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Efficacy of CRISPR-Cas9 CAR T-Cell Therapy vs. Blinatumomab in the Treatment of Acute Lymphoblastic Leukemia

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Abstract

Introduction: With immunotherapy drugs such as Blinatumomab (Blincyto), the current overall survival rate of relapsed/refractory B Lymphocyte Acute Lymphoblastic Leukemia (B-ALL) is 7.8 months in adults. However, with CRISPR-Cas9 gene editing, CAR T-cell therapy may extend survival. By knocking out T-cell receptors (TCRs) and HLA class I receptors on T-cells, CRISPR-Cas9 edited CAR T-cells could become a universal treatment alternative for B-ALL. This study compares the efficacy of CRISPR-Cas9 edited CAR T-cell therapy, a new era of CAR T-cell therapy, to the treatment of relapsed/ refractory B-ALL with Blinatumomab in mice.

Methods: A cohort of 30 NSG mice receiving xenografts from an 18-24 year old patient with relapsed B-ALL will be randomly assigned to one of three groups, where they will receive treatment with: (1) mock transduced T-cells, (2) Blincyto, or (3) CRISPR-Cas9 edited TRAC- and B2M- knockout CAR T-cells. Another cohort of 30 NSG mice, receiving human skin xenografts from a healthy donor, will also be assigned to one of the groups and treated accordingly. Tumour shrinkage will be analyzed through *in vivo* antibody fluorescent imaging in the first cohort. Graft Vs Host Disease (GvHD) development will be assessed in the second cohort.

Results: It is expected that mice treated with CRISPR-Cas9 edited CAR T-cells will be more effective at eliminating tumours. Compared to mice treated with Blincyto or mock-transfused T-cells, these mice will exhibit the highest overall response rates, complete response rates and tumour shrinkage rates, and the lowest relapse rates. It is also expected that these mice will not develop GvHD.

Discussion: The use of CRISPR-Cas9 edited CAR T-cells in the treatment of B-ALL reveals a new facet to cancer therapeutics. The efficacy of this treatment will be demonstrated through greater tumour shrinkage rates and higher overall response rates than treatment with Blincyto, the current standard treatment drug for B-ALL.

Conclusion: Current treatments involving Blinatumomab or conventional CAR T-cell therapy are expensive and therefore inaccessible to many. These results could demonstrate that CRISPR-Cas9 edited CAR T-cell therapy is viable as a safe, universal and more affordable alternative for relapsed/refractory B-ALL treatment.

Keywords: CRISPR-Cas9; CAR T-cell therapy; Blinatumomab; liquid tumours; acute lymphoblastic leukemia; relapsed/refractory B-ALL; Blincyto

Introduction

The second most common type of leukemia in adults is Acute Lymphoblastic Leukemia (ALL), with over 6500 cases a year in the US [1]. Approximately 75% of these cases are due to a defect in B-cell lymphoblasts [1]. This type of cancer, known as precursor B-cell ALL (B-ALL), is a highly aggressive, rapidly growing leukemia in which too many immature white blood cells (B-cells) are found within the bone marrow and blood [1]. Treatment for relapsed/refractory B-ALL is particularly difficult and most are immunotherapy based; currently, the two popular immune-based therapeutic interventions implemented are Blinatumomab (Blincyto) and chimeric antigen receptor (CAR) T-cell Therapy (specifically Kymriah) [2]. Blincyto is a constructed monoclonal antibody, bi-specific T-cell engager (BiTEs), that targets the CD19 antigen present on B-cells [2]. Blincyto binds to the CD19 present on the leukemic cells and the CD3 antigen on immune system cells, bringing them in proximity such that the immune system can target and eliminate the cancerous cells [1].

This drug has been FDA approved since 2014, increasing the overall survival rate for relapsed/refractory B-ALL to 7.8 months (versus the normal 4 months with chemotherapy) [1]. Over the past several years, another therapy known as CAR T-cell Therapy has also come onto the scene that has the potential to further increase the survival rate.

CAR T-cell therapy involves genetically engineering naturally occurring T-cells to recognize and attach to a specific antigen. In this process, the patient's own T-cells are isolated [3]. Then, using a deactivated virus, the T-cells are engineered to show chimeric antigens receptors (CARs) on their surface [3]. These CARs can recognize antigen-presenting cells and in the case of B-ALL, CD19 is the main antigen being targeted [3].

This treatment has promising results, showing 80-100% remission rates in patients with refractory/relapsed B-ALL [4]. However, there are several key downfalls to this treatment. One of the biggest drawbacks of this therapy is that the T-cells harvested from the patients are often substandard in quality and insufficient in quantity [4].

Other problems include CAR T-cell exhaustion, uncontrollable proliferation, potential self-killing and tumour suppressive microenvironments [4]. This is where new genome techniques can be effective, particularly CRISPR-Cas9. Cas9 is an endonuclease, guided by an RNA molecule, and used to form DNA double strand breaks (DSB) at a targeted location [4]. During the repair of these DSB, scientists can either knock out genes by causing multiple DSB or insert specific genes [4]. In order to create a T-cell treatment effective for everyone, the logical way is to form universal T-cells of sorts that bypasses the aforementioned deficits. In order to do this, CRISPR-Cas9 is used to knockout two specific proteins: endogenous T-cell receptors (TCRs) and beta-2-microglobulin [4]. TCRs are responsible for inducing graft-versus-host disease (GvHD) leading to the patient's body rejecting the allogeneic T-cells, thus knocking them out may remove this threat [4]. The beta-2-microglobulin subunit is part of a larger human leukocyte antigen class 1 proteins (HLA-1), which are responsible for acting as cell surface markers to differentiate self cells and non-self cells [4]. The body can recognize foreign or mismatched HLA markers, and initiate GvHD [4]. By knocking out both the TRAC and B2M genes, the first coding for TCRs and the latter for HLA-1, the CAR T-cells formulated are now universal and can be used by a multitude of patients [4]. However, it is extremely important to test how the efficacy of universal CRISPR-Cas9 edited CAR T-cells compares to that of standardized immunotherapies used to treat ALL, such as Blincyto. This study aims to compare the efficacies of the new and developing technique with an already established therapy, elucidating whether CRISPR Cas-9 CAR T-cell therapy is more or less effective compared to Blincyto.

Methods

CRISPR-Cas9-edited CAR T-cell and Mock-Transfused T-cell Preparation

The methods used for this procedure will follow the protocol reported by Liu et al. [6], who produced TRAC/B2M double knockout CAR T-cells (DKO T-cells) with 95% efficiency.

Umbilical cord blood samples will be collected from healthy donors at a local hospital [6]. Density gradient centrifugation will be used to remove mononuclear cells from these samples, following which a commercially available human T-cell enrichment kit will be used for T-cell isolation [6]. Dynabeads coated with anti-CD3 and anti-CD28 antibodies [7] will be used to activate and expand the T-cells, which will then be cultured in TheraPEAK™ X-VIVO™ 15 medium (Lonza) [8] with supplements as described by Liu et al [6]. This medium is typically used to optimize the proliferation of CD3+ T-cells [8]. Finally, with a commercially available kit, the Trypan Blue Exclusion Test will be used to determine the viability of the obtained CD3+ T-cells [6].

The gene sequence encoding second generation anti-CD19 CARs will be established as described by Liu et al [6], and introduced to the CD3+ T-cells through a lentiviral vector three days after they activate [6]. This sequence will also contain a GFP sequence so that, following transduction, CAR+ T-cells can be identified through eGFP-expression [6].

Two days after transduction, CRISPR-Cas9 electroporation will be conducted [6]. As described by Liu et al. [6], a mixture of Cas9 protein and the required guide RNAs (sgRNAs) - one for knocking out TRAC and two for B2M - will be electroporated into the T-cells. Finally, 10 days after electroporation, successfully generated DKO cells will be harvested and enriched with magnetic bead-mediated negative selection [6]. For this technique, PE-conjugated antibodies and anti-PE microbeads from a commercially available PE positive selection kit will be used to magnetically label TRAC+ and B2M+ T-cells [6]. When placed in a magnetic field, the unlabelled DKO cells will rise into a supernatant fluid while the labelled, PE-positive TRAC+ and B2M+ cells will remain below the fluid in a test tube [6]. This will allow for the extraction of DKO cells [6].

For the preparation of mock-transfused T-cells, the aforementioned procedure will be replicated without the following procedures: (1) transduction to generate the second generation anti-CD19 CARs [5]; and (2) CRISPR-Cas9 electroporation [6]

Peripheral Mononuclear Blood Cell (PBMC) Preparation and T-cell Enrichment for Blincyto

The methods used for this procedure will follow the protocol reported by Dreier et al. [10], who enriched T-cells from PBMCs specifically for use with Blincyto *in vitro*.

Blood samples will be collected from healthy donors at a local hospital [10]. Density centrifugation will be used to extract the buffy coats from these samples, and Ficoll density-gradient centrifugation will be used to separate PBMCs from the buffy coats [10]. Next, an erythrocyte lysis buffer and a round of centrifugation will be used to remove the erythrocytes and thrombocytes from the solution [10]. Finally, T-cells will be isolated from the unstimulated PBMCs through commercially available enrichment column kits [10].

Animal Models

30 male and 30 female 6-week old NOD-*scid* IL2Rgamma^{null} (NSG) mice [11] will be used as analogs for leukemic humans. The mice will be placed on a 12 light/ 12 dark cycle with 60-80% humidity at room temperature, as recommended by The Jackson Laboratory [11].

Establishment of B-ALL Patient-Derived Xenografts

The methods and materials used for this procedure will follow the protocol reported by Weigert et al. [12]

B-ALL cells will be obtained from a relapsed 18-24 year old patient and engrafted into mice via tail-vein injections without prior irradiation [12]. Tumor growth will be assessed through *in vivo* antibody fluorescent imaging, with anti-CD22 as the fluorescent antibody [13]. Once the bone marrow blasts of the entire cohort exceed 30%, the mice will be considered leukemic [12] and the experiment will begin.

Establishment of Skin Xenografts

The methods and materials used for this procedure will follow the protocol reported by Shanmugam et al. [14], who used a normal human skin xenograft procedure to study wound healing in mice.

Normal human dermal cells will be harvested during an elective abdominoplasty procedure from a healthy donor [14]. Skin on the dorsal regions of mice will be removed from two graft beds, and replaced with full thickness human epidermal xenografts [14]. The experiment will begin 90 days from engraftment, once graft viability is confirmed [14].

Experimental Design

At t=0, where t is measured in days from xenograft establishment, a cohort of 15 male and 15 female mice (with only patient-derived xenografts) will be randomly assigned to one of three treatments group: (1) a control group treated with mock transduced T-cells, (2) an experimental group treated with DKO T-cells or (3) an experimental group treated with Blincyto.

Similar to the study by Liu et al. [6], mice in the control group will receive an intraperitoneal injection of mock-transduced T-cells once at t=3, and mice in the DKO T-cell group will receive an intraperitoneal injection of the DKO T-cells once at t=3. In addition, similarly to the study by Drieier et al. [10], mice in the third group will receive intravenous injections of Blincyto through the tail vein once daily from t=0 to t=4.

Tumor shrinkage will be assessed weekly through *in vivo* antibody fluorescent imaging using anti-CD22 antibodies [13]. Moribund mice will be euthanized as per standard protocol [10].

Concurrently, at t=0, another cohort of 15 male and 15 female mice (with only human skin xenografts) will also be randomly assigned to one of the three treatment groups, and treatment will commence as previously outlined. The clinical signs of GvHD, including but not limited to weight loss and fur loss, will be assessed daily and scored on the same scale used by Sadeghi et al [16]. Severely moribund mice will be euthanized as per standard protocol.

The experiment will be considered complete 6 months after the last treatment dose is received. Mice that undergo successful treatment, but later experience deterioration, will be declared as relapsed. At the endpoint of the study, overall survival, tumor shrinkage, overall response rates (OR), complete response rates (CR), relapse rates and GvHD development will be compared across all six groups of mice.

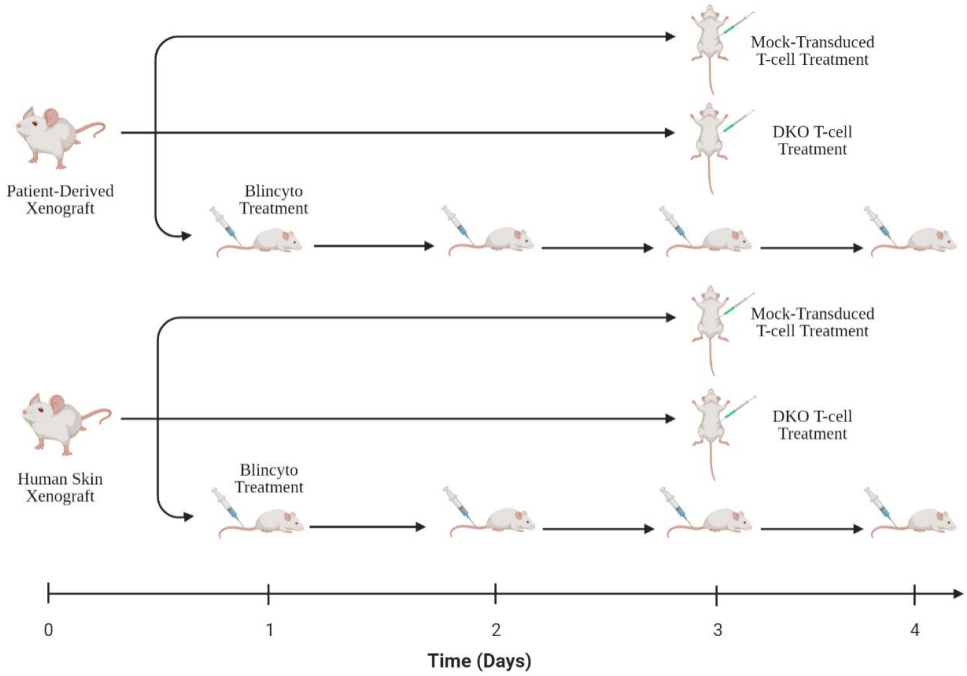

Figure 1. Dosing Regime for Mouse Treatment**.** Mice from both the patient-derived xenograft and human skin xenograft will receive Blincyto treatment through a tail-vein injection from days one to four. Similarly, mice from both xenografts will receive DKO T-cell treatment and mock transfused T-cell treatment on day 3 through an intraperitoneal injection.

Results

It is expected that DKO T-cells will be more effective than Blincyto at clearing tumors. If this is indeed the case, then OR rates, CR rates, overall survival and tumor shrinkage will be higher for mice treated with CRISPR-Cas9 edited CAR T-cell therapy than mice treated with Blincyto or mock-transfused T-cells. Additionally, it is also expected that relapse rates measured 6 months after the last tumor is eliminated will also be lower in the mice treated with CRISPR-Cas9 edited CAR T-cell therapy. No sex-based differences are expected to occur in any of the treatment groups.

Furthermore, since TRAC and B2M are knocked out in the CRISPR-Cas9 CAR T-cells, GvHD is not expected to develop in the DKO group. However, GvHD is expected to develop in the control group receiving mock-transfused T-cells.

The correlation between the high CR rate and tumor shrinkage rate is expected to be the strongest, indicating the efficacy of the DKO T-cells at clearing tumours. Following the treatment, relapse rates assessed after 6 months are expected to be the lowest for mice in this treatment group.

Discussion

This study aims to compare the in vivo efficacy of CRISPR-Cas9 edited CAR T-cell therapy with the conventional B-ALL treatment option, Blincyto. The findings of this study display the efficacy and safety of immunotherapy treatments for patients with B-ALL. Precise in vivo antibody fluorescent imaging measurements allow for a qualitative comparison of tumor shrinkage. The OR and CR rates, which are dictated by the overall survival rate and tumor shrinkage rates, quantify the efficacy of each treatment. If the tumour shrinkage rates and survival rates for the DKO T-cell treatment are higher than the Blincyto treatment, then CRISPR-Cas9-edited CAR T-cell treatment can be considered a promising immunotherapy alternative for B-ALL.

Tumor shrinkage is not expected to occur in control group mice, as their treatment is only being limited to the mock transfused T-cells. Since recovery is not expected to occur, the relapse rates for this group are also anticipated to be zero.

The relapse rates assessed 6 months after the elimination of the last tumor will provide insight on the post-operational efficacy of each treatment. Given that relapsed B-ALL is associated with the largest mortality rate in all age groups, if the long-term results indicate that CRISPR-Cas9 edited CAR T-cell therapy is more effective than Blincyto, a promising revolutionary new treatment can be formed [15].

Additionally, the mice were separated into two cohorts depending on whether they underwent the patient-derived xenograft or human skin xenograft procedure. The main purpose of dividing the mice into cohorts is to be able to accurately assess tumor shrinkage and GvHD development independently and together as a function of DKO cells.

Future studies involving CRISPR-Cas9 edited CAR T-cell therapy should focus on the effects of the therapy in humans. Additionally, a major function of the CRISPR-Cas9 system is to form universal CAR T-cell therapy, helping to reduce GvHD commonly seen in T-cell based treatments [16]. Future studies should closely monitor variation in patient responses to the same type of treatment, alongside the rates of overall and complete remission. CRISPR-Cas9 edited CAR T-cell therapy can also act as a bridgeway between multiple treatments. For example, this treatment will effectively wipe out a large chunk of B-cells present in the patient, after which stem cell transplants can be done effectively [4]. Future studies should focus on the impact that this therapy has in combination with other current therapies, and its effect on patient response. Lastly, regular CAR T-cell therapy lacks potential for solid tumour treatment, thus future studies shall focus on modifying CRISPR-Cas9 edited CAR T-cell therapy to treat various solid tumours such as lymphomas and carcinomas [4].

There is one major limitation to this study - the mice were engrafted with B-ALL cells that were extracted from only one patient, thereby potentially hindering the generalizability of the results. Since the findings of this study do not account for variations in the results due to y patient sex, age, ethnicity and/ or comorbidities, the influence of such factors were not analyzed. Future studies could also investigate whether these factors significantly predict the success of CRISPR-Cas9 edited CAR T-cell therapy.

Conclusions

This study compares the B-ALL treatment efficacy of CRISPR-Cas9 edited CAR T-cell therapy to that of Blincyto, the conventional immunotherapy drug used. CRISPR-Cas9 edited CAR T-cell therapy is expected to provide a greater reduction in tumor size and larger rate of survival than Blincyto as it "more potently targets and neutralizes CD19 antigen expressing elements of tumorigenic cells." If the results are as expected, CRISPR Cas9 CAR T-cell therapy will provide a universal technique to destroy tumor cells in all patients, resulting in safer and more efficient treatment. Future directions for research in this area include longitudinal clinical trials in human B-ALL patients, with a focus on measuring overall survival rates, response rates and relapse rates following CRISPR-Cas9 edited CAR T-cell therapy.

List of Abbreviations Used

BiTes: bi-specific T-cell engager
CRISPR: clustered regularly interspaced short palindromic sequences
CAR: chimeric antigen receptor T-cell
TCR: T-cell receptor
HLA: human leukocyte antigen
ALL: acute lymphoblastic leukemia
B-ALL: B-cell acute lymphoblastic leukemia
TRAC: trans-activating CRISPR RNA
B2M: beta-2-microglobulin
CD22: cluster of differentiation 22
GvHD: graft versus host disease
CD19: cluster of differentiation 19
CD3: cluster of differentiation 3
FDA: The Food and Drug Administration
sgRNA: small guide RNA
DNA: deoxyribose nucleic acid
RNA: ribonucleic acid
DSB: DNA double strand breaks
GFP: green fluorescent protein
PBMC: peripheral blood mononuclear cell
DKO: double knock-out
OR: overall response
CR: complete response
NSG: NOD-scid IL2Rgamma^{null}

Conflicts of Interest

The author(s) declare that they have no conflict of interests.

Ethics Approval and/or Participant Consent

This study will be approved by the McMaster Research Ethics Board. Patient consent and confidentiality will be obtained and maintained during the collection of patient-derived materials. Additionally, all mice will be cared for in accordance with the ethical standards outlined by the Canadian Council for Animal Care.

Authors' Contributions

KR: Acquired an extensive amount of information for the experiment, contributed to the design and planning of the study, drafted the manuscript and gave final approval of the version to be published.
SR: Contributed to the design of the study, outlined the interpretation of the data, designed relevant images, drafted the manuscript and gave final approval of the version to be published.
SS: Contributed to the design and planning of the study, outlined the expected analysis of the data, drafted the manuscript and gave final approval of the version to be published.

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