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Immunology

Generation of molecular-targeting helix-loop-helix peptides for inhibition of the interaction between cytotoxic T-lymphocyte-associated protein 4 and B7 in the dog

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ABSTRACT. Blocking the interaction between CD28 and B7 by cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) is a potent immune checkpoint that prevents damage to host tissues from excessive immune responses. However, it also significantly diminishes immune responses against cancers and allows cancer cell growth. This study found that recombinant (*r*) human (h) CTLA-4 specifically binds to canine dendritic cells (DCs) and suppresses the responses of canine T cells to allogeneic DCs. ERY2-4, a peptide targeting *r*hCTLA-4 selected from a yeast-displayed library of helix-loop-helix (HLH) peptides and improved to have a binding affinity to *r*hCTLA-4 as strong as that of *r*hB7, inhibited the binding of *r*hCTLA-4 to canine DCs. Furthermore, the targeting peptide significantly enhanced the response of canine T cells to allogeneic DCs. These results suggest that the CTLA-4-targeting peptide enhances canine T cell activity by blocking the interaction between canine CTLA-4 on T cells and canine B7 on DCs. This study demonstrates the generation of a new type of immune checkpoint inhibitor, which may be applicable to cancer therapy in dogs.

KEYWORDS: B7, cytotoxic T-lymphocyte-associated protein 4, immune checkpoint, molecular-targeting peptide

Co-stimulatory signals generated by the interaction of T cells and dendritic cells (DCs), a typical antigen presenting cells, are required to activate immune responses. The most effective co-stimulatory signal is generated by the binding of CD28 on T cells with B7 on DCs [13]. However, following activation by CD28, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) is expressed on T cells, which wrest B7 from CD28 and generates suppressive signals to inhibit immune responses [1, 19]. Hence, CTLA-4 and programmed death-1 (PD-1) [6] play a role in protecting normal tissues from damage by excess immune responses as immune checkpoints. However, immune checkpoints restrict immune responses against tumors and allow their growth.

Recently, monoclonal antibodies (mAbs) that block this checkpoint have been developed as immunotherapeutic strategies for human and veterinary cancers [5, 12, 14, 16, 22]. However, these mAbs have high molecular weights and complex structures, making them difficult to synthesize, have high immunogenicity, require framework changes from murine Ig to those of the animals to be treated, and it is difficult to improve their affinity to their respective targets. Moreover, the anti-CTLA-4 mAb kills CTLs against cancer via Ab-dependent cell cytotoxicity when CTLs temporally express CTLA-4 after activation [11]. Such limitations of mAbs have prompted an extensive investigation into alternative binders [4].

A *de novo* designed small molecule is a molecular-targeting peptide with a helix-loop-helix (HLH) structure which has a molecular

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weight of approximately 4000 Da, high *in vivo* stability, and a high possibility of improving its affinity to its target [9]. Therefore, it is a promising alternative to cancer therapy. We previously developed an HLH peptide targeting recombinant (*r*) human (h) CTLA-4, which specifically binds to *r*hCTLA-4 with high affinity and inhibits the interaction between *r*hCTLA-4 and hB7 [17]. Because of its low molecular weight (4181.4 Da), the hCTLA-4-targeting peptide is expected to be non-immunogenic regardless of the species, similar to other HLH peptides [9, 18]. This study examined whether the hCTLA-4-targeting peptide could react with canine immune cells for future applications in cancer therapy.

MATERIALS AND METHODS

Animals

Female beagles aged 2–6 years were purchased from Oriental Yeast Co., Ltd. and housed according to the guidelines of national institute of health, the local Institutional Animal Care and Use regulations, and accepted veterinary medical practice. The study protocol was approved by the Animal Experiment Committee of Osaka Prefecture University (approval no. 19-79). The dogs were fed commercial canine food once daily and provided water *ad libitum*.

Reagents and antibodies

Isolation of CTLA-4-targeting peptides was performed using the following reagents and Abs: *r*hCTLA-4-Ig (Abatacept; Bristol Myers Squibb, New York, NY, USA), a chimeric protein composed of the extracellular domain of *r*hCTLA-4 and a constant region of hIgG, the yeast cell surface display vector pYD1 system (Invitrogen, Carlsbad, CA, USA), QIAquick gel extraction kit (Qiagen, Hilden, Germany), fluorescein isothiocyanate (FITC)-goat anti-hIgG-Fc Ab (Jackson ImmunoResearch, Baltimore, MA, USA), Alexa Fluor® 647 (AF647)-goat anti-hIgG-Fc Ab (Jackson ImmunoResearch), anti-FLAG mAb (Sigma-Aldrich, St. Louis, MO, USA), and AF488-goat anti-mouse IgG Ab (Thermo Fisher Scientific, Waltham, MA, USA). Canine DCs were detected using PE-hamster anti-mouse CD80 mAb (e-Bioscience, San Diego, CA, USA) and AF647-anti-cCD40 mAb (AbD Sertec, Oxford, UK). Peripheral blood mononuclear cells (PBMCs) were analyzed using FITC-anti-cCD3 mAb (AbD Sertec), PE-Cy5-anti-h CD14 mAb (AbD Sertec), and PE-anti-cCD21 mAb. AF488-conjugated streptavidin was purchased from Invitrogen. The cross-reactivity of all these mAbs with canine DCs was confirmed by their respective manufacturers.

Generation of hCTLA-4-targeting peptides

Molecular targeting peptides were developed as described by Mudiyanselage *et al*. [17]. An HLH peptide library, ΔPTA-12RC-2, which has 3.0×10^8 variants, was used in this study (Supplementary Fig. 1). A clone of yeast (*Saccharomyces cerevisiae*), EBY100, was transformed with the plasmid pYD11-BxXN, which contains nucleotides encoding ΔPTA-12RC-2 and a FLAG tag peptide, and expanded in culture. The peptide library and a FLAG tag are displayed on the extracellular surface (Supplementary Fig. 2A). After incubation with *r*hCTLA-4 Ig and anti-FLAG mAb, followed by secondary incubation with AF647-goat-anti-hIgG-Fc Ab and AF488-goat anti-mouse IgG Ab, the EBY100 displaying the hCTLA-4-targeting peptide was isolated via fluorescence-activated cell sorting (FACS) using FACSAriaIII (Beckton & Dickinson, San Jose, CA, USA) (Supplementary Fig. 2B). The peptide Y-2 (Supplementary Fig. 1), which showed specific binding activity to hCTLA-4, was identified; however, the affinity of *r*hCTLA-4 to Y-2 displayed on EBY100 was very low [dissociation constant (K_D)=1.53 μM] compared to the affinity of hCTLA-4 to B7-1 (K_D=278 nM), evaluated using Biacore T200™ (Cytiva, Tokyo, Japan). Therefore, to improve binding affinity, random mutations were introduced into the Y-2 peptide by error-prone PCR. Thereafter, ERY2-4, the mutant peptides with the highest affinity for CTLA-4, (Supplementary Fig. 1) were isolated by FACS as described above. ERY2-4 was used as the CTLA-4-targeting peptide and was synthesized by standard Fmoc solid-phase methods. The synthesized ERY2-4 showed a significantly high affinity to *r*hCTLA-4 (K_D=196.8 nM), comparable to that of B7-1. However, it had no affinity for other proteins, such as human tumor necrosis factor-α and epithelial growth factor, hIgG-Fc, and anti-hCD80 mAb. Moreover, ERY2-4, as compared with B7-1, exhibited a significantly lower affinity to CD28, which generates activation signals by binding to B7 [17].

Preparation of cells

Canine DCs were prepared from peripheral blood (PB) monocytes, as described previously [8]. PB monocytes were isolated by magnetic cell sorting using anti-hCD14 microbeads (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany). To induce their differentiation into DCs, the isolated PB monocytes were incubated with rcGM-CSF (R&D Systems Inc., Minneapolis, MN, USA) and *r*cIL-4 (R&D Systems) for seven days. More than 95% of the resulting cells showed high expression of CD40 and CD80, which are co-stimulatory molecules highly expressed on DCs.

As previously described [21], the T cell fraction was prepared by incubating PBMCs in a nylon wool column (Wako Pure Chemical Co., Ltd., Osaka, Japan). Seventy-eight percent of the T cell fraction expressed CD3. Moreover, the population did not express CD14, a marker of monocytes, or CD21, a marker of B cells.

Flow cytometry

The following assays were performed in flow cytometry (FCM).

To estimate the binding ability of *r*hCTLA-4 to canine DCs, various concentrations of *r*hCTLA-4 Ig were incubated with DCs for 20 min at room temperature (RT), washed, and incubated with FITC-goat anti-hIgG-Fc Ab and AF647-anti-cCD40 mAb on ice for 30 min. After incubation, *r*hCTLA-4-bound DCs were detected as FITC-labeled cells in the $CD40^+$ population. The binding of

*r*hCTLA-4 to canine DCs was evaluated by the median fluorescence intensity (MFI) of FITC. In some experiments, to rule out the possibility of *r*hCTLA-4 binding to canine DCs via the Fc receptor, *r*hCTLA-4 was biotinylated using an EZ-Link Sulfo-NHS-Biotin kit (ThermoFisher Scientific), and the biotinylated *r*hCTLA-4 (100 nM) was mixed with a larger amount (300, 1,000, or 3,000 nM) of hIgG (Novus Biologicals, Centennial, CO, USA) and incubated with DCs for 20 min at RT. After incubation, the DCs were washed and incubated with Alexa Fluor 488-streptavidin on ice for 30 min.

To evaluate the inhibitory effect of the CTLA-4-targeting peptide ERY2-4 against *r*hCTLA-4 in binding to B7, canine DCs were incubated with various concentrations of ERY2-4 for 30 min at RT in the presence of a constant amount of *r*hCTLA-4 Ig (30 nM). After washing, the DCs were incubated with FITC-goat anti-hIgG-Fc Ab and AF647-mouse anti-cCD40 mAb on ice for 30 min. The binding of *r*hCTLA-4 to canine DCs was evaluated as described above. The inhibitory effect of ERY2-4 was detected as a decrease in MFI. The HLH peptide YT1-S, which did not show any binding activity to *r*hCTLA-4 [17], was used as a negative control.

FCM was performed using an S3 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) or CytoFLEX (Beckman Coulter, Brea, CA, USA). The results were analyzed using the associated software (Prosort-ver.1.5, Bio-Rad or CytExpert, Beckman Coulter).

Mixed lymphocyte reaction assay

In the mixed lymphocyte reaction (MLR), T cells (2×10^5) were cultured with canine DCs (2×10^4) in flat-bottom 96-well culture plates in triplicate. The reaction was evaluated based on lymphocyte proliferation. In some experiments, [^{3}H]-thymidine (PerkinElmer Inc., Waltham, MA, USA) was added on day 3 of MLR culture and incubated for 16 hr. Lymphocyte proliferation was evaluated by measuring the radioactivity (cpm) of [^{3}H]-thymidine incorporated into the DNA of the cells, as previously reported [17]. In other experiments, according to the report by Austyn *et al*. [2], the reaction was evaluated by counting cell clusters in the MLR culture using a phase-contrast microscope (Nikon, Tokyo, Japan).

To examine the inhibitory activity of *r*hCTLA-4 against MLR, different concentrations of *r*hCTLA-4 Ig were added to the cultures. The same concentration of hIgG was used as a control. To examine the function of the hCTLA-4-targeting peptide ERY2-4, 30 µM ERY2-4 was added to the MLR. The same concentration of YT1-S or phosphate buffered saline (PBS) was used as the control.

Statistics

Results of experiments with more than three groups were compared using the Tukey-Kramer multiple comparison test. Data from experiments with two groups were compared using Student's *t*-test. The significance level was set at $P<0.05$.

RESULTS

rhCTLA-4 interacted with canine DCs

First, we examined the reactivity of *r*hCTLA-4 in canine immune cells. As shown in Fig. 1A, the fluorescence intensity of DCs was higher when treated with fluorescence-labeled *r*hCTLA-4, as observed in FCM. As shown in Fig. 1B, the fluorescence intensity of DCs increased in a *r*hCTLA-4 dose-dependent manner, whereas that of the T cell fraction did not. To rule out the possibility of *r*hCTLA-4 binding to canine DCs via the Fc receptor, *r*hCTLA-4 (100 nM) was mixed with a higher concentration of hIgG (300, 1,000, and 3,000 nM) and incubated with DCs. The binding intensity of *r*hCTLA-4 to DCs did not change regardless of the concentration of hIgG, as evaluated via fluorescence intensity analysis in FCM (Supplementary Fig. 3). These results suggest that *r*hCTLA-4 does not bind to DCs via the Fc receptor. Moreover, *r*hCTLA-4 treatment significantly suppressed the proliferative response of canine T cells to allogeneic canine DCs (Fig. 2).

hCTLA-4-targeting peptide inhibited the interaction of rhCTLA-4 to canine DCs

As shown in Fig. 3A, the binding of 30 nM *r*hCTLA-4 to canine DCs was observed through an increase in fluorescence intensity that was completely decreased to the baseline by adding 30 µM ERY2-4. In contrast, the fluorescence intensity did not decrease with the addition of the same concentration of the control peptide YT1-S, which was previously shown not to interact with *r*hCTLA-4 [17]. Moreover, as shown in Fig. 3B, the fluorescence intensity decreased as the dose of ERY2-4 was increased. In contrast, the fluorescence intensity was not affected by the YT1-S dose.

hCTLA-4-targeting peptide enhanced the interaction between canine DCs and T cells

Finally, we examined whether the hCTLA-4-targeting peptide inhibits the interaction between canine CTLA-4 and B7 in allogeneic MLR. As shown in Fig. 4A, there were clusters in MLR cultures in which canine T lymphocytes reacted to allogeneic canine DCs. However, these clusters were not found in the cultures of the T cell fraction despite the addition of ERY2-4. As shown in Fig. 4B, the number of clusters was notably higher in cultures treated with ERY2-4 than in those treated with YT1-S or PBS at any culture point examined. The number of clusters in the ERY2-4-treated culture significantly increased between days 2 and 3 and then plateaued. However, the clusters in cultures treated with the control peptide YT1-S or PBS did not significantly increase during this period.

In agreement with the results of cluster counts, the proliferation of responding cells, evaluated through the incorporation of [^{3}H]-thymidine into DNA on day 4 of MLR, was significantly higher in the ERY2-4-treated cultures than in the YT1-S- or PBS-treated cultures (Supplementary Fig. 4).

DISCUSSION

This study examined the reactivity of *r*hCTLA-4, a target protein in canine immune cells, and found that *r*hCTLA-4 specifically

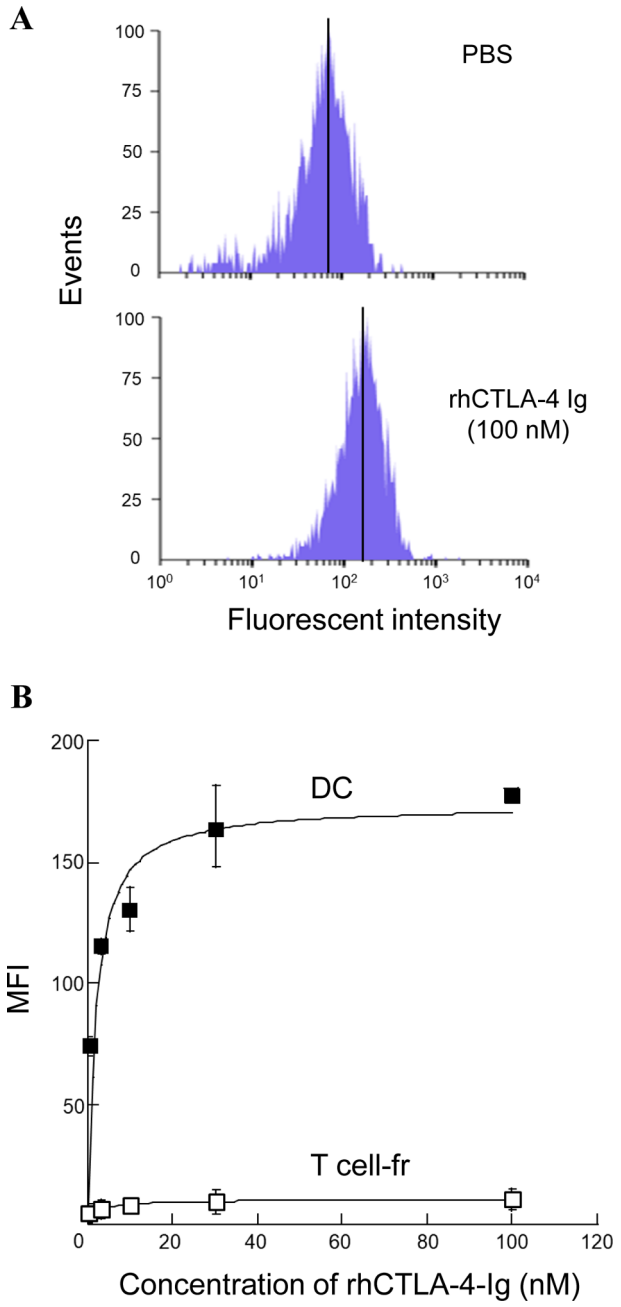

Fig. 1. The binding activity of recombinant (*r*) human (h) cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) to canine dendritic cells (DCs). (**A**) Fluorescence intensity of DCs incubated with *r*hCTLA-4 Ig or phosphate buffered saline (PBS) followed by incubation with fluorescein isothiocyanate (FITC)-labeled anti-hIgG Fc Ab. The cross-points of the x-axis and the vertical black lines are the medians of fluorescent intensity (MFI). Representative results are shown. (**B**) MFI of canine DCs and the canine T cell-fraction (fr) incubated with the indicated concentrations of *r*hCTLA-4 Ig and FITC-labeled anti-hIgG Fc Ab. Experiments were independently carried out three times. Results were expressed as mean ± SD.

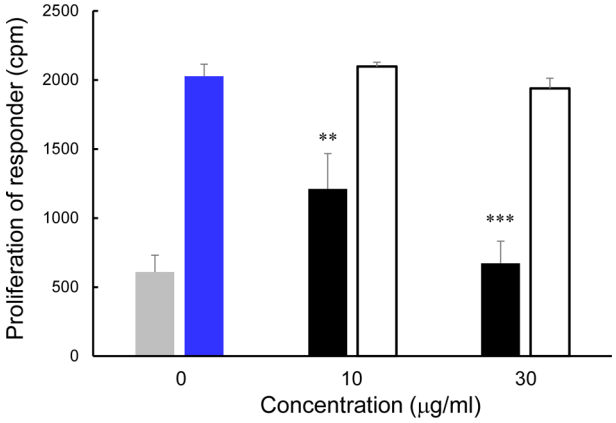

Fig. 2. Suppressive activity of recombinant (*r*) human (h) cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) in the canine mixed lymphocyte reaction. Cells of the T cell fraction were cultured with allogeneic canine dendritic cells (DCs) for 4 days in the presence of the indicated concentrations of *r*hCTLA-4 Ig or hIgG. The proliferation of responder cells was evaluated by the incorporation of [^{3}H]-thymidine into the DNA (cpm). The gray column indicates the proliferative responses in cultures without DCs; the blue column indicates the response in cultures with DCs, but without *r*hCTLA-4 Ig or hIgG; the black columns indicate the responses in cultures with *r*hCTLA-4 Ig; and the open columns indicate the responses in the cultures with hIgG. Experiments were independently carried out three times. Results were expressed as mean ± SD. ***P*<0.01 vs. responses without *r*hCTLA-4 Ig or hIgG and vs. responses with hIgG at the same concentration. ****P*<0.001 vs. responses without *r*hCTLA-4 Ig or hIgG and vs. responses with hIgG at the same concentration.

binds to canine DCs and inhibits the interaction between canine DCs and canine T cells, similar to human MLR [7]. These results suggest that *r*hCTLA-4 binds to canine B7 or other co-stimulatory molecules on DCs and inhibits their interaction with their agonist expressed on canine T cells, such as CD28. Similar to a previous report using human DCs [17], the hCTLA-4-targeting peptide ERY2-4

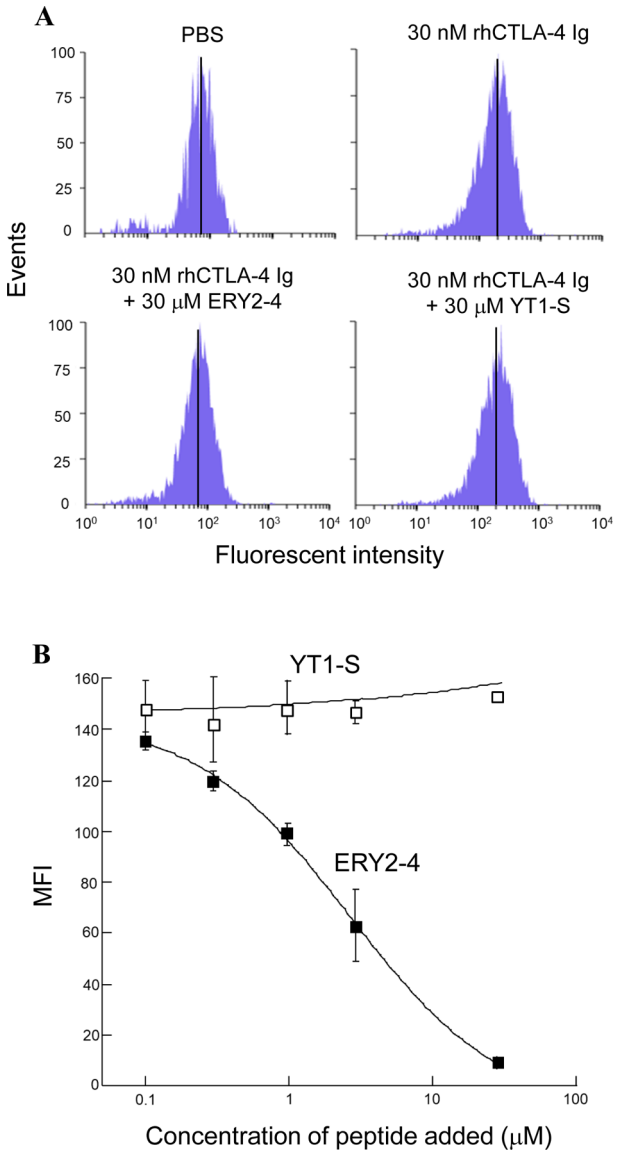

Fig. 3. Effect of ERY2-4 treatment on the binding of recombinant (*r*) human (h) cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) to canine dendritic cells (DCs). (**A**) The fluorescence intensity of canine DCs incubated with the indicated reagents and fluorescein isothiocyanate (FITC)-labeled anti-hIgG Fc Ab. The cross-points of the x-axis and the vertical black lines represent the median fluorescent intensity (MFI). Representative results are shown. (**B**) MFI of DCs incubated with 30 nM *r*hCTLA-4 Ig and FITC-labeled anti-hIgG Fc Ab in the presence of the indicated concentrations of ERY2-4 or YT1-S. Experiments were independently carried out three times. Results are expressed as mean ± SD.

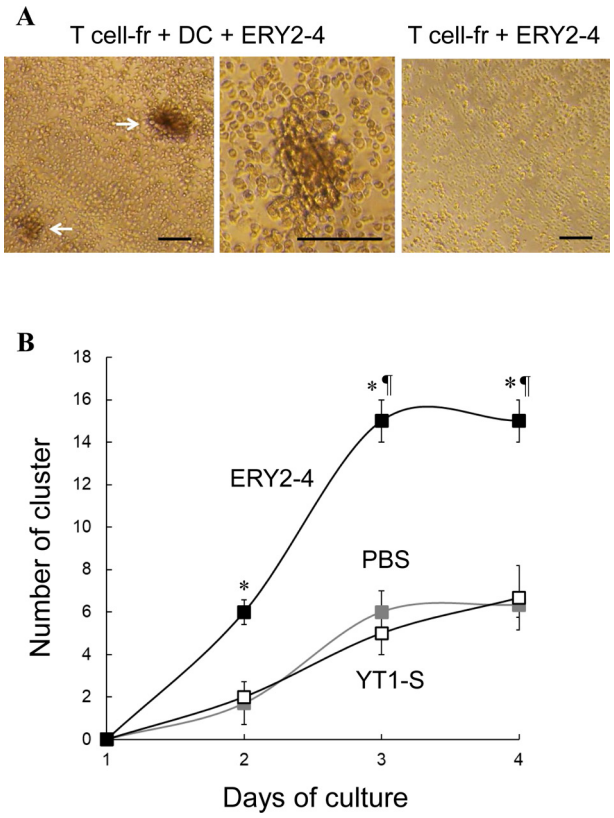

Fig. 4. Effect of ERY2-4 on the canine mixed lymphocyte reaction (MLR). (**A**) Representative photomicrographs of the cultures at day 3, including the indicated cells and peptides. Arrows indicate the clusters of responding cells. The size of the bars indicates 100 μm. (**B**) The number of clusters in the MLR cultures treated with ERY2-4, YT1-S, or phosphate buffered saline (PBS). Experiments were performed independently three times. Results are expressed as mean ± SD. **P*<0.05 vs. the number of clusters in the cultures treated with YT1-S or PBS on the same day of culture. ¶*P*<0.05 vs. the number of clusters on day 2.

inhibited the binding of *r*hCTLA-4 to canine DCs in a dose-dependent manner that completely decreased to the baseline at the same concentration that it did against the binding of *r*hCTLA-4 to human DCs. These results suggest that *r*hCTLA-4 may bind to canine B7 on DCs and inhibit the interaction between canine B7 and CD28 on T cells.

Similar to a previous report using human DCs and human T cells, the hCTLA-4-targeting peptide ERY2-4 enhanced MLRs in canine DCs and canine T cells. Austyn *et al.* reported the bulk of the proliferative response is due to increasing cluster formation of T cells [2]. Since we sought to observe changes in the proliferative response over time, we counted the T cell clusters and found that ERY2-4 significantly enhanced the proliferative response between days 2 and 3 of MLR. In a mouse study, the expression of CTLA-4 occurred on T cells immediately after the stimulation of CD28 by binding with B7. CTLA-4 expression increased, peaked at

48 hr after stimulation, and then decreased thereafter [19]. Considering some time lag caused by differences in stimulation design, the inhibitory effect of ERY2-4 against CTLA-4 may be maximal from days 2 to 3 of MLR in agreement with the results of the cluster counts. Furthermore, the results in culture counts on day 4 of the MLR were consistent with those evaluated by incorporation of [^{3}H] -thymidine. Therefore, these data indicate a high possibility that the hCTLA-4-targeting peptide ERY2-4 inhibits the interaction between canine CTLA-4 and canine B7 and significantly enhances the immune responses of canine T cells.

Blocking CTLA-4 sometimes induces autoimmune-like diseases [12]. CTLA-4 could be expressed on CTL and is enhanced after their activation [13]; therefore, CTLA-4 plays a role in the negative feedback of immune responses. Combined with effective anti-tumor vaccination using DCs [8, 15, 20], blocking CTLA-4 by a targeting peptide will more specifically affect the anti-tumor response, with a concomitant decrease in the prevalence of adverse effects.

In this study, 30 µM ERY2-4 was required to completely inhibit the binding of 30 nM *r*hCTLA-4-Ig, a 1,000-fold difference. Because *r*hCTLA-4-Ig is a dimer of extracellular domain of hCTLA-4 and B7 on DCs are thought to form dimer or oligomer [3, 10], multivalent binding of *r*hCTLA-4-Ig and B7 is expected on DCs with significantly higher binding affinity than monomeric interaction that occurs between ERY2-4 and *r*hCTLA-4-Ig. This difference in affinity may require a significantly larger amount of ERY2-4 to completely inhibit the binding of *r*hCTLA-4-Ig to DC. Therefore, further studies are ongoing to produce targeting peptides with an increased affinity for canine CTLA-4 for clinical applications in cancer therapy.

CONFLICT OF INTEREST. The authors have no conflicts of interest.

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