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Research paper

Engineered cells for costimulatory enhancement combined with IL-21 enhance the generation of PD-1-disrupted CTLs for adoptive immunotherapy

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ABSTRACT

Blockade of the immune cell checkpoint inhibitors programmed death 1 (PD-1) and programmed death-ligand 1 (PD-L1) has become a powerful tool in cancer treatment, which is effective across various solid cancer types and hematologic malignancies. Our previous studies showed that by reducing immune tolerance, clustered regularly interspaced short palindromic repeats-associated protein 9 (CRISPR-Cas9) modified cytotoxic T lymphocytes (CTLs) rank highly in terms of immune responses and cytotoxicity. In this study, a genetically modified K562 cell line with surface expression of 4-1BBL was developed to expand PD-1-disrupted CTLs in vitro for further adoptive immunotherapy against cancer. Our findings demonstrate that after a long-term, up to 28 days, engineered cells for costimulatory enhancement (ECCE) combined with IL-21 promote the expansion of PD-1-disrupted CTLs with a less differentiated "young" phenotype, enhanced immune response and superior cytotoxic effector characteristics. These new in vitro conditions represent a nimble and cost-effective approach to developing PD-1-disrupted CTLs with improved therapeutic potential.

1. Introduction

The successful use of T-cell checkpoint inhibitors, especially programmed death 1 (PD-1), has been used to enhance immunity in solid tumors and obtain durable clinical responses with an acceptable safety profile [1–5]. From the more advanced point of view, the cell-intrinsic disruption of immune checkpoints by gene targeting in T-cells is likely to display a better safety profile than the systemic administration of a blocking antibody [6]. Our laboratory pioneered a gene editing approach using clustered regularly interspaced short palindromic repeats-associated protein 9 (CRISPR-Cas9) to disrupt PD-1 on primary T cells [7]. We demonstrated that by reducing immune tolerance, CRISPR-Cas9-modified cytotoxic T lymphocytes (CTLs) ranked above other reagents in terms of immune responses and cytotoxicity [8].

The Epstein-Barr virus (EBV), a type of herpesvirus, is one of the most common viral agents infecting humans; it is estimated that 90% of the world population has been exposed to this virus before adolescence. EBV is associated with a variety of malignancies, such as Hodgkin's disease, Burkitt's lymphoma, nasopharyngeal carcinoma and gastric carcinoma. EBV-associated gastric cancer (EBVaGC) comprises nearly 10% of all gastric carcinomas worldwide, with over 80,000 new cases being diagnosed each year. An earlier study suggested that EBV infection might be involved in the early stages of gastric carcinogenesis [9]. Other available studies showed that the sensitization of peripheral blood lymphocytes with a peptide derived from latent membrane protein 2A (LMP2A) can induce a CTL response against EBVaGC cells [10]. Accordingly, EBVaGC is susceptible to the LMP2A-targeting immunotherapy.

Effective adoptive immunotherapy depends on high enough number of tumor-specific T lymphocytes with the appropriate phenotypic characteristics and potent effector functions [11]. To generate sufficient number of PD-1-disrupted CTLs for adoptive cell transfer, extensive *ex vivo* expansion of T cells is required. We have previously demonstrated that PD-1-disrupted CTLs can be activated and *ex vivo* expanded when cultured with IL-2, IL-7, and IL-15 [7,8]. To facilitate further PD-1-disrupted CTLs activation and obtain sufficient number of them with increased antitumor activity, we investigated and compared the effects of 4-1BBL-expressing K562 cells and IL-21 on the expansion behavior, phenotype and function of PD-1-disrupted CTLs.

K562 is a human erythroleukemic cell line that was derived from a patient with chronic myelogenous leukemia in blastic crisis [12]. The K562 cell line offers several advantages over other cells, in generating

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engineered cells. (i) K562 cells express intercellular adhesion molecule 1 (ICAM-1; CD54), lymphocyte function-associated antigen 3 (LFA-3; CD58), and B7-H3 molecules (CD276), which improve the interaction and stimulation of T cells [13–15]. (ii) The absence of endogenously expressed human leukocyte antigen (HLA) molecules, with the possible exception of HLA-C, reduces the incidence of unintended allogeneic T-cell responses [16]. K562 cells are also suitable for cell expansion and transgene expression with lentivirus. (iii) The K562-derived engineered cells can be pre-irradiated (100 Gy) prior to thawing and used in co-culture with T cells and thus offers an attractive platform that avoids the need, inconvenience and lot-to-lot variation associated with the use allogeneic peripheral blood mononuclear cells (PBMC). K562 cell line is usually chosen as a scaffold for artificial antigen-presenting cells (aAPCs). Several other groups are currently conducting clinical trials of adoptive cell transfer, utilizing aAPCs derived from K562, thus further confirming the versatility and utility of this cell line. [17]. Receptor 4-1BB (CD137) is a member of the tumor necrosis factor (TNF) receptor superfamily that has T-cell costimulatory functions [18]. Signaling through 4-1BB by its natural ligand, 4-1BB-L, or agonistic antibody, promotes the expansion of T cells, sustains their survival and enhances their cytolytic effector functions [19,20]. Some researchers have used aAPCs derived from K562 cells engineered to express T-cell receptor (TCR), to expand CD8T cells through 4-1BB signaling. [13,16]. However, the multiple TCR stimulations that drive T-cell differentiation toward an exhausted terminal effector state are associated with impaired antitumor function in mouse models [21,22]. Friedman engineered the 4-1BBL-expressing K562 cells without T-cell receptor, called engineered cells for costimulatory enhancement (ECCE), which can only deliver the co-stimulation signal but not the TCR signal, thus limiting the differentiation of effector T-cells [23].

IL-21, the most recent member of the common γ-chain (γc) receptor cytokine family, has a role in innate and adaptive immunity [24–26]. Previous studies demonstrated a significant role for IL-21 in the primary Ag-specific human CTL response and supported the use of IL-21 in the *ex vivo* generation of potent Ag-specific CTLs for adoptive therapy or as an adjuvant cytokine for *in vivo* immunization against tumor antigens [27]. Importantly, T cells generated in the presence of IL-21 showed a higher antitumor activity in *in vivo* experimental models [28–30]. Besides, other studies show that the IL-21-mediated expansion of antigen-specific CTLs involves the suppression of Foxp3-expressing cells and reversal of the inhibition of tumor-associated antigen-specific CTL generation *in vitro* [31]. Actually, IL-21 acts synergistically with IL-7 and IL-15 to promote proliferation and survival of both memory and naïve T cells [32,33].

As IL-21 has never been combined with ECCE to stimulate CRISPR-Cas9-modified CTLs, we investigated the effects of IL-21 and ECCE on the expansion behavior, phenotype and function of PD-1-disrupted CTLs. Our findings demonstrate that ECCE combined with IL-21 promote the expansion of PD-1-disrupted CTLs with a less differentiated “young” phenotype, enhanced immune response and superior cytotoxic effector characteristics. This new method represents a promising platform for the expansion of PD-1-disrupted CTLs for use in adoptive immunotherapy.

2. Materials and methods

2.1. Plasmid construction, tumor cell lines, and peptide

The Cas9 expression construct pST1374-Cas9-*N*-NLS-Flag-linker (Addgene 44758) and pGL3-U6-sgRNA-hPD-1 (Addgene 51133) vectors were constructed as previously described [7,8]. The expression of Epstein-Barr virus in the gastric cancer cell line AGS-EBV was verified by the surface expression of the target antigen LMP2A. ECCE is a single clone with strong surface expression of 4-1BBL (CD137L) generated by retroviral transduction of K562 cells. ECCE was generously donated by Dr. Hongming Hu (Earle A. Chiles Research Institute, Providence Portland Medical Center, Portland, OR, USA), and were subjected to 100 Gy of γ-irradiation before being used. The peptides used for the stimulation of T cells, namely HLA-A02 restricted LMP2A 356–364 (FLYALALLL) and LMP2A 426–434 (CLGGLLTMV), were chemically synthesized at China Peptides (Shanghai, China) and achieved over 98% of purity.

2.2. Preparation of primary human PBMCs

Apheresis specimens were collected from gastric cancer patients. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation on a Ficoll density gradient and suspended in AIM-V medium (Gibico, USA). Cells were frozen in 90% FBS serum (Gibico, USA), and 10% dimethyl sulfoxide (Sigma, USA). All PBMCs were used for experiments or stored in a secure liquid nitrogen freezer until use.

2.3. Generation of hPD-1-sgRNA-disrupted T cells by electroporation

The hPD-1-sgRNA-disrupted T cells were prepared using PBMCs from gastric cancer patients as described previously [7,8]. Cells were transfected with the appropriate plasmids with Nucleo-fector II (Lonza, Germany) using the Amaxa Human T cells Nucleofector Kit, VPA-1002 (Lonza, Germany). A total of 5–10 × 10^6 cells were washed with DPBS by centrifuging at 800 rpm for 5 m, suspended in a 100 F06DL transfection buffer and then transferred into the electroporation cuvette. Program T-007 was selected for both high transfection and high efficiency. After electroporation, cells were re-suspended in 500 μL pre-warmed AIM-V medium containing 10% FBS, transferred into a six-well cell plate and incubated at 37 ˚C in 5% CO2. The transfection efficiency was evaluated by fluorescent counts 24 h after electroporation.

2.4. Generation of human mature dendritic cells

Dendritic cells (DCs) were generated from monocytes enriched by adherence for 2 h, and cultured in AIM-V medium containing human GM-CSF (500 U/ml, Peprotech) and IL-4 (500 U/mL, Peprotech) to obtain immature DCs. To obtain mature DCs (mDCs), fresh complete medium containing LPS (10 ng /mL, Sigma) and IFN-γ (500 U/mL, Peprotech) was added to the culture on day 5. The culture was continued for an additional 24 h. As described previously, these mDCs possess the ability to present antigen peptide and express CD80, CD86, HLA-DR and CD11c.

2.5. Generation of PD-1-disrupted LMP2a-CTLs

Mature DCs were pulsed by peptide (10 μg/mL) for 4–6 h at 37 ˚C, washed with pre-warmed PBS and then incubated with hPD-1-sgRNA-disrupted T cells at a ratio of 1:10 in complete AIM-V medium supplemented with: IL-7 (25 ng/mL), IL-15 (10 ng/mL), or IL-21 (30 ng/mL). Between 4–8 × 10^6 cells per well were plated in six well-plates. After 24 h, half of the cells were co-cultured with irradiated ECCE at a ratio of 1:2 (hPD-1-sgRNA-disrupted T cells: ECCE). Cytokines and medium were replaced every 2 to 3 d. For re-stimulation, autologous DCs were pulsed with peptide (10 μg/mL) for 4–6 h and added to the cultured cells for another 7 d. Fresh complete medium containing cytokines was added every 2–3 d until cells were used for further phenotypic and functional analysis. The groups were in turn named as IL-7/15, IL7/15/21, IL-7/15 + ECCE, IL7/15/21 + ECCE.

2.6. Flow cytometry

A FACSAriar cytometer (BD Bioscience, San Jose, CA, USA) was used to perform fluorescent expression analysis. CTLs were harvested and stained with mouse anti-human antibody labeled by fluorescence for 30 m in 4 ˚C in the dark as follows: CD3-FITC (HIT3a, BD Bioscience), CD4-Per-Cy5.5 (RPA-T4, BD Bioscience), CD8-Per-Cy5.5