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What costimulatory domains in chimeric antigen receptor T cells induce the strongest killing response and increase serial killing efficacy against pediatric rhabdomyosarcomas and osteosarcomas?

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Senior Thesis Research Proposal:

What costimulatory domains in chimeric antigen receptor T cells induce the strongest killing response and increase serial killing efficacy against pediatric rhabdomyosarcomas and osteosarcomas?

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Introduction

Pediatric osteosarcoma and rhabdomyosarcoma are two of the most common malignant musculoskeletal tumors in children. Osteosarcomas make up roughly 2% of childhood cancers and 3% of childhood cancers are rhabdomyosarcomas (American Cancer Society). Currently, common treatments for pediatric osteosarcomas and rhabdomyosarcomas include surgery, chemotherapy, and radiotherapy. These treatments have varying levels of effectiveness and can also have adverse side effects. The side effects of the current treatments include infection, nausea, vomiting, fever, pain, and exhaustion. Immunotherapies such as chimeric antigen receptor (CAR) T cell therapy have gained the interest of researchers as a potential treatment for various cancer types, including pediatric osteosarcoma and rhabdomyosarcoma (Kulemzin, S. V., et al. 2017). CAR T cell therapy consists of genetically modified T cells altered to encode a receptor that identifies and attacks cancer cells (Barret, D. M., et al. 2014).

Naturally, the immune system is capable of attacking cancer cells to a certain extent, just as they do with other foreign invaders such as viruses and bacteria (Kosti, P., Maher, J., Arnold, J.. 2018). T cells, a vital component of the immune system, have receptors on their surface which bind to the antigens found on cancer cells and pathogens. Cancer cells, however, can adapt to this and defend themselves either by exhausting the T cells so they no longer function properly or by various methods of blocking antigen recognition. The development of CAR T cell therapies has created an override to the defense mechanisms of cancer cells by enhancing the abilities of the immune system to recognize and destroy cancer cells (Jindal, V., et al. 2018).

There are several components to CAR T cells that are necessary to their function. The two most important structural features of CARs are the antigen recognition domain and the signaling domain. The antigen recognition domain allows CAR T cells to recognize cancer cells by using a

single-chain variable fragment (scFv) taken from antibody heavy and light chains or the extracellular domain of a receptor that recognizes tumor associated antigens (Kershaw et al. 2013). To activate the T cells after tumor cell recognition, most CARs utilize the CD3 zeta chain (CD3z) of the T cell receptor (TCR) complex as the main signaling domain (Kershaw et al., 2013). This builds the foundation for the signaling domain structure of CAR T cells and it has been found that all CARs require the CD3z signaling domain for CAR T cell function (Kershaw et al., 2013). Extra costimulatory domains derived from various molecules can also be added to the CD3z chain to increase efficacy in vivo. CARs that only contain the CD3z chain without costimulatory domains are referred to as first generation CARs. Adding one or more intracellular costimulatory domains to create second, third, and fourth generation CARs increases T cell activation, proliferation, persistence, and cytokine production. The basic structure of CAR T cells and the addition of costimulatory domains can be seen in Figure 1.

There are several costimulatory domains that are currently being studied for use in CARs. Some of these costimulatory domains are CD28, 4-1BB, Dap10, GITR, and OX40. Each costimulatory domain acts differently and may induce a unique response (Weinkove, R., et al. 2019). Preclinical experiments have found that while CARs with a CD28 domain have higher cytokine release than those with 4-1BB or Dap10 domains, these three costimulatory domains induce similar tumor killing activity in vitro (Kintz H., et al. 2020; Cappell, K., and Kochenderfer, J. 2021). However, CARs with 4-1BB or Dap10 domains induce longer T cell survival in vivo compared to CD28 costimulatory domains. Other studies have found the incorporation of a GITR domain increases secretion of the proinflammatory cytokine IFN-gamma (Golubovskaya, V., et al. 2018). OX40 domain containing CAR T cells have also been found to be effective specifically

in their ability to enhance the induction of immune memory responses and T cell survival in vivo (Tan, J., et al. 2022).

One major effector function that CAR costimulatory domains alter is killing of tumor cells. Perforin, granzymes, and Fas Ligand (FasL) are three major proteins involved in the CAR T cell killing mechanisms (Benmebarek, M., et al. 2019) (Figure 2). Perforin functions by creating pores in the tumor cell membrane which disturbs homeostasis ultimately leading to cell death (Osińska I. et al. 2014). Granzymes (Granzyme A and B) are a type of enzyme found in T cells that can induce apoptosis and therefore can kill tumor cells (Chowdhury, D., and Lieberman, J. 2009). Usually, perforin and granzyme work together because after perforin is released, granzymes can then enter the tumor cell and induce apoptosis and cell death (Trapani, J. 2001). FasL also contributes to a T cell's ability to kill tumor cells by triggering apoptosis upon stimulation of the Fas receptor on tumor cells (Volpe, E. et al. 2016). In addition, once a T cell enters the site of the tumor, it needs to be able to effectively kill one tumor cell after another in a process called serial killing. Previous research has shown that CAR T cells that contain the Dap10 or 4-1BB costimulatory domains may be better at serial killing and this would likely result in better overall anti-tumor efficacy in patients (Kintz H., et al. 2020). Changing the costimulatory domain that is included in the CAR T cell may have a positive or negative effect on these tumor killing mechanisms (Campana, D., Schwarz, H., Imai, C. 2014) and maximizing the activity of these killing mechanisms is essential for increasing CAR T cell efficacy. Furthermore, combining costimulatory domains may help further increase the killing efficacy of CAR T cells (Lynch, A., et al. 2017).

While CAR T cell therapies have shown great success in treating hematopoietic malignancies including leukemia, lymphoma, and multiple myeloma, it is proving to be more

difficult to target solid tumors, including pediatric osteosarcoma and rhabdomyosarcoma, with CAR T-cell therapy. Some of the reasons for this are that tumor associated antigens that can be targeted on solid tumors are also commonly found on healthy tissues (Wang, Y. D. et al. 2017; Makni-Maalej, K., et al. 2023), T cells are unable to reach the tumor site, and the immunosuppressive environment of solid tumors (Parriott, G., et al. 2020). To combat this, the chimeric PD1 receptor (chPD1) was developed because many solid tumors express PD1 ligands. Targeting the PD1 ligands on the surface of cancer cells may resolve the issue of finding a unique tumor associated antigen but the other obstacles for treating solid tumors with CAR T cells remain. The addition of costimulatory domains to chPD1 could potentially assist in the trafficking of T cells to the tumor site, increasing *in vivo* persistence, reducing the inhibition encountered in the immunosuppressive tumor environment, and maximizing the release of cytotoxic proteins and induction of tumor cell killing (Kintz H., et al. 2020).

Specific Aims

While it is clear that each costimulatory domain in a CAR T cell induces a unique set of anti-tumor mechanisms, which costimulatory domain is ideal for maximum cancer killing is still unknown. Furthermore, it is important to develop new CAR T cell therapies that efficiently kill pediatric osteosarcoma and rhabdomyosarcoma tumor cells. Therefore, the specific aims of my Longwood Senior Thesis project are as follows:

> *Aim 1*: How do costimulatory domains (CD28, 4-1BB, Dap10, GITR, and OX-40) compare to one another in their induction of the expression and release of cytotoxic proteins against pediatric rhabdomyosarcomas and osteosarcomas?

Aim 2: How do costimulatory domains affect serial killing efficacy of CAR T cells against pediatric rhabdomyosarcomas and osteosarcomas?

MATERIALS AND METHODS

Student researchers in the lab of Dr. Amorette Barber often complete the following procedures successfully. Appropriate precautions and techniques will be performed to promote sterility of samples. Lab safety protocols will be followed at all times.

T cell Transduction: Frozen, naïve splenocytes from C57BL/6 mice will be thawed and stimulated with ConA for 18 hours in a 25 cm^2 flask. T cells will be genetically engineered to express chPD1 receptors containing different costimulatory domains either CD28, 4-1BB, Dap10, GITR, and OX40. T cells will also be transduced to express a wtPD1 receptor as a control. T cells will be expanded with 25 IU/mL of IL-2 for an additional 6 days.

T cell Viability and Proliferation assays: T cell survival will be measured using a Lactate Dehydrogenase (LDH) release assay (Pierce) according to the manufacturer's instructions. This colorimetric assay measures the release of the enzyme lactate dehydrogenase (LDH) that is released upon cell death. T cell proliferation will be measured using an MTT cell proliferation assay (Promega) according to the manufacturer's instructions. This colorimetric assay measures mitochondria metabolism and cell proliferation.

Cytotoxicity assay: T cells will be cultured with pediatric osteosarcoma and rhabdomyosarcoma cell lines (K7, K7mt WT, M3-9-M, and Rh5) for 6 hours and killing of tumor cells will be measured using an LDH release assay (Pierce) according to manufacturer's instructions.

Serial Cytotoxicity assay: T cells will be cultured with pediatric osteosarcoma and rhabdomyosarcoma cell lines (K7, K7mt WT, M3-9-M, and Rh5) for 6 hours and killing of tumor cells will be measured using an LDH release assay (Pierce) according to manufacturer's instructions. To analyze the ability of chDP1 T cells to serially kill tumor cells, T cells will be restimulated by culturing T cells without tumor cells for 7 or 14 days and then used in a second cytotoxicity assay.

Detection of proteins involved in tumor cytotoxicity: T cells will be cultured with pediatric osteosarcoma and rhabdomyosarcoma cell lines (K7, K7mt WT, M3-9-M, and Rh5) for 6 hours and levels of cytotoxic proteins will be analyzed. Expression of FasL on the surface of T cells and perforin and granzyme inside T cells will be analyzed by flow cytometry. Recent release of perforin/granzyme granules will be measured by detecting LAMP1/CD107a on the cell surface using flow cytometry and by using perforin and granzyme specific ELISAs. Flow cytometry and ELISAs measure the presence of proteins on the cell surface or that have been secreted, respectively.

Statistical analysis: Samples will be analyzed in triplicate and will be repeated using T cells from three separate mice. This will ensure biological reproducibility and the ability to calculate statistical significance.

TIMELINE

FALL 2023	
Week 1-5	Transduction of T cells, proliferation, viability, and cytotoxicity assays
Week 6-9	Serial killing assays
Week 10-13	Tumor cytotoxicity protein assays
Week 14-15	Data analysis/ Progress report
SPRING 2024	
Week 1-3	Data analysis
Week 4-5	Preparation of poster for presentation at a conference
Week 4-12	LST thesis writing
Week 14-15	Oral defense preparation

Thesis Defense Committee Members

Dr. Wade Znosko (Department of Biological and Environmental Sciences)

Dr. Bjoern Ludwar (Department of Biological and Environmental Sciences)

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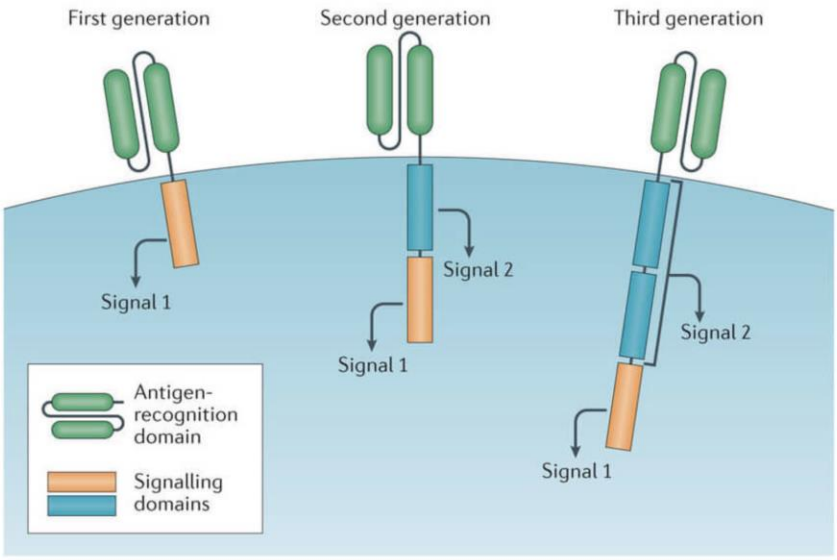

Figure 1. Chimeric Antigen Receptors. (Brentjens R, et al. 2016)

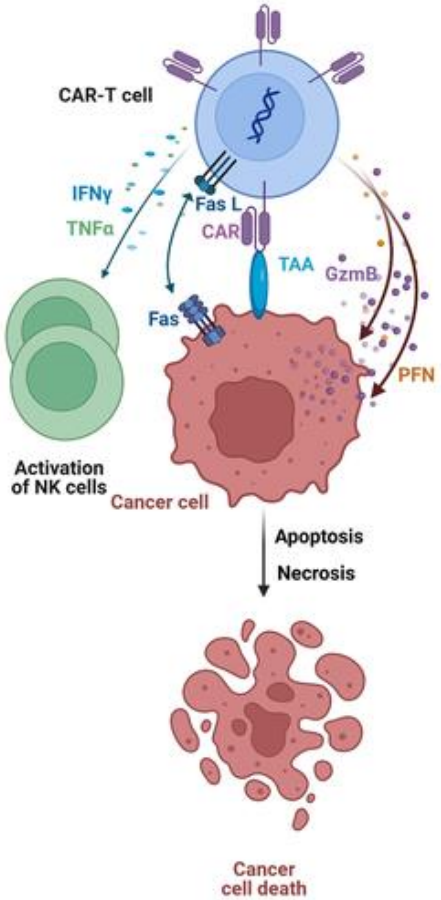

Figure 2. Killing mechanisms of CAR T cells. (Makni-Maalej, K, et al. 2023)

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