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Quality Control and Nonclinical Research on CAR-T Cell Products: General Principles and Key Issues

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ABSTRACT

Adoptive cell therapy using chimeric antigen receptor T (CAR-T) cells, which is a promising cancer immunotherapy strategy, has been developing very rapidly in recent years. CAR-T cells are genetically modified T cells that can specifically recognize tumor specific antigens on the surface of tumor cells, and then effectively kill tumor cells. At present, exciting results are being achieved in clinical applications of CAR-T cells for patients with hematological malignancies. The research and development of CAR-T cells for various targets and for the treatment of solid tumors have become a hot topic worldwide, so an increasing number of investigational new drug applications (INDAs) and new drug applications (NDAs) of CAR-T cell products are expected to be submitted in future. The quality control and nonclinical research of these products are of great significance in ensuring the safety and effectiveness of these products; however, they also present great challenges and difficulties. This article discusses the general principles of and key issues regarding the quality control and nonclinical research of CAR-T cell products based on their product characteristics and on relevant guidelines for gene and cell therapy products.

1. Introduction

Chimeric antigen receptor T (CAR-T) cells are genetically engineered T cells, which usually express chimeric antigen receptors (CARs) that can recognize specific tumor antigens and then activate the immune system to eliminate tumors. A CAR typically contains three domains: an extracellular domain (e.g., single-chain fragment variable (scFv)) that recognizes a tumor-associated antigen; a signal transduction domain (e.g., CD3ζ); and intracellular co-stimulatory domains (e.g., that can be derived from CD28, 4-1BB, OX40, etc.). The usual method of CAR-T cell immunotherapy is to isolate T cells from the patient's collected blood, which are then genetically modified in a good manufacturing practice (GMP) facility. The CAR genes are delivered to T cells by viral vectors (retroviral vectors or lentiviral vectors), transposon systems (e.g., Sleeping Beauty), or direct message RNA (mRNA) transduction, and are expressed on the surface of T cells. These modified T cells are amplified and then infused to the donor patient. They can identify and kill tumor cells specifically and efficiently, while avoiding damage to normal tissues.

In clinical trials for the treatment of B-cell malignancies (B-cell acute lymphoblastic leukemia and B-cell lymphoma, etc.), CAR-T cells targeting CD19 exhibited exhilarating efficacy and few side effects [1,2]. On August 31, 2017, Novartis AG announced that tisagenlecleucel (previously known as CTL019, under the trade name Kymriah), a CAR-T cell product targeting CD19 that had been developed by the company, was approved by the US Food and Drug Administration (FDA) for the treatment of precursor B-cell acute lymphoblastic leukemia [3]. Less than two months later, the FDA approved another CAR-T cell product under the trade name Yescarta (axicabtagene ciloleucel)—a CAR-T cell therapy developed by Kite Pharma that also targets CD19 for the treatment of adult patients with certain types of large B-cell lymphoma [4]. The research and development of CAR-T cell therapy for other malignancies is also in progress [5,6].

There are currently more than 500 CAR-T clinical trials underway. For hematological malignancies, more clinical trials are needed to investigate new therapeutic targets and new treatment combinations [7,8]. For solid tumors, it is necessary to optimize imperfect targets or explore better targets, and to overcome the obstacles to T cell function in the tumor microenvironment [9–11]. China is conducting the second-largest amount of CAR-T clinical research in the world, second only to the United States.

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As of June 2018, CAR-T clinical trials in China accounted for about 30% of the global total. In terms of its scope and extent in current clinical research, CAR-T has become an irreplaceable new immunotherapy that will be an important cornerstone of cancer treatment in the future.

As more and more investigational new drug applications (INDAs) and new drug applications (NDAs) of CAR-T cell products are submitted, settling the quality control, effectiveness and safety studies, and related regulatory issues of CAR-T cell products has become an urgent matter. Although current technical guidelines for cell therapy and gene therapy products at home and abroad contain some general descriptions that are relevant [12–16], these are macroscopic and lack the technical requirements and specifications of CAR-T cell products [17]. Based on these guidelines and on the characteristics of CAR-T cell products themselves, this paper further explores the general principles of and key issues regarding quality control and nonclinical research of CAR-T cell products.

2. General principles for the quality control and nonclinical research of CAR-T cells

Fig. 1 summarizes the general principles for the quality control and nonclinical research of CAR-T cell therapies.

2.1. Quality control

At present, no uniform technical standards exist for CAR-T cell products. Since differences exist in the CAR design, gene introduction, cell culture, and cell purification technologies from each manufacturer, the quality control of CAR-T cells should take the specific production process and product characteristics into account. The production process of CAR-T cells should meet the requirements of current good manufacturing practices (CGMPs). The aim of CGMPs is to provide a framework to ensure high-quality production in well-controlled facilities and equipment by well-trained and regularly trained staff, while requiring a wide range of systems that document all aspects of operation in order to demonstrate continuous compliance. The quality control of CAR-T cell products should include a full range of tests on quality, safety, and effectiveness, based on relevant guidelines and comprehensive consideration of the products' characteristics as biological products, cell products, and gene therapy products. As a living "drug," a CAR-T cell has a complex preparation process, and requires whole-process quality control. The quality control of CAR-T cells should include inspection of the materials used in production, process control, and a release test of the finished products. The corresponding test items and their acceptable criteria should be set respectively. In addition, production process validation and stability studies are essential for CAR-T cell products.

2.1.1. Control of production materials

The production materials for CAR-T cells are the substances or materials used in the preparation of the cell therapy product, which include cells, vectors for gene modification, culture mediums, cytokines, various additive components, cryoprotectants, excipients, and so forth. The materials used for production are directly related to the quality of the products. Therefore, researchers should establish a good and standardized quality management system for production materials, including risk assessment of their use, auditing of suppliers, and quality testing.

If T cells are provided by allogeneic donors, the source of the donor cells should meet the requirements of relevant national laws, regulations, and ethics. Procedures for the acquisition, transportation, sorting, testing, and preservation of donor cells should be well studied and validated. On this basis, explicit specifications and requirements should be formulated for aspects such as the characteristics, culture conditions, generation, growth characteristics, preservation status, preservation conditions, and quality

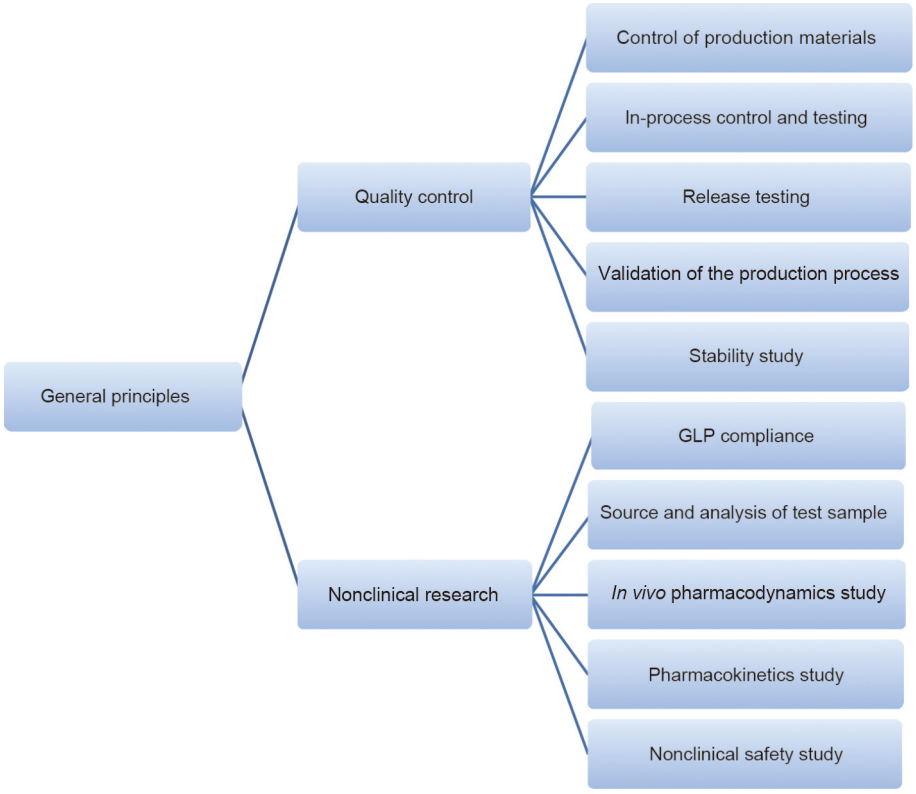

Fig. 1. An organizational graphic depicting the general principles of quality control and nonclinical research for CAR-T cell therapies. GLP: good laboratory practice.

testing of donor cells. In principle, cell banks should be established for the preservation and production of donor cells, if feasible. The tiers of a cell bank would depend on full consideration of the cell characteristics, production, and clinical application. Cell bank testing standards should be established, and should meet basic requirements for safety, quality controllability, and/or efficacy. Autologous donors do not require qualification, and the screening and testing of pathogens are advisory but not mandatory. The cells used in the production process, such as those used to produce viral vectors, should meet certain basic requirements: a clear source and culture history, controllable safety risks, adequate quality for production processes, and well-managed cell banks [18,19].

At present, gene modification in the production of CAR-T cells is mainly achieved by viral vectors (retroviral vectors or lentiviral vectors), transposition subsystems, or direct mRNA electroporation, so that CARs are expressed on the surface of T cells in order to specifically recognize and bind antigens on the surface of tumor cells, and finally kill the tumor cells. Although the vector for gene modification appears to be a raw material in the production process of CAR-T cells, it enters into the human body in essence as a component of the final product. Relevant requirements for the quality control of vectors will be discussed in detail later in this article.

Each substance used in the production of CAR-T cells, such as the culture medium, serum, cytokines, enzymes, antibodies, antibiotics, and magnetic beads, should be clearly defined and assessed for applicability. The identity, purity, bacterial endotoxin, sterility, adventitious factor, and biological activity should be tested in order to demonstrate quality [20]. It is advisable to use culture media that are as simple as possible, and to avoid the use of ingredients from human or animal origins, or agents that may cause allergies, such as β-lactam antibiotics. If a commercial medium is used, the supplier should be qualified, and should provide information about the ingredients along with relevant quality certificates. Aside from special circumstances, the use of human or animal-derived sera should be avoided as far as possible in the culture of T cells, and the use of allogeneic human serum or plasma must be forbidden. If the use of animal serum is unavoidable, it is necessary to ensure that it is free of specific animal virus contamination. It must be strictly prohibited to use bovine serum from the endemic area of a spongiform encephalopathy. If the culture medium contains human blood-derived components such as albumin, transferrin, and various cytokines, it is necessary to clarify the source, batch number, and quality certification report, and to use products that have been approved by a regulatory agency for clinical application whenever possible. The ingredients in cell therapy products should meet the relevant requirements of pharmaceutical excipients, and a priority should be put on those that are approved for human use; otherwise, a full-scale study and evaluation must be conducted.

2.1.2. In-process control and testing

In-process control and testing should be performed during the production of CAR-T cells. In-process control monitors the production process; this includes monitoring process parameters and achieving the objectives of process control. On the basis of fully understanding the overall process and the cumulative experience of production, researchers should clarify the key production steps in the process control and define the limits of sensitive parameters in order to avoid process deviation. In-process testing monitors the quality of cells during the preparation process; this involves testing key quality attributes of the product at critical steps or intermediate product levels. By establishing test methods and acceptance criteria for the in-process testing, in combination with the release testing of final products, control of the overall process and product quality of CAR-T cells can be achieved in order to ensure the repeatability of the production process and batch-to-batch consistency of the final products [21,22].

2.1.3. Release testing

Prior to the release of CAR-T cell products, appropriate tests must be carried out to ensure that the products meet the specified release criteria. The basic principle of the release criteria is that adequate tests must be conducted in order to ensure that the product's identity, purity, safety, and effectiveness meet the requirements. These characteristics of the cell products should be tested by means of various methods, with the products being released only if they are qualified and certificated. A certificate that summarizes the test methods that have been used, the corresponding test results, and the acceptable range should be provided. The methods that are used in release testing should be well studied and validated. Comprehensive validation is necessary, particularly for new methods. For methods that are included in the pharmacopoeia, validation of applicability is required. A simplified release test procedure can be carried out when a complete quality control test procedure is not possible due to the short shelf life of the product. In this case, the reason for a simplified release test procedure should be clearly described and explained; more importantly, the missing information for the release must be appropriately supplemented by reasonable in-process testing and more extensive process validation.

The quality control test items of CAR-T cells should be based on product quality research and on a full understanding of the production processes and clinical indications, while taking into account the characteristics of products and current scientific understanding and consensus. With deepening research in this field (from preclinical to clinical stages), process-related information can gradually be accumulated, and test methods should gradually be improved to meet the quality control requirements of the various clinical development stages. It is suggested that the quality control requirements of samples for confirmatory clinical trials should be consistent with marketed products. Quality control items should generally include product identification, biological efficacy, purity, impurities, cell number (i.e., live cell count, number of functional cells, etc.), and general detection (e.g., sterility, mycoplasma, endotoxin, and appearance) [23]. The establishment of acceptance criteria should be based on test data on all batches in preclinical studies, clinical studies, and validation tests, together with other related data (e.g., stability studies, literature reports, and experience).

2.1.4. Validation of the production process

For the whole process of CAR-T cell product preparation, comprehensive process characterisation and process validation of multiple continuous batches (at least three batches) of production are required. On this basis, appropriate process parameters and quality standards should be established to ensure the effective control of each process. For process validation batches, the intermediate products and finished products should be tested, analyzed, and identified more extensively than regular batches, thus providing a basis for setting up test items and quality standards for in-process testing and for the release testing of regular batches [24].

2.1.5. Stability study

In the processes of the production and use of CAR-T cell products, well-controlled cold chain transport and storage play an important role in ensuring the quality of cell products and preventing the occurrence of bacteria and mycoplasma contamination. For samples that need to be temporarily preserved during the production process, stability studies are required to verify their storage conditions and storage period. The basic principles of stability studies of CAR-T cell products can refer to the related

requirements of the general biological product. A reasonable research approach should be designed according to product characteristics, the needs of clinical use, and the specific conditions of preservation, packaging, and transportation. Taking frozen cell products as an example, it is necessary to carry out a freezing and thawing study under simulated scenarios (such as a cell recovery process). It is recommended that the investigative items include cell characteristics, biological potency, cell purity, number and ratio of living cells, cell count, and safety-related tests [25].

2.2. Nonclinical research

Nonclinical research of cell therapy products should include *in vitro* and *in vivo* pharmacodynamics studies (antitumor activity), pharmacokinetics studies (proliferation, distribution, and persistence of CAR-T cells in the body), and safety studies in animals. The *in vitro* antitumor activity of CAR-T cells is closely related to their potency/biological activity, which will be described in the Section 3.3 potency test. This section only discusses *in vivo* animal studies.

2.2.1. Good laboratory practice compliance

In vivo pharmacodynamics and pharmacokinetics studies of CAR-T cell therapy products could be performed under non-good laboratory practice (GLP) conditions; however, for nonclinical safety evaluation, they should meet GLP requirements. Certain special indicators included in both the safety evaluation and pharmacodynamic studies can also be tested under non-GLP conditions; however, it is necessary to guarantee the reliability and integrity of test results and assess the impact on the overall safety evaluation [26,27].

2.2.2. Source of test sample

In general, it is unnecessary to use a patient's blood to prepare CAR-T cell products for nonclinical research; instead, CAR-T cells derived from healthy donors can be used. A proof-of-concept study using animal-derived surrogate products should also play a supporting role [28].

2.2.3. Analysis of test sample

Given the particularity of the preparation process for CAR-T cell products, a complete quality analysis report and stability study data of the test samples should be provided. The stability study should cover all dosing concentrations, and should simulate all transport processes and treatment operations before administration to animals. If treatment operations such as resuscitation and resuspension are required before administration, then at least the cell morphology, total number of living cells, cell viability, color, and foreign substances should be tested before administration to animals.

2.2.4. In vivo *pharmacodynamics study*

At present, transplanted tumor models of immunodeficient mice are employed to study the inhibitory effect of CAR-T cells on tumors. For CAR-T cells targeting lymphocytic leukemia or lymphoma, human-derived cell lines (e.g., Raji, Daudi, Nalm-6, and Jeko-1) or stable genetically modified cell lines presenting specific targets (e.g., CD19-K562 and CD20-K562) can be used to establish a transplanted lymphoma model in immunodeficient rodents [29–32]. Mouse-derived cell lines such as A20 can also be used to build models to evaluate mouse-derived CAR-T cells. Since T cells that are not transfected with CAR have nonspecific killing effects on tumor cells, it is best to set up a T cell control group (i.e., non-transduced CAR-T cells, mock transduced T cells, or hapten-specific CAR-T cells) in addition to a solvent control group.

Routine pharmacological or pathological methods to detect tumor-related parameters (e.g., tumor volume, tumor weight, and colonization site of tumor cells in animals) and animal median survival time can be used. The most straightforward method for studying the pharmacodynamics of CAR-T cells is to detect luciferase-expressing tumor cells using bioluminescent imaging (BLI) technology. Other methods include: ① flow cytometry to detect the number of tumor cells in the animal; and ② flow cytometry, enzyme-linked immunosorbent assays (ELISA), meso scale discovery (MSD), and other immunological methods to detect changes in tumor-associated cytokines in serum, and thus indirectly reflect the pharmacodynamics results [33].

2.2.5. Pharmacokinetics study

In nonclinical research, it is important to elucidate the *in vivo* process of the cells as much as possible, as this is vital to the evaluation of cell activity and safety. Due to immunological rejection, human-derived CAR-T cells are usually studied using immunodeficient animals. In general, CAR-T cells will proliferate in large quantities and play their biological roles in the presence of tumor cells *in vivo*. Therefore, the most commonly used pharmacokinetic models of CAR-T cells are transplanted tumor models. Non-tumor-bearing mouse models can be used as a control in bio-distribution studies.

Pharmacokinetics studies of CAR-T cells mainly focus on their proliferation, distribution, and persistence *in vivo*. Optional techniques include imaging technology, flow cytometry, immunohistochemistry, quantitative polymerase chain reaction (PCR) technology, and so forth. Different methods are suitable for different samples and testing purposes. The imaging method can visually detect the *in vivo* distribution of CAR-T cells; it requires cell labeling, such as radioisotope labeling, genetic modification labeling (e.g., green fluorescent protein or luciferase), or nanoparticle labeling (e.g., iron-glucan nanoparticles). Flow cytometry can detect CAR-T cells in the blood, bone marrow, and spleen of animals [31]. The immunohistochemical method can detect $CD3^+$ cells or CAR^+ cells in the spleen and other organs, thereby revealing the distribution and accumulation of human T cells in organs. The Q-PCR method can detect the level of DNA or RNA from human-derived CAR-T cells in all types of samples. At present, certain other techniques, such as *in situ* hybridization, have been developed to detect the tissue distribution of CAR-T cells.

2.2.6. Nonclinical safety study

According to clinical studies, the major safety risks of CAR-T cells include cytokine release syndrome (CRS) [34], neurotoxicity [35], and B-cell depletion [36]. In addition, the potential risk of tumorigenesis/tumorigenicity of transgenic cells is a critical consideration. No uniform standard exists regarding the extent of the prediction of adverse reactions. At the same time, toxicology studies in animals present some challenging problems, such as animal models, species specificity, and so forth.

In addition to routine toxicological indices (i.e., clinical symptoms, body weight, food intake, serum biochemistry, hematology, gross pathology, and histopathology), animal safety studies include immunotoxicity indices, such as graft-versus-host reaction (GVHR), peripheral blood cell count and phenotypic analysis, and cytokine levels in serum (e.g., interleukin (IL)-2, IL-6, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α) [37–40]. In addition, if possible, safety pharmacology, local tolerance, and other studies can be carried out simultaneously or separately. Conventional genetic toxicity combination studies and reproductive toxicity studies are generally not recommended. Tumorigenic studies also require special considerations.

3. Key issues

Fig. 2 summarized the key issues for the quality control and nonclinical research of CAR-T cell therapies.

3.1. Quality control of vectors for gene modification

A CAR-T cell product is a combination of gene therapy and cell therapy technology. As a tool for gene therapy, a vector may bring risks such as insertion mutation, replication of a competent virus, adventitious factor contamination, and so forth. Meanwhile, the genetic material it carries is an important component of a CAR-T cell, as it provides the basis for the strong activity of T cells to identify and kill tumor cells. Therefore, the quality of the vector is very important; it is totally different from the other raw materials used in production, and should be managed as a component of the product. The production and quality control of vectors for genetic modification should meet the requirements of current guidelines for gene therapy products. Furthermore, years of research on gene therapy products in China and abroad have brought us many experiences and documents on quality control that can be used for reference [41–43].

The vector for gene modification should be a clinical-grade product that has been produced under CGMP conditions, with a high quality and that has passed comprehensive quality tests. The production process must be validated to ensure its reproducibility on the production of vectors with high safety, efficiency, and quality consistency by means of controlled process flows. The key raw materials commonly used for vector production are cells, media and serum, and plasmids. Each of these must come from an approved supplier and should undergo rigorous testing procedures to reduce the risk of introducing adventitious factors into the production process. A cell bank system is required for the retrovirus packaging for stably transfected cells. Both a bacterial seed bank and a cell bank system are required for lentiviral vectors that are prepared by the transient transfection method. All banks must be managed and tested according to the corresponding regulatory requirements. The established cell bank, virus seed bank, bacterial seed bank, and animal-derived components in the culture medium all require extensive testing. The vector harvested in the production process should be purified and preserved using a suitable formulation. A stability study should also be carried out in order to ensure the stability of the vector. For retroviral/lentiviral vectors [44] and transposon plasmid systems [45], routine test items in the quality control of final vector products are listed in Table 1. Quality control items and corresponding standards should be established according to the characteristics of the individual vector and its production process. In addition, since the vector for the gene modification of CAR-T cells does not enter the patient directly, but undergoes dilution and rinses along with the host cells, it is necessary to establish a reasonable quality standard according to the results of process validation and risk evaluation.

3.2. Microbiological safety

The microbiological safety risks of CAR-T cells may involve contamination from bacteria, fungi, mycoplasma, adventitious viruses, and replication-competent viruses derived from vector production. The problem of microbial safety requires a great deal of concern and focus because it is easy for microbiological contamination to occur during the production process, and because the final cellular products cannot be decontaminated. The microbiological safety of CAR-T cells is mainly assured by rigorous testing of the materials for production, strict control of the production process in accordance with CGMP requirements, and microbiological examination of the products. The following is a brief description of sterility test, mycoplasma examination, replication-competent virus detection, and rapid microbiological detection.

3.2.1. Sterility test

At present, the classic approach for sterility test is the 14-day culture method that is described in the pharmacopoeia of most countries. However, since CAR-T cell products often need to be infused into patients shortly after production, alternative quick-detection methods such as gram staining or acridine orange staining are required in process control and release testing. Before a particular quick method is fully validated, two methods must be carried out in parallel. For each batch of cultured CAR-T cells, a sterility test should be performed before the infusion to patients.

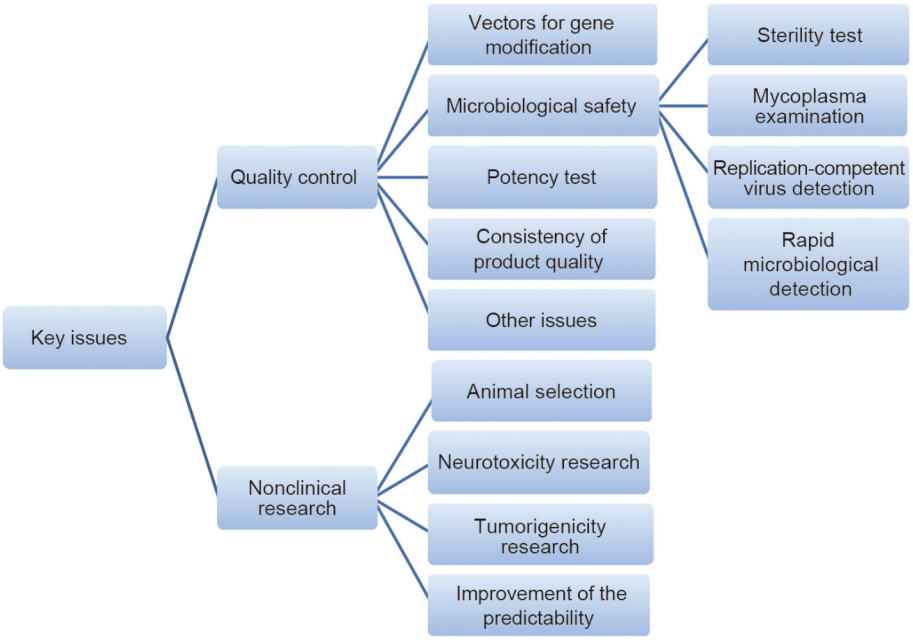

Fig. 2. An organizational graphic depicting the key issues in quality control and nonclinical research for CAR-T cell therapies.

Table 1
Common quality control items of vectors for the gene modification of CAR-T cells.

Category	Assays of retroviral/lentiviral vectors	Assays of transposon plasmid vectors
Identity	RT-PCR sequencing	Restriction enzyme mapping PCR Sequencing
Potency/concentration	Infectious/transducing titer Particle number (P24 ELISA) Particle to infectious/transducing titer ratio Transgene expression Bioactivity/functionality	Concentration Transgene expression Bioactivity/functionality
Purity	Host cell protein Host cell DNA Residual reagents (antibiotics, BSA, benzonase, etc.)	A_{260}/A_{280} HPLC purity (including ratio of supercoiled DNA) Host cell protein Host cell DNA Bacterial RNA Antibiotics
Safety	Sterility Bacterial endotoxin RCR/RCL Adventitious virus Mycoplasma	Sterility Bacterial endotoxin
Physiochemical characteristics	Appearance pH Osmolality Particle size distribution	Appearance pH Osmolality

RT-PCR: reverse transcription polymerase chain reaction; BSA: bovine serum albumin; HPLC: high performance liquid chromatography; RCR: replication-competent retrovirus; RCL: replication-competent lentivirus.

It is recommended to perform a sterility test on samples taken from culture supernatant at regular intervals after three or four days of culture, covering 48–72 h before release. If contamination is found in the early stages of cell preparation, further preparation should be terminated. Although the results of rapid detection could support product release, it is still necessary to keep track of the 14-day culture test and monitor the patient's condition. Emergency-response solutions must be designed in case the results obtained after cell infusion conflict with the results of the quick release test. Formal procedures are usually required in order to inform the subject's doctor to identify infected tissues and organs, determine the sensitivity to antibiotics, and carry out the treatment in a timely and effective manner. If the sterility test yields positive results, the production process should be inspected in time, and corresponding corrective/preventive actions (CAPA) should be proposed.

3.2.2. Mycoplasma examination

Mycoplasma contamination may come from several different sources, among which the use of ingredients derived from animal serum and a contaminated facility environment for cell culture (especially when it is an open-culture system) may be the most common. It is recommended to carry out mycoplasma examination at a high-risk production stage. For example, both the cells and the culture supernatant should be tested after the enrichment of harvested culture and before cell rinsing. Since CAR-T cell products often need to be manufactured and returned to patients within a short period of time, the detection of mycoplasma using the culture method is usually not feasible. Therefore, it is necessary to adopt a PCR-based method or another rapid alternative method. These methods need to be validated in order to ensure proper sensitivity and specificity.

3.2.3. Replication-competent virus detection

Although the retroviral and lentiviral vectors are designed to be replication defective, there is still a possibility that recombination will occur during production or after infusion into the patient, resulting in the generation of new replication-competent retroviruses/lentiviruses (RCRs/RCLs). The FDA guidelines for RCR/RCL detection in cell products and patients recommend that RCRs/RCLs in viral vectors, cell products, and post-infusion patients be detected using appropriate biological and/or molecular assays [46,47]. Biological assays are usually used to screen for RCRs in vector products, while the monitoring of post-infusion patients relies on molecular or serological tests [48,49]. Molecular and serological tests are faster than biological assays and consume fewer resources; however, they also easily give false positives. The most commonly used RCR bioassays are the extended S^+/L^- assay and the marker rescue assay [50–52]. The most common method of RCL bioassay is performed by amplifying potential RCLs to a higher titer in packaging cells at the stage of proliferation, followed by an ELISA or molecular assay [47,53–55]. Thus far, no positive RCR/RCL result has been reported in viral vectors, transduced cell products, or recipient patients. As a result, some researchers have suggested that it may be time to revise the requirements for RCR/RCL monitoring in FDA guidelines [56–59].

3.2.4. Rapid microbiological detection

Most cell-based products have a limited shelf life. In some cases, the transduced cells are frozen so that all tests can be finished before the release of the products. However, in most cases, it is not possible to complete traditional sterility tests, mycoplasma tests, RCL, and adventitious virus tests prior to administration. Therefore, it is extremely necessary to explore corresponding rapid detection methods.

At present, many kinds of rapid microbial detection instruments are used in clinical blood culture testing, food hygiene, and applications in other industries [60]. These instruments, which are usually based on technologies such as carbon dioxide sensors, adenosine triphosphate (ATP) bioluminescence, and flow cytometry of fluorescently labeled organisms, may be used to replace the current sterility test. As a substitute for traditional methods, nucleic acid testing (NAT) is currently being used in mycoplasma examination [61,62] and RCL detection [63]. For the detection of adventitious virus, massively parallel sequencing or deep sequencing can be used to detect multiple DNA sequences from different viruses, and may therefore be used as an alternative to traditional methods [64–66]. However, extensive and in-depth optimization and validation should be conducted before these methods obtain the approval of relevant national regulatory agencies, in order to prove their compatibility with or superiority to standard test methods.

3.3. Potency test

A potency test is used to determine whether a CAR-T cell product has the expected therapeutic ability, which is closely related to the efficacy of the product [67,68]. This test is a quantitative determination of the product-related biological functions, and ensures the quality and consistency of released batches. It also provides an important basis for stability studies and comparability evaluations after process changes. Many factors can affect the efficacy/activity of CAR-T cells, including transfection efficiency, CAR structure, gene vectors, culture conditions, cell types, and cell proportions. Items that are commonly tested include the number of transduced T cells and the function of antigen-specific T cells

(e.g., IFN-γ production and cytotoxic effect). The expression of CAR on the cell surface is a key feature of active CAR-T cell products. The proportion of CAR^+ cells is an important index for activity, which can be detected by flow cytometry and PCR. The corresponding release criteria should be established in order to ensure that the final products have enough CAR^+ T cells to work effectively. If possible, a reference batch of cells with an assigned potency value should be established and used to calibrate subsequent tests.

The function of antigen-specific T cells is often evaluated by detecting the killing ability of CAR-T cell products to target antigen-positive tumor cells *in vitro*. The classic method is a ^{51}Cr release assay. To avoid the risk of radioactivity, alternative methods such as bis(acetoxymethyl)-2,2′:6′,2″-terpyridine-6,6″-dicar-boxylate (BATDA) or carboxyfluorescein diacetate succinimidyl ester (CFSE) fluorescent dye labeling were developed. Due to the complex features of CAR-T cell products, it is often difficult to evaluate efficacy from a single potency test. Therefore, it is suggested that diverse assays be developed during the early stages of product development so that the most effective testing method can be selected based on the accumulated experience.

The mechanism of CAR-T cell therapy is manifold and complex, and is not completely clear. Current IFN-γ production and cytotoxicity assays still cannot exactly predict the clinical efficacy and safety of the product. Therefore, it is necessary to carry out comprehensive analyses of CAR-T cell products, including exploring the roles of different subtypes of T cells and analyzing the interaction mechanism between CAR-T cells and the tumor microenvironment. This will permit the development of more or better potency test methods that reflect the complete efficacy of CAR-T cells more accurately. In addition, considering the amplification of CAR-T cells *in vivo*, it is necessary to assess the ratio of CAR^+ T cells to total T cells and the correlation between the copy number of CAR and the efficacy. Xiong et al. [69] recently evaluated the quality of CAR-mediated immunological synapse (IS) by detecting the content of F-actin, clustering of tumor antigen, polarization of lytic granules (LGs), and distribution of key signaling molecules within the IS, and thus better predicting the effectiveness of CAR-T modified cells.

3.4. Consistency of product quality

The quality of CAR-T cell products depends on a perfect quality control system in order to ensure consistency between different production batches. Starting materials, intermediate products, and final products should be strictly tested during the production process to ensure that they meet acceptable criteria. The starting materials of CAR-T cells are mainly T cells from patients and vectors for gene modification. It is difficult to accurately define the consistency of cell sources due to individual differences among donors, which makes it difficult to ensure the consistency of final products. The control indexes of donors include not only the physical condition (i.e., classification and stage of tumor, time interval between collection of initial sample and current treatment regimen, routine physical examination items associated with the treatment, etc.), but also the total number of peripheral blood cells or T cells with a specific phenotype, pathogen screening, and so on. Since the vector for gene modification can be produced in large amounts under GMP conditions, quality consistency is particularly important for ensuring the stability of transfection efficiency and the consistency of final CAR-T cell products. Based on their experience in using lentiviral vectors produced by Oxford BioMedica, Novartis AG found that consistent vector quality can minimize site-to-site variation during the subsequent production of CAR-T cells [70]. In addition, due to the complexity of the production process of CAR-T cells *in vitro*, good process control and testing can effectively control various factors in the production process that directly or indirectly affect product quality in order to ensure that the obtained product meets the expected requirements, and thus promote the consistency of the product. At present, some advanced process analysis techniques have emerged, including various biosensors [71], image analysis techniques [72], automatic flow cytometry [73,74], metabolic flux analysis [75], and so forth. Further study is needed before these techniques can be applied to the CAR-T cell production process, where they will eventually improve the quality control level and establish a quality-by-design (QbD) strategy for CAR-T cell products, as was done for recombinant monoclonal antibodies and other biological products [76]. In addition, the accession of automated production technology will better promote consistency of production [77,78]. The basic requirement for ensuring the consistency of products is to control various factors, both in the manufacturing process and in the final products, such as transduction efficiency, copy number of expression vectors, level of CAR expression, phenotype and maturity of CAR-T cells, and functional capabilities such as cytokine release and target-cell killing. Process validation can provide scientific evidence for the continuous production of products with consistent quality.

3.5. Other issues in quality control

In tests of CAR-T cells and vectors for gene modification, such as the detection of cell surface markers by flow cytometry, the CAR-T cell potency test, the virus titer test, and so on, reference materials are required for the development, validation, and application of analytical methods [79]. Fully characterized products are usually used as reference standards to evaluate products from different manufacturers or batches; this is necessary to ensure product quality and production consistency, and to determine the appropriate clinical dosage. However, there is currently a lack of approved reference materials and mature experience in establishing reference materials for cell products. Therefore, cooperation among different standard-setting bodies, manufacturers, and academic institutions is necessary in order to establish reference materials at regional, national, and international levels.

In addition, the cost of quality control accounts for a large proportion of the total cost of CAR-T production. Thus, it is necessary to optimize and improve the efficiency and cost-effectiveness of quality control testing. Establishing a simple, effective, and cost-efficient quality control test system will have a huge impact on promoting the application of CAR-T cell therapy to more patients.

3.6. Animal selection in nonclinical research

As an individualized human-derived cell product, nonclinical research of CAR-T cells should be performed on the relevant species. When human-derived CAR-T cells are administered to an animal with normal immune function, rejection will occur, resulting in the elimination of human-derived cells or the emergence of a GVHR. Therefore, a genetically immunodeficient animal may be a better option. The use of immunosuppressive agents may also eliminate the immune responses to heterologous cells, but may interfere with the evaluation of drug efficacy or toxicological effects, making it necessary to assess the evaluation results with extra care. Humanized mice with a partial human immune function, which are created by implanting immunodeficient mice with human hematopoietic cells, lymphocytes, or tissues, have been preliminarily used in the evaluation of CAR-T cell products [80]. However, as the current humanized mouse model is still imperfect, its application is in the exploratory stage, and cannot be fully expanded to nonclinical studies [81]. The use of animal-derived CAR-T cells instead of human-derived cells can also be considered for confirmatory studies in animals [82].

At present, transplanted tumor models based on immunodeficient mice have been widely used to study the inhibitory effect of CAR-T cells on tumors. Studies on the tumor-inhibitory effect of mouse CAR-T cell products using a homologous mouse model and a mouse model transduced with a human tumor-associated antigen gene, respectively, have also been reported. The two models have complete immune systems, so they reflect the host's immune response to CAR-T cells to a certain extent. These are proof-of-concept studies. For the use of CAR-T cells against lymphocytic leukemia or lymphoma, human-derived cells (e.g., Raji, Daudi, Nalm-6, or Jeko-1) or stable cell lines with specific targets introduced by genetic engineering (e.g., CD19-K562 or CD20-K562) can be used to establish a transplanted lymphoma model in immunodeficient rodents [29–32]. Mouse-derived cells such as A20 can also be used to build mouse-derived models for the evaluation of mouse-derived CAR-T cells.

Each of the animal models currently used in nonclinical safety studies on CAR-T cells has advantages and drawbacks. The homologous mouse model and the transgenic mice expressing human tumor-associated antigen (TAA) model both have an intact immune system, but are only suitable for mouse-derived CAR-T cells. The mouse model with xenograft tumors can be used as a disease animal model to simulate the action process of CAR-T cells, but without a host immune system, the cascade reaction caused by CRS in the human body cannot be completely simulated, and the off-target toxicity cannot be detected. The non-tumor bearing immune deficient mouse model does not produce an immune rejection, and CAR-T cells can survive in the body for a long time; therefore, it is more suitable for research on the overall safety risk and tumorigenesis/tumorigenicity of CAR-T cell products. The immune-system-reconstruction mouse model can simulate the human immune system, but difficulties in the unification and standardization of this model remain, including the following: Various sources of human-derived $CD34^+$ hematopoietic stem/progenitor cells (HSPCs) were used to build the models; there are large individual differences in reconstructed mice; the reconstructed immune system may reject CAR-T cells; there is a risk of myeloid cell hypoplasia; and so forth. Non-human primates (e.g., monkeys) have a similar immune system and physiology to humans, so they should be more suitable animal models for predicting the safety of CAR-T cells in humans. However, the problem of immunogenicity makes it impossible to study long-term toxicity of CAR-T cells in monkeys. Therefore, there have been few reports on the application of this model in studies of CAR-T cells.

3.7. Neurotoxicity research

At present, no appropriate animal models or methods can well predict the neurotoxicity of CAR-T cells before clinical trial. A conventional safety pharmacology functional observation battery (FOB) study may not be suitable for CAR-T cells. Some studies suggested that the neurotoxicity of CAR-T cell therapy may be related to the increase of cytokine levels [83]. Therefore, the combination of neurotoxicity research with other toxicity studies may be considered as much as possible, with a focus on observing the neurotoxicity at the time point when CRS appears in clinical research. The significance of neurotoxicity research conducted in an animal model that does not mimic CRS well needs to be carefully considered.

3.8. Tumorigenicity research

Because they are a terminally differentiated somatic cell therapeutic product, the risk of tumorigenicity of CAR-T cells is theoretically low. *In vitro* soft agar cloning assays often exhibit negative results. In genome insertion site analysis, because the insertion is random, the insertion sites for each patient may be different. Even if the insertion site is a potential tumor-causing site, its tumorigenicity cannot be detected with the death of CAR-T cells or with immune attack on CAR-T cells *in vivo*. However, for CAR-T cell products introduced with foreign genes, the tumorigenicity risk must be preliminarily evaluated by means of insertion site analysis of viral vectors, *in vitro* analysis of cell immortalization proliferation, *in vivo* analysis of abnormal/ectopic proliferative diseases (e.g., hyperplasia and tumor), and so forth. *In vivo* tumorigenicity studies can be carried out along with long-term animal toxicology studies, and can be completed during clinical trials.

3.9. Improvement of the predictability of nonclinical research

At present, an urgent problem to be solved in nonclinical research on CAR-T cells is the extent to which the safety risk in clinical research can be predicted. If there is no relevant animal model to simulate the main biological process of human-derived cells *in vivo*, the significance of animal studies to predict human risk will be greatly reduced. Since CAR-T cells are highly individualized treatment products, and since some CAR-T cell therapies such as anti-CD19 CAR-T cell therapy have already gained a great deal of clinical safety experience, animal experimental research for these types of CAR-T cell product should focus on biological distribution and mechanism/activity validation. For example, animal homologous cell products can be used for pharmacodynamics and toxicity studies in animal models; or, xenograft models with human tumors in immunodeficient animals can be used to study human-derived CAR-T cells. The final safety and effectiveness evaluation should depend on clinical trials as the most important source of evidence.

4. Conclusion

As a new type of complex biological product, more and more CAR-T cell products will enter clinical trials and the market with the deepening of research and development in this field. Quality control and nonclinical research are important for ensuring the safety and effectiveness of these products; however, they also present great challenges and difficulties. This article put forward general principles of and key issues regarding the specific characteristics of CAR-T cells and related technical guidelines for cell therapy and gene therapy, with the aim of providing a useful reference for the research, development, and production of related products.

Compliance with ethics guidelines

Yonghong Li, Yan Huo, Lei Yu, and Junzhi Wang declare that they have no conflict of interest or financial conflicts to disclose.

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