

ENHANCING THE POTENCIES OF CHIMERIC ANTIGEN RECEPTOR T CELL (CAR T CELL) BY CRISPR/CAS9 SYSTEM TO ERADICATE RETINOBLASTOMA

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ABSTRACT

Retinoblastoma (RB) is the most common primary intraocular malignancy of childhood. There is no therapies that can eradicate specifically the whole cancer cells without any side effects. The disialoganglioside 2 (GD2), one of the cancer's cell markers that can be treated using immunotherapy, is expressed in RB. Through this fact, immunotherapy based on chimeric antigen receptor (CAR)-engineered T cells targeting cancer-specific antigens has shown great potential in treating this cancer. Although in recent studies show that immune cells are not able to destroy cancer cells because in every cancer cells there is protein programmed death ligand 1 (PD-L1). This literature review also shows the potential technology using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-CRISPR associated protein (Cas9) method to silence PD-1 in CAR T cell, so PD-L1 can not deactivate CAR T Cell through PD-1 signaling. The combination using CAR T cell and CRISPR-Cas9 will be the great therapy to eradicate RB without any side effect.

Keywords: CAR T Cells, CRISPR, GD2, PD-1, PD-L1, Retinoblastoma.

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INTRODUCTION

Retinoblastoma (RB) is the most common primary intraocular malignancy of childhood [1]. RB usually occurs in children younger than five years of age by epidemiological survey of consultations at the eye unit of the Douala General Hospital over the last 20 years (from January 1, 1990 to December 31, 2009). In children aged 0 to 10 years, the major causes of bilateral blindness were: cataract (51.2%), cortical blindness (16.3%), congenital glaucoma (10%), RB (7.5%), and posterior uveitis (2.6%); meanwhile the major causes of unilateral blindness included cataract (32.2%), RB (15.7%), complicated hyphemas (12.3%), wounds of the eyeball, and unspecified oculo-orbital tumors (8.9%) each[2]. According to the report from the Pediatric Health Department RSK Dharmas Indonesia in 2013, RB was the second leading cause of death due to cancer in children after leukemia. Four of six cases were reported death because of RB [3]. In developing countries with poor education, socio-economic conditions and health care systems, delayed diagnosis and suboptimal care constitute the common problem. Through the analysis of various cases, complications cause by RB are bleeding in the eyeballs, glaucoma, eye ablation, and the most dangerous is partial blindness or total blindness[4].

Nowadays, RB treatment is aimed at salvage of (1) life, (2) the eye and (3) vision in this order. Primary eye preserving treatments include combinations of chemotherapy, either systemic by intravenous injection or local through selective intra-arterial chemotherapy (SIAC) or intra-vitreous chemotherapy (IVitC), external irradiation, brachytherapy, cryotherapy and/or laser therapy. However, those therapies increase the risk for secondary malignancies in heritable patients and therefore currently avoided as first-line therapy [5]. Patients with poor prognosis often face resistant relapses after chemo and radiation therapies. Overall, there is no therapies

that can eradicate specifically the whole cancer cells without any side effects. The only option that we have is to increase the potencies of patients immune system and crush the cancer cells exclude the normal cells [6].

The GD2 is highly expressed in neuroblastoma and also several other pediatric as well as adult cancers, for example in melanoma, osteosarcoma, uterine leiomyosarcoma, small cell lung cancer, and RB [7]. As GD2 expression is high on tumor cells, GD2 has become an interesting target for immunotherapeutic approaches including monoclonal antibodies (mAbs) and more recently chimeric antigen receptors (CARs) [8]. Immunotherapy based on chimeric antigen receptor (CAR)-engineered T cells targeting cancer-specific antigens has shown great potential in treating hematological malignancies. This literature review aims to identify RB- specific surface antigens and investigate the RB tumor targeting function of a 4th generation CAR-T cells with specificity for GD2 protein [6,7].

In recent studies show that immune cells are not able to destroy cancer cells because every cancer cells have protein programmed death ligand 1 (PD-L1). PD-L1 is able to deactivate performance of T cells via the PD-1 gluing mechanism expressed by T cells. PD-L1 expressed by tumor cells. Editing T-cell becomes CAR T Cell and silencing PD-1 expression on CAR T Cells can be done using CRISPR-Cas9 system [9,10].

METHODS

The method that is used technically review of resources such as articles and journals in SpringerLink, Journal of Hematology and Oncology, Molecular Medicine Report, etc published all around the last 10 years. Most of articles are written in English. Our search terms included “CAR T Cells”, “CRISPR”, “GD2”, “PD-1”, “PD-L1”, and “Retinoblastoma”.

RESULT AND DISCUSSION

Retinoblastoma

RB is a malignant tumor of the developing retina that occurs in children, usually before age five years. RB develops from cells that have cancer-predisposing variants in both copies of RB1. RB may be unifocal or multifocal. About 60% of affected individuals have unilateral RB with a mean age of diagnosis of 24 months; about 40% have bilateral RB with a mean age of diagnosis of 15 months [5,11]. RB should be suspected in individuals with leukocoria, strabismus, change in eye appearance, and reduce visual acuity [1,5]. Early diagnosis and treatment of RB and non-ocular tumors can reduce morbidity and increase longevity. Treatment options depend on tumor stage, number of tumor foci (unifocal, unilateral multifocal, or bilateral), localization and size of the tumor(s) within the eye(s), presence of vitreous seeding, the potential for useful vision, the extent and kind of extraocular extension, and the resources available[11].

GD2 detection in Retinoblastoma

RB is kind of cancer that seems like neuroblastoma. With immunohisto- chemistry test both of RB and neuroblastoma give same positivity to the same marker, GD2. GD2 are the most frequently used molecular markers for such a purpose in other pediatric malignancies that share a similar antigenic profile with RB, such as neuroblastoma [6]. Gangliosides are involved in normal biological functions including cell adhesion, cell- cell interactions and proliferation [12]. Specific alterations in the expression of GD2 after neoplastic transformation, which are likely involved in the metastatic phenotype of malignant cells, have been found, particularly in tumors of neural crest- derived tissues. One of the most extensively studied molecular markers for the detection in neuroblastoma is the transcript of GD2 synthase gene. GD2 synthase is the key enzyme required for the synthesis of the GD2, and is commonly expressed in normal tissues such as the brain and in several tumor types, as previously mentioned [4,6,13].

On the testing in eight tumor bank samples from different RB patients in order to preliminarily examine the potential clinical value of the present RT-PCR assay in detecting disseminated tumor cells. When nested-PCR was performed, a clear GD2 synthase band was detected. He was treated with adjuvant systemic chemotherapy and orbital irradiation according to the institutional protocol, and has been in complete remission for 25 months. The patient with stage 4a disease had GD2-positive cells by immunocytology in the bone marrow and showed a faint band corresponding to GD2 synthase mRNA visible in the first round of RT-PCR amplification in the CSF sample at diagnosis and a clear band in the NESTED-PCR. Also on the analyzing the expression of GD2 synthase in human tumor samples from another three patients from the tumor bank in order to verify whether GD2 synthase was expressed as in the cell lines. As shown in Fig. 1, only one sample was positive in the first round of amplification (lane t7). Using a second round of amplification, all tumor samples were positive for Gd2 synthase, as expected (Fig. 1) [4,14,15].

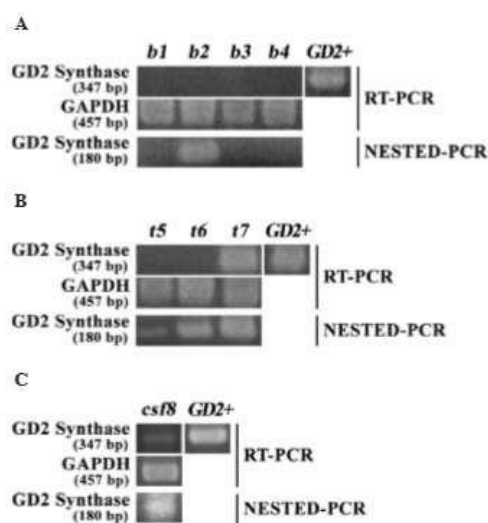


Figure 1. Molecular detection of GD2 synthase mRNA by RT-PCR and NESTED-PCR in samples from RB patients. Optimization of molecular detection of GD2 synthase mRNA in retinoblastoma [4].

Chimeric Antigen Receptor T Cells (CAR T cells)

Chimeric Antigen Receptor (CAR) is a synthetic receptor expressed by T cells. CAR receptors in T cells consist of extracellular single-chain variable fragment (scFv), transmembrane domain, and intracellular part of immunoreceptor tyrosine-based activation motifs (ITAMs) [12]. The fragment of scFv acts as a tumor-associated receptor antigens (TAAs) receptor expressed by tumor cells. CAR receptors can specifically bind to the surface antigen of cancer cells (Table 1) such as the Epidermal Growth Factor Receptor (EGFR), the Human Epidermal Growth Factor Receptor (HER2), Mesothelin (MSLN), Prostate-Specific Membrane Antigen (PSMA), and Carcinoembryonic Antigen (CEA). The target of TAA for CAR T in RB is GD2 [12,16]. This binding results in a transducer signal on T cells via CD3 ξ or Fc receptor- γ . This signaling process enhance the effector function of T cell as a cancer cell crusher [17].

The CAR receptor on T cells has 3 generations with different specific function. The 1st generation CAR receptors are activated through signals from CD3 ξ and Fc receptor- γ . The 2nd and 3rd generation receptors have a co-stimulator domain (CD28, 4-1BB, or OX40) that function as an expansion of antitumor activity and serve as a cytokine secretory inducer such as IL-2, TNF α , and IFN- γ . The CAR receptor structure of each generation is a derivative of T

Cells Receptor (TCR) which consists of antigen binding domains, Transmembrane-domain (TM) and Immune-receptor Tyrosine based Activation Motifs (ITAMs) [12,18]

Table 1. Tumor-surface specific antigens receptor of CAR T Cell[12].

Antigen	Full name	Disease
EGFR	Epidermal growth factor receptor	NSCLC, epithelial carcinoma, glioma
EGFRvIII	Variant III of the epidermal growth factor receptor	Glioblastoma
HER2	Human epidermal growth factor receptor 2	Ovarian cancer, breast cancer, glioblastoma, colon cancer, osteosarcoma, medulloblastoma
MSLN	Mesothelin	Mesothelioma, ovarian cancer, pancreatic adenocarcinoma
PSMA	Prostate-specific membrane antigen	Prostate cancer
CEA	Carcinoembryonic antigen	Pancreatic adenocarcinoma, breast cancer, colorectal carcinoma
GD2	Disialoganglioside 2	Neuroblastoma, melanoma

CAR T Cells recognize specifically the antigen presented by tumor cells. When the CAR receptor specifically binds TAAs, T cells are activated through ITAMs phosphorylation so that T cells secrete cytokines, proliferate, and show cytotoxic effects on tumor cells. The chimeric immunoreceptor actively demonstrates cytotoxic effects via 2 pathways, namely (1) granule secretion of perforin and granzyme and (2) activation of death receptor signaling via Fas / Fas-ligand (Fas-L) or TNF / TNF-R [19]. Studies and research from Yu Shengnan et. al suggests that multireceptor signaling on CAR T cells increases amplification, cytokine production, and cytotoxic effects on T cells, and reduces antigen-induced cell death (AICD) in vitro and in vivo[20].

This modified T cell increases the proliferation and secretion of cytokines from Th1 cells, including IL-2, IFN- γ , IL-12, and TNF. The CD28coreceptor plays an important role in the mechanism of cytokine secretion, proliferation of T cells, and lyse the target tumor cells [21]. IL-12 induced CAR T cells induces cytotoxic T cell activity [22]. Other transgenic cytokines such as IFN- γ secreted by CAR T cells contribute as independent antigens that will destroy tumor cells through signaling mechanisms IFN- γ R, IFN- γ receptors on membrane of tumor cells [23]. Going forward, the clinical application of CAR T cells can be enhanced by strengthening the function of CAR T cells through co- activation of macrophages and NK cells [12,23].

Some cancers are very difficult to cure with chemotherapy conventional. The remaining tumors in some cases are aided by an immunomodulatory checkpoint to maintain an imbalance between balance of immunity and proliferation of cancer cells. Impurities of the checkpoint immunity, such as anti PD-1 / PD-L1, is a new class of resistor serves as a tumor cell suppressor factor through modulation of cell immunity on tumor cell interactions. The inhibitor quickly becomes an approach a promising therapeutic cancer that produces an anti-response tumor with extraordinary side effects. Today, more than 4 antibody inhibitors have been commercialized to target PD-1, PD- L1, and CTLA-4. Despite the great success and efficiency of the anti-PD therapy response is limited to specific types of cancer, which leads to weakening and

heterogeneous expression of PD-1 / PD-L1. In addition to the pattern of reinforcement mechanisms on tumor immunity and therapeutic results of cancer therapy there are also reviews the current process in clinical trials, a combination of drug therapy and safety immunotherapy and other checkpoint inhibitors in the future for various types cancer[17,24].

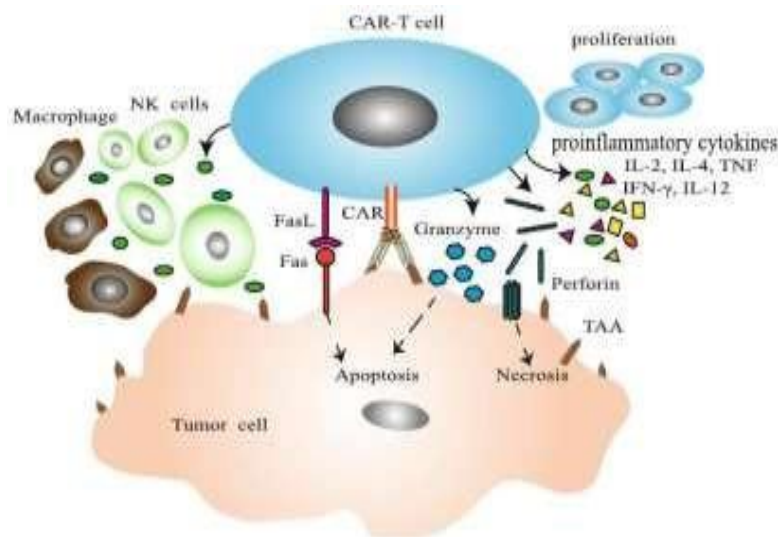


Figure 2. CAR T cells specifically recognize TAAs does not depend on MHC proteins. T cells were activated through ITAMs phosphorylation pathway followed by increased secretion of cytokines (IL-2, IL-4, IFN- γ , IL-12, dan TNF), proliferation of t cells, and cytotoxic effects. Activated T cells and CAR T cells increasing of cytotoxic effects through perforin and granzyme and through Fas/FasL receptor path. CAR T cells antitumor activity is stronger than TCR caused co- stimulator [12].

Increased Potential of CAR T Cells in destroying RB through silencing the PD-1 receptor by CRISPR / Cas9 System

CAR T Cells show promising clinical results in coping leukemia and lipoma including solid tumors including RB. Methodgenetic programming, CRISPR / Cas9 has now been applied to T cellsprimer. CAR T cells are lymphocytes resulting from genetic engineering producing synthetic receptors containing scFv fragments and costimulators.The PD-1 / PD-L1 complex regulates the T cell function. PD-1 acts as a marker of T cell exhaustion. The expression of PD-1 and PD-L1 on tumor cells becomes markers poor prognosis. Research conducted by Rupp et. al aims see if by silencing the PD-1-producing gene (pdcd1) on CAR T Cells through the CRISPR / Cas9 Method, will enhance the anti-tumor effect. Ablation pdcd1 genes specific to CAR T cells are the safest way todayto enhance the effect of anti-tumor or tumor immuno-suppression.This silencing is done simultaneously by silencing the T cell receptor(TCR) to prevent the occurrence of autoreactive T cells [9,25-28].

The first experiment was performed to find out the PD-L1 expression in the cell tumors capable of deactivating CAR T cells in vitro. Transduction CD19 + cell K562 (myelogenous leukemia cell) with expressed lentiviral vector human PD-L1 to form CD19 + PD-L1 + cell K562. Cell K562 has MHC proteins are hard to detect [9,25-28].

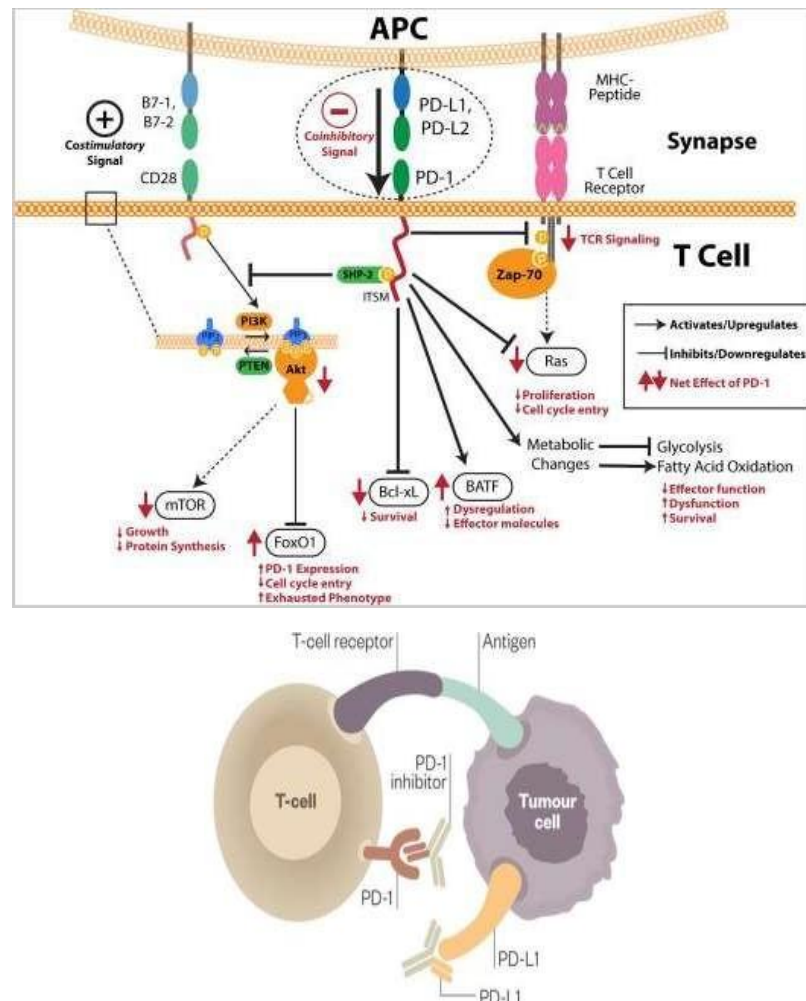


Figure 3. PD-1 receptor signaling pathway with PD-L1 ligand. Signals from PD-L1 cause T cell dysfunction through increased SHP-2 activity as an anti-proliferation factor, and cell survival. Signals from the PD-1 / PD-L1 complex cause dysregulation of T cells and changes in cell metabolism leading to systemic cell dysfunction.

Modified PD-1 CAR T Cells Receptors Increase Potential Antitumor.

To determine the effect of PD-1 deficiency on T cell function, Rupp et.al tested on anti-CD19 CAR T cells with engineered receptors in vitro as in the previous picture, the disturbance causes pcd1 degradation of CD107a degranulation and ending in CD8+ CAR T cell defects cell.

The in vivo test of pdccl1 effect on CAR T cells was performed by injection mice in subcutaneous (SC) with CD19 + PD-L1 + K562 cells. Tumor before injection with Cas9 or edited PD-1 (unedited) cell anti-CD19 CAR T. As a result, 100% of mice that received CAR T injection edited PD-1 cells can destroy tumors. The results of this study show CRISPR targeting pdccl1 locus may increase the CAR's anti-tumor potential T cells in vivo. Pdccl1 can be efficiently silenced in CAR T cells using Cas9 ribonucleo-protein (Cas9 RNPs). PD-1 silencing is done with cell culture anti CD3 / CD28 (reducing the effects of autoactivity) for 48 hours. After that, the nucleophase process is performed to deliver RNP Cas9 into T cells. Cas9 RNP serves to silence pdccl1 so that T cells do not reveal it PD-1. The next step performs CAR transduction with the help of vectors lentiviral. The end result is a CAR T-cell without a PD-17 receptor. This cell able to increase the efficacy of anti-tumor effect and increase the effects of degranulation CD8 + cells [9,29-30].

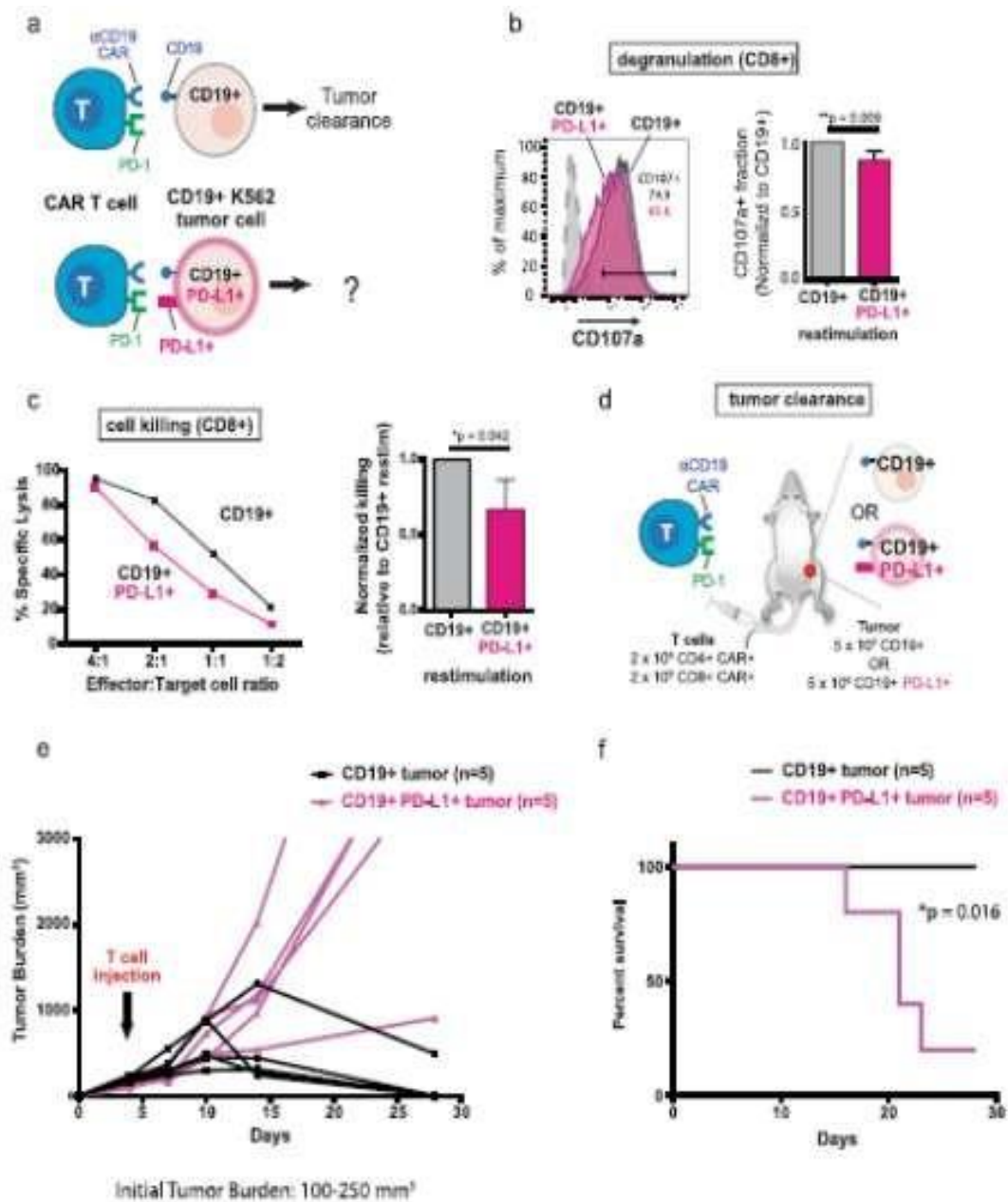


Figure 4. (A) shows the effect of PD-L1 on anti-CD19+ cells that have PD-1 receptors. (B) shows decreased CD8+ degranulation effects when PD-L1 is present in tumor cells. (C) shows a CD8+ chart lyse cells with and without PD-L1. (D) administration of 2 treatments which is given to mice. (E) higher cancer cell count when there is PD-L1. (F) percentage survival of T cells indicates the number of T cells decreased on the 20th day due to the presence of PD-L1[9].

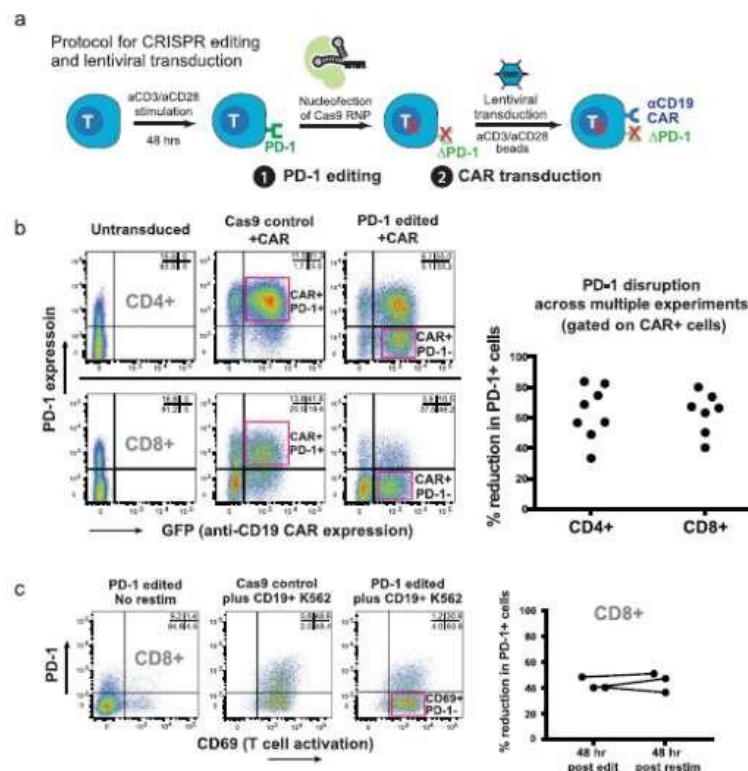


Figure 5. (A) Indicates PD-1 silencing protocol with CRISPR and transduction CAR with lentiviral vector. (B) Efficiency of PD-1 deletion and CAR transduction in primary T cells. PD-1 staining on the cell surface and CAR transduction was performed 48 hours after editing. In the right pane, individual dots show independent editing experiments. (C) CAR T cells which edited stable when cultured. Reduction percentage of PD-1 receptor is measured using flow cytometry based on the staining that has been done at PD-1 on the cell surface[9].

CONCLUSIONS

Based on the analysis and synthesis of problems that have been described based on the literature review, the appropriate immunotherapy for Retinoblastoma is CAR T cells. Increasing potential of CAR T cells can be done by silencing PD-1 receptors on CAR T cells with CRISPR/Cas9 system. The combination using CAR T cell and CRISPR-Cas9 will be the great therapy to eradicate RB without any side effect.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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