Comparison of CAR-T cell generations for the treatment of glioblastoma by tumor cell reduction

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Abstract

Currently, glioblastoma is one of the deadliest forms of brain cancer due to its location in the nervous system and high rate of recurrence. There have not been many effective treatments developed to combat glioblastoma. Immunotherapies such as CAR-T cell therapy hold promise as a potential treatment for solid tumors due to past successes in treating liquid tumors. However, due to the nature of glioblastoma, there are still adjustments to be made in order for CAR-T cell therapy to become a successful treatment. This study analyzed bioluminescent data from xenograft mouse models of glioblastoma in order to compare the effectiveness between 2nd-generation and 3rd-generation CAR-T cells in reducing tumor volume. The results suggested that 3rd-generation CAR-T cells were more effective than 2nd-generation CAR-T cell treatments in these preclinical models. Because these results are not a full representation of CAR-T cells in a clinical setting, further research must be conducted.

Introduction

Glioblastoma

Glioblastoma multiforme (GBM), also called glioblastoma, is the most common and aggressive type of malignant glioma, or brain and spinal cord tumor. Gliomas represent 80% of all malignant primary brain tumors in adults (Maxwell et al., 2017). In the United States, there are about 6 glioma cases per 100,000 people, with glioblastoma consisting of 50% of the cases (Lim et al., 2018). Tumors commonly originate from mutations in glial stem cells and persist due to immune dysregulation or similarities between the tumor cells and healthy cells (Bagley et al., 2018; Maxwell et al., 2017; Lim et al., 2018). This combination of stem cell mutations and

immune dysregulation creates an immunosuppressive environment that makes it difficult for the immune system to detect tumor cells and initiate a response. Once affected, patients experience a poor quality of life due to neurologic deficits, personality changes, delirium, seizures, and headaches; as well as nausea, vomiting, and bowel and bladder problems. At best, they can receive symptom control through drug treatments that mostly alleviate pain and seizures (Oberndorfer et al., 2008).

Patients with GBM experience high mortality rates and poor prognosis despite treatment (Oberndorfer et al., 2008). The most common chemotherapy treatment is Temodar (temozolomide), which destroys GBM tumors by damaging DNA to trigger cell death (Young et al., 2015). One study demonstrated a 10.4% improvement in 2-year overall survival with radiotherapy and 26.5% improvement with chemoradiotherapy using Temodar (Stupp et al., 2005). However, others question whether the improvements are due to the intervention or a result of the intensive care provided to patients who were a part of the trial (Lim et al., 2018).

According to the 2017 European Association for Neuro-Oncology, the goal of surgical treatments is to entirely remove the tumor without harming too many healthy cells (Lim et al., 2018). Surgical methods of treatment, including but not limited to needle biopsies, awake craniotomies, and standard craniotomies, are associated with longer survival time, but quality of life remains poor despite the large amounts of funding, preparation, and cooperation invested in treatment (Mauer et al., 2015).

Unfortunately, these standard-of-care treatments using radiotherapy, chemotherapy, and surgery are ineffective against most GBMs. The treatments typically result in off-target toxicities, such as damage to healthy nerves or lowered white blood cell counts. Additionally,

treatments are not accurate and miss target cells, thus leading to tumor recurrence (Choi et al., 2018; Mirzaei et al., 2017). These off-target effects are of greater concern in the context of gliomas, as nerve tissues are more difficult to replace compared to other cells, such as endothelial cells. Due to the high toxicity profile and low accuracy of current treatments, GBM is described as incurable (Bielamowicz et al., 2017).

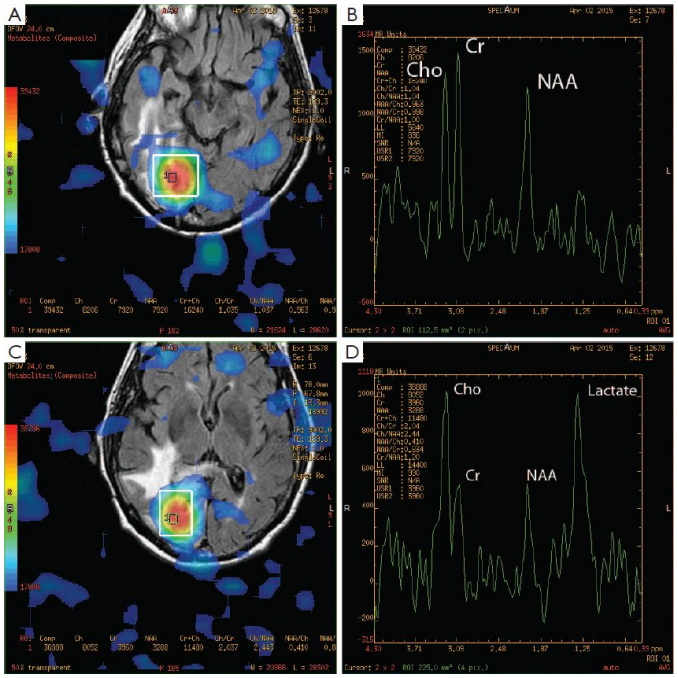


Figure 1. Example of an area of interest detected through MR spectroscopy, which denotes a possible glioblastoma tumor. Retrieved from Young et al., 2015

CAR-T cell therapy

Immunotherapy is an emerging form of therapy that utilizes the immune system to target and attack cancer cells. This treatment is potentially more effective than current methods, as it is not localized and may be able to detect cells that are missed by past treatments. One type of immunotherapy is chimeric antigen receptor (CAR)-T cell therapy, which involves immune

system cells that are engineered to target specific tumor cells by detecting protein receptors expressed on the outside of tumor cells.

CAR-T cell therapy relies on the nature of the adaptive immune system, which consists of T and B lymphocytes. The T lymphocytes, or T cells, are divided into either cytotoxic CD8+ T cells, which directly lyse damaged or target cells, or T helper CD4+ cells, which control the rest of the immune response (Wilcox et al., 2018). In a typical immune response, the immune system recognizes antigens expressed by damaged or harmful cells through the B cells and T cells. The main difference between these lymphocytes is that B cells can secrete receptors, whereas the T cells must present them on the cell surface. Additionally, T cells require other cells to present the antigen through major histocompatibility (MHC) molecules to activate and recognize the molecule on other cells. Once activated, the T cells can then attack cells with the target antigen or help activate other immune cells (Actor, 2014).

The process of engineering CAR-T cells involves obtaining the patient's own blood cells and modifying them to express a recombinant receptor called a chimeric antigen receptor (CAR) (see Fig. 2). A CAR is composed of an extracellular single-chain variable fragment (scFv) fusion protein, a transmembrane domain, and an intracellular cytosolic signaling domain CD3 chain, which essentially combines the T cells with antibody-like abilities to recognize antigens (Maxwell et al., 2017; Finocchiaro & Pellegatta, 2014). The most important aspect of this structure is that it allows for the CARs to recognize antigens independently from the MHC (Yong et al., 2017). This is important as patients with different MHCs may have different responses if the treatment relies on antigen presentation (Sadelain et al., 2018). Once the CAR-T cell identifies the antigen target, it activates an immune response that leads to cytokine release,

cytolytic degranulation, and T-cell proliferation, which are all processes involved in killing the tumor cells (Bagley et al., 2018).

Engineered CAR-T cells are potentially more effective than other immunotherapies in cancer treatment such as vaccines since CARs do not require antigen presentation and stimulation of the MHC (O'Rourke et al., 2017). Another type of immunotherapy uses transduced T-cell receptors (TCR), which are similar to CAR-T cells. One benefit of using TCRs is that they can be used to target intracellular receptors, which CAR-T cells cannot (Sadelain et al., 2018). However, TCRs have limited effect against antigens that avoid MHC presentation, such as those found on certain tumors (Yong et al., 2017).

CAR-T cell therapy has undergone much development, beginning with the first-generation CARs that consist of a singular CD3ζ (a protein that allows for T cell activation and cytotoxic function). Next, second-generation CAR-T cells have an added a co-stimulatory domain such as CD28, which are usually found on antigen-presenting cells rather than T-cells. Thus, they allow the newly engineered T-cells to induce full T-cell activation, target killing, and long-term persistence (Abate-Daga & Davila, 2016). Third generation CARs typically include an additional co-stimulatory domain (Maxwell et al., 2017; Yong et al., 2017). Although performing much better than first-generation CARs, there is still room to improve the technology.

Currently, CAR-T cells have been FDA approved for treating two liquid tumors: leukemia with Kymriah, approved on August 30, 2017, and lymphoma with Yescarta and Kymriah, approved on October 22, 2017 and May 1, 2018, respectively. Researchers have already conducted studies that investigate the success of CAR-T cell treatments in other various clinical studies, such as neuroblastoma and multiple myeloma (Yong et al., 2017).

Certain characteristics in CAR-T cells give them better potential to treat GBM rather than other methods. As mentioned previously, CAR-T cell therapy allows for tumor targeting without needing the MHC complex, thus allowing it to seek out missable target cells (Yong et al., 2017; Sadelain et al., 2018). Additionally, CAR-T cells can cross the blood-brain barrier, unlike many drug treatments, allowing them to access both the brain and the rest of the body (Bagley et al., 2018). This is especially important for targeting multiple tumor sites or administering CAR-T cells through the veins because it is more effective in reducing tumor growth than directly through the tumor (Mirzaei et al., 2017; Brown et al., 2016).

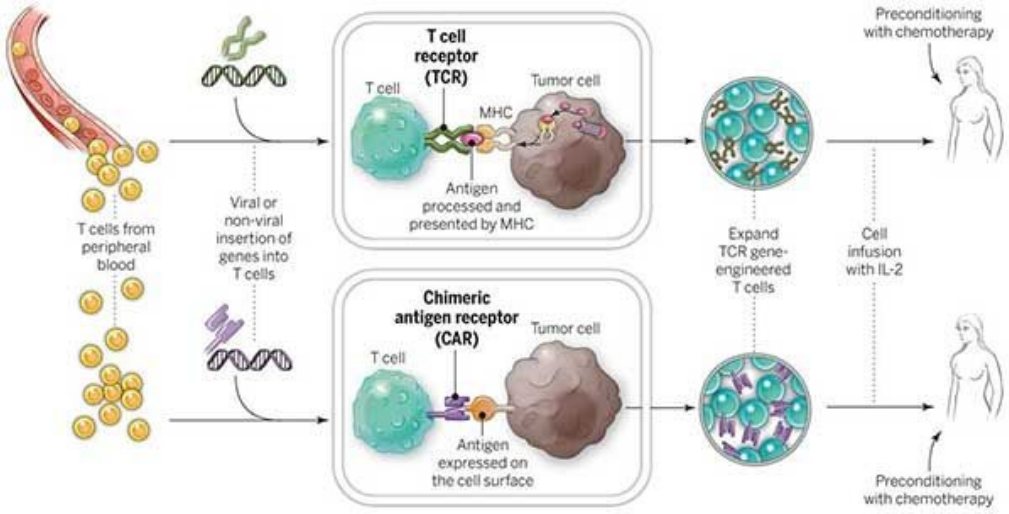


Figure 2. Process of generating a TCR or a CAR. Genetic material is inserted into the extracted T cells, then allowed to proliferate until ready to infuse within the patient. The type of receptor depends on the genetic material inserted. Retrieved from "CAR-T Cells," 2017

Challenges of CAR-T Cell Therapy

However, there are multiple challenges in treating solid tumors with the same therapy in a clinical setting due to a hostile tumor microenvironment, T cell trafficking difficulties, and heterogeneous gene expression. These difficulties can result in off-site toxicity from the immune response or the recurrence and persistence of the tumor, causing the therapy to become

ineffective or even detrimental to the patient. The issue becomes more concerning due to the high cost and preparation necessary to perform the treatment (Mirzaei et al., 2017).

As mentioned before, one of the major concerns with CAR-T cell therapy for GBM is heterogeneous gene expression. Different types of receptors expressed on GBM tumors include mutation associated neoantigens (MANA) and tumor-associated antigens (TAA). Epidermal growth factor receptor variant III (EGFRvIII) mutations are the most common MANA, which means that they are antigens unique to GBM and not presented on healthy cells. Dutoit et al. estimate that it can be found in about 20-50% of GBMs, whereas Mirzaei et al. estimate it to be expressed in ~30% of glioma cells (2016; 2017). Its frequency is likely due to how the EGFRvIII mutation interferes with the epidermal growth factor (EGFR) pathway. This pathway is linked to colorectal cancer, non-small cell lung cancer, and pancreatic cancer, thus linking EGFRvIII to oncogenesis in GBM (Westphal, Maire, & Lamszus, 2017). The other common targets are TAA such as human epidermal growth factor receptor 2 (HER2), interleukin-13 receptor α2 (IL-13Rα2), and ephrin type-A receptor 2 (EphA2), which are merely overexpressed in GBM tumor cells (Choi et al., 2018; Krenciute et al., 2017). They can still be found, although in fewer amounts, on healthy cells. Their lack of specificity may cause very sensitive CAR-T cells to target healthy cells possessing these antigens. Even so, they are still potential candidates for antigen targeting, as they differentiate tumor cells from normal cells. Another notable aspect of many antigen targets, such as IL-13Rα2, is that they are commonly an indicator of poor patient prognosis when expressed on the tumor (Mirzaei et al., 2017). However, much of the research investigating both types of targets note that despite their initial successes, it is difficult to create a specific response and avoid antigen escape at the same time, as avoiding more healthy cells

increases the risk of missing tumor cells (Jena, Dotti, & Cooper, 2010). There are existing methods to make CAR-T cells more precise and effective, such as by targeting multiple antigens at once, but there have not been many studies discussing their extent (Maxwell et al., 2017).

CAR-T cells may also indirectly trigger adverse responses through the immune system. The most concerning of these responses is cytokine release syndrome (CRS), which causes fever, tachycardia, vascular leak, oliguria, hypotension, and even multiorgan failure. CRS results from a hyperactivated response of immune cells within normal tissues. A similar immune reaction is an autoimmune reaction, where the immune cells target and attack normal tissues (Weber et al., 2015). These are indirect responses because they result from the rest of the immune system responding to the initial CAR-T cell signal rather than the actions of the CAR-T cells themselves. Fortunately, many of these symptoms can be managed through anti-inflammatory drugs or prevented by adding suicide genes to CAR-T cells, which would kill the cells before potential toxicity (Dutoit et al., 2016).

Purpose

The purpose of this study is to investigate CAR-T cell therapy as a viable option for the treatment of GBM based on results of studies using xenograft mice models by comparing the effectiveness between two generations of CAR-T cells. In the past, GBM has been difficult to treat using conventional methods of surgery, radiation, and chemotherapy. Because immunotherapy is a systemic therapy that has the potential to remove tumors undetected by past treatments, it could provide a novel form of treatment for gliomas and other cancers that are difficult to treat. To date, there are not many studies comparing the direct efficacy of each

generation of CAR-T cells. This paper will evaluate the performance of CAR-T cells targeting the same target receptor but different generations.

This study carries importance because it aims to make CAR-T cell therapy a more viable approach for solid tumors than immunotherapies in the past. As such, it will also bring hope to other areas of CAR-T cell research that are focusing on solid tumors or cancers with heterogeneous antigen expression. Additionally, GBM has a high mortality rate and is difficult to treat with current methods, so CAR-T cell therapy may prove to be more effective or efficient in eliminating tumor cells. In comparison with the current methods of treatment, CAR-T cell therapy has the potential to greatly improve patient outcome, but they must be optimized in preclinical studies in order to determine its effectiveness to treat glioblastoma patients.

Research Question

The questions used for this study is the following: Using a xenograft mouse model and targeting one target antigen, which method for CAR-T cell therapy of GBM will result in the greatest decrease in tumor cell count? To answer this question, this study will conduct a statistical analysis on the percentage of tumor reduction from multiple studies covering multiple generations of CAR-T cell therapy. Based on the results, a conclusion will be drawn regarding the efficacy of CAR-T cell therapies in reducing glioblastoma cells.

Hypotheses

Alternative Hypothesis

Considering that newer generation CAR-T cells have a greater number of co-stimulatory domains than older generations, newer generation CAR-T cells are hypothesized to perform significantly better than past generations in reducing GBM tumor volume.

Null Hypothesis

There is no difference between the different CAR-T cell generations in reducing GBM tumor volume. This would suggest that the added co-stimulatory domains have little to no effect in improving the antitumor response.

Methods

Data Sources

A systematic literature review was conducted using data published in peer-reviewed papers detailing experiments using CAR-T cell therapy to target GBM tumor cells. A literature review involves collecting data from multiple peer-reviewed sources and utilizing statistical analysis to calculate trends and make predictions, making it most suitable for this study. Statistical analysis was done through Microsoft Excel to find the mean and standard deviation of CAR-T cell performance. The data could not be collected through an experiment due to the limited time and lack of resources at a high school level. A survey would not have been able to collect quantitative data on the effectiveness of CAR-T cells targeting GBM cells even if patients could be contacted in the first place by a high school student. The research was too recent and specific to use secondary sources as the main source of data for analysis.

Papers were gathered primarily from PubMed, EBSCOhost, and Google Scholar. Initially, papers were found using keywords such as “CAR-T cell” “chimeric antigen receptor,” “chimeric antigen receptor T cell,” “glioblastoma,” “glioblastoma multiforme,” etc. The papers were then cross-referenced to find additional literature through the reference sections.

Criteria

The statistical analysis focused on full-text research involving xenograft animal models specifically discussing antigen expression and tumor size reduction. Papers were not strictly excluded based on year, as CAR-T trials are fairly recent and thus are all pertinent. Systematic reviews, meta-analyses, clinical trials, and *in vitro* studies were utilized as a reference, but were not included in the statistical analysis.

The evaluation was not based on the tumor microenvironment as there are many specific variables within the microenvironment that carry significance but do not have enough research material to study individually at a high school level. Additionally, the research did not use animal cell lines of GBM, as they do not fully represent the heterogeneity of human GBM antigens, an important aspect of immune evasion in GBM, and make the analysis too broad (Morgan et al., 2012). Based on the results, if there was a clear pattern of certain antigens being more effective targets than others, certain methods were hypothesized to increase efficacy, such as targeting multiple antigens at once (Pituch et al., 2018).

Papers were excluded if they did not contain quantitative data on the change in tumor size. This was represented by using bioluminescence measurements as a surrogate for tumor volume. Initially, when researchers xenograft the tumor cells into a mouse, they attach

bioluminescent proteins to the cells. After allowing the tumor to proliferate, they measure the bioluminescent intensity (photons/sec) of the mice. By continually imaging the mice over a certain period of time, increases and decreases of tumor volume can be calculated using the bioluminescent data obtained.

There were also criteria used to arrange eligible papers (see Table 1). After being determined as eligible, studies were organized based on the methods used to cultivate CAR-T cells and to conduct experimentation. This was due to the possibility of confounding variables due to differences methodologies. Although all variables listed were important to reduce confounding variables, more emphasis was placed on the methods used to generate CAR-T cells, as their growth *in vitro* greatly affects their performance within a xenograft model.

Table 1. Criteria for categorizing papers. Certain parameters, such as the type of study and the CAR-T generation, were used as strict criteria for the paper's eligibility. Others were used to arrange studies of similar methodologies with each other for comparisons.

	Examples
Type of study	Animal model, xenograft, in vitro
Tumor cells	U373, Patient,
Blood samples	Healthy donors, GBM patients
Generation of CAR-T	1st, 2nd, 3rd (4th in dev)
Co-stimulatory domain	OX40 (TNFRSF4), ICOS, CD28ζ, 4-1BB (CD137)
Target receptor	EGFRvIII, HER2, IL13Rα2, EphA2
Type of lymphocyte	CD4, CD8

Statistical Analysis

In this study, data was collected by obtaining quantitative bioluminescence data from studies analyzing tumor reduction due to CAR-T cell therapy. The independent variable in question was the generation of CAR-T cell and the dependent variable is the tumor reduction.

Most studies used three groups of mice: a control group with tumor cells and phosphate buffered saline (PBS), a control group with tumor cells and untransduced T-cells (UTD), and a treatment group with tumor cells and engineered CAR-T cells.

In order to standardize the units, the bioluminescence data was taken and calculated using percent treatment/control values (%T/C) using the following formula:

$$\%T/C = 100 \times T/\Delta C \text{ if } \Delta T \geq 0$$

$$\%\text{ Regression} = 100 \times T/T_{Initial} \text{ if } \Delta T < 0$$

T=Tumor volumes of UTD/CAR-T group on final day

$T_{initial}$= Tumor volumes of UTD/CAR-T group on initial day

$\Delta T = T - T_{initial}$

C = Mean tumor volume of PBS control group on final day

ΔC = Mean tumor volume of PBS control group difference between final and initial days

This conversion was necessary because different studies may use a differing amount of tumor cells when conducting xenograft experiments. Additionally, if the ΔT was negative, it would signify a decrease in tumor volume as a result of the treatment (Johnson et al., 2015).

Using these units, one-tailed two-sample t-tests were run using Google Sheets, then repeated using a TI-89 Titanium calculator for verification. A t-test was used rather than the Wilcoxon-Mann-Whitney rank sum test, which has commonly been used in previous studies to compare the UTD control groups and CAR-T cell treatment groups. This is because the rank-sum test may state that there is a statistically significant difference between the means of the groups if there are differences in distributions and shape even if the means are not actually different. This would create a false positive in the results (Bartlett, 2014; Wild & Seber, 2000).

In all tests, p-values were calculated to determine whether results were significant and whether to reject the null hypothesis. If a p-value $\leq .05$, there was strong evidence suggesting a difference between the two groups and the null hypothesis was rejected.

Results

Search results yielded more than 35,000 studies discussing GBM and more than 100 pertaining to both GBM and CAR-T cell therapy. Ultimately, after screening each study's abstract, 19 were considered eligible for the study, two of which were used (see Fig. 3). These articles were chosen as they detailed CAR-T cell therapy for GBM and evaluated the tumor reduction of each treatment within a xenograft model. Finally, three articles could be used in data analysis based on the availability of raw data tables. However, one paper had significant deviations from the other two, such as the different target receptors, tumor cell origins, and engineering processes, which ultimately led to it being excluded from the statistical analysis (Wang et al., 2018).

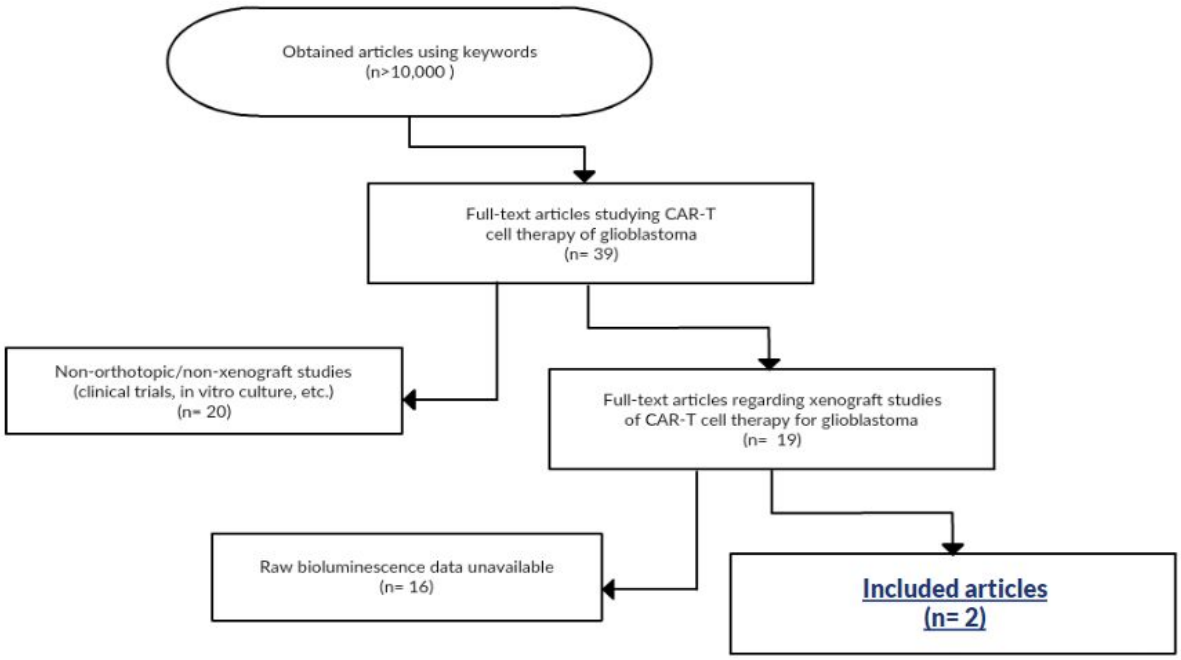


Figure 3. Flow diagram of article selection process for data analysis

Article Comparison

The two included studies, conducted by Johnson et al. (2015) and Miao et al. (2014) were selected due to their similarities in method and access to raw bioluminescence data, provided by original authors through direct contact or published supplementary data tables. Both studies conducted an orthotopic xenograft mouse model of a GBM treatment, then measured the bioluminescence data. In both studies, researchers used T-cells derived from the blood of healthy donors and used EGFRvIII as their potential target. To conduct the xenograft model, the studies obtained NOD scid gamma (NSG) mice, which were immunodeficient mice that could not produce their own T and B cells. The tumor was inserted through the cranium of the mouse, and after a few days of incubation, the PBS or CAR-T cells were injected intravenously through the tail.

The main variable that differentiated the two studies were the generations of CAR-T cells used. Johnson et al. used a CD28 co-stimulatory domain when designing the receptor on the CAR-T cell whereas Miao et al. used both the CD28 and 41BB co-stimulatory domain. Because co-stimulatory domains enhance T cell survival, the statistical analysis aimed to investigate differences in T cell performance between the two constructs (Sadelain et al., 2018).

However, there were small differences between the study methodologies, such as the volume of initial tumor cells, volume of CAR-T cells, and duration of tumor incubation before treatment (see Table 2). For the purpose of analysis, most small differences were not accommodated for within the analysis. The possible sources of error that may arise from these confounding variables are addressed later in this paper.

Table 2. Variables considered in comparing studies. These three studies all provided raw data for statistical analysis. However, due to the differences in the study conducted by Wang et al., namely the different target antigen receptors, it was excluded from statistical analysis.

Reference	Johnson et al. 2015	Miao et al., 2014	Wang et al., 2018
Type of study	Xenograft mouse model Orthotopic Subcutaneous	Xenograft mouse model Orthotopic	Xenograft mouse model Orthotopic Subcutaneous
Tumor cells	U87 U87-EGFRvIII	U87MG/(withEGFR) D-270 (Patient)	Patients (Patient) PBT103-2 (Patient)
Blood samples	Purchased	Healthy donors	Patient samples
Generation of CAR	2nd	3rd	2nd
Costimulatory domain	4-1BB	hCD28-41BB-CD3ζ	4-1BB (CD137)
Target receptor	EGFRvIII	EGFRvIII	IL13Rα2
CD4/ CD8?	Both	Both	Both, separate
Units of measurement	BLI (originally BLI*10^5)	BLI	BLI (originally log(BLI))
Amount tumor injected	5*10^4 cells	1*10^4 cells	1*10^5 cells
Amount treatment injected	4*10^6 cells	5.0-10*10^6 cells	0.5-1.0*10^6 cells

Bioluminescent Measurements

Prior to injection, the tumor cells were transduced to express luciferase and green fluorescent protein (GFP), allowing the tumor cells to emit light as a surrogate for tumor volume. This light was then imaged and quantified as photons per second to compare the tumor amount throughout the study. The values were then converted to %T/△C in order to standardize the data in relation to the amount of CAR-T cells administered. An increase in bioluminescence and %T/△C suggested an increase in tumor volume and a decrease in bioluminescence and %T/△C suggested a decrease in tumor volume. In turn, a significant decrease in tumor volume in the treatment group but not in the control groups implied that the EGFRvIII- targeting CAR T-cells were effective in tracking and killing tumor cells.

Table 3. Bioluminescence data (photons/sec) of xenograft mice models, days after T-cell delivery for 2nd-generation CAR-T cells. This study used PBS as a control to emulate no treatment for the xenografted tumors and UTD as a control to emulate a basic human immune response without the engineered CAR-T cells. 2173 was used as the CAR-T cell treatment group. Mice were humanely euthanized as according to Institutional Animal Care and Use Committee (IACUC)–approved protocols. Retrieved from Johnson et al., 2015.

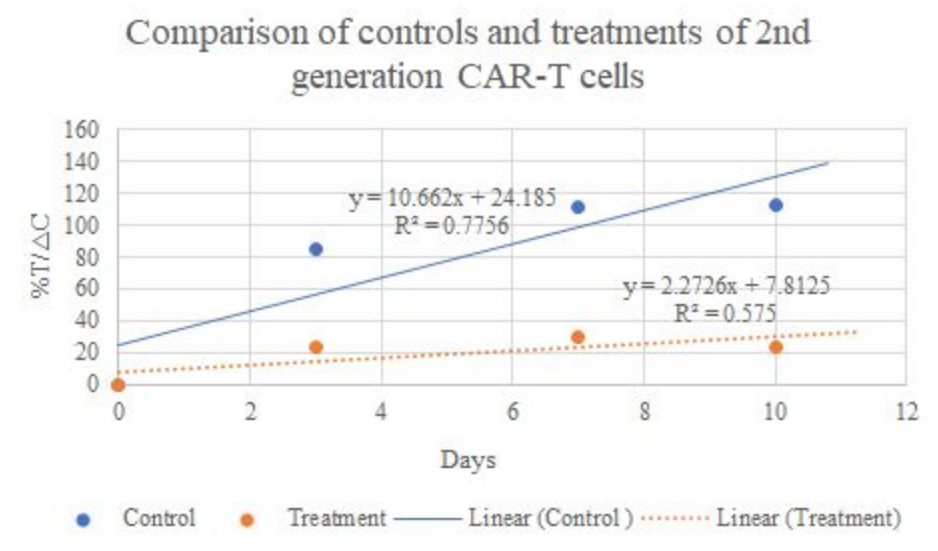
	PBS (n=9)	**UTD (n=10)**		**2173 (n=10)**	
	BLI	BLI	T/△C	BLI	T/△C
Day 0*					
AVE	429000	557500		361300	
SEM	98241.47	113802.28		66331.75	
Day 3					
AVE	2251333.33	2110369.9	85.21	781629.9	23.07
SEM	1033944	481968.43	65.46	219230.97	23.29
△C	1822333.33				
Day 7					
AVE	16567777.8	18579061	111.67	5216000	30.08
SEM	7281991.33	5485022.78	76.63	1464729.1	27.48
△C	16138777.8				
Day 10					
AVE	76262888.9	86319982.9	113.09	18225100	23.56
SEM	28423062.7	31608436.3	77.85	5162579.9	21.36
△C	75833888.9				

*The first measurements were made the day of administering the treatment, so no %T/△C data was calculated for this day.

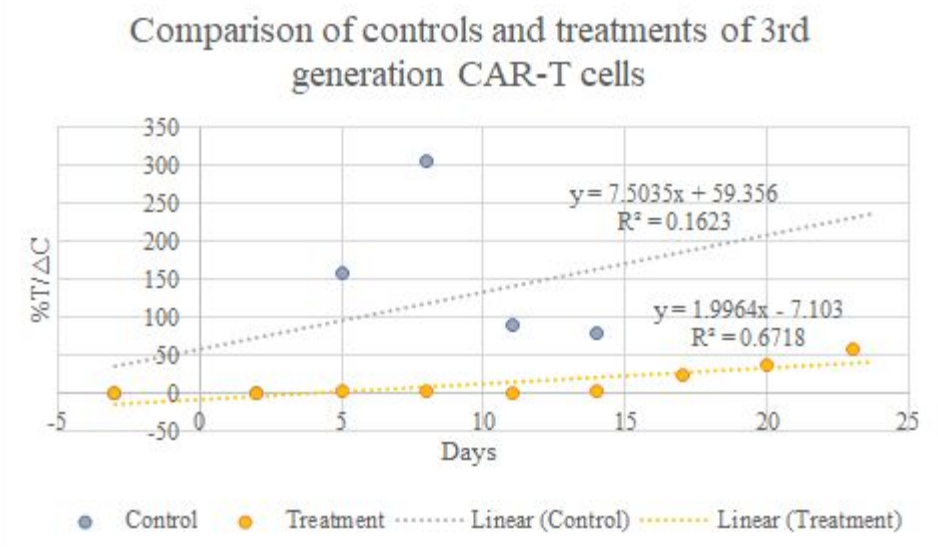
Table 4. Bioluminescence data (photons/sec) of xenograft mice models, days after T-cell delivery for 3rd-generation CAR-T cells. This study used a control group with no treatment for the xenografted tumors (n=10) and a control group with nonspecific CAR-T cells to emulate a basic human immune response without engineered CAR-T cells (n=10). EGFRvIII+ CAR-T cells were used as the treatment group (n=10). Mice were humanely euthanized as according to IACUC–approved protocols. By day 20, no living mice remained in either the untreated and control CAR-T cell groups, so there is no data from these groups. Original data retrieved from Miao et al., 2014.

	Untreated (n=10)	**Control CAR T cells (n=10)**		**EGFRvIII+ CAR T cells (n=10)**	
	BLI	BLI	T/△C	BLI	T/△C
Day -3**					
AVE	1286315	1965390		199993	
SD	914249.83	2286129.06		393295.06	
Day 5					
AVE	7887180	12402600	158.12	403762	3.08
SD	6778357.54	11962947.08	149.64	1054400.76	10.16
△C	6600865				
Day 8					
AVE	167835000	511679000	306.04	7058500	4.21
SD	75030412.69	504862962.7	301.91	13505535.5	8.31
△C	166548685				
Day 11					
AVE	1091500000	994014285.7	91.08	13131270	1.186
SD	722849761.1	426655045.8	39.09	15494743.52	1.40
△C	1090213685				
Day 14					
AVE	2787800000	2188666667	78.52	65092544.44	2.30
SD	1082768766	1779456752	63.86	92436006.01	3.30
△C	2786513685				
Day 17***					
AVE				641885000	23.03
SD				570666881.2	20.47
Day 17 △C	2786513685				
Day 20					
AVE				1047466667	37.58
SD				1241869849	44.55
Day 17 △C	2786513685				
Day 23					
AVE				1625550000	58.34
SD				1516673335	54.43
Day 17 △C	2786513685				

** The first measurements were made 3 days before administering the treatment, so no %T/△C data was calculated for this day.
*** By day 20, all NSG mice in both control groups had passed away. As such, no BLI data was available from that day onwards and %T/△C concentrations were referenced based from day 17, the last recorded values from the control mice.



a)



b)

Figure 4. Graph of quantitative data comparing the PBS control with the CAR-T cell treatment in terms of tumor growth over time using %T/△C. The control group for both studies have a visually greater increase in tumor cells than the treatment group, thus suggesting that the CAR-T cell treatment group for both studies were more effective in combating tumor cells than if it were not present. (a) The data, from Table 3, depicts the performance of 2nd-generation CAR-T cells compared to the control. (b) The data, from Table 4, depicts the performance of 2nd-generation CAR-T cells compared to the control. The treatment group also survived much longer than the control group for this experiment and both the control and treatment group in Figure 5a.

The data points from Tables 3 and 4, originating from studies conducted by Johnson et al. and Miao et al., were plotted on a scatter plot with a regression line to compare the trends of the respective treatment and control groups. Next, a one-tailed two-sample T-test was run to first compare the control and treatment of the respective studies, then to compare the difference between the 2nd-generation and 3rd-generation treatments. The %T/△C values were provided from supplementary data from Johnson et al., whereas %T/△C values from Miao et al. were calculated by raw data supplied from direct contact with the researcher.

When graphing %T/△C against time in the study conducted by Johnson et al. (see Fig. 4a), the slope was calculated for the control (10.662) and the 2nd-generation treatment (2.273). Both lines showed a positive slope, but the control group had a constant higher %T/△C value than the treatment group. Both groups of mice survived a maximum of 10 days before euthanization. A t-test was run comparing the %T/△C of the control group and 2nd-generation treatment group on day 10, as this was the final day that bioluminescent measurements were obtained before euthanization of all mice. This t-test suggested that the lower values of the 2nd-generation CAR-T cell treatment group in comparison with the control group were likely statistically significant ($p = 0.003$).

This process was repeated using data from Miao et al. (see Fig. 4b), which yielded slopes for the control (7.504) and 3rd-generation treatment (1.996). Both lines also showed a positive slope, although there was a significant drop in the control group after day 8. However, the control group of mice still had a consistently high %T/△C value in comparison with the treatment group. Before day 14, the change in tumor volume of the treatment group remained close to 0 but gradually began increasing afterward. In this study, the control group survived a

maximum of 14 days, whereas the treatment group survived a maximum of 23 days. As with the 2nd-generation treatment, another t-test was run comparing the %T/△C of the control group and 3rd-generation treatment group on day 11. Although both the control and treatment of this study had survived past this point, day 11 was the closest time to day 10, and so would be best to compare with the day 10 results of Johnson et al. The t-test suggested that lower values of the 3rd-generation CAR-T cell treatment group in comparison with the control group were likely statistically significant ($p = 0.004$).

Finally, a one-tailed two-sample T-test was run between the %T/△C of the day 10 2nd-generation CAR-T cell treatment and the day 11 3rd-generation CAR-T cell treatment. The p-value obtained suggested that the 3rd-generation CAR-T cell treatment had significantly more tumor reduction than that of the 2nd-generation CAR-T cells ($p= 0.005$).

Discussion

By looking at the average BLI and %T/△C values, it is apparent that both treatments had greater performance than their respective controls. The strange pattern in the control group of the 3rd-generation study is likely due to the mice with high tumor volumes being euthanized, and thus left mice with lower tumor volume in the group. As a result, it may seem as if both treatments showed similar performance in comparison with their controls. However, there are several distinctions that suggest a greater antitumor response in the 3rd-generation CAR-T cell treatment.

Firstly, the 3rd-generation treatment group mice survived much longer than any of the controls or treatments within both studies. Those treated with the 3rd-generation CAR-T cells

had survived 23 days, whereas others survived for 10-15 days. Since both studies used the same criteria for euthanization, this meant that mice in the treatment group had the least harm from tumor burden in comparison with other groups of mice. Although the mice were still eventually euthanized as a result of disease burden, these results supported 3rd-generation treatments as superior in terms of helping survival against GBM tumors.

Additionally, 3rd-generation CAR-T cell treatment had a lower %T/△C value (AVE= 1.19) on day 11 than that of the 2nd-generation CAR-T treatment (AVE= 23.56). After conducting a t-test to determine significance in tumor reduction, the difference was found to be statistically significant ($p= 0.005$) As such, it is likely that the lower %T/△C value was attributed to differences in treatment rather than random chance.

Sources of Error

As a high school researcher, the amount of obtainable raw data was limited. A total of 3 raw data tables were gathered by emailing the corresponding authors of various papers that studied CAR-T cell treatment of GBM. Due to this, the current research may be too limited in sample size to make accurate extrapolations from.

There are also inconsistencies within research methods. The process of delivering CAR-T cells alone can have many variables, such as the time of delivery, the location of delivery, and the amount delivered. As stated above, there was a limited number of papers, making it difficult to draw a comparison between every single variable that could possibly be compared.

One interesting detail to note is that U87MG, a common GBM cell line to use in xenograft or in vitro studies, has an unknown source (Allen et al., 2016). There had been a

misidentification of some similar cell lines in the past, and as such researchers do not know if there are other details about them that may cause the cell lines to differ from the tumor of origin. This may result in variances in results depending on the type of tumor cell line used. It is possible to argue that this makes GBM cell lines more representative of heterogeneous GBM cases, but researchers should be wary of the discrepancies between cell lines.

Conclusion

Both generations of CAR-T cells demonstrated antitumor effects in their respective xenograft model studies. This study supports the hypothesis suggesting the greater performance of 3rd-generation CAR-T cells in comparison with older generations. The differences between the regression lines and the p-values obtained from t-tests demonstrate the greater tumor reduction in 3rd-generation CAR-T cells. As a result, it suggests that the 3rd-generation CAR-T cells will potentially have a greater antitumor response in clinical settings as well, although this conclusion cannot be made without further preclinical and clinical studies.

Further Work

Further research analyzing the performance of different target receptors should be conducted under identical parameters, such as the protocol for the incubation of CAR-T cells or the form of gene transfer.

Additionally, this paper only discussed the efficacy of CAR-T cell treatment in a preclinical setting. In the future, it will be beneficial to observe the patterns in clinical trials, as this may reveal other unknown variables. Clinical trials demonstrate the possible off-target

toxicities, which are a critical factor in determining the benefits of a treatment. They may also demonstrate adjustments to make in order to use CAR-T cells as an actual treatment in clinical settings, such as the ideal amount of CAR-T cells to be administered in a human.

Currently, there are a few clinical trials that have been conducted for treating GBM with CAR-T cells. These studies have targeted various antigens, such as IL-13Rα2, HER2, and EGFRvIII (Ahmed et al., 2018; Brown et al., 2015; Brown et al., 2016; O'Rourke et al., 2017). However, all studies demonstrated a recurrence of some sort after treatment. This is likely due to the tumor heterogeneity allowing some cells without the target antigen to escape T-cell detection. As such, improvements to the co-stimulatory domains within future generations may not be enough to make CAR-T cells clinically viable to treat GBM.

In the future, it may be beneficial to investigate the efficacy of different target antigen receptors to improve not only CAR-T cells but other forms of immunotherapy as well. By targeting an antigen that is expressed on a large proportion of tumor cells, it lowers the chances of tumor recurrence. Initially, this research aimed to investigate the differences between such receptors, but not enough data was collected to justify the research and perform the statistical analysis. Currently, there are studies investigating possibilities to improve the performance of CAR-T cells by changing the targets, such as targeting multiple receptors at once (Bielamowicz et al., 2017; Hedge et al., 2013; Hedge et al., 2016). These studies have demonstrated a greater anti-tumor response than just targeting a single antigen alone.

3 of 100,00 people are diagnosed with GBM in the United States (Lim et al., 2018). As of current, there is no effective treatment for GBM that targets a majority of tumor cells without damaging healthy cells. GBM patients can only receive symptom control for the many severe

detrimental effects of the disease(Oberndorfer et al., 2008). As such, this CAR-T cell research carries importance as a potential method to specifically target GBM without causing off-target toxicities.

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