Optimization of Culture Conditions for the Generation of Canine CD20-CAR-T Cells for Adoptive Immunotherapy

OSAMU SAKAI, HIROKA YAMAMOTO, MASAYA IGASE and TAKUYA MIZUNO

*Laboratory of Molecular Diagnostics and Therapeutics,
Joint Graduate school of Veterinary Medicine, Yamaguchi University, Yoshida, Japan*

Abstract. *Background/Aim:* Chimeric antigen receptor (CAR) T cell therapy targeting CD20 has the potential to become a promising novel treatment for canine B cell lymphoid malignancy. However, the optimal approach for producing potent CAR-T cells with favorable phenotype for dogs remains unknown. In this study, we assessed several culture conditions and their effects on the phenotypic characteristics of CD20-CAR-T cells. *Materials and Methods:* Canine CAR-T cells were generated by incubating with several mitogens in the presence or absence of Akt inhibitor. Gene transduction efficiency and phenotypic characteristics were determined by flow cytometry. *Results:* Comparison of several kinds of mitogens revealed that stimulation with phytohemagglutinin has high transduction efficacy, whereas stimulation with concanavalin A was superior in memory T cell formation. Akt inhibition at the initial stage of CAR-T production tended to enhance transduction efficiency and memory T cell formation. *Conclusion:* This study provides a significant insight into the understanding of the *ex vivo* expansion of canine T cells in adoptive immunotherapy.

Canine cancer is clinically important because it can be a promising and relevant model for human cancer (1). Current research has reported similarities between human and canine cancer types such as soft tissue sarcoma, osteosarcoma, and lymphoma (2-4). Consequently, models of canine cancer can be useful especially in the field of immunotherapy, because

immunocompetent dogs have an immunosuppressive cancer microenvironment that enables evaluations of the complex interactions between cancer and immune cells.

Canine B cell lymphoma is one of the most common hematopoietic neoplasms in veterinary medicine. Multi-drug chemotherapy, as a standard treatment, is effective in the majority of cases, however, treatment is often challenging because of relapsed or refractory disease, and drug resistance. Therefore, it is important to identify a novel therapeutic agent. As with human medicine, novel immunotherapy, such as adoptive immunotherapy and monoclonal antibody therapy, has been developed in canine medicine (5-7).

The adoptive transfer of CAR-T cells is a novel cancer immunotherapy that can redirect patient's T cells to attack cancer cells. CAR-T cell therapy seems promising especially in hematopoietic tumors, and additional approaches have been tested to conquer solid tumor (8). CD19-redirectioned CAR-T cell therapy is a promising strategy for children and adults with B cell malignancies (9-12), and CD20-redirectioned CAR-T cell therapy is considered a new approach for canine B cell lymphoma (5, 7). The efficacy of infused CAR-T cells is associated with many factors, such as CAR-positive expression rate, proliferative response, and the persistence of infused cells *in vivo* (13, 14). However, the manufacturing of CAR-T cells is a complicated process that involves *ex vivo* cell activation, gene modification and expansion, where many factors can influence the quality of the final products. Studies indicate that less-differentiated T cells with memory phenotype correlate with the *in vivo* proliferation and persistence of CAR-T cells, thus leading to good clinical outcomes (15, 16). Various strategies have been employed to maintain the memory phenotype of CAR-T cells (13, 14). These strategies include cytokine modulation, small molecule inhibitors that regulate transcription or metabolic transformation, immune checkpoint blockade, epigenetic modification, or costimulatory domain modification. Several small molecules have been identified to arrest T cells at the memory T cell stage, and recent studies have highlighted the importance of the Akt pathway in the regulation of T cell

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Correspondence to: Takuya Mizuno, Laboratory of Molecular Diagnostics and Therapeutics, Joint Graduate school of Veterinary Medicine, Yamaguchi University, 1677-1 Yoshida, Yamaguchi 753-8515, Japan. Tel: +81 839335894, e-mail: mizutaku@yamaguchi-u.ac.jp

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Table I. List of culture conditions for CAR-T cells.

Condition	Cell stimulation	Akt inhibitor	Clture medium	Number of donors*
#1	PHA ConA PMA/Iono	-	R10	2
#2		-		
	PHA	DMSO Akt-inhibitor VIII GDC-0068 (1 µg/ml) GDC-0068 (10 µg/ml)	R10	2
	ConA	DMSO Akt-inhibitor VIII GDC-0068 (1 µg/ml) GDC-0068 (10 µg/ml)	R10	3
#3		-		
	PHA		R10 LymphoONE OpTmizer (SR 0%) OpTmizer (SR 2%) OpTmizer (SR 5%)	2
	ConA		R10 LymphoONE OpTmizer (SR 0%) OpTmizer (SR 2%) OpTmizer (SR 5%)	3

*Each donor was used once for each experimental condition. PHA: Phytohemagglutinin; ConA: concanavalin A; PMA: phorbol myristate acetate; Iono: ionomycin; SR: serum replacement.

differentiation and memory formation (13, 17). In addition, protocols for the generation of CAR-T cells using selective Akt inhibitors have been reported (18, 19).

Additional factors that have been found to influence *ex vivo* culture of T cells include mitogens for cell stimulation and culture media. Mitogens are widely used to stimulate lymphocytes in culture. Panjwani *et al.* used ConA stimulation to prepare canine CAR-T cells (5) and recently they have reported a more efficient method using anti-canine CD3 and anti-canine CD28 antibodies (7, 20), yet its effects on T cell phenotype remain unknown. In general, culture medium is complemented with serum to support cell growth. Serum provides factors that sustain cell expansion and proliferation. However, in the setting of adoptive immunotherapy, the use of serum is associated with concerns about the potential risk of contamination and immunogenicity. Therefore, a serum-free medium optimized for expansion of human T cells has been developed and used to expand CAR-T cells (21). Various studies have also demonstrated that serum-free media improve memory subset formation and *in vivo* antitumor function (22, 23).

The purpose of this study was to determine the culture conditions and cell stimulation protocol that can produce potent CAR-T cells. In our previous study, we have reported optimal transduction protocol to generate canine CAR-T

cells (24). We also examined several culture conditions, which have been widely used in human T cell culture, however, the culture conditions to produce CAR-T cells with favorable phenotype for adoptive immunotherapy remained unclear. In this study, we evaluated phenotypic effects of the following culture conditions: mitogen modulation, Akt inhibition at the initial stage of T cell stimulation, and use of serum-free media. Consequently, we used transduction efficiency, memory subset formation, and the expression of activation/exhaustion markers to assess the effects of culture conditions. Our results provide supporting information on canine T cell culturing for adoptive immunotherapy.

Materials and Methods

Cells. Retroviral packaging cell lines Plat-E and PG13, were cultured in D10 complete medium (Dulbecco's modified Eagle's medium supplemented with high glucose, 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 µg/ml streptomycin, and 55 µM 2-mercaptoethanol). Plat-E cells were kindly provided from Dr. Kitamura (Institute of Medical Science, University of Tokyo, Tokyo, Japan). PG13 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). All cell lines were tested for mycoplasma contamination by e-Myco™ Plus Mycoplasma PCR detection kit (iNtRON Biotechnology, Inc., Burlington, MA, USA) in our laboratory, and cultured in a humidified incubator at 37°C and 5% CO₂.

CAR construction and retrovirus production. We used a third-generation CAR construct for all experimental processes that is described in our previous study (24). To obtain a PG13 cell line that is stably producing viruses, retrovirus particles were generated by transient transfection of Plat-E cells with the CAR-encoding retrovirus vector. Supernatants containing the retrovirus were then collected following 48 h, and were used to transduce PG13 cells for retroviral transduction of T cells. More specifically, the retroviral production from the PG13 producer cell line was conducted as follows. First, 3×10^6 transduced PG13 cells were seeded in T75 flasks and cultured in a CO₂ incubator at 37°C. Twenty-four hours later, the culture media were changed with fresh DMEM containing 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, and 5 mM sodium butyrate and incubated for 24 h. Finally, the retrovirus-containing supernatants were filtered through 0.45 µm filters and stored at -80°C until later use.

Cell stimulation, transduction, and expansion of CAR-T cells. All animal studies were conducted in accordance with the Yamaguchi University Animal Care and Use Committee Regulations. All blood samples were obtained from healthy beagle dogs that were previously kept as blood donors in the Yamaguchi University Animal Medical Center. Peripheral blood mononuclear cells (PBMCs) were isolated using Lymphoprep (Axis-Shield, Oslo, Norway) gradient centrifugation and were then stimulated in the presence of 200 U/ml of recombinant human IL-2 (Proleukin; Novartis, Basel, Switzerland) for 72 h.

To evaluate the phenotypic effect, various culture conditions (Table I) were tested during CAR-T manufacturing as follows: mitogens (condition #1), Akt inhibitors (condition #2), and culture media (condition #3). In condition #1, cells were stimulated with phytohemagglutinin (PHA, 5 µg/ml; Sigma, St. Louis, MO, USA), concanavalin A (ConA, 5 µg/ml; Calbiochem, San Diego, CA, USA), or phorbol myristate acetate (PMA, 25 ng/ml; Calbiochem) plus ionomycin (Iono, 1 µg/ml; Calbiochem) for the first 72 h. In condition #2, cells were stimulated with PHA or ConA, and Akt inhibitors were added in the first 120 h: Akt Inhibitor VIII (12 µM; Cayman Chemical, Ann Arbor, MI, USA) or GDC-0068 (1 or 10 µM; Cayman Chemical). R10 complete medium (RPMI1640 supplemented with 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin, and 55 µM 2-mercaptoethanol) was used for culture of PBMCs in conditions #1 and #2. In condition #3, in addition to R10 complete medium, two serum-free media, LymphoONE T cell Expansion Xeno-Free Medium (LymphoONE; TaKaRa Bio, Shiga, Japan) and CTS OpTmizer T cell Expansion SFM (OpTmizer; Thermo Fisher Scientific, Waltham, MA, USA), were also used according to manufacturer's instructions. CTS immune cell serum replacement (SR; 2 or 5%; Thermo Fisher Scientific) was added to the OpTmizer as indicated.

Retroviral transduction was performed following the initial stimulation using a recombinant human fibronectin fragment (RetroNectin; TaKaRa Bio, Shiga, Japan). Non-treated 24-well culture plates were coated with 0.5 ml of RetroNectin solution (10 µg/ml) diluted with phosphate buffered saline (PBS) for 2 h at room temperature in accordance with the manufacturer's instructions. After removal of the RetroNectin solution, all plates were washed with PBS. Consequently, 0.5 ml of retrovirus-containing supernatants were added to the RetroNectin-coated plate, and a virus-bound plate was prepared using centrifugation methods (2,000×g for 2 h at 4°C). A total of 2.5×10^5 stimulated T cells was added to the virus-bound

plates and incubated in a 37°C, 5% CO₂ incubator for 24 h, followed by a second virus infection in the same manner. To promote contact between T cells and viral particles, plates were centrifuged at 500×g for 1 min after the second infection.

Finally, CAR-T cells were subsequently expanded with 200 U/ml IL-2 for 6 days following retroviral transduction. Non-transduced T cells, used as controls, were stimulated with PHA or ConA and expanded in parallel in the presence of 200 U/ml IL-2.

Flow cytometry. CAR-T cells were collected and washed with PBS, followed by incubation with Fixable Viability Dye eFluor 780 (eBioscience, Inc. Vienna, Austria) for 30 min on ice. Then, cells were resuspended in the fluorescence-activated cell sorting (FACS) buffer (PBS containing 2% FBS and 0.1% NaN₃). A total of 2×10^5 cells was stained with each antibody for 30 min on ice. After incubation, cells were washed and fixed in 1% paraformaldehyde and were stored until analysis. For T cell phenotyping, the following antibodies were used: mouse anti-dog CD3 (clone CA17.2A12; dilution 1:500), mouse anti-dog CD21 RPE (clone CA2.1D6; dilution 1:20) and mouse anti-human CD62L RPE (clone FMC46; Bio-Rad Laboratories, Inc. Hercules, CA, USA; dilution 1:20); rat anti-dog CD8α APC (clone YCATE55.9; eBioscience; dilution 1:20); mouse anti-dog CD4 (clone CA13.1E4; dilution 1:4), and mouse anti-dog CD45RA (clone CA21.4B3; undiluted) kindly provided by P.F. Moore (University of California, Davis, CA, USA). Anti-human CD62L antibody was confirmed to cross-react with canine cells in a previous report (25). We used anti-cPD-1 (clone 4F12-E6; concentration 10 µg/ml) and anti-cPD-L1 (clone G11-6; concentration 10 µg/ml) antibodies that were prepared in our laboratory for the analysis of PD-1 and PD-L1 expression on CAR-T cells (26). Purified rat IgG2a antibody (clone RTK2758; BioLegend, San Diego, CA, USA; concentration 10 µg/ml) was used as isotype control for anti-cPD-1 and anti-cPD-L1 antibodies.

Samples were then analyzed using an Accuri C6 (BD Biosciences, San Diego, CA, USA), and data were obtained with the FlowJo software (BD Biosciences).

Results

Firstly, we evaluated the effect of mitogens on the phenotype of CAR-T cells (condition #1). Figure 1 shows the transduction efficiency using cells from two healthy donors; in which the results were similar between the two. Stimulation with PHA resulted in the highest gene transduction rate (67.2% in donor #1 and 32.8% in donor #2), followed by stimulation with ConA (49.1% in donor #1 and 28.4% in donor #2) and PMA plus ionomycin (26.2% in donor #1 and 10.6% in donor #2) (Figure 1A). To evaluate the T cell differentiation status, we assessed the expression of CD45RA and CD62L, and CAR-T cells were classified into four differentiation subsets: stem cell memory T cells (Tscm, CD45RA+CD62L+), central memory T cells (Tcm, CD45RA-CD62L+), effector memory T cells (Tem, CD45RA-CD62L-), and effector T cells (Teff, CD45RA+CD62L-). Phenotypic analysis of CAR-T cells revealed that CAR-T cells generated by ConA stimulation or PMA plus ionomycin showed the higher number of Tscm

Figure 1. The effect of three types of mitogens on phenotypic characteristics of CAR-T cells. PBMCs were stimulated with either of the three mitogens (PHA, ConA, or PMA plus ionomycin) and were transduced with a third-generation CAR construct. Transduction efficiency was assessed by Venus expression using flow cytometry. In addition, CAR-expressing cells were stained with antibodies to identify the respective phenotypes, and were then analyzed by flow cytometry. Each experiment was independently performed using two healthy dogs. (A) Transduction efficiency of CAR. (B) T cell memory subset analysis based on CD45RA and CD62L expression. (C) Expression levels of PD-1 and PD-L1 in CAR-T cells. CAR-T: Chimeric antigen receptor T cells; PHA: phytohemagglutinin; ConA: concanavalin A; PMA: phorbol myristate acetate; Iono: ionomycin; Teff: effector T cells; Tem: effector memory T cells; Tcm: central memory T cells; Tscm: stem cell memory T cells.

Figure 2. Effect of Akt inhibition during T cell stimulation. PBMCs were stimulated with PHA or ConA, and were retrovirally transduced with third-generation CAR. Akt Inhibitor VIII (12 μ M) or GDC-0068 (1 or 10 μ M), were added to the culture medium in the first 120 h during CAR-T cell manufacturing. No-addition control (control) and vehicle control (0.05% DMSO) were stimulated and transduced in parallel. Transduction efficiency was assessed by Venus expression using flow cytometry. CAR-expressing cells were stained with antibodies to identify the respective phenotypes, and were then analyzed by flow cytometry. Each experiment was independently performed using five healthy dogs (PHA, $n=2$; ConA, $n=3$), and the representative data are shown. (A) Transduction efficiency of CAR. (B) T cell memory subset analysis based on CD45RA and CD62L expression. (C) Expression levels of PD-1 and PD-L1 in CAR-T cells. CAR-T: Chimeric antigen receptor T cells; PHA: phytohemagglutinin; ConA: concanavalin A; Akt-VIII: Akt Inhibitor VIII; GDC-1: 1 μ M GDC-0068; GDC-10: 10 μ M GDC-0068; Teff: effector T cells; Tem: effector memory T cells; Tcm: central memory T cells; Tscm: stem cell memory T cells.

Figure 3. CAR-T cell production using serum-free media. PBMCs were stimulated using PHA or ConA, and were transduced with a third-generation CAR. R10 complete medium or two serum-free media were used during all culture periods, and serum replacement (SR) was added to the OpTmizer medium at the indicated amount. (A) Fold expansion during the cell expansion period. The total cell numbers of Day 10 were compared with those of Day 3. Control shows the results of non-transduced PBMCs. Bars indicate average, and error bars indicate standard deviation. (B) Transduction efficiency was assessed by Venus expression using flow cytometry. Each experiment was independently performed using two healthy dogs, and representative data are shown. PHA: Phytohemagglutinin; ConA: concanavalin A; R10: R10 complete medium; LymphoONE: LymphoONE T cell expansion Xeno-free medium; OpTmizer: CTS OpTmizer T cell Expansion SFM; SR: CTS immune cell serum replacement.

subset, as compared with PHA stimulation (Figure 1B). Stimulation with ConA tended to decrease PD-1 and PD-L1 expression in both donors (Figure 1C).

Next, we assessed how Akt inhibition influenced the phenotype of canine CAR-T cells (condition #2). In this experiment, PBMCs from five donors were used: PBMCs

from two donors were stimulated with PHA, and the remaining PBMCs were stimulated with ConA. Since the results from the two PHA-stimulated PBMCs and the results from the two of the three ConA-stimulated PBMCs showed similar patterns, the representative data from three donors are shown in Figure 2. In all samples from the five donors,

irrespective of stimulation, Akt inhibition improved transduction rate (Figure 2A). As shown in Figure 2B, Akt inhibition by the Akt inhibitor VIII resulted in an increased number of Tscm subset in both samples from PHA-stimulated PBMCs, and in one of the three samples from ConA-stimulated PBMCs (donor #1). At the same time, we did not observe any increase in the Tscm subset by Akt inhibition in two of the three samples from ConA-stimulated PBMCs (Figure 2B, donor #2). Decreased expression of PD-1 by Akt inhibition was observed in both samples from the PHA-stimulated PBMCs, and in one of the three samples from the ConA-stimulated PBMCs (Figure 2C, PHA and donor #1). Akt inhibition by the Akt Inhibitor VIII demonstrated a greater decrease in PD-1 expression than in GDC-0068. Furthermore, we did not observe any decrease in the expression of PD-1 and PD-L1 by Akt inhibition in two of the three samples from the ConA-stimulated PBMCs (Figure 2C, donor #2).

Finally, our study examined whether serum-free media could be used for canine CAR-T generation (condition #3). Instead of using R10 complete medium, the LymphoONE and OpTmizer serum-free media were used, and serum replacement was added to OpTmizer to evaluate the effect on canine T cells. As a result, PBMCs from three of the four donors could expand in LymphoONE medium, whereas almost no cell growth was observed in the OpTmizer medium (Figure 3A). Moreover, PBMCs from two donors exhibited superior cell expansion in LymphoONE medium compared to the R10 complete medium (Figure 3A, donor #2 and #4). However, FACS analysis of the collected cells revealed that the CAR-expression levels were low in serum-free conditions, especially in the LymphoONE medium (Figure 3B).

Discussion

We have reported the basic optimization of canine CAR-T generation in a previous study whose results revealed an optimal transduction protocol to generate CAR-expressing canine T cells (24). However, the culture conditions and cell stimulation protocol that can produce potent CAR-T cells with favorable phenotype for adoptive immunotherapy remained elusive. In this study, we further investigated the effects of the following factors: mitogens, small molecule inhibitors, and serum-free medium (Table I). We used transduction efficiency, differentiation status of T cells, and T cell exhaustion to assess the phenotypic effects of these factors. The mitogens used in this study are widely used to stimulate lymphocytes, however, there are no studies that compare the effects of these mitogens to generate the CAR-T cells. Although there is one report that describes the activating effects of these mitogens on canine B and T lymphocytes (27), the effects on transduction efficiency and cell phenotype are still unclear. Our results indicate that the

effects on transduction efficiency and cell phenotype vary depending on each mitogen, and that stimulation with Con A and PMA plus ionomycin enhances memory T cell formation. Adoptive immunotherapy against canine cancer has been not established, however, it may be useful to use different mitogens depending on the cases.

The changes observed in this study, such as the increase in transduction efficiency and memory subset, and the decrease in PD-1 expression, can contribute to the clinical efficacy of the CAR-T cell therapy. Zhang *et al.* have also reported that Akt inhibition enhanced CAR-expression rate and memory phenotype (19). This study verifies these results; however, our findings indicate that the effects of Akt inhibition are different among individual dogs, especially with regard to ConA-stimulated PBMCs. Further studies are required to assess whether Akt inhibition contributes to the clinical efficacy of the canine CAR-T cell therapy. During preparation of this manuscript, Mason's group published an efficient transduction method for canine CAR-T using magnetic beads coated with anti-canine CD3 and anti-canine CD28 antibodies, but they mainly focused on the gene transduction efficiency and cytotoxic functions (7, 20), and not on the phenotypic characteristics, which are also important for the maintenance *in vivo* after CAR-T injection.

Furthermore, the results from the cells cultured in OpTmizer medium demonstrated that the addition of serum replacement facilitates decreased CAR-expression levels. These results indicate that these serum-free media and serum replacement, optimized for human cell culture, are not suitable for the preparation of canine CAR-T cells. However, it is still plausible that the LymphoONE medium could be used for alternative adoptive cell therapies. Canine cell culture using serum-free medium is reported in cell lines and mesenchymal stem cells (MSCs); however, to this date, there are no data on the culture of canine PBMCs using serum-free medium (28, 29). Development of novel serum-free medium that is optimized to expand canine PBMCs may help in the generation of more effective canine CAR-T cells.

Conclusively, this study presented the effects of mitogens on CAR-T cell phenotype and demonstrated that Akt inhibition enhanced CAR-expression rate and memory phenotype. As mentioned above, reports have suggested many biomarkers, which correlated to safety and efficacy in CAR-T cell therapy. It is unclear whether these markers are valid in canine CAR-T cell therapy. Our results provide novel information that can be significantly useful in CAR-T cell therapy, but also in other adoptive immunotherapies for canine cancer.

Conflicts of Interest

The Authors declare that there are no conflicts of interest regarding this study.

Authors' Contributions

O.S. and H.Y. carried out the experiments. O.S. wrote the manuscript in consultation with M.I. and T.M. T.M. supervised the project.

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