Utilizing a Proximity Dependent Labeling Strategy to Study Cancer-Immune Intercellular Interactions In Vitro and In Vivo

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Abbreviations

EXCELL: Enzyme-mediated intercellular proximity labeling;
DC: Dendritic cell;
ICI: Immune checkpoint inhibitor;
SrtA: Sortase A;
TIL: Tumor infiltrating lymphocyte
TME: Tumor microenvironment

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Abstract

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, which is able to label 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, both in vitro and in vivo, without requiring pre-engineering of the immune cells. We also highlight its ability to detect various types of immune cell subpopulations in vivo that have migrated out of tumor into the spleen, providing insights into the role of peripheral T cell recruitment in tumor progression. 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.
Significance Statement

The EXCELL method holds promise for detecting immune cell interactions with cancer cells, both *in vitro* and *in vivo*. It has important implications for studying immune tumor cell dynamics and potentially uncover novel subtypes of immune cells within the TME, both prior to and during immunotherapeutic interventions.
Introduction

Cell-cell interactions play a critical role in organ development, homeostasis, and immune surveillance (Armingol et al., 2021). The immune system 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 (Armingol et al., 2021). Notably, immune cells play a key role in surveilling cancer, and when this process fails, it can increase the risk of cancer initiation and progression (Schreiber et al., 2011; O'Donnell et al., 2019). Therefore, the intercellular interaction between immune cells and cancer cells are crucial in determining cancer status, progression, treatment response, and the constitution of tumor microenvironment (TME).

The TME is a complex ecosystem composed of immune cells, blood cells, fibroblasts, blood vessels, and lymphocytes (Anderson and Simon, 2020). Tumors are classified based on their TME landscape, ranging from “hot” tumors that are rich in tumor infiltrating lymphocytes (TIL) to “cold” tumors devoid of immune infiltrate (Lanitis et al., 2017). 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 (Zhang and Zhang, 2020). It remains challenging to investigate which types of cells are in close proximity to tumor cells and are in direct contact with tumor cells in dynamic TMEs before and during therapies.

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 (O’Donnell et al., 2019). 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 (Darvin et al., 2018).
ICIs have the ability to block inhibitory interactions and reactivate effector immune cells (Darvin et al., 2018; He and Xu, 2020). ICIs have shown efficacy in many cancers including melanoma, non-small cell lung cancer, and many others (Huang and Zappasodi, 2022; Yang et al., 2022). However, only a small percentage of patients respond to ICIs and there is high inter-patient and intra-cancer variability in response to ICI treatment (Haslam et al., 2020; Valero et al., 2021). 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.

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. 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 (Maffuid, et al., 2023). There are several methods available for studying cell-cell interactions, including proximity-dependent techniques such as synNotch (Morsut et al., 2016), FucoID (Liu et al., 2020), LIPSTIC (Pasqual et al., 2018) and G-BaToN (Tang et al., 2020), microscopy and imaging techniques (Rust et al., 2006), and bioinformatic techniques for molecular profiling (Newman et al., 2019) such as ligand-receptor pair analysis (Pasqual et al., 2018; Tang et al., 2020). The choice of method will depend on the specific research question, the type of cells being studied, and the available resources.

Bioinformatics techniques primarily serve to deduce intercellular communications within tissue environments, whereas the other approaches focus on interactions based on proximity, specifically cell-cell contact. 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. In this study, we utilized the enzyme-mediated intercellular proximity labeling (EXCELL) technique to study...
cancer cell-immune cell interactions both in vitro and in vivo (Ge et al., 2019). 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 (Theile et al., 2013). SrtA recognizes substrate proteins with a specific motif called LPTXG (Chen et al., 2016). 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 (Ge et al., 2019). This increases the labeling potential on the interacting cell (Chen et al., 2016). The objective of this study is to test the application of EXCELL technique in tumor systems to gain a better understanding of and characterize the TILs of the TMEs in vivo.
Materials and Methods

Reagents and Antibodies

Antibodies included FITC anti-human CD20 (Cat #980202) and PE anti-DYKDDDDK Tag Antibody (Cat# 637309) from BioLegend, and Streptavidin Alexa Fluor 674 Conjugate (Cat # S21374) from ThermoFisher. Lipofectamine 3000 Transfection Reagent (Cat # L3000015) and enzyme free cell dissociation buffer (Cat # 13151014), Puromycin (Cat # A1113803), and Custom TaqMan Probe for mgSrtA (Cat # 4331348) were obtained from ThermoFisher.

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 Infection-Transfection

Plasmid pcDNA3.1-CD40L-mgSrtA was packaged into Lentivirus via GeneTech 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 amplified in PCR 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 titrated to measure
below 10^8 IFU/ml as determined by p24 ELISA. Of note, the plasmid comes with a N-terminal 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 centrifuged at 1,000 x g for 20 min at room temperature and placed into incubator. The morning after transfection, media in the 12-well plate was changed. 48 hrs post transfection. Antibiotic selection using Puromycin was added at 1ug/mL to kill the cells that do not contain the desired construct. 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 re-suspended 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. Research reported in this publication was supported in part by the North Carolina Biotech Center Institutional Support Grant 2012-IDG-1006.

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. 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 were 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.

**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.
Results

**EXCELL Approach and Generation of Stable Cell Lines**

The enzyme mgSrtA is present on the surface of cancer cells. As shown in Figure 1, when an immune cell comes in contact with 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.

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. Since there was no commercially available antibody for mgSrtA detection by flow cytometry, a custom TaqMan probe was created to measure mgSrtA gene expression via RT-qPCR. RT-qPCR data shows that mgSrtA gene expression was highly present in both cell lines, HEK293T and MC38, compared to their respective control (Figure 2b). Both HEK293T and MC38 cells expressed a significantly higher expression of mgSrtA compared to their respective controls.

**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. Raj 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 Raj 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 (Ge et al., 2019). 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.

**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 Raj B cells after directly contact with HEK293T and MC38 cells at various cell concentrations. Raj B cells were co-cultured with HEK293T and MC38 cells at different ratios, ranging from 100,000 cells to 1 million Raj B cells, while keeping a constant number of 1 million HEK293T or MC38 cells. The results showed that the number of Raj B cells that were biotinylated increased with the increasing concentration of Raj B cells in the cell-cell interaction (Figure 4a) (n=3/group), indicating a concentration-dependent labeling of Raj B cells upon direct ligand-receptor interaction.
Next, time-dependent proximity labeling between Raj 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 Raj 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 (Ge et al., 2019). This time-dependent increase in Raj 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.

**Proximity dependent intercellular 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 5a. 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.

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 5b. 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 (Lv et al., 2021, Liu et al., 2019).

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% (Figure 5c), while the control mouse only had 0.4% biotin-labeled cells. 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.

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 5d). Moreover, the fraction of these lymphocytes appeared to be similar across individual animals. These findings suggest that the labeled immune cells that have interacted with tumor cells could migrate in 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

Utilizing the same system as the original work by Ge et al., our study successfully demonstrated that the EXCELL method possesses crucial quantitative attributes, including concentration- and time-dependency, when it comes to detecting intercellular interactions. Significantly, our research highlighted its potential in detecting interactions between tumor and immune cells, particularly those involving with primary immune cells. In this particular animal model, as the tumor reaches a considerable size, it is anticipated that the lymphocytes that have infiltrated into these larger tumors lose their functionality and are confined in terms of their spatial distribution. As a result, the significance of characterizing these active cells, which can engage closely with tumor cells, diminishes (Lv et al., 2021). Consequently, we isolated splenocytes to analyze intercellular interactions. Our investigation shed light on the potential of EXCELL to track cell migration across anatomical sites (e.g., primary vs. metastatic lesions vs. lymphoid tissues), a vital aspect regarding where and how tumor-specific effector cells reinvigorated during immunotherapy. The identification of labeled immune cell subtypes through EXCELL opens the door to subsequent applications, like molecular characterization, to unveil the types and characteristics of immune cells that have engaged with tumor cells.

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 (Pasqual et al., 2018) and G-BaToN (Tang et al., 2020), 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 (Spitzer et al., 2017; Yost et al., 2021). 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 (Wei et al., 2018), we may be able to establish biomarkers that predict response and discover new targets for combination therapies.
Although the EXCELL method has great potential, there are several limitations that need to be considered in the context of oncology research. One limitation is the potential for cell subpopulation bias, as the mgSrtA enzyme can label various cell surface proteins that contain a monoglycine residue at the N-terminus. This promiscuous nature of the mgSrtA enzyme’s ability to bind to monoglycine residues did result in some self-labeling of the cancer cell. The possibility of self-labeling among tumor cells could potentially impede the accurate detection of labeled TILs within TME, even though we didn't evaluate TILs within the TME in our study. An additional sorting step utilizing immune cell markers (such as CD3 for T cells or CD19 for B cells) becomes necessary to effectively filter out self-labeled tumor cells. It's important to note that our evaluation of immune cells was focused on the spleen, where the presence of tumor cells is anticipated to be minimal due to the unlikelihood of tumor cell migration, or metastasis into the spleen, within the observation window.

Furthermore, there exists a limitation pertaining to the selectivity of the label process, ensuring the labeling of only close and durable cell-cell interactions. While the time- and cell density-dependency of labeling has been demonstrated, it is still unclear which types of cell-cell interactions are preferentially labeled. This ambiguity could impact the accuracy of the EXCELL method in detecting specific cell-cell interactions crucial for tumor immunity. To overcome these limitations, future studies could focus on optimizing the labeling specificity and sensitivity of the EXCELL method by identifying and targeting specific cell surface proteins that are preferentially expressed on immune cells that interact with cancer cells. This could also involve comparing the labeling efficiency and specificity of the EXCELL method with other proximity-dependent techniques, such as LIPSTIC and G-BaToN, which may provide complementary insights into cell-cell interactions in tumors.
In summary, the EXCELL method shows considerable promise in detecting immune cell interactions with cancer cells, both \textit{in vitro} and \textit{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 are capable of migrating 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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Authorship Contributions

Participated in research design: Maffuid, K and Cao, Y

Conducted experiments: Maffuid, K and Cao, Y

Contributed new reagents or analytic tools: Maffuid, K and Cao, Y

Performed data analysis: Maffuid, K

Wrote or contributed to the writing of the manuscript: Maffuid, K and Cao, Y
References


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**Data Availability Statement**

The authors declare that all the data supporting the finding of this study are contained within the paper.
Figure Legends

Figure 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.

Figure 2: Generation of Stable HEK293T- and MC38-mgSrtA Expressing Cell Lines
a. 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). **** p < 0.001

Figure 3: Imaging Immune-Tumor Intercellular Interactions. Confocal microscopy images were captured to observe the cell-cell interactions between Raj 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.

Figure 4: Cell Density- (a) and Time- (b) Dependent Labeling of Raj B Cells
a. The graph presents the number of Raj B cells that were biotinylated after interacting with HEK293T or MC38 mgSrtA expressing cells, with different amounts indicated in the legend
(n=3/group). ** p < 0.01. b. The graph illustrates the percentage of biotinylated Raj B cells labeled over time, ranging from zero to two hours, following interaction with either HEK293T or MC38 cells. # indicated that there was no biological replication rather technical replications.

**Figure 5: Detecting Proximity Dependent Immune – Tumor Cell Interaction in Vivo.**

a. 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. b. The workflow of the pilot animal study is presented. c. 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 are shown in red, along with the percentage of biotin-labeled (+) cells among the biotin-labeled (+) splenocytes, represented in green. d. The mean and standard deviation of the cell types characterized from biotin-labeled (+) splenocytes from mgSrtA-expressing mice are presented. The cell types are stratified by individual mgSrtA-expressing mouse. * p < 0.05.
Fig 2.
Fig. 4

**HEK293T: RB Interaction**

- Control
- 100K Cells
- 250K Cells
- 500K Cells
- 1M Cells

**MC38: RB Interaction**

- Control
- 100K Cells
- 250K Cells
- 500K Cells
- 1M Cells

**b.**

- Percent of Biotinylated Raji B Cells
- 0
- 15min
- 30min
- 1hr
- 2hr

**Percent of Biotinylated Raji B Cells**

- 0
- 15min
- 30min
- 1hr
- 2hr