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Visualization and Characterization of the Immunological Synapse Between Chlorotoxin Chimeric Antigen (CLTX-CAR) Redirected T Cells and Targeted Glioblastoma Tumors

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Visualization and Characterization of the Immunological Synapse Between Chlorotoxin Chimeric Antigen (CLTX-CAR) Redirected T Cells and Targeted Glioblastoma Tumors

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Abstract

Chimeric Antigen Receptor T (CAR-T) cells have demonstrated anti-tumor activity against aggressive and invasive cancers such as glioblastoma (GBM); however, clinical response rates remain low in clinical trial studies. Tumor heterogeneity and tumor microenvironment conditions pose significant challenges for treatment of GBM, thus continuous optimization of CAR-T cell therapies and identification of novel, widely expressed, and highly specific GBM antigens are vital to better patient outcomes. A newly developed CAR-T cell construct incorporating chlorotoxin (CLTX) as the targeting domain exhibited broad GBM-targeting capabilities and elicited potent cytotoxic effects during preclinical studies and is currently being tested in a phase I clinical trial (NCT04214392) for recurrent GBM. Since much is still unknown about the binding mechanism and cytotoxic action of CLTX-CAR-T cells, characterization of the immunological synapse is important to be able to quantify and visualize structural, functional and signaling cascading components of CAR-T cell cytotoxicity. The purpose of this research project was to investigate the morphology of the immunological synapse between the novel CLTX-CAR-T cells and GBM tumor cells using key structural components such as receptor aggregation, actin accumulation, cytotoxic granule convergence and mitochondrial organizing center polarization to indicate effective cytotoxic activity and potentially predict clinical performance. Through confocal microscopy, visualization of the CLTX-CAR-T cell immunological synapse provided evidence of significant cytotoxic activity and matrix metaoproteinase-2 (MMP2) receptor localization at CLTX-CAR-T cells contact sites during tumor cell killing. These findings further our understanding of the CLTX-CAR-T cell binding complex and enhance the potential for optimizing the clinical efficacy of CLTX-CAR-T cells, thereby contributing to better treatment outcomes for patients with glioblastoma.

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Introduction

Glioblastoma

Glioblastoma Multiforme (GBM) is a fast growing and extremely aggressive malignant brain tumor, which remains incurable with current available treatment options (Alexander & Cloughesy, 2017). GBM is a type of glioma, a solid brain tumor that develops from dysregulated cell growth in glial cells. More specifically, GBM is categorized as a grade IV astrocytoma due to its aggressive nature and origin in astrocytes, a specialized glial cell that is involved with maintaining neuronal homeostasis and metabolism (Thakkar, 2020). The clinical presentation of GBM involves neurological changes in mood, executive dysfunction, memory disorders, and alteration of language which result from the high prevalence of GBM tumors in the frontal or temporal lobes as well as the corpus callosum (Alexander & Cloughesy, 2017).

Despite over half a century of research and numerous therapeutic attempts, GBM has been successful in evading both conventional and novel therapeutic interventions, with an extremely grim median overall survival rate of 15 months and a 5-year survival rate of 5% (Alexander & Cloughesy, 2017). The standard treatment approach for GBM includes surgical resection followed by radiation and chemotherapy (Gesundheit et al., 2020). These standard treatment strategies pose significant challenges against GBM due to the brain's limited capacity for repair, the migration of malignant cells, the disruption of local blood supply around the tumor, the accumulation of fluid surrounding tumor masses, tumor induced seizures, and toxicity from treatments. Additionally, the diffuse nature of GBM prevents complete resection of the tumor, which can result in poor clinical outcomes (Thakkar, 2020). Glioma cells migrate through the white matter tracts and along blood vessels of the brain, which leads to recurrence of the

tumor at distant sites even after successful surgical intervention. The limited success of classic therapeutic techniques has resulted in the development of more innovative strategies including targeted immunotherapies. These contemporary solutions provide more individualized strategies for targeting GBM tumors, a key component for attacking the more invasive and diffuse cell phenotypes (Holland, 2000).

Prominent advances in immuno-oncology have significantly contributed to our clinical understanding of GBM and have uncovered potential treatment strategies such as Chimeric Antigen Receptor (CAR) T cell therapies (Wang et al., 2020). A recent case study of a GBM patient showed complete regression of intracranial tumors while under a CAR T cell clinical trial at City of Hope. This result established that CAR T cell therapies can mediate anti-tumor activity against solid tumors, such as GBM, and demonstrated significant promise for improving patient outcomes. (Brown et al., 2016). Aside from a few individual successes , GBM-targeted immunotherapies display relatively low clinical response rates (Davenport et al., 2019). This is likely attributed to cell heterogeneity, complex tumor microenvironment interactions, and limited patient immune system invasion. Additionally, GBM tumors contain self-renewing and tumorigenic cancer stem-like cells which contribute to tumor resurgence following treatment (Lathia et al., 2015). Therefore, further optimization of immunotherapies including CAR T cells will provide opportunities to improve patient outcomes and hopefully find cures for patients with GBM (Brown et al., 2020).

CAR T Cell Immunotherapy

Immunotherapy is a cancer therapy that involves the harnessing of immune cells to selectively target and kill tumor cells (Liu et al., 2020). There are many types of cancer immunotherapies including targeted antibodies, cancer vaccines, tumor-infecting viruses, checkpoint inhibitors and adoptive T cell transfer, which all involve the use of an individual's immune system to fight cancer. The development and clinical use of immunotherapies has been rapidly increasing due to its high cancer fighting potential through tumor specificity and adaptation against recurrent tumors (Cohen, 2020).

Harnessing an individual's own immune system is a powerful tool, since a common mechanism of cancer growth and expansion involves immune evasion Strohl and Naso, 2019). The purpose of the immune system is to distinguish 'non-self' pathogens from the body; however, cancer is formed from our own cells through dysregulated cell growth, which presents a challenge for the immune system to target and kill cancer cells. Aside from these innate challenges, cancer cells adopt mechanisms of immunosuppression that further exacerbates immune system evasion (Fesnak et al., 2016).

By downregulating the expression of the major histocompatibility complex class I (MHC-I) molecules, cancer cells are able to evade the adaptive immune system. Normally, T cells use their T cell receptors (TCRs) to bind to MHC-I receptors and recognize bound cells as dysfunctional or 'non-self'. Once this recognition occurs, the bound T cell forms a structured immunological synapse and releases cytotoxic proteins to kill the targeted cell. Without the MHC-I molecules presented on cancer cells, T cells are unable to recognize and kill cancer cells, thus providing a mechanism for evasion of a patient's immune system (Strohl and Naso, 2019). Additionally, the tumor microenvironment further induces immunosuppression through physical

barriers, metabolic checkpoints, and a hostile immunological environment. The creation of a dense extracellular matrix and dysfunctional epithelial cells prevents T cells from binding to surface receptors at the tumor core. Hypoxic conditions also suppress immune cell activity as well as promote cancer cell proliferation. Lastly, the release of immunosuppressive cytokines prevents immune system activation (Watanbe, 2018). Understanding the mechanisms of immunosuppression in cancer are important to developing effective immunotherapies that can selectively target cancer cells despite barriers to anti-tumor immune responses.

The development of Chimeric Antigen Receptor (CAR) T cell immunotherapies is a growing area of cancer research due to its generation of targeted anti-tumor immunity. CAR T cell therapy redirects the cytotoxic ability of T cells towards tumor cells, independent of MHC recognition, thus circumventing one mechanism of immune evasion by cancer cells. This anti-tumor immunity is possible by engineering T cells to express synthetic immunoreceptors called CARs (Wang et al., 2020). CARs are composed of three functional components: the antigen-binding domain, intracellular signaling domain, and the extracellular linker, which work together to recapitulate endogenous T cell function while selectivity targeting tumor cells (Figure 1). Typically, the antigen binding domain is composed of a single variable chain fragment derived from a monoclonal antibody, and is capable of MHC-I-independent binding to surface antigens on the target cancer cell. The intracellular signaling domain consists of a co-stimulatory component and CD3ζ activation domain, both important for CAR T cell persistence and cytotoxic activation. The extracellular linker connects the antigen-binding domain to the intracellular domain and controls the immunological synapse formation and CAR stability (Priceman et al., 2015). The generation of antitumor immunity by CAR T cells provides a

mechanism by which to address aggressive GBM characteristics such as heterogenous antigen expression and immune system evasion (Fesnak, June, & Levine, 2016).

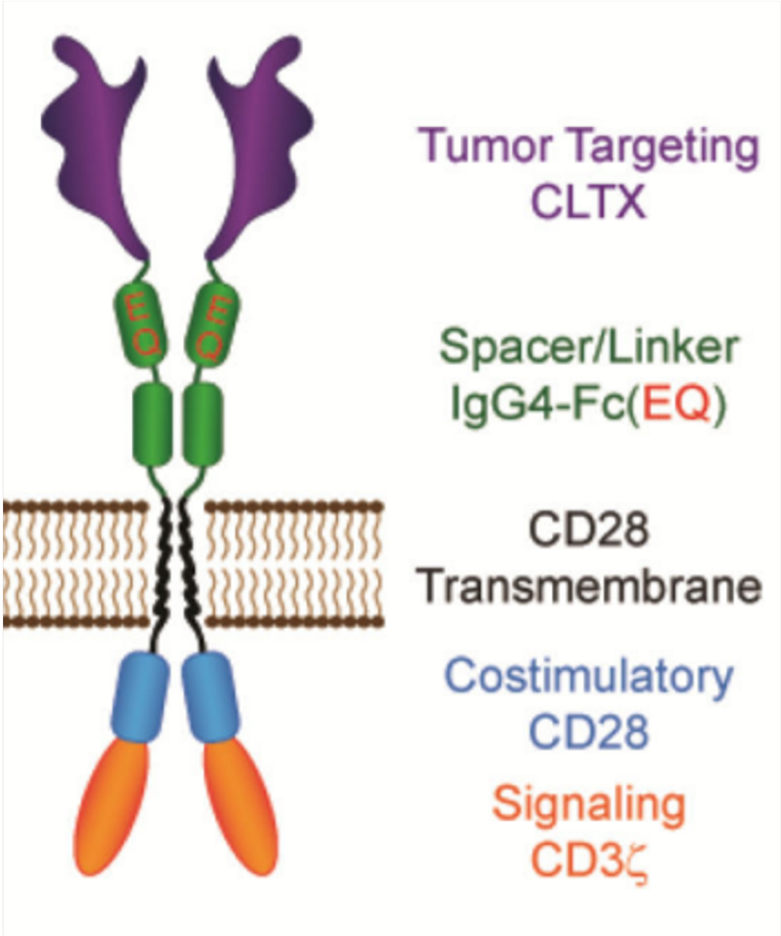

Figure 1. Diagram of a CAR incorporating a CLTX tumor targeting domain, an IgG4-Fc spacer domain with EQ mutations, a CD4 transmembrane domain, and intracellular costimulatory and signaling domains (CD28 and CD3ζ). Image credited to "Chlorotoxin-Directed CAR T cells for Specific and Effective Targeting of Glioblastoma" by Wang. et al. 2020. *Sci. Transl. Med.* 12(533).

CAR T cell therapies have demonstrated strong clinical efficacy against several hematological cancers with increased relapse-free survival of patients. However, GBM-targeted CAR T cell therapies display relatively low clinical response rates in comparison (Davenport et al., 2019). This discrepancy in clinical performance of CAR T therapies is attributed to high

tumor heterogeneity, antigen escape, and the immunosuppressive microenvironment which are major drivers of immunotherapy evasion (Priceman et al., 2015). Across patient samples, there are many GBM sub-classifications and genetic variations which suggest highly variable antigen expression and plasticity between cellular states. Specifically, recently developed CAR T cell treatments directed against GBM antigens IL13Ra2 and EGFRvIII , have resulted in tumor resurgence because the non-homogenous expression of these antigen targets allows the tumor to adapt to the selective pressure of the immunotherapy. Therefore, continuous progress toward optimizing CAR T cell therapies as well as identifying novel, widely expressed, and highly specific GBM antigens is vital to better patient outcomes (Wang et al. 2020).

The Novel CLTX-CAR T Cell

Current research investigating glioblastoma target antigens has identified chlorotoxin (CLTX) as a successful GBM binding component for CAR T cell constructs (Wang et al., 2020). CLTX is a 36-amino acid peptide component of the venom of the death stalker scorpion *Leiurus quinquestrias*. Injection of arthropods with CLTX induces paralysis as a result of inhibiting chloride leak channels in arthropod muscles (DeBin et al., 1993). Chloride leak channels are important for mediating changes in the extracellular brain environment which promote glioma migration and invasion along the white matter tracks and blood vessels in the brain. Other chloride channel inhibitors such as DIDS and tamoxifen have been shown to inhibit glioma cell invasion *in vitro* (Deshane, 2003). Thus, CLTX's chloride channel inhibiting properties have been proposed to mediate anti-tumorigenic effects to specifically target aggressive gliomas such as GBM. (Lyons et al., 2002). Additionally, the administration of radiolabeled CLTX in mouse xenografts and patients with aggressive gliomas indicated high tumor specificity with minimal

cross reactivity with normal brain tissue. Furthermore, patients treated with CLTX experienced no significant adverse effects, demonstrating the safety of CLTX treatments (Mamelak et al., 2006). Thus, the discovery of CLTX as a safe and GBM specific targeting agent suggested its use in the development of immunotherapy treatments.

The CAR T cell construct developed by the collaboration of the Brown and Barish laboratories at City of Hope incorporates CLTX as the targeting domain (Figure 1) and has exhibited broad GBM-targeting capabilities and elicited potent cytotoxic effects during preclinical studies. Investigation of this CLTX-CAR T cell construct showed binding across a wider range of patient tumor samples compared to CAR T cells targeting IL13Ra2, HER2, and EGFR tumor antigens. Additionally, the administration of CLTX-CAR T cell in mouse models resulted in anti-tumorigenic effects while remaining non-toxic. These preclinical results provide strong evidence for potential clinical effectiveness, and thus the CLTX-CAR T cell treatment is currently being tested in a clinical trial at City of Hope (NCT04214392) for recurrent glioblastoma (Wang et al., 2020).

Despite these discoveries, the composition and structure of the cell surface complex recognized by CLTX is still unknown, and thus is an important focus of current research due to its implications for the optimization of the CLTX-CAR (Aftabizadeh et al., 2021). Previous studies have suggested that metalloproteinase-2 (MMP2) is the primary receptor for CLTX on the surface of GBM tumors. MMP2 expression is widely upregulated in gliomas therefore providing evidence for selective binding affinity for GBM (Deshane et al., 2003). Additionally, genetic knockdowns of MMP2 expressions *in vitro* provided potential confirmation that MMP2 is required for CLTX-CAR T cell activation. Since CLTX binding in gliomas results in decreased tumor invasion mediated by changes in the extracellular environment and MMP2

activation is involved in the remodeling of extracellular matrix proteins, these findings fit with previous literature that MMP2 activation induced by CLTX binding leads to antitumorigenic effects (Kasai et al., 2012). However, MMP2 may not be the only receptor involved with CLTX-CAR T cell binding, as ClC-3, MMP14, neuropilin-1, and annexin-A2 interactions have also been observed. Thus the exact mechanism with CLTX is unknown. For example, evidence has suggested that MMP14 plays a role in MMP2 activation, leading to subsequent CLTX binding. A comprehensive understanding of the CLTX binding complex and the mechanism of its cytotoxic activity is therefore important to the optimization of the CLTX-CAR T cell therapies as a potential cure for glioblastoma (Aftabizadeh et al., 2021).

Immunological Synapses

Cytotoxic T cell activation and killing is facilitated by the formation of the immunological synapse, the complex cellular structure that forms at the interface between a T cell and an antigen-bearing cell (Dustin, 2014). Characterizing the structure and morphology of the immunological synapse contributes to our understanding of CAR T cell cytotoxicity. To characterize the immunological synapse in CLTX-CAR T cells, we first need to understand the structure of a conventional immunological synapse (Xiong et al., 2018).

The term ‘synapse’ is derived from the Greek word for ‘connection’’, thus we can understand the immunological synapse as the junction of immune cell activity. Conceptually, this term highlights the dynamic state, information flow and modulation of cellular response that characterizes various types of synapses (Huppa & Davis, 2003). The immunological synapse structure and function mirrors that of the neuronal synapse in that the pre and postsynaptic cells interact via the release of chemical messengers into the synaptic cleft. As such, the mechanisms that regulate exocytosis of neurotransmitters in the neuronal synapse belong to the same protein

family as the immunological synapse, illustrating the conserved function of these structures. Through the study of synapses and their conserved secretory pathways, the cytotoxic activity of T cells, especially those targeting tumor cells via immunotherapies, can be further investigated (Saint Basile et al., 2010).

In characterizing the interaction between an immune cell and its target cell, there are three broad stages of immune synapse formation: initiation, effector and termination (Figure 2). These stages are observed in all types of T cells and involve the direct contact with the target cell via antigen recognition, T cell polarization and signaling across the synaptic cleft (Dustin, 2014). The initiation stage directs the recognition and ligation of the receptor (TCR or CAR) to the target cell. The effector stage follows this binding and initiates the polarization of T cell components towards the synapse. Accumulation of filamentous actin, convergence of lytic granules, polarization of the microtubule organizing center (MTOC), and the release of chemical signals into the synapse characterize the effector stage of immune cell function. Finally, the termination stage involves apoptosis of the target cell induced by cytotoxin release and the separation of the immune cell from the target cell (Mukherjee et al., 2017). Since the mechanism of immunological synapse formation is conserved among T cells, detailed analysis of synaptic structures can be used for comparative analysis of T cell cytotoxicity (Xiong, et al., 2018)

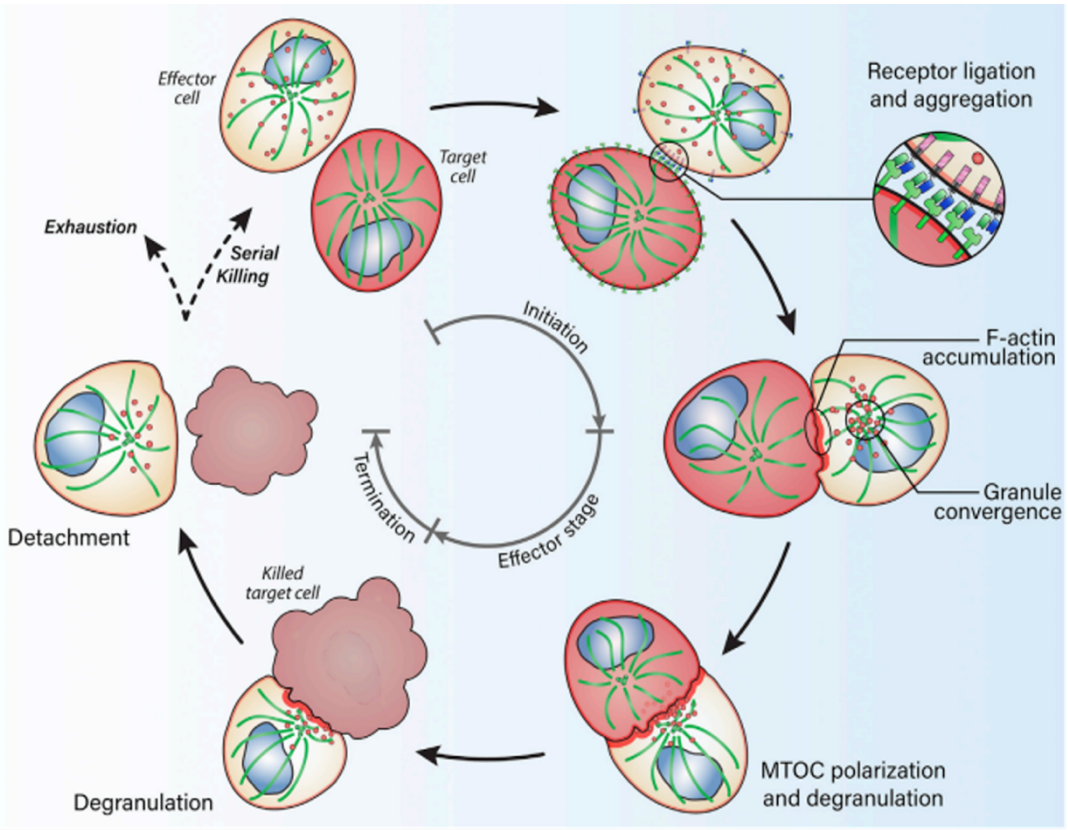

Figure 2. Schematic Representation of the killing of a target cell by a CAR-T cell effector. Image credited to "Quantitative Imaging Approaches to Study the CAR Immunological Synapse" by Mukherjee, M. et al. 2017. *Mol. Ther.* 25 (8): 1757-1768.

Despite the mechanistic similarities of immunological synapse formation, the TCR and CAR T cells exhibit structural and kinetic differences in the immunological synapse due to differences in the mechanotransducive processes associated with receptor binding (Li et al., 2020). The classic structure of the TCR T cell immune synapse is composed of three supramolecular organization complexes (SMAC) that form a well-organized "bull's eye" structure which sequentially activate downstream signaling cascades initiating T cell function (Figure 3). Each component of these SMACs has a distinct function and location that contribute to mechanotransduction in the effector stage of immunological synapse formation (Li et al., 2020). The distal SMAC exhibits lamellipodium behavior, retracting and protruding to facilitate T cell motility, whereas the peripheral SMAC exhibits lamellum behavior with abrupt changes in filamentous actin to reorganize the cytoskeletal network within the T cell. Lastly, the central

SMAC contains the recognition domains which the T cell uses to bind to target cells (Dustin, 2014). Unlike the immunological synapse structure in the endogenous TCR T cells, CAR T cell immunological synapses are significantly less organized and contain a reduced cytoskeletal network (Figure 3).

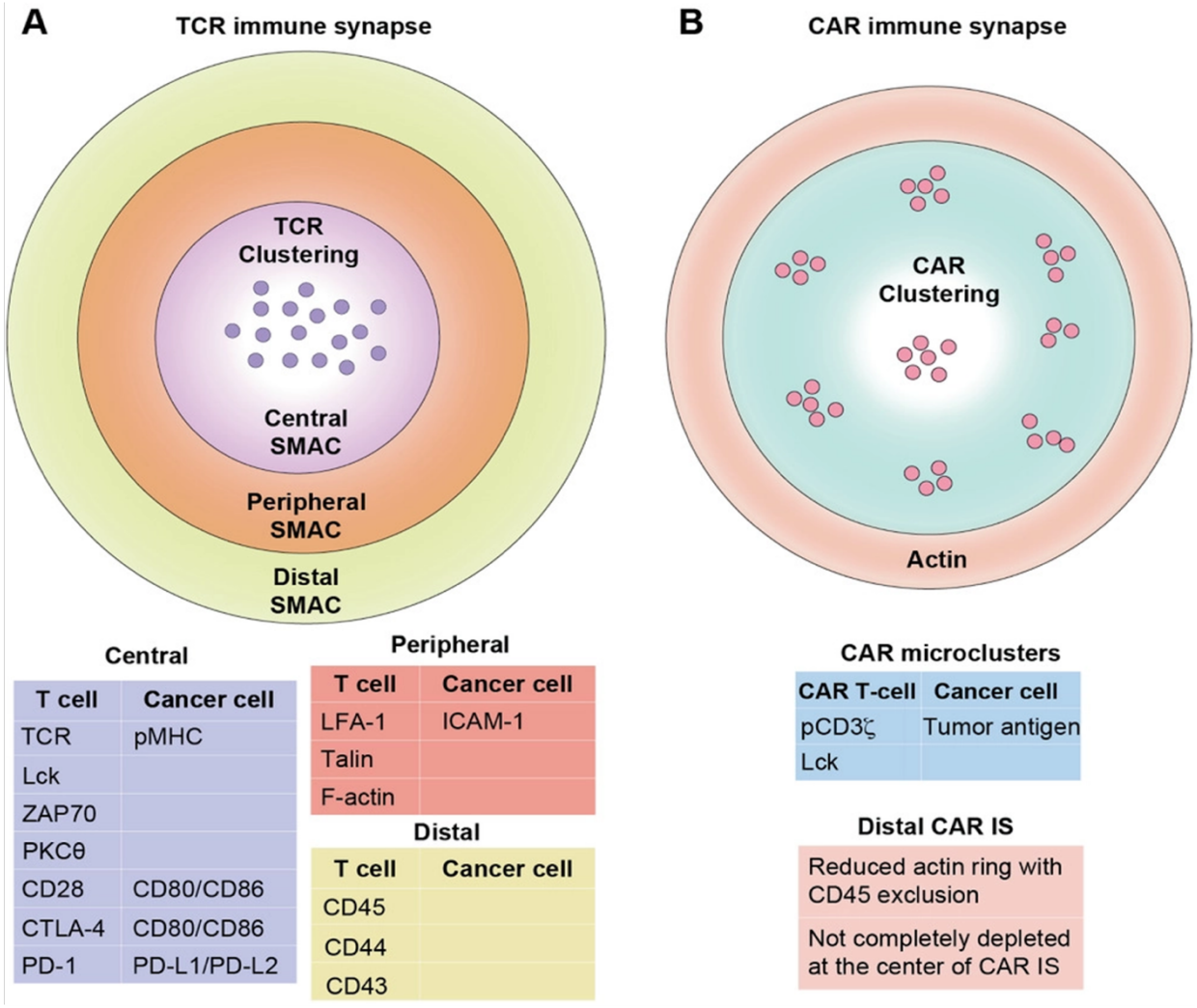

Figure 3. The classical (left) and nonclassical (right) immunological synapses. Image credited to "The CAR T Cell Mechanoimmunology at a Glance" Li, R et al., 2020 *Adv. Sci* 7(24): 20002628-20002645.

Experimental evidence suggests that mechanosignaling in CAR T cells is significantly faster and higher intensity, resulting in the rapid recruitment and release of cytotoxic granules. This increase in cellular kinetics has been hypothesized to cause the lack of organized SMAC regions in the immunological synapses formed by CAR T cells (Davenport et al., 2018). These differences in immunological synapse structure are important for understanding the functional mechanisms of cytotoxicity in T cells, thus understanding the character of these structures and their role in the stages of immunological synapse formation can be used in the optimization of CAR T cells (Xiong et al., 2018).

Using Immunological Synapse to Potentially Predict Clinical Efficacy

For a CAR T cell therapy to be approved for use in clinical trials, pre-clinical testing and optimization is necessary to determine both its safety and efficacy. The *in vitro* approaches currently used to assess CAR T cell efficacy include: immunophenotyping, proliferation and cytokine release, chromium release, long-term killing assays, and interferon gamma production However, the conventional tools used in immunological analysis are time consuming, labor intensive and costly (Xiong et al., 2018). Additionally, these immunological analyses do not recapitulate the *in vivo* conditions that may influence clinical efficacy such as the tumor microenvironment and sample heterogeneogeneity (Liu et al., 2020).

Researchers have recently proposed a novel approach to predict the clinical efficacy of CAR T cells using the formation of the immunological synapse. The assessment of the immunological synapse may serve as a predictive measure of effective cytotoxic activity. The conserved mechanism of immunological synapse formation act as a structural reference for T cell cytotoxicity, allowing for the comparison T cell mediated killing across different targets.

Thus, key structural components of these distinct stages are used as quantifiable metrics to characterize T cell cytotoxicity (Mukherjee et al., 2017). Previous research used the visualization of the immunological synapse as a quantifiable parameter for cytotoxic activity in CAR T cells. Through studying the immunological synapse in two different CAR T cells, researchers were able to compare the clinical efficacy between the two CAR T cell constructs which led to further optimization of these constructs (Xiao et al., 2018). Thus, to further optimize the novel CLTX-CAR T cell, characterization of the immunological synapse is necessary.

Since the mechanism of immunological synapse formation is conserved across all T cells, four key structural parameters which characterize the stages of immunological synapse development (initiation, effector and termination) can be used to characterize CAR T cell cytotoxicity. The structural parameters (Figure 4): receptor aggregation, actin accumulation, granule convergence, and microtubule organizing center polarization have been previously established indicators of effective CAR T cell killing (Mukherjee et al., 2017).

Receptor aggregation marks the initiation stage of immunological synapse formation since the CAR construct binds to specific antigens on the surface of the target cell. Following the initiation stage of immunological synapse formation, cytoskeletal rearrangements are necessary to facilitate cytotoxic activity through structural changes. The active immunological synapse structure relies on the physical forces exerted by actin to organize and induce cytotoxic vesicle transport in the CAR T cell, thus actin accumulation at the synapse is one of the most important indicators for cytotoxic activity in T cells (Comrie & Bukhardt, 2016).

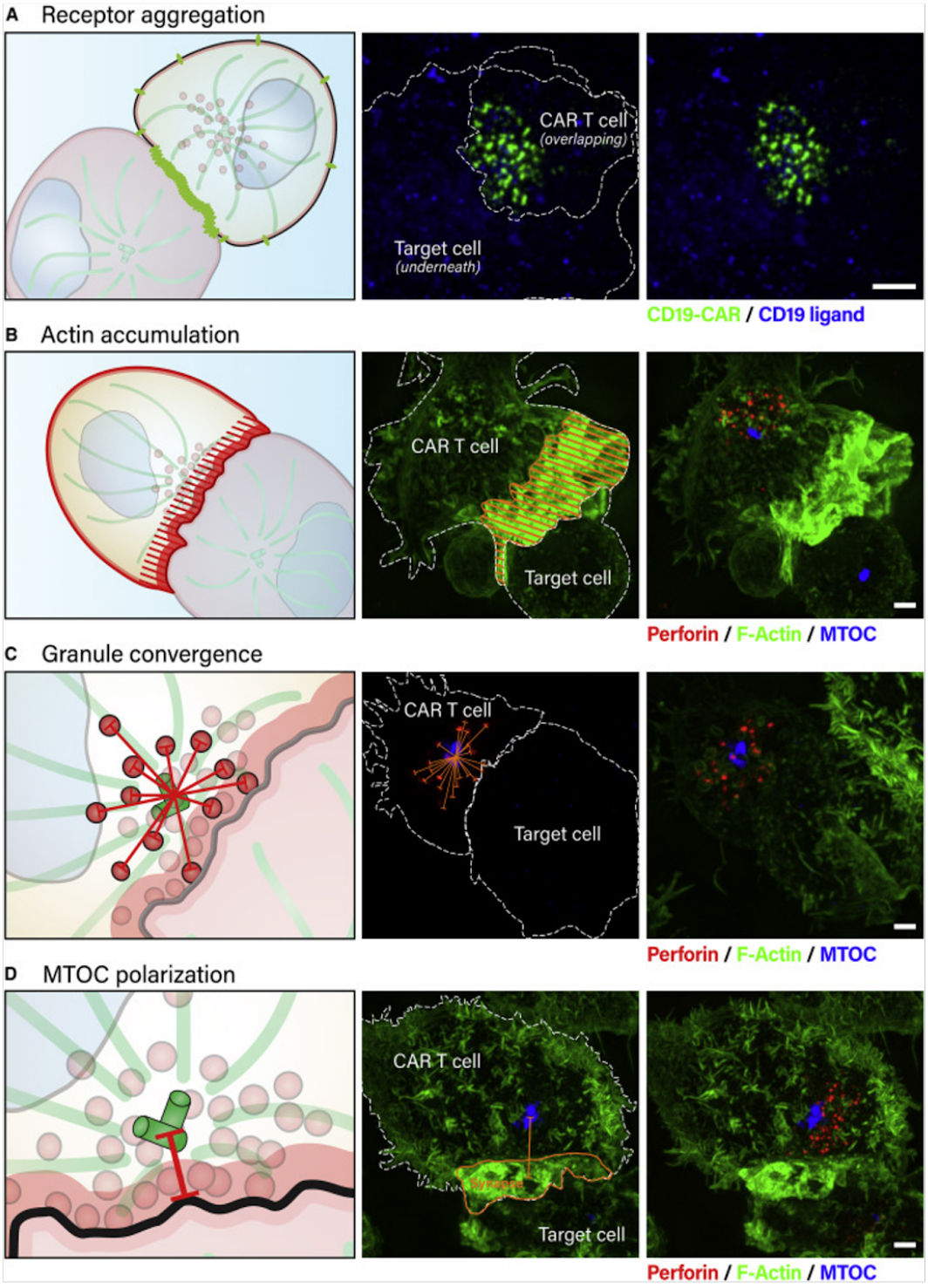

Figure 4. Schematics and microscopy images demonstrating four quantifiable parameters of immunological synapse formation between target cells and CAR-T cells. Image credited to "Quantitative Imaging Approaches to Study the CAR Immunological Synapse" by Mukherjee, M. et al. 2017. *Mol. Ther.* 25 (8): 1757-1768.

Similarly, to the actin rearrangements, MTOC polarization contributes to exocytosis in the CAR T cell through the mobilization of cytoskeletal networks to facilitate protein transport and cytotoxin release (Saint Basile et al., 2010). Therefore, the effector stage of immunological synapse formation is characterized by the exocytosis of cytotoxic granules into the immunological synapse which stimulate the apoptotic pathway in the target cell. Since the T cell granule secretory pathway involves the migration of these cytotoxins in vesicles along the cytoskeletal network, granule convergence occurs at the synaptic terminal in response to actin and MTOC rearrangements Thus, key structural features involved with CAR T cell activation and cytotoxicity potentially enable us to predict the clinical efficacy of the novel CLTX-CAR T cell and shape its further optimization for future applications to improve patient outcomes.

The focus of this research project is to characterize the structure and morphology of the immunological synapse formed by these novel CLTX-CAR T cells. Using confocal microscopy, key structural components of the CLTX-CAR T immunological synapse were imaged and compared to the known immunological synapse structure of endogenous T cells and other types of CAR T cells. Since CAR mediated immunological synapse formation has been previously characterized by receptor aggregation, actin accumulation, cytotoxin release and MTOC polarization, I hypothesized that the CLTX-CAR T cell will also display these morphological changes if the cell is actively and effectively killing tumor cells. Additionally, I predicted that CLTX-CAR T cells will be bound to areas of tumor cells which contain MMP2 and MMP14, since they have been thought to be associated with the CLTX binding mechanism. Through this study, we can further our understanding of CLTX binding in GBM tumors as well as potentially use immunological synapse formation to evaluate the efficacy of the novel CAR as a potential immunotherapy in clinical applications.

Methods

Experimental Design

In this study, I evaluated the potential clinical efficacy of the novel CLTX-CAR T cell against GBM tumor cells through visualization of the immunological synapse onto target tumor cells. Using this experimental framework, the purpose of the experiment was to capture the novel CLTX-CAR T cell immunological synapse to evaluate its tumor cytotoxicity and further investigate the tumor receptors involved in synaptic binding. First, CLTX-CAR T cells were phenotyped for T cell composition and CAR expression using flow cytometry. Upon determination of sufficient CAR expression, the immunological synapses were formed by co-culturing patient derived brain tumor cells with CLTX-CAR T cells. Then, CLTX-CAR T and PBT cells were stained for immunological synapse markers and CLTX binding receptors using immunofluorescence techniques (Figure 5). Lastly, the stained samples were imaged using confocal microscopy and analyzed for immunological synapse key characteristics and binding interactions. Overall, this experimental design, visualizing the CLTX-CAR T cell immunological synapse, helped answer research questions surrounding CLTX-CAR T cell efficacy against GBM tumors and the CLTX and tumor cell binding interactions at the synapse.

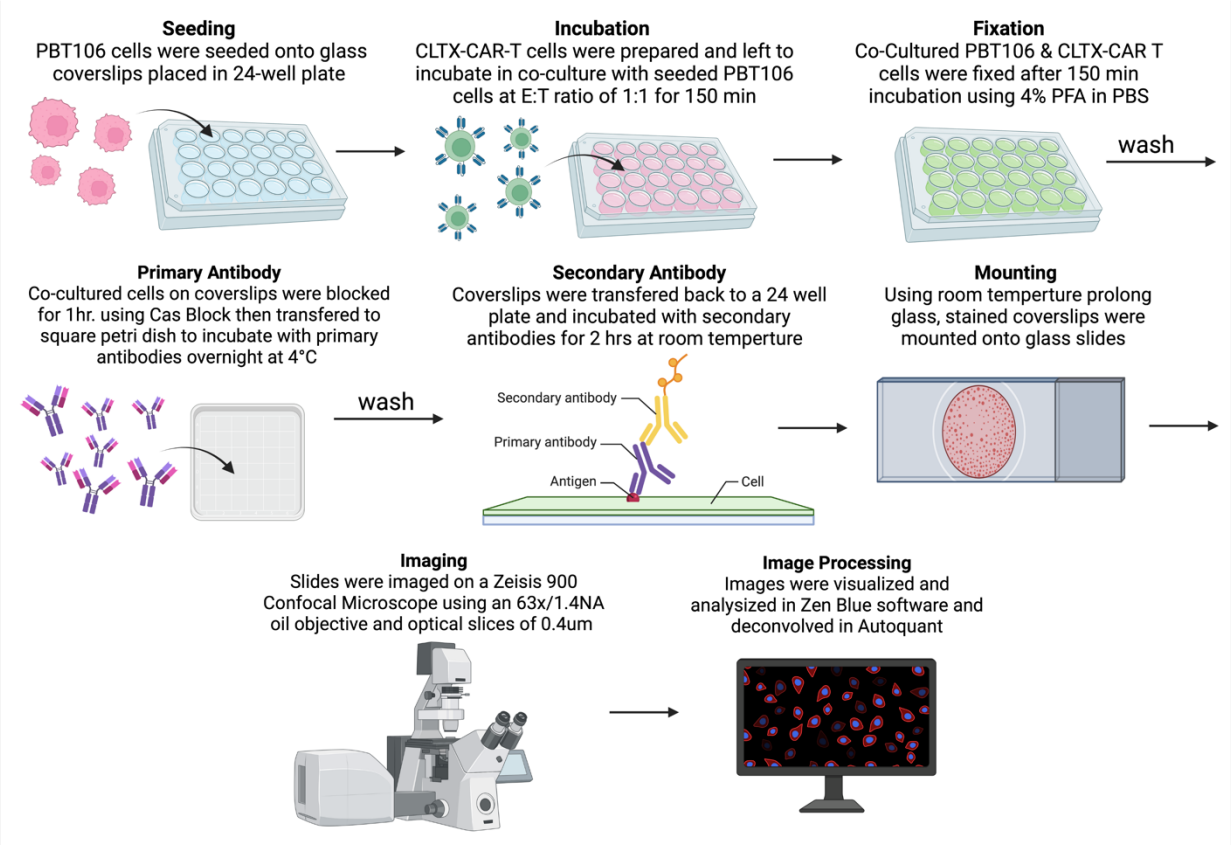

Figure 5. Schematic of the experimental design and procedure of visualizing the immunological synapse formed between CLTX-CAR T cells and PBT106 cells

Isolation of primary brain tumor cell lines and CLTX CAR T cells

Prior to my experiment, the Brown laboratory created the cell lines and CLTX-CAR T cells used in this experiment using the following protocols. Primary brain tumor (PBT) cells were obtained from GBM resections at City of Hope under protocols approved by the City of Hope Internal Review Board. Resected brain tumor specimens were dissociated using a human tumor dissociation kit (Miltenyi Biotech Inc) and were expanded in neural basal stem cell media (Stem Cell Technologies) to generate PBT106 cells. The PBT106 cells were cultured in neurobasal stem cell media with the addition of epidermal growth factor (EGF) and fibroblast growth factor (FGF) to promote cell proliferation.

The CLTX-CAR T cell was developed by the Brown laboratory using established protocols for CAR T cell construction. The CLTX-CAR construct contains a tumor targeting CLTX peptide, an IgG4-Fc spacer, a costimulatory CD28 transmembrane domain, and a signaling, cytoplasmic domain of human CD3ζ (Figure 1). A truncated CD19 was also introduced into the construct to induce and prolong CAR activation. To produce CAR T cells, blood products were obtained from healthy donors under protocols approved by the City of Hope Internal Review Board. Peripheral blood mononuclear cells (PBMCs) were isolated using density gradient centrifugation over Fiscol-Paque (GE Healthcare) and then underwent sequential rounds of AutoMACS depletion to remove CD14 and CD25 expressing cells. To generate CAR T cell products, T cells were stimulated with Dynabeads (Human T expander, Invitrogen) at a 1:3 T cell to bead ratio and transduced with CAR encoding lentivirus. At 7 days following transduction, Dynabeads were removed from cultures using DynaMag-50 magnet (Invitrogen). CAR expressing T cells were enriched by positive selection using anti-CD19 magnetic beads. CAR T cell cultures (cF17982) were incubated at 37°C and 5% CO_2, and maintained in hematopoietic serum free media (Lonza, X-Vivo).

Cell Passage and Seeding

Using the tumor cell lines and CAR-T cells created by the Brown laboratory, I independently cultured and imaged these cells using the following protocol (Figure 5). To prepare PBT106 cells for co-culturing with CLTX-CAR T cells, PBT106 cells were passaged to prevent over proliferation of tumor cells prior to seeding. Then, Accutase (Stem Cell Technologies) was added into a flask of PBT106 cells to break down cell adhesions. Cells were viewed under a Zeiss ICM 405 light microscope to ensure full dissociation of cells. Then, cells

were centrifuged down for 5 min on 1500 rcf and re-suspended into proliferation neurobasal stem cell media (Stem Cell Technologies). Cell concentration was counted using a Nexcelom cell counter to prepare a PBT106 cell solution yielding 30,000 cells per well.

Using forceps, circular glass coverslips were placed into two 24-well plates (48 wells total). Poly-L-ornithine (1:500 dilution; Sigma Aldrich) was added into each well in order to enhance cell attachment to the glass surface of the cover slip and left to incubate for 1hr in 37°C, 5% CO_2 conditions. After the incubation period, coverslips were rinsed with TC grade H_2O. Laminin (10 µl/mL dilution) was added into each well to support cellular growth through the maintenance of the extracellular matrix and left to incubate for 2 hrs in 37°C, 5% CO_2 conditions. The prepared PBT106 cell solution was added to the coated coverslips and left to incubate for 2 days in 37°C, 5% CO_2 conditions. Following the PBT106 cell incubation period, CLTX-CAR T cells were counted using a Nexcelom cell counter and prepared to a desired E:T ratio of 1:1. To co-culture the PBT106 and CAR T cells, 1 mL of CLTX- CAR T cells were added to the seeded coverslips. Co-cultured cells were fixed in 4% PFA after 150 min, the optimal binding time as determined in preliminary experiments. After fixation, CAS block was added to co-cultured cells for 1 hr to prevent non-specific binding of primary antibodies.

Immunofluorescence and Confocal Microscopy

Immunofluorescence is a technique that allows for the detection and localization of cellular antigens (Lm, et al., 2019), and was used for the visualization of key structural components of the immunological synapse between PBT106 and CLTX-CAR T cells. The co-cultured PBT106 and CLTX-CAR T cells were stained with the following primary antibodies:

Granzyme B (Invitrogen, GB11, 1:100), CD3 (Invitrogen, OKT3, 1:25), phosphorylated-CD3ζ (Abcam, EP7762Y, 1:100), Pericentrin (Abcam, ab448, 1:1800) MMP2 (Invitrogen, 2C1-1D12, 1:100) MMP14 (R&D Systems, 128527, 1:100), Annexin A2 (Abcam, D1/274.5, 1:50).

On a 3x3 square petri dish lined with Parafilm, each coverslip was incubated with 20 ul of primary antibody master mix for 24 hrs at 20°C. After 24 hrs, coverslips were removed from the square petri-dishes using forceps and placed back into the 24 well plates for washing with TBS and secondary antibody incubation. The coverslips were incubated with Alexa Fluor secondary antibodies and AF647-conjugated-phalloidin for 2 hrs. After the coverslips were fully stained with primary and secondary antibodies, coverslips were mounted onto slides using Prolong glass. A 48-hour mounting period allowed for optimal polymerization of the mounting medium. Slides were imaged using a Zeiss LSM-900 Airyscan confocal microscope. Optical slices of 0.4 um for z-stacks obtained with a Planachromat 63x/1.40 NA oil objective were used to capture the immunological synapse interface and morphology. Images were visualized and analyzed in Zen software (Zeisis) after deconvolution (Autoquant software).

Flow Cytometry

Flow cytometry is a quantitative tool used in single cell analysis, and is used in this experiment to phenotype the CLTX-CAR T cell populations. In this method, cells are suspended in a buffer solution and passed through a series of lasers to be quantified by the light scatter and fluorescent parameters (Drescher et al., 2021). The following protocol was used to make the experimental, compensation and control samples used in the analysis.

Ultra-Compensation beads, spherical particles used for single-color compensation controls, were prepared by adding the respective dilutions of fluorophore conjugated antibody per drop of compensation bead: CD45RA-FITC 5 µL; CD4-PerCP-Cy5.5 1.25 µL; CD62L-APC 5 µL; CD8-AF700 1.25 µL; CD3-APC-Cy7 1.25 µL, CD19 1.25 µL. The ultra-compensation beads are used to set the voltages to obtain an accurate fluorescence signal (Tung, et al., 2007). These compensation samples were incubated in the dark for 15 min then centrifuged down for 5 min at 524 rcf (relative centrifugal force). FSS buffer was added to all beads following incubation to create a suspension medium for the cells. Then, 6 million CLTX-CAR T cells were added to a microcentrifuge tube containing an FSS buffer. CAR T cells were centrifuged down for 5 min at 524 rcf at a final volume of 300 µL of cell solution. Next, Heat-shock 50/50 controls were prepared by subjecting half of a tube of CLTX-CAR T cells to a heated water bath at 42°C. The purpose of the heat shock 50/50 control is to provide a gating control for dead cells during analysis (Tung, et al., 2007). The CLTX-CAR T cells used in the flow cytometric analysis were blocked using Fc block then stained with the same amount of antibody used during the preparation of compensation beads. Finally, three fluorescence minus one (FMO) controls (CD45RA FMO, CD62L FMO, and CD19 FMO) were also prepared using the same antibody concentrations as the stained sample, but with the exclusion of the respective antibody. FMO controls are used to set the upper boundary of background signal, therefore contributing to accurate gating of positive cell populations (Feher, et al., 2014).

Samples were run on the Attune NxT and analyzed in FCS Express. Figure 6a shows the gating procedure used to prepare the flow cytometry data for further analysis. Using the forward scatter (FSC) and side scatter (SSC) scales, the size and granularity of the cells, respectively, were measured and used in the gating analysis (Drescher et al., 2021). The FSC and SSC were

used to gate cells from debris in Figure 6a1. Next, singlets were selected from doublets and triplet cells detected by the attune using side scatter area and height scales. Finally, live cells were selected and gated from dead cells using DAPI exclusion criteria.

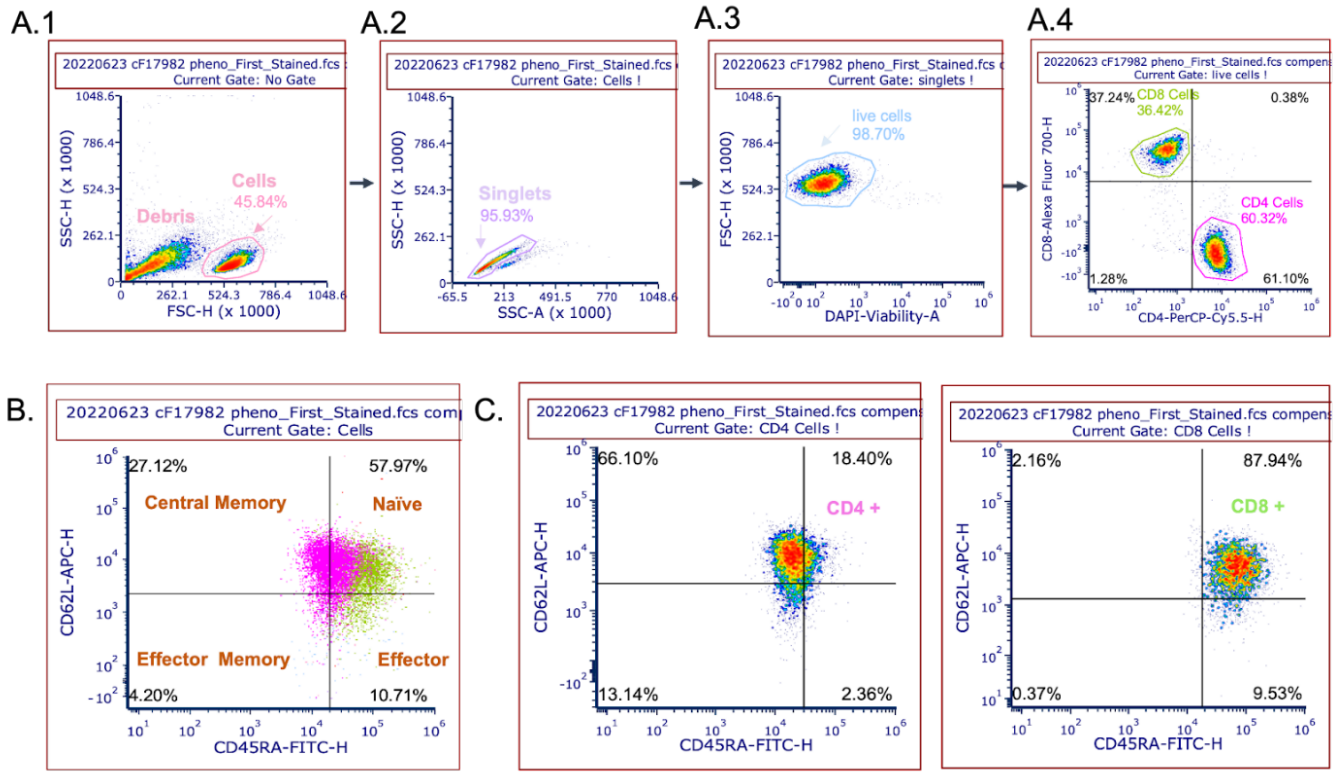

Figure 6. Flow cytometry phenotyping of CLTX-CAR T cells shows active CAR-T cell populations. A. Outlines the gating procedure used in the analysis. In A1, cells were gated from debris using SSC-H and FSC-H. In A2, singlets were gated from doublets using SSC-A & SSC-H. In A3, live cells were gated from dead cells using DAPI exclusion. In A4, CD4+ helper T cells and CD8+ cytotoxic T cells were subdivided. B. CD62L and CD45RA were used to determine the cell type of CD8+ and CD4+ cells, with Central Memory and Naïve cells as the predominant cell types. C. CD19 positivity was used to determine the CAR transfection rate of the CD4+ (left) and CD8+ (right) cells.

Results

CLTX-CAR T cells have sufficient CAR expression for cytotoxic activity

Since CAR T cell populations are heterogeneous, a preliminary phenotyping experiment using flow cytometry was used to confirm the T cell composition and CAR expression of the CLTX-CAR T cells used in the subsequent experiments. Majority of the cells in the sample were central memory and naïve T cells, with 27% of cells being central memory and 58% being naïve cells (Figure 6B). This cellular composition is to be expected with CAR T cell generation since central memory and naïve cells are less differentiated cells which contributes to higher proliferative potential and long-term persistence (Stock, Schmitt, & Sellner, 2019)

There are two main types of T cells, CD8+ and CD4+, which are important for the characterization of adequate immune response as well as facilitate tumor targeting capabilities through different pathways. CD8+ cells are cytotoxic T cells which means that they are directly involved with the targeting and killing of target (GBM) cells. CD4+ cells are helper T cells which promote associated pathways for immune system memory (Stock, Schmitt, & Sellner, 2019). By phenotyping the CD4+ and CD8+ CLTX-CAR T cells, we can characterize their immune responses. In this phenotyping experiment, we found that he ratio of CD4+/CD8+ CLTX-CAR T cells would produce an adequate immune response to be used in subsequent experiments. Additionally, 88% of CD8+ cells were positive for CD62L, thus CD8+ cells had a more naïve phenotype than the CD4+ cells. This indicates that the T cells directly involved with tumor killing were less differentiated, contributing to the longevity of cytotoxic response.

Determination of the CAR positivity rate is important for ensuring proper transfection of the T cells with the CAR construct. Thus, the CAR positivity rate, as determined by expression of a CD19 marker, was 37% for all live cells, 43% for CD4+ cells, and 47% for CD8+ cells (Figure 6C). This level of CD19 positivity is consistent with other CLTX-CAR T cells lines produced by the Brown lab and thus the CLTX-CAR T cells have a high enough transfection rate to be considered active and cytotoxic in subsequent experiments.

The immunological synapse is an effective way to measure cytotoxicity of the CLTX-CAR T cell suggesting clinical efficacy against glioblastoma

Confocal imaging of the immunological synapse formed between CLTX-CAR T cells and tumor cells (PBT106) showed morphological changes in key structural components. Immunological synapses are characterized by the following T cell features: receptor aggregation, actin accumulation, granule convergence, and MTOC polarization which have been previously established indicators of effective CAR T cell killing (Mukherjee et al., 2017).

The immunological synapse was characterized through differences in cellular morphology and interfacing structure between the CLTX-CAR T and tumor cells. Figure 7A shows two CLTX-CAR T cells (left) binding and attacking a singular PBT106 cell. The CLTX-CAR T cells are much smaller than the singular PBT106 cell and include structural components indicative of CAR T cell cytotoxicity such as the polarization of cytotoxins (Figure 7B) and cytoskeletal rearrangements (Figure 7C) towards the synaptic interface. This comprehensive view of the cytotoxic action in CLTX-CAR T cells, is necessary to interpret more magnified images visualizing the immunological synapse allowing us to characterize effective cytotoxicity as the integration of receptor binding, cytoskeletal rearrangement and cytotoxin release. As such,

the expression, and interactions of these immunological synapse characteristics in the visualized PBT106 and CLTX-CAR T cell junction support the conclusion that visualizing immunological synapse is an effective way to measure cytotoxicity of CLTX CAR T cell and thus potentially predict its clinical efficacy.

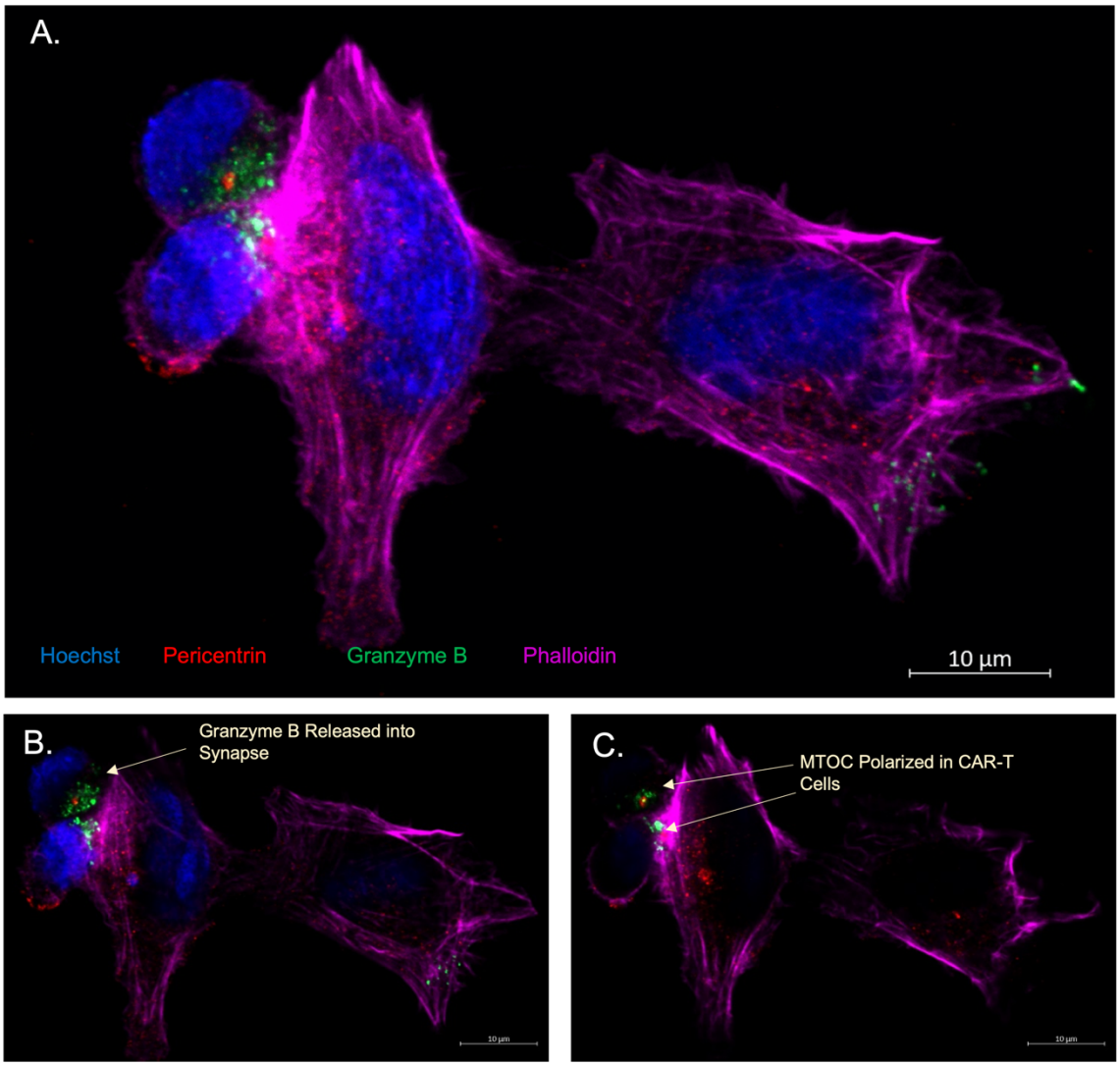

Figure 7. Immunological synapse formation after 150 min after adding CLTX-CAR T cells to dissociated PBT106 cells (E:T = 1:1) A. Composite, maximum projection image of the immunological synapse formed between two CLTX-CAR T cells and one PBT106 cell B. Granzyme B convergence illustrates the release of cytotoxins into the synapse C. Pericentrin marking the MTOC is polarized toward the synapse to help facilitate the release cytotoxic granules

i. Microtubule Organizing Center Polarization

The microtubule organizing center (MTOC) regulates cytoskeletal rearrangements, thus its polarization is indicative of morphological changes in the CLTX-CAR T cell which contribute to cytotoxic activity. As with actin accumulation, the MTOC facilitates the release of cytotoxic granules by generating and organizing a microtubule network for transport of vesicles containing these cytotoxins to transport to the synapse. Therefore, the MTOC polarizes with the release of cytotoxins during the effector stage of immunological synapse development to closely regulate granule convergence (Saint Basile, Menasche & Fischer, 2010). Figure 7c shows the expression of pericentrin, a MTOC marker, polarized in two attacking CAR T cells in close proximity to granzyme B and the accumulating actin at the interface. This MTOC polarization observed in the imaged immunological synapses is indicative of the cytoskeletal rearrangements that are required to release cytotoxic granules to kill tumor cells.

ii. Receptor Aggregation

Endogenous and engineered T cell receptors (CD3ε and CD3ζ, respectively) are polarized towards the synaptic interface within the CLTX-CAR T cell. CD3ε is a component of the native T cell receptor complex (TCR) involved with binding and recognition of immunologic targets leading to downstream signaling cascades. CD3ζ is a component of the engineered CLTX-CAR TCR, therefore any polarization of this CD3ζ indicates activation of the CAR complex as well as the structural changes of the synapse that indicate cytotoxicity (Xiong et al., 2018). Figure 8b shows the polarization of phosphorylated-CD3ζ at the synaptic interface, co-localizing with granzyme B, marking vesicles containing the cytotoxin. The phosphorylation of the CD3ζ

antibody used in the experiment is important to note because the CAR T cells may already be partially activated prior to binding. Nonetheless, receptor aggregation at the synapse and co-localization with granzyme B, is indicative of the initiation stage of immunological synapse development, providing evidence of cytotoxic activity of the CLTX-CAR T cell.

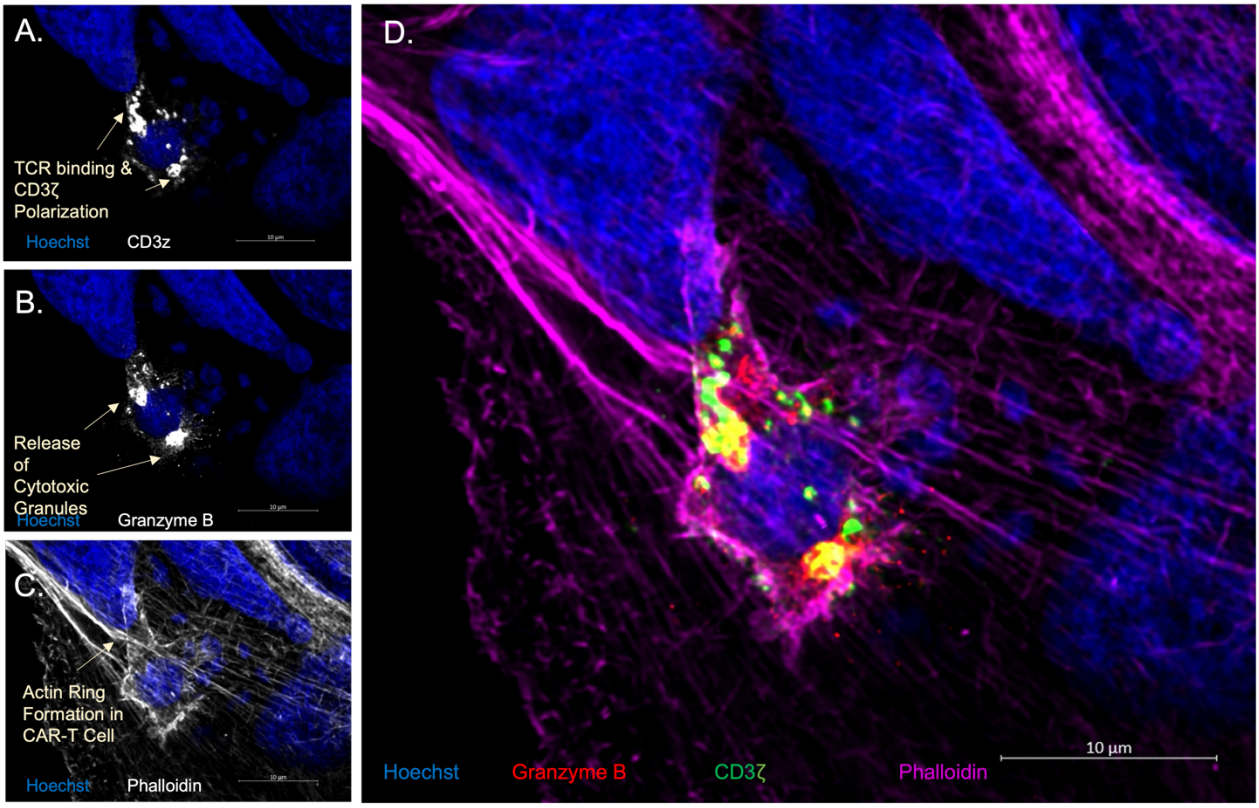

Figure 8. Immunological synapse formation after 150 min after adding CLTX-CAR T cells to dissociated PBT106 cells (E:T = 1:1). A. Phosphorylated CD3ζ polarization characterizing T cell binding and CAR activation B. Granzyme B convergence illustrates the release of cytotoxins into the synapse C. Localization of phalloidin marks an F-actin submembrane ring to facilitate cytotoxic activity. D. Maximum Intensity image of the immunological synapse stained with Granzyme B, phosphorylated CD3ζ, and Phalloidin.

iii. Actin Accumulation

To facilitate the release of cytotoxins at the synapse, cytoskeletal rearrangements induced by changes in actin are necessary for signaling, transport, and release of vesicles containing cytotoxic granules (Comrie & Burkhardt, 2016). Actin morphology indicating cytotoxic activity in CAR T cells can be characterized in two ways, actin accumulation at the synaptic interface and submembrane actin ring formation. Significant cytoskeletal rearrangements and changes in actin morphology in the CLTX-CAR T cell were observed in all imaged immunological synapses. Figure 8c and 8d show the localization of phalloidin binding, marking F-actin, forming a well-defined actin ring surrounding the CLTX-CAR T cell bound to a tumor cell. This CLTX-CAR T cell also has a long actin-containing protrusion along the middle-left of the tumor cell, which increases its surface area at the immunological synapse therefore allowing for more direct contact for cytotoxin release. The CLTX-CAR T cell in Figure 9 displays a similar actin morphology, using cytoskeletal protrusions to enhance its cytotoxic abilities. The actin protrusion formed in this CLTX-CAR T cell is similar to the dSMAC featured in endogenous T cells, forming a lamellipodium to assist with T cell motility (Figure 9c). Unlike what is seen in Figures 7 and 9, the CAR T cell in Figure 7 has dense actin accumulation yet lacks a clearly defined submembrane actin ring. This could be attributed to the dual attack of two CAR T cells in which membrane overlap leads to apparent dense localization at the immunological synapse. Overall, actin accumulation is a clear indicator of cytotoxic activity in the CLTX-CAR T cells and is an integral part of the effector stage of immunological synapse development since cytoskeletal rearrangements accompany cytotoxin release.

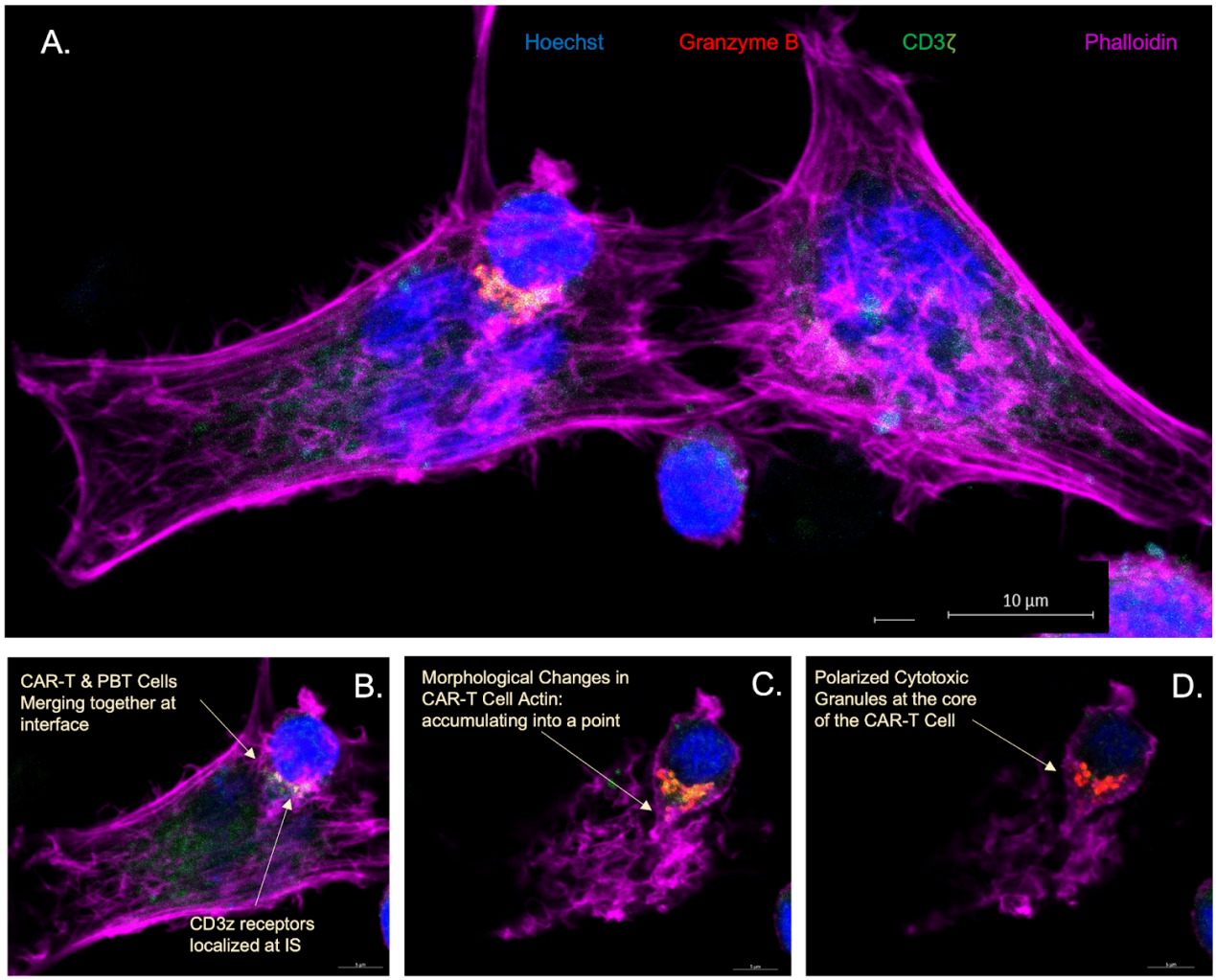

Figure 9. Immunological synapse formation after 150 min after adding CLTX-CAR T cells to dissociated PBT106 cells (E:T = 1:1). A. Maximum Intensity image of the immunological synapse stained with Granzyme B, CD3ζ, and Phalloidin. B. CD3ζ polarization characterizing T cell binding and CAR activation C. Localization of phalloidin marks an F-actin submembrane ring to facilitate cytotoxic activity. D. Maximum Intensity image of the immunological synapse stained with Granzyme B, CD3ζ, and Phalloidin. D. Granzyme B convergence illustrates the release of cytotoxins into the synapse

iv. Cytotoxic Granule Convergence

Cytotoxic granule release was visualized through the convergence of these molecules towards the synapse, the interface between the CLTX-CAR T and tumor cells. The exocytosis of cytotoxic granules into the immunological synapse is mediated by vesicle transport along cytoskeletal networks and initiates an apoptotic pathway in the tumor cell (Mukherjee et al.,

2017). Thus, co-localization with receptor and actin expression is to be expected with effective cytotoxic activity. As mentioned earlier, these cytotoxins co-localized with the expression of CD3ζ and followed morphological patterns of actin accumulation, therefore functioning as the culmination of cytotoxic activity for these CLTX-CAR T cells. All imaged immunological synapses illustrate this cytotoxin release through significant expression of granzyme B, clustered at the synaptic cleft of the CLTX-CAR T cell. Figures 7b, 8c, and 9c capture clear, punctate expression of granzyme B at the synaptic interface. Therefore, the release of granzyme B is a clear indication of active cytotoxic activity by the CLTX-CAR T cell.

The co-localization of MMP2 and MMP-14 in the immunological synapse supports previous studies on the CLTX binding mechanism

To better understand the binding mechanism of the CLTX construct, we stained for a few proposed tumor receptors of CLTX: MMP2, MP14 and AnnexinA2. Consistent with previous studies, MMP2 co-localized with MMP14 near the PBT106 nuclei and higher CLTX-CAR T cell binding was seen in these regions of higher MMP2 and MMP14 expression (Figure 10). Phosphorylated-CD3ζ receptor polarization follows the presence of MMP2 and MMP14 and is aligned with the structure of CAR T cell receptor aggregation in the synaptic interface. Figure 10 illustrates the co-localization of MMP2 and MMP14 in puncta surrounding the nuclei of tumor cells. The CLTX-CAR T cells in Figures 10a and 10b were observed binding to these areas of high MMP2 and MMP14 expression, consistent with current hypotheses findings of the CLTX binding mechanism.

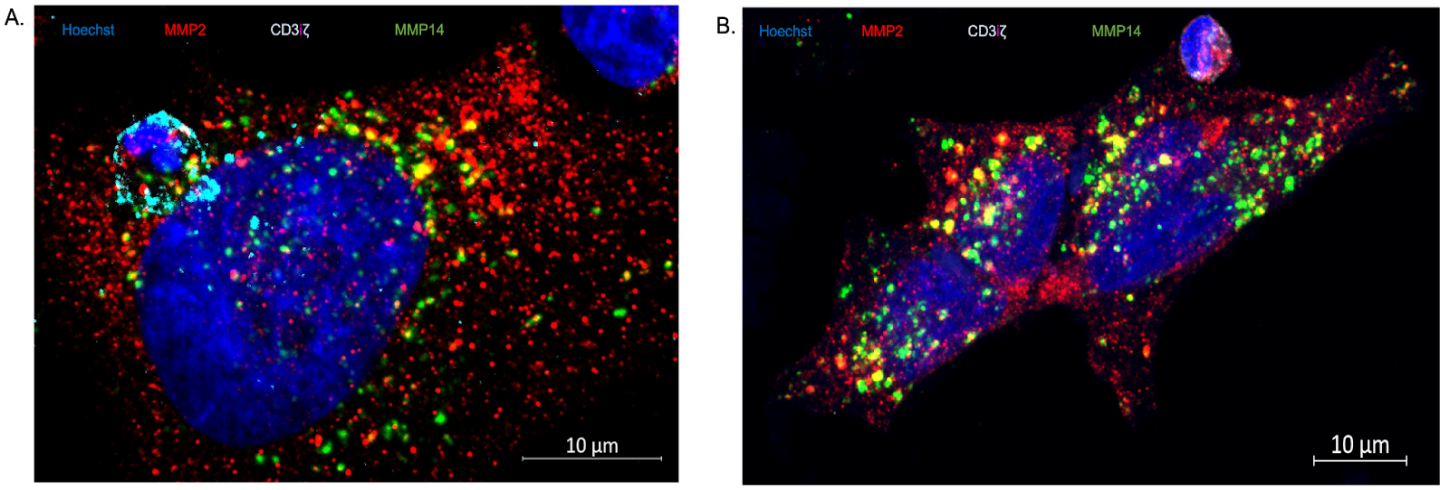

Figure 10. Co-localization of MMP2 and MMP14 in the immunological synapse formed between CLTX-CAR T and PBT106 cells. Phosphorylated CD3ζ polarization, present in high areas of MMP2 and MMP14 expression suggesting a possible binding mechanism for CLTX-CAR T cells

Possible selective presence of MMP2 in CLTX-CAR T cells suggests a role in T cell priming and/or T cell exhaustion

Since the serial killing capacity of CLTX-CAR T cells is not well described, the appearance of MMP2 within CLTX-CAR T cells can be an important influence on the cytotoxic behaviors of these cells. The presence of MMP2 was observed in some but not all CLTX-CAR T cells, which suggests that MMP2 receptors might be endocytosed by the CLTX CAR T cells during repeated tumor cell contacts. This selective uptake of MMP2 receptors could help facilitate easier binding to tumor cells, faster killing mechanisms due to continued activation, or conversely potential T cell exhaustion. Since the binding mechanism of CLTX-CAR T cells is thought to involve MMP2 co-localization with MMP14 and/or other molecules in the target tumor cell membrane, it is not clear why MMP2 but not other potential components of the CLTX-CAR T cell receptor (MMP14, annexin A2) are not also found. Figure 11 illustrates the

appearance of MMP2 but not annexin A2 in the CLTX-CAR T cell. Whether MMP2 in CLTX-CAR T cells is a result of selective uptake or de novo expression is a very interesting question that will require additional research to further understand its potential role in influencing serial killing.

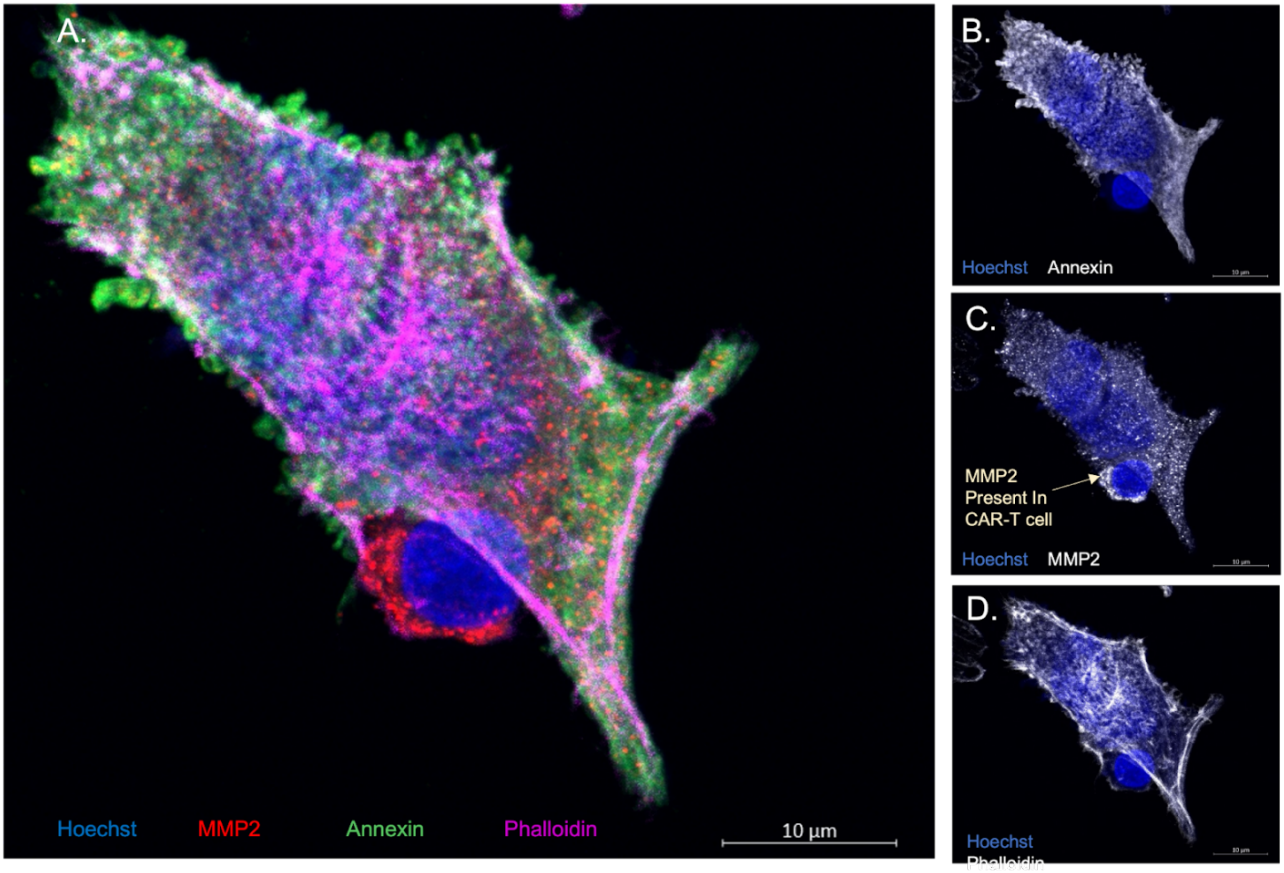

Figure 11. Possible selective presence of MMP2 in CLTX-CAR T cells. A. Maximum Intensity image of the immunological synapse stained with MMP2, Annexin A2 and Phalloidin. B. Expression of Annexin A2 localized to the PBT106 cell, with no expression in the CLTX-CAR T cell. C. MMP2 expression is higher in the CLTX-CAR T cell, suggesting possible selective endocytosis of receptors. D. Localization of phalloidin marks an F-actin submembrane ring to facilitate cytotoxic activity

Discussion

Overall, this study was able to further our current understanding of the novel CLTX-CAR T cells through the visualization and characterization of immunological synapses formed between the CLTX CAR T cells and GBM samples. Specifically, CLTX-CAR T cells exhibited cytotoxic activity as demonstrated by morphological changes of immunological synaptic structures. In addition, visualization of the CLTX-CAR T cell interface with GBM cells supports current hypotheses on the CLTX binding mechanism, an important area of research for CLTX-CAR T cell optimization. Of particular interest, CLTX-CAR T cells displayed MMP2 receptors of an unknown origin which could have potential implications for CAR T cell exhaustion and/or priming. Future, experimentation will shape our understanding of this expression and its possible role in serial killing. Therefore, the characterization of the CLTX-CAR T cell immunological synapse can potentially be used to assess the clinical efficacy of this novel immunotherapy and further optimize therapeutic outcomes for patients with GBM.

Tumor resurgence remains a significant barrier to the treatment of GBM, thus the development and optimization of targeted therapies are vital to better clinical outcomes. Complex features of GBM tumors such as cell heterogeneity, tumor microenvironment interactions, immune system evasion and cancer stem cell (CSC) dynamics are responsible for aggressive GBM phenotypes and low clinical responses to current treatment options (Lathia et al., 2015). By using patient-derived tumor lines in our analysis of CLTX-CAR T cells, we are able to evaluate the cytotoxicity of CLTX-CAR T cells against CSC and non-CSC populations. The evaluation of immunological synapse formation provides insight into the immune system dynamics that might modulate therapeutic responses. Furthermore, this study design, investigating the CLTX-CAR T cell binding mechanism and immunological synapse formation,

helps further our understanding of the potential clinical efficacy of the novel immunotherapy against GBM tumors. Lastly, by expanding the possible options for preclinical testing of novel CAR T cells, we can further optimize CAR T cell treatments to create better clinical outcomes for patients suffering from aggressive cancers.

Previous studies have identified four key structural components of the immunological synapse to quantify the cytotoxic activity of CAR-T cell interactions: receptor aggregation, actin accumulation, granule convergence, and microtubule organizing center polarization. These features of the immunological synapse are involved in the conserved T cell cytotoxic pathway, and thus enable us to draw conclusions about the potential clinical efficacy of engineered T cells (Mukherjee et al., 2017). As a result, the confocal imaging of the immunological synapse formed between CLTX-CAR T cells and PBT106 tumor cells showed morphological changes in key structural components thus suggesting activity and potential clinical efficacy against GBM. In terms of CLTX-CAR T cell activation, endogenous and engineered T cell receptors aggregated towards the synaptic interface and co-localized with cytotoxic granule markers, which indicate the initiation of cytotoxic activity. Additionally, significant cytoskeletal rearrangements characterized by actin accumulation at the synaptic interface and submembrane actin ring indicate cytotoxic activity through their role in facilitating cytotoxin release. The Microtubule Organizing Center (MTOC) supports this cytoskeletal rearrangement, and its polarization towards the synaptic interface further indicates effective cytotoxic activity. Finally, the visualization of cytotoxic granule release into the synaptic cleft between the CLTX-CAR T and tumor cells clearly demonstrates cytotoxic activity.

Aside from potentially predicting clinical efficacy of the CLTX CAR T cell, the imaging data containing CLTX-CAR T and PBT106 cells provided important insight into the CLTX

binding mechanism. Research suggests that the membrane-associated metalloproteinase-2 (MMP2) is a critical component for CLTX binding and promotes inhibits GBM cell migration and invasion. However, it remains unclear whether this binding interaction of CLTX and MMP2 is direct or indirect, since other receptor proteins (ClC-3, MMP14, neuropilin-1, and annexin-A2) have also been shown to play a role in CLTX binding and function (Wang et al., 2020). Thus, this study aimed to clarify the binding mechanism of the CLTX-CAR T cell to better understand its anti-tumorigenic effects against GBM. The results of this study are consistent with previous studies, demonstrating MMP2 as a binding agent for CLTX along with the association of MMP14. The co-localization of MMP2 and MMP14 created areas for CLTX-CAR T cell binding which is consistent with the current hypothesis of the binding mechanism.

Interestingly, MMP2 was visualized in some but not all of the CLTX-CAR T cells, suggesting a possible selective endocytosis of receptors during repeated cell contacts. This is particularly fascinating because not much is known about the serial killing mechanism of the CLTX-CAR T cell, thus the presence of MMP2 receptors in CLTX-CAR T cells could have an important role in T priming and/or exhaustion, two important factors influencing effective cytotoxicity. Since the binding mechanism of CLTX-CAR T cells is thought to involve MMP2 co-localized with MMP14 and/or other molecules in the target tumor cell membrane, it is not clear why MMP2 but not other potential components of the CLTX-CAR T cell receptor are not also identified. Thus, future research is needed to determine whether this MMP2 expression in CLTX-CAR T cells is a result of selective uptake or de novo expression as well as to evaluate the mechanistic and functional role of the possible MMP2 uptake.

Future experiments will characterize the CLTX-CAR T cell binding, priming and exhaustion mechanisms through the formation of the immunological synapse. Comparative studies of immunological synapse structure between CLTX, L13Ra2 and EGFRvII targeted CAR-T cells will demonstrate differences in cytotoxicity and potential clinical efficacy. These comparative studies will allow for the optimization of these CAR T cell therapies currently being used in GBM clinical trials and thus improve clinical outcomes. Additionally, CLTX-CAR T binding assays will also provide insight into the complex binding mechanism of the CLTX construct allowing for more effective targeting of GBM. Lastly, we hope to resolve unanswered questions pertaining to the potential selective MMP2 expression in CLTX-CAR T cells. Future research to evaluate the basis of this expression will help continue our understanding on CAR T priming and exhaustion. Therefore, continued research into the binding and cytotoxic action of CLTX-CAR T cells is imperative to its clinical application in patients with GBM.

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