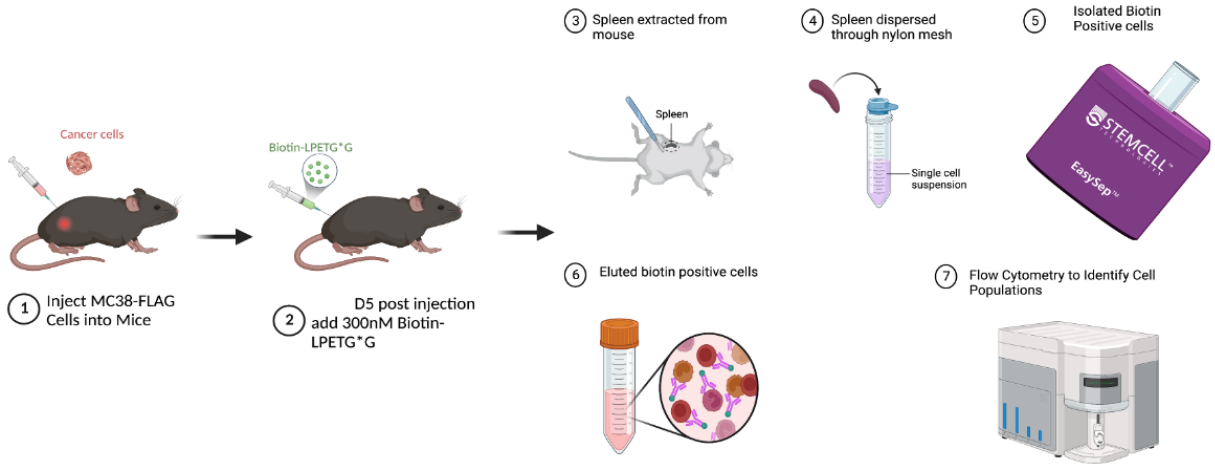
The streptavidin staining reveals the biotinylated lymphocytes and splenocytes, depicted in blue, compared to the control cells shown in red. The bar graphs illustrate the percentage of biotinylated lymphocytes and splenocytes, represented in blue, compared to the control cells indicated in red.

Testing EXCELL in a pilot in vivo study

Following the successful demonstration of EXCELL's ability to label immune cells in vitro, an *in vivo* xenograft study was conducted to evaluate its translatability. To examine cell-cell labeling *in vivo*, MC38-mgSrtA-expressing cell lines were implanted into five C57BL/6 mice, with one additional mouse receiving WT MC38 as a negative control. The study design is shown in Figure 3.2. It's important to note that the animals were sacrificed for splenocyte

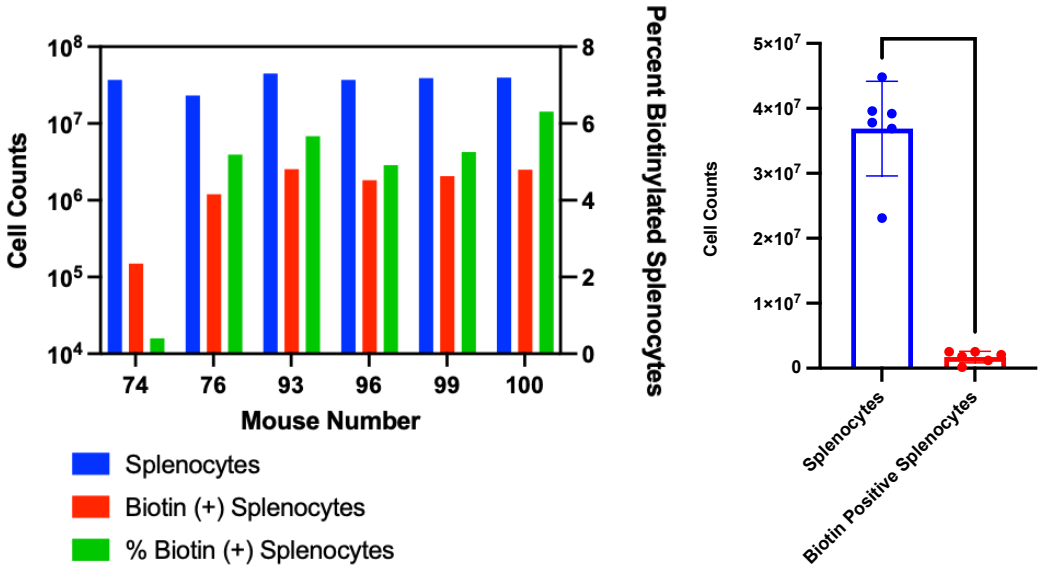
harvesting on the 5th day after inoculation, at a point when the tumor xenograft was palpable but not yet measurable. This timeframe aligned with the initiation of immunotherapy intervention studies in these models, a period characterized by heightened anti-tumor immune activity.[12,13]

Figure 3.2: EXCELL Method *in vivo*



To isolate the small population of biotin-positive cells from the splenocytes and minimize the amount of fluorophore present in the antibody panel, a biotin positive selection kit was used. After processing the splenocytes through the kit, the sample group of mice showed a median positive biotin population of 5.25%, while the control mouse only had 0.4% biotin-labeled cells (Figure 3.3). These results demonstrate a significant increase in biotin-positive immune cells compared to the control mouse, indicating EXCELL's potential for effective *in vivo* cell labeling.

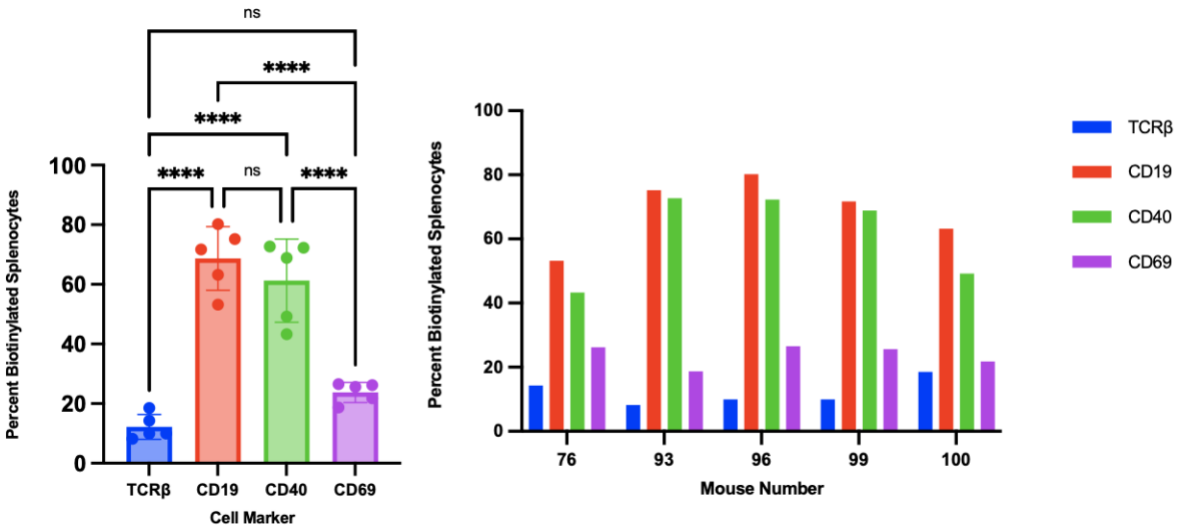
Figure 3.3: Isolated Splenocytes from pilot *in vivo* Study



The murine cell counts from the pilot in vivo study are provided. It includes the number of splenocytes isolated from MC38-WT and MC39-mgSrtA expressing mice, displayed in blue. Additionally, the biotin-labeled (+) splenocytes post StemCell Biotin isolation kit is shown in red, along with the percentage of biotin-labeled (+) cells represented in green. A student T Test was performed to compare the two groups with **** representing statistical significance of P<0.0001.

The study results indicate that tumors expressing MC38-mgSrtA are capable of effectively labeling immune cells that have interacted with tumor cells in vivo. After sorting, the biotinylated immune cells were further characterized by identifying common cell markers on T and B cells using flow cytometry. TCRβ and CD69 markers were used to identify T cells and activated T cells, respectively, while CD19 and CD40 were used to identify B cells, as these cell types play important roles in immunotherapy and are commonly found in the spleen. The results showed that the relative percentage of biotin-positive cells for TCRβ, CD69, CD40, and CD19 markers were 9.95%, 25.6%, 68.9%, and 71.7%, respectively (Figure 3.4).

Figure 3.4: Biotinylated Immune Cell Subtypes



The mean and standard deviation of the cell types (n=5) characterized from biotin-labeled (+) splenocytes from mgSrtA-expressing mice are presented as well as each cell characterization of the individual mice. One way ANOVA with multiple comparisons was performed to analyze the amount of each cell type present. Statistical significance was observed between some groups as denoted by **** representing $P<0.0001$ and ns representing $P>0.05$.

Moreover, the fraction of these lymphocytes appeared to be similar across individual animals. These findings provided suggest that the labeled immune cells that have interacted with tumor cells could migrate to the spleen, including activated T and B cells. Discovery of these migratory cell types suggests an immune response to the xenograft tumor and provides preliminary evidence that EXCELL can characterize the status of a TME with the cell types that are labeled.

Discussion

The EXCELL method has demonstrated its applicability in detecting and characterizing immune-tumor cell interactions through preliminary *in vivo* data. A notable aspect of EXCELL is its ability to label primary immune cells without the need for pre-engineering. This feature provides the potential to identify and characterize naturally occurring immune cells in the TME,

as well as those capable of trafficking back to lymphoid tissues. Furthermore, the cells that are labeled are immune cells that are trafficking to the tumor site on their own accord without any engineered signaling from the tumor cell. This could lead to valuable insights into the types of cells that interact with tumors at various stages of development and the locations these cells traffic to post-interaction. Overall, EXCELL holds promise as a powerful tool for investigating immune-tumor interactions and could yield important insights into the mechanisms underlying cancer progression and development.

Unlike other methods such as LIPSTIC[8] and G-BaToN[9], without pre-engineering the interacting immune cells, the EXCELL method offers a more representative view of the immune response at their natural state within the TMEs. In particular, the EXCELL method can facilitate the detection and sorting of immune cell subtypes that interact with tumor cells, which is beneficial for investigating immune cell heterogeneity in different TMEs. Our study demonstrated the ability of the EXCELL method to detect various immune cell types interacting with tumor cells. Identification of these novel subpopulations of major immune cell infiltrate populations such as T cells, B cells, NK cells, and DC cells could aid in the identification of new targets for immunotherapy or in the stratification of patients based on their immune profiles.

Identifying immune cells that can migrate between anatomical sites has significant implications for comprehending the immune response to cancer immunotherapy.[14,15] Recent research indicates that the T cell response to immune checkpoint blockade may originate outside the tumor and depend on recruiting peripheral T cells. By using the EXCELL approach to track immune cells that move from the tumor to the spleen and vice versa, we can improve our understanding of how the immune response to cancer immunotherapy is generated and maintained. This could have essential implications for patient selection and the development of

combination treatment strategies. For instance, if we can identify specific subpopulations of immune cells that are recruited from peripheral tissues and play a crucial role in the response to immunotherapy[16], we may be able to establish biomarkers that predict response and discover new targets for combination therapies.

As promising as the pilot *in vivo* study is there are some limitations that need to be addressed in future studies. The results of this study justify that a larger study be done that is statistically powered. This follow-on study would ideally include samples that are taken from the TME and other secondary lymphoid structures as well as a full negative mouse control population. Furthermore, in our pilot study we only isolated and tested immune infiltrate from the spleen and did not test the tumor itself or the other secondary lymphoid structure the lymph node. This will be an important step moving forward to truly understand the immune landscape within the tumor itself and how that compares or contrasts to the immune landscape in the secondary lymphoid organs. There will also need to be a larger immune cell panel developed for flow cytometry hopefully to fully capture innate and adaptive immune cell subtypes present in the TME and lymphoid organs. This panel should include DCs (CD11c), neutrophils (Ly6G), NK cells (NK1.1), and macrophages (CD68). From this full panel of both innate and adaptive immune cells, it will be interesting to see the types of immune cells that are labeled to then make a more specific panel that will have more specific markers for each cell type. The only control that was used in the pilot study was to control for any biotin labeling in a non-tumor bearing mouse. While this in an important control, in any future mouse studies there will need to be an inclusion of a full negative control to compare the composition of immune cells labeled in the spleen and lymph node of a tumor bearing mouse to the composition of immune cells in a healthy mouse. This will be important to help uncover cells that are present under normal

environments compared to cancerous environments. For example, in our pilot *in vivo* study we showed that activated biotinylated T cells were present in the spleen as denoted by the marker CD69, having a control that can show if any T cells are active in a normal mouse or that they are activated post xenograft. This will help to identify the cell types that are present during the formation of the TME compared to baseline immune cells present. Importantly, while mouse models play a role as a preclinical model, they are a limitation in studying immune cells and personalized medicines like immunotherapy that are tailored to humans. This is due largely to the differences in immune cell composition and immune cell markers between human and mice cells (Table 3.1).

Table 3.1: Comparison of Mice and Human Cell Markers[17,18]

Cell Type	Mouse	Human
Neutrophils	F4/80^{med} and Ly-6G+	CD16+ and CD66b+ or CD15/SSEA1+
Eosinophils	CD193+	CD193+ and siglec-8+ and CD16-
Monocyte MDSC	Ly-6C+ and Arginase+	CD15- and CD14+ and HLA-DR-
Polymorphonuclear MDSC	Ly6G+ and arginase+	CD15+ and CD14- and HLA-DR-
Conventional DC	CD11c+ and MHCII+	CD11c+ and HLA-DR+
Activated Conventional DC	CD83+	CD83+
cDC1	XCR1+ or CLEC9A+	XCR1 or CLEC9A+
Migratory DCs	CD103+	-
cDC2	CD11b+ or SIRPα+	CD1c+ or SIRPα+
Macrophage	F4/80high	CD68+ and HLA-DR+ and CD11c-
M1-like	CD86+ or CD80+	CD86+ and CD80+
M2-like	CD163+ or CD206+	CD163+ or CD206+
T cell	CD3+	CD3+
Cytotoxic T cell	CD8+	CD8+
Helper T cell	CD4+	CD4+
Th1	T-Bet+ and IFNγ+	T-Bet+ and IFNγ+
Th2	GATA-3+ and IL-4+	GATA-3+ and IL-4+
Th17	RORγt+ and IL-17+	RORγt+ and IL-17+

Th9	PU.1+ and IL-9+	PU.1+ and IL-9+
Tfh	Bcl-6+ and CXCR5+ and IL-21+	Bcl-6+ and CXCR5+ and IL-21+
Treg	FoxP3+ and CD25+	FoxP3+ and CD25+
Activated T cell	CD69+ or CD25+	CD69+ and CD25+
Naïve T cell	CD44- and CD62L+	CD45RA+ and CD62L+ or CCR7-
Effector Memory T cell	IL7Rα+ and CD44+ and CD62L2-	CD45R0+ and CD62L- or CCR7-
Central Memory T cell	IL7Rα+ and CD44+ and CD62L+	CD45R0+ and CD62L+ or CCR7+
Effector T Cell	CD44+ and CD62L-	CD45RA+ and CD62L- or CCR7-
Progenitor Exhausted T Cell	TCF1/TCF7+ and PD-1^{high} and TIM-3^{high}	TCF1/TCF7+ and PD-1^{high} and TIM-3^{high}
Terminally Exhausted T Cell	Tox/Tox2+ and PD-1^{high} and TIGIT+	Tox/Tox2+ and PD-1^{high} and TIGIT+
NK Cells	NK1.1+ or NKp46+ or NKG2D+ and CD3-	CD56+ and CD3-
Activated NK Cells	CD69+	CD69+
Cytotoxic NK Cells	Granzyme+ or Perforin+	Granzyme+ or Perforin+
B Cells	CD19+ or CD40+	CD19+
Naïve	IgD+ and CD27-	IgD+ and CD27-
Switched Memory B Cells	IgD- and CD27+	IgD- and CD27+
Unswitched B Cells	IgD+ and CD27+	IgD+ and CD27+

Mice models may not be the best preclinical model to study these types of personalized therapies due to the differences between human and mice immune systems specifically immune system development, response to immune challenges, and immune cell activation. While mice and humans have similar immune systems there are some significant variations between the two. These include the lymphocyte to neutrophil ratio, FcR expression, Ig isotope differences, immune cell differentiation and function, stimulatory receptors, and chemokine and cytokine composition.[19,20] Potential reasons for the differences between mice and humans have to do with

the fact that laboratory mice are bred for experimental purposes, kept in a sterile aseptic environment, and they are never challenged with any type of pathogen.

While these techniques are important to preventing contamination and maintain a constant testing species for experiments, it means that their immune systems are not as evolved as humans, and therefore are effectively a naïve immune system. Laboratory mice do not have as many different populations of memory T cells, such as effector differentiated or mucosal distributed.[19,20] This implies that the immune system of mice is more representative of a neonate that an adult human. This thought leads to an important question: are the use of mouse *in vivo* models a good representation to study immunotherapies and other personalized medicines. Current data show the limitations of current models as data are not correlative in transitioning from mice to humans.[19,20] To address some of these differences, a study performed by Beura et al. compared the immune systems of feral mice, pet store mice, lab mice to humans to assess which had the most similar immune system to humans.[21] Researchers found that laboratory mice after co-incubation with pet store mice and wild mice had immune system composition changes that resulted in laboratory mice having immune systems more aligned with human adults than neonates.[21] Specifically, co-incubating the laboratory mice with pet store or feral mice increased the numbers of circulated and tissue resident CD8+ T cells, differentiated CD4+ T cell subtypes, and others immune cell differentiation.[21] Data generated from this study showcases that co-housing laboratory mice with non-laboratory mice can provide the laboratory mice with a physiological exposure to natural mouse pathogens[21]. In turn, this matures the laboratory mice's immune system to a more reflective immune system to compare with human adults. While it is important to note that exposing laboratory mice to these pathogens results in a non-sterile vivarium, utilizing the appropriate vivarium controls to create a "dirty" colony of mice could

potentially progress and aid in the development of immunotherapies and other personalized medicine.

In summary, the EXCELL method shows considerable promise in detecting immune cell interactions with cancer cells, both *in vitro* and *in vivo*. This technique holds the promise of providing insights into the behaviors of immune cells within the TME, thereby potentially facilitating advancements in cancer immunotherapy strategies. By utilizing this approach, we may be able to address pertinent questions such as the specific subtypes of immune cells engaged in interactions with tumor cells and their underlying molecular characteristics during immunotherapy. By distinguishing the gene expression features between "interacting" and "non-interacting" cells, we could significantly enhance our comprehension of immune cell subpopulations that are functionally capable of reaching tumors and establishing contact. Furthermore, this method might help us understand whether effector cells that have engaged with tumor cells can migrate across metastatic lesions, from primary tumors to tumor-draining lymph nodes or to lymphoid tissues. Nevertheless, additional research and validation studies are necessary to fully establish the usefulness and generalizability of the EXCELL method in other contexts.

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CHAPTER 4: CONCLUSION[1]

Summary

Cell-cell interactions are critical in cell development, organ system function, tissue homeostasis, and interaction of the immune system with foreign pathogens[1]. Immune cell communication is vital and is composed of a variety of cell types that communicate with one another to ensure homeostasis. Immune cells are a key player in immunosurveillance constantly protecting the body from cancer development and metastasis. However, when this process fails there in an increased risk for cancer progression leading to the development of the TME[2]. Cellular communication is of the upmost importance in the TME as communication shapes and sets the TME landscape. Understanding the presence and characteristics of TILs in the TME has the potential to provide important insights into tumor progression as well as patient response to therapy. This chapter will summarize the key research findings and discuss the potential contribution and value of these discovery to the ongoing work for improving immunotherapy for cancer. This chapter will also discuss the impact the EXCELL method can have in future research.

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Introduction

Immunotherapy revolutionized cancer with ICI treatment showing efficacy in numerous cancers.[3] However, only a small percentage of patients respond to ICI with high inter-patient and intra-cancer variability. Therefore, understanding the complex interactions between immune cells and cancer cells in the TME is critical in moving cancer immunotherapy forward. It is also important to understand the TME composition to better understand the issues with immunotherapy and to help improve patient response. As reviewed and discussed in this dissertation, there are numerous immune cell types that are present in TMEs that contribute to both pro- and anti- tumor mechanism cues from the microenvironment. Furthermore, most of the cells within the TME exhibit plasticity, and the subtype and state of the immune cells present influence the "hot" or "cold" status of the TME. The current methods to uncover interactions in the TME include proximity dependent labeling, microscopy, and bioinformatic approaches. An issue with the current methods is their inability to determine unknown immune cell subtypes, and need a preconceived idea of immune cells that would be present in a TME. This provided an unmet need to develop a method that can detect immune cells interacting with cancer cells on their own accord without any pre-engineering of the immune cells both in vitro and in vivo.

The questions that were sought to answer in this dissertation were twofold: 1) can primary cells be labeled and 2) can those labeled cells be tracked. We developed a method that significantly improves the detection of immune cells showcased through flow cytometry, confocal microscopy, and a pilot in vivo study. This method provides a potential tool for immunologists and cancer researchers to determine different immune cell types that are present in tumor microenvironments.

Overall Findings

The EXCELL method was developed to label immune cell both in vitro and in vivo. Notably, a pilot in vivo study using EXCELL was able to label interacting immune cells without pre-engineering, synthetic infiltrations, or artificial gradient to the tumor present. To our knowledge this is the first time the EXCELL method has been used in vivo to label immune cells after interacting with cancer cells. Utilizing EXCELL with the described biotin isolation kit shows potential to help uncover some of these immune cell subsets including T and B cells. Understanding the tumor infiltrating immune cells and their implications in the TME can be extremely beneficial for comprehending the immune response to immunotherapy.

Furthermore, EXCELLs ability to covalently label the immune cells that are then trafficked back to the spleen shows the potential for tracking cells from primary tumor site to a distal organ, and therefore could be a tool to study metastatic spread. From this we can improve our understanding of how the immune cells respond to immunotherapy, and which immune cell types of traffic from the primary tumor to secondary legions during metastasis. Furthermore, this method has the potential to uncover cell type present at different stages of the TME development which, could help provide context to when “hot” tumors turn “cold” or vice versa. This is beneficial for investigating immune cell heterogeneity in different tumor microenvironments. If we can identify specific subpopulations of immune cells recruited from peripheral tissues and play a critical role in the response to immunotherapy biomarkers can potentially be discovered to either help predict patient response and/or discover new targets for combination therapies. From these findings, EXCELL shows promise as a powerful tool to investigate immune-tumor cell interactions to inform new cell biomarkers and immunotherapy developments. Overall, EXCELL

has implications for studying immune tumor cell dynamics and potentially uncovering novel subtypes of immune cells within the TME.

Future Research and Directions

The method development that occurred during this dissertation showcased the ability of EXCELL to label immune cells in an in vivo setting. This provides a launch pad for very exciting experiments and studies that can be done utilizing this method. First, limitations of this study include the small sample size that was used for the pilot in vivo study, and we only assessed the biotinylated immune cells that were localized to the spleen. In future trials, it will be important to extract the tumor, the tumor draining lymph node, and take blood for circulating immune cells. This set of samples from the mice will provide a comprehensive look at the immune cells present at different anatomical locations. Other components of this larger mouse study include assessing the immune cell composition in the TME and other secondary lymphoid organs of mice that are responders and non-responders post immunotherapy treatment.

Furthermore, it would be informative to compare the immune cells that are labeled in two different TMEs. For example, comparing the labeled immune cells of MC38-mgSrtA tumor bearing mice with B16F10-mgSrtA expressing mice. In human populations both colorectal and melanoma patients have high intra-patient variability in response to immunotherapy. Comparing the labeled immune infiltrate between two different xenografted tumors in the same type of mice has the potential to provide some context into how different immune cells play a role in different cancer types.

Another set of experiments could use the EXCELL model to study the immune infiltrate of “cold” tumors versus “hot” tumors. Murine cell lines such as Pan02 a pancreatic cancer cell line and 4T1 a triple negative breast cancer cell line is referred to as cold or cool tumors.[4] MC38

is regarded as “warm” tumor model and can be used to compare against either the pancreatic or breast cancer mouse model.[5] Furthermore, MC38 can be mutated to have loss of function mechanisms to make the xenograft more resistant to therapy replicating a cooler TME.[5] This set of experiments will be important to study the different immune infiltrates in cold and hot tumors murine models. Furthermore, it has the potential to see if it is possible to turn a “cool” or “cold” tumor “warm” or “hot” and which immune cells are labeled with biotin in each of these TMEs. Due to EXCELLs ability to potentially label subsets of cells, this set of experiments has the potential to uncover TME immune landscapes in hot versus cold tumors.

EXCELL also as the potential to be utilized during the drug screening phase. During my Ph.D. training, I collaborated with chemists in the structural genomics consortium to screen novel compounds across 10 cancer cell lines to determine what drug candidates should move forward.[6–9] Taking experience from this process, I think EXCELL has the potential to assist in testing the efficacy of immunotherapies through an immunotherapy drug screen. To this end, various immunotherapies can be administered to in vivo, or ex vivo models of EXCELL. Hypothetically, “cold” tumor models and “hot” tumor models will be treated with various immunotherapies, and EXCELL could potentially be used to see which subsets of immune cells are present in the TME during treatment. This could have implications for determining if immunotherapies can cause an influx or re-engaging of cytotoxic cells turning a cold tumor hot. This set of experiments also has the potential to study smaller subsets of immune cells like myeloid derived suppressor cells, dendritic cells, and helper T cell subsets and how these cells respond to therapies. Furthermore, it could help determine novel immunotherapy candidates to move forward with.

The EXCELL method can also be used to inform bioinformatic platforms and mathematical models such as quantitative systems pharmacology (QSP) and agent-based models (ABM). QSP models can integrate interacting components of large system in a mechanistic manner through ordinary differential equations. ABMs can be used to account for the spatio-temporal aspects of the environment that is being modeled.[10] These two model systems together and apart can account for the changes and dynamics between cells within the TME. The EXCELL method can help to provide parameters including number of immune cells, the types of immune cells, and others to build out a mathematical model representing the TME. From this model, simulations can be run showing how the TME will respond to different immunotherapies or how the different immune cells dictate the TME.

EXCELL also has the potential to be utilized in more physiologically relevant preclinical models that have similar disease and patient specific targets that are predictive to humans. Non-human primates (NHP) share more similarities with humans than mice models such as immune cell populations, immune regulatory mechanisms, and physiology.[11] NHP have also been used as preclinical models for cancer as well[12]. NHP are similar in incidence and pathology to humans and naturally develop cancers with colorectal and breast cancers as the highest incidences in a similar fashion to humans.[11] In comparison to classification of immune cells between mice and humans, the immune cell populations between humans and NHP have similar incidences of CD4+ and CD8+ T cells, B cells, DC cells, NK cells, and macrophages.[11] Interestingly, healthy NHPs have been utilized as a preclinical model to determine the pharmacokinetic and pharmacodynamic parameters and safety in chemotherapies and immunotherapies.[11] For example, cancer free NHP have been used in the testing of bispecific antibodies, and some PD-1 and PD-L1 inhibitors. Cancer free NHPs are utilized to test the toxicity of the drug trastuzumab

since HER2, the target of trastuzumab, is highly expressed in NHP and not present in mice.[13] This means that NHP are the only preclinical model that can be used to test this drug. As mentioned above, NHP can naturally develop cancer making them an interesting preclinical model to be used to study immunotherapies in. The Primate Center Initiative at Wake Forest University is currently working to recruit a cohort of NHPs that have naturally developed cancer to be used for immunotherapy studies.[11] There have been some studies done in tumor bearing NHPs specifically looking at fibroblast activation protein α (FAP) targeted bispecific antibodies.[11,14] FAP is highly expressed on the surface of CAFs in 90% of human epithelial cancers and is a target of importance for cancer treatment because of its expression in the TME, and is vastly different between mice and humans.[11]To study FAP targeted therapy in mice, murine-specific bispecific FAP antibodies are needed in comparison to NHPs where the same FAP-bispecific that is used in humans be tested in NHPs.[11] Using EXCELL in a preclinical NHP would be a better translational preclinical model to humans for a multitude of reasons. One major reason is that in preclinical mice most of the immunotherapies that are given to them are murine specific and not the same immunotherapies that will be administered to humans. In NHP the human immunotherapies can be used. To this end, immunotherapies would be administered to NHP with EXCELL expressing tumors. The immune cells labeled from this set of experiments could be better informative of the cell types that may be present in a human TME. These examples showcase that NHPs are a better translational preclinical model due to the similar cancer incidence, drugs being tested, and similar immune composition.

However, as mentioned above NHPs are a very protected population and are rarely used in preclinical research. Furthermore, the sample size of a NHP cohort compared to a mouse cohort would be vastly different with a much smaller samples size as well as the cost to maintain

a NHP cohort. These limitations aside, as personalized medicine continues to grow and be used to treat cancer and other immunomodulating diseases, using a preclinical model that is more physiologically relevant to humans can help in the discovery of biomarkers, testing mechanisms of immunotherapies, and better predict patient outcomes.

Closing Summary

This dissertation uses an optimized method of EXCELL, to investigate immune cell-cancer cell interactions in the TME. We explored the EXCELL method through the time and concentration dependent labeling of immune cells, confocal microscopy imaging of labeled immune cells, labeling of primary cells, and a pilot in vivo study. These findings improve the methodology of studying tumor infiltrating immune cells that interact with tumor cells without any form of gradient. EXCELL method has the potential to help explore the immune landscape in the TME, find new biomarkers, and assist in the advancement of immunotherapy.

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