PRECLINICAL STUDIES USING CAR-T CELLS

Lucila Nassif Kerbauy¹, Juliana Aparecida Preto de Godoy¹*, Raquel de Melo Alves Paiva¹, Andrea Tiemi Kondo¹, Oswaldo Keith Okamoto², Martín Bonamino³

¹ Department of Hemotherapy and Cell Therapy, Hospital Israelita Albert Einstein, São Paulo, SP, Brazil.
² Human Genome and Stem Cell Research Center, Department of Genetics and Evolutionary Biology, Biosciences Institute, University of São Paulo (USP), Sao Paulo, Brazil.
³ Molecular Carcinogenesis Program - Research Coordination - Brazilian National Cancer Institute (INCA), Rio de Janeiro, Brazil
*these authors contributed equally to this article.

Correspondence to: ju.pgodoy@gmail.com

ABSTRACT

The therapy with genetically modified T cells to express chimeric antigen receptors (CAR) is a promising strategy for immunotherapy against cancer. CAR-T cells can specifically recognize antigens on the surface of tumor cells and then effectively kill those cells. Several researchers have presented the development of CAR-T cells for various hematological targets and the treatment of solid tumors. Quality control and preclinical evaluation of these products are essential to demonstrate their safety and efficacy and allow development to the clinical trial phase. This chapter will present relevant guidelines regarding pre-clinical research of CAR-T cell products. Preclinical research on cell therapy products should include in vitro and in vivo pharmacodynamics studies (antitumor activity), pharmacokinetics (proliferation, distribution, and persistence of CAR-T cells in vivo), and animal safety studies.

Keywords: Immunotherapy, Adoptive.

OBJECTIVE

Describe relevant requirements for preclinical studies (in vitro and in vivo) in order to evaluate the functionality and safety of CAR-T cells.

INTRODUCTION

The therapy with genetically modified T cells to express chimeric antigen receptors (CAR) is a promising strategy for immunotherapy against cancer. CAR-T cells can specifically recognize antigens on the surface of tumor cells and then effectively kill those cells1,2. Several researchers have presented the development of CAR-T cells for various hematological targets and the treatment of solid tumors2-4. Quality control and preclinical evaluation of these products are essential to demonstrate their safety and efficacy and allow development to the clinical trial phase. This chapter will present relevant guidelines regarding pre-clinical research of CAR-T cell products.

Preclinical research on cell therapy products should include in vitro and in vivo pharmacodynamics studies (antitumor activity), pharmacokinetics (proliferation, distribution, and persistence of CAR-T cells in vivo), and animal safety studies.

- Good Manufacturing Practices

It is recommended that pharmacodynamics and pharmacokinetics studies as well as in vivo safety analysis of CAR-T cell products be carried out under
good manufacturing practices (GMP) conditions. If GMP conditions cannot be met at this stage of CAR-T cell development, pre-clinical experiments must follow the guidelines of good laboratory practices (GLP). This experimental rigor is necessary to guarantee the reliability, integrity, and traceability of the trial results to obtain a final preclinical study report. The results should be robust and adequate to support the analysis of the advanced cell therapy product development by the research team, regulatory agencies, and other stakeholders.

- **Starting material and reagents**

Starting material to be used in CAR-T cells manufacturing for animal studies does not necessarily need to be derived from a patient; healthy donor samples can also be used in this case. In addition, animal-derived reagents should be avoided or replaced with clinical, pharmaceutical, or GMP-certified products whenever possible. Thus, the homogeneity of the manufacturing process and a better comparability of the final advanced cell therapy product results can be later evaluated regarding safety and efficacy in clinical protocols.

- **Analysis of the cells before infusion**

The manufacturing process of CAR-T cells for animal studies must be accompanied by a complete analysis of the quality and stability of the product. An immunophenotypic evaluation is recommended for detecting CAR expression and T lymphocyte cell populations. It is also necessary to perform a functional evaluation of the antitumor activity of CAR-T cells through potency tests (detailed below) and tests to detect possible contamination of the cell preparation by microorganisms.

The stability study must include all the dosages that will be administered, the expected storage temperatures, and the transport process simulation before animal administration. Complementary analyses must be performed at the time of administration of CAR-T cells product to verify cell viability, total number of cells, and particles presence in suspension.

- **In vitro potency assays**

The potency assay is used to determine the effectiveness of the manufactured CAR-T cells. Among the factors that can affect CAR-T cells activity are the type of vector and its design, the transduction rate of T cells, the manufacturing process, the source of the starting material, the percentage of cell populations at the beginning of the culture process, expansion profile ex vivo and T cell phenotype at the end of the expansion, including effector, memory or exhausted populations, among others.

The percentage of CAR-T cells in the final product is an essential factor in determining the effectiveness of antitumor activity; this analysis is usually performed by flow cytometry. The classical method for determining the efficacy of CAR-T cells is the chromium release assay. It is based on the use of Chromium isotope loaded into target cells and measuring its release to assess T cell-mediated cytotoxicity. Alternative methods based on fluorescent agents, such as carboxylfluorescein diacetate (CFSE), have been used to avoid radioisotopes, labeling the target cells and evaluating the target the effector/target ratios before and after the period of co-cultivation.

Another example is the use of calcein, also practical for the analysis of cytotoxicity mediated by CAR-T cells. Calcein can tag target cells, and its release into the culture medium represents cell death under attack by CAR-T cells. Its detection/quantification can be performed in fluorimeters.

The functionality and antitumor activity of CAR-T cells can also be evaluated by detecting the production of specific cytokines such as INF-γ, TNF-α, IL2, in addition to other proteins associated with the cytotoxic activity of T cells, such as perforin and granzyme. Flow cytometry can detect these molecules after co-culture of CAR-T cells with tumor cells. Alternatively, cytokines can also be identified by enzyme-linked immunosorbent assay (ELISA), bead-based detection panels (with cytometer readout), and enzyme immunosorbt assay (ELISpot).

- **Animal model selection**

When administered to animals with a normally functioning immune system, the host can eliminate the human cells. Previous studies have shown that using immunodeficient mice, such as NOD/SCID or NOD/SCID knockout gamma chain (NSG), is appropriate. The grafting and growth of hematologic neoplasia and human immune system cells such as T lymphocytes are possible and pertinent in this animal model, preserving the characteristics of the primary tumors and the human cells used for the treatment. Human T cells can cause a xenogeneic response in animals, causing graft-versus-host disease. An animal variation that can avoid this effect is NSG mice that are knockouts for the invariant beta chain MHC class I, which prevents the expression of MHC class I in the cell membrane.
Another animal model widely used for this purpose is based on humanized mice, with partial human immune function or producing cytokines that help the engraftment of human hematopoietic cells. This animal model was previously established by administering human hematopoietic cells, lymphocytes, or tissues in immunodeficient mice. The use of immunosuppressive agents to eliminate the immune response to cells is less recommended since these agents can interfere in evaluating efficacy or toxicity, requiring careful analysis. The use of animal-derived CAR-T rather than human cells may also be considered for animal studies. However, potential differences between the functionality of murine and human CAR-T cells may compromise the analysis and interpretation of results. The use of murine cells expressing CARs in these immunocompetent mouse models is recommended when the tumor microenvironment must be reproduced, as is the case especially for solid tumors. Models that determine, as far as possible, the natural history of the disease, such as transgenic animals such as MMTV-PyMT (mouse mammary tumor virus - Polyoma Virus middle T antigen) for breast cancer, are recommended especially for the evaluation of 4th generation CARs that are also intended to modulate the microenvironment.

Clinical signs of animals should be recorded throughout the in vivo study, which includes, but are not limited to: changes in skin and fur; changes in attitude, posture, and reaction to handling, eating, and stool patterns, as well as stereotypies (e.g., excessive licking, repetitive movement) or abnormal behaviors (e.g., self-mutilation).

Other animals such as primates, pigs, and rats can be used to evaluate the pharmacodynamics and pharmacokinetics of CAR-T cells. For all models, it is necessary to consider an adequate weight and age pattern, healthy animals, and preferably SPF (Specific-pathogen-free). The food and environment must remain constant during the preclinical trial. These more complex models, in general, are not widely used and have limited exploration from the regulatory point of view. In addition, in the design of the in vivo study, other factors that may affect the study’s outcome must be evaluated, as described in Table 1.

**Table 1 - Factors to be considered for the in vivo study using CAR-T cells.**

<table>
<thead>
<tr>
<th>Vector</th>
<th>Animal Model</th>
<th>Experimental Design</th>
<th>Advanced Therapy Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>integration</td>
<td>immune response</td>
<td>number of animals</td>
<td>dose</td>
</tr>
<tr>
<td>transduction</td>
<td>pre-existing immunity</td>
<td>control groups</td>
<td>fresh/thawed product</td>
</tr>
<tr>
<td>replication</td>
<td></td>
<td></td>
<td>cell viability</td>
</tr>
<tr>
<td>infectivity</td>
<td>route of administration</td>
<td></td>
<td>potency</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>treatment regimen</td>
</tr>
</tbody>
</table>

**- Pharmacodynamics/Efficacy Study in vivo**

As described, experimental models with immunodeficient mice for xenotransplantation of human cells are the most commonly used to evaluate the antitumor effect of CAR-T cells. In the case of CAR-T cells used to treat lymphocytic leukemia or lymphoma, cell lines derived from human tumors (e.g., Raji cells – Burkitt’s lymphoma) or genetically modified cell lines that express the molecular target under analysis can be used for the establishment of an animal model of the target disease. In this approach, it is essential to establish appropriate control groups: a group with unmodified T cells, since they may affect tumor cells and a group that only receives the infusion of tumor cells. Increasingly, tumor cell lines expressing reporter genes such as green fluorescent protein (GFP) or luciferase are being used. With these reporters, tumor cells can be easily detected in the mouse by flow cytometry or immunohistochemistry (GFP) or even by imaging the animal with ultra-sensitive cameras to detect bioluminescence in case of grafted animals with luciferase-expressing tumors that have received luciferin substrate injections. This last technique, in particular, allows the longitudinal follow-up of the same animals, quantifying the tumor burden, and has been the method of choice in preclinical studies to validate CAR-T products.

Other complementary assessments are equally important, such as analyzing the number of tumor cells in the animal by flow cytometry, macroscopic and microscopic analysis of the mouse’s organs, and de-
Detecting tumor-associated cytokines in the animal's serum. In the case of subcutaneously grafted tumors, calculating the tumor volume using a caliper is also a standard practice. Table 2 presents some of the general parameters for evaluating the pharmacodynamics/efficacy of CAR-T cells. If the mice show any signs of suffering, severe pain, or are moribund, they should be euthanized, and the effectiveness of the treatment will also be evaluated through the survival curve.

**TABLE 2** - Parameters commonly evaluated in the analysis of the pharmacodynamics/efficacy of CAR-T cells

<table>
<thead>
<tr>
<th>Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor volume</td>
</tr>
<tr>
<td>Tumor weight</td>
</tr>
<tr>
<td>Location of tumor cells</td>
</tr>
<tr>
<td>Tumor cell analysis - bioluminescence</td>
</tr>
<tr>
<td>Serum cytokine analysis</td>
</tr>
<tr>
<td>Global animal survival</td>
</tr>
<tr>
<td>Macroscopic and microscopic analysis of organs</td>
</tr>
<tr>
<td>Effective dose with less toxicity</td>
</tr>
</tbody>
</table>

- **Pharmacokinetic study in vivo**

The *in vivo* study is essential for the preliminary analysis of the safety and efficacy of the produced CAR-T cells. The pharmacokinetic study includes analysis of proliferation, distribution, and persistence of CAR-T cells *in vivo* (Table 3). The CAR-T cells can also be detected in the animal body by preclinical imaging tests, using, for example, CAR-T cells labeled with fluorochromes, nanoparticles, or radioisotopes, in addition to their modification with different versions of luciferase. Flow cytometry can also be applied to detect CAR-T cells in these experimental animal tissues and organs. The PCR technique can detect trace amounts of DNA or RNA from CAR-T cells in tissue or organ samples, as well as hybridization *in situ* can also be used to analyze the presence of CAR-T cells in tissues.

Pharmacokinetics also analyze the excretion of the advanced cell therapy product and the risk of transmission to the germline.

**TABLE 3** - Evaluation of the pharmacokinetic profile in preclinical in vivo studies.

<table>
<thead>
<tr>
<th>Biodistribution</th>
<th>Persistence</th>
<th>Clearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose</td>
<td>Cells duration</td>
<td>Excretion cells</td>
</tr>
<tr>
<td>Route of administration</td>
<td>Level of expression</td>
<td></td>
</tr>
<tr>
<td>Treatment regimen</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- **Safety in vivo study**

Safety of the treatment with CAR-T cells *in vivo* must be analyzed against the following items:

- Immunogenicity
- Risk of transmission to the germline
- Immunotoxicity
- Genotoxicity
- Tumorigenicity
- Tropism and biodistribution

In addition, routine toxicological indices such as clinical symptoms, body weight, food intake rate, serum biochemistry, hematology, and histopathology should be analyzed. Figure 1 shows a basic outline of a preclinical study for safety assessment.
It is essential to highlight that, although widely used to assess the potency of the antitumor effect of CAR-T cells, models of immunodeficient mice grafted with human tumors are not models that allow the prediction of CAR-T off-target effects, since cross-reactivity between antigens of mice and humans is not guaranteed. The pattern of antigen distribution can also vary between the two species, not guaranteeing that results in the preclinical model can be transposed to the result in clinical trials. Likewise, these models are not suitable for assessing cytokine release syndrome (CRS) and neurotoxicity. Adaptations such as pre-engraftment of NSG animals that produce human cytokines with human hematopoietic cells can be made to create a framework that reproduces the interrelationship between CAR-T cells and the human myeloid compartment. When these models receive the tumor and CAR-T cells, it becomes possible to reproduce some aspects of CRS and neurotoxicity\(^5\), serving as an investigational model, although still little used due to its complexity.

REFERENCES


