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

Tharanga M.R. Ramanayake Mudiyanselage¹, Daisuke Fujiwara², Masataka Michigami², Shunichi Watanabe¹, Zhengmao Ye², Atsuko Uyeda², Ryoji Kanegi¹, Shingo Hatoya¹, Ikuo Fujii², Kikuya Sugiura¹

¹Department of Advanced Pathobiology, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1-58 Rinku-ourai-kita, Izumisano, Osaka 598-8531, Japan
²Department of Biological Science, Graduate School of Science, Osaka Prefecture University, 1-1 Gakuen-cho, Naka-ku, Sakai, Osaka 599-8531, Japan

*Present affiliation: Department of Medical Laboratory Science, Faculty of Allied Health Sciences, University of Ruhuna, Matara, Sri Lanka

*Correspondence to:
Kikuya Sugiura
Department of Advanced Pathobiology
Graduate School of Veterinary Science
Osaka Metropolitan University
1-58 Rinku-ourai-kita, Izumisano, Osaka 598-8531, Japan
E-mail: sugiurak@omu.ac.jp
Phone: +81-72-463-5374
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 rhCTLA-4 selected from a yeast-displayed library of helix-loop-helix (HLH) peptides and improved to have a binding affinity to rhCTLA-4 as strong as that of rhB7, inhibited the binding of rhCTLA-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.
INTRODUCTION

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 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 rhCTLA-4 with high affinity and inhibits the interaction between rhCTLA-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: rhCTLA-4-Ig (Abatacept; Bristol Myers Squibb, New York, NY, USA), a chimeric protein composed of the extracellular domain of rhCTLA-4 and a constant region of hIgG (Bristol-Myers Squibb), 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-human 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 $3.0 \times 10^8$ 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 rhlCTLA-4 Ig and anti-FLAG mAb, followed by secondary incubation with AF647-goat-anti-human IgG-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 rhlCTLA-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 rhlCTLA-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, hlgG-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 rcIL-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 population 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 rhCTLA-4 to canine DCs, various concentrations of rhCTLA-4 Ig were incubated with DCs for 20 min at room temperature (RT), washed, and incubated with FITC-goat anti-human IgG-Fc Ab and AF647-anti-cCD40 mAb on ice for 30 min. After incubation, rhCTLA-4-bound DCs were detected as FITC-labeled cells in the CD40+ population. The binding of rhCTLA-4 to canine DCs was evaluated by the median fluorescence intensity (MFI) of FITC. In some experiments, to rule out the possibility of rhCTLA-4 binding to canine DCs via the Fc receptor, rhCTLA-4 was biotinylated using an EZ-Link Sulfo-NHS-Biotin kit (ThermoFisher Scientific), and the biotinylated rhCTLA-4 (100 nM) was mixed with a larger amount (300, 1000, or 3000 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 rhCTLA-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 rhCTLA-4 Ig (30 nM). After washing, the DCs were incubated with FITC-goat anti-human IgG-Fc Ab and AF647-mouse anti-cCD40 mAb on ice for 30 min. The binding of rhCTLA-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 rhCTLA-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 h. 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 rhCTLA-4 against MLR, different concentrations of rhCTLA-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 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 rhCTLA-4 in canine immune cells. As shown in Fig. 1A, the fluorescence intensity of DCs was higher when treated with fluorescence-labeled rhCTLA-4, as observed in FCM. As shown in Fig. 1B, the fluorescence intensity of DCs increased in a rhCTLA-4 dose-dependent manner, whereas that of the T cell fraction did not. To rule out the possibility of rhCTLA-4 binding to canine DCs via the Fc receptor, rhCTLA-4 (100 nM) was mixed with a higher concentration of hIgG (300, 1000, and 3000 nM) and incubated with DCs. The binding intensity of rhCTLA-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 rhCTLA-4 does not bind to DCs via the Fc receptor. Moreover, rhCTLA-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 rhCTLA-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 rhCTLA-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 rhCTLA-4, a target protein in canine immune cells, and found that rhCTLA-4 specifically binds to canine DCs and inhibits the interaction between canine DCs and canine T cells, similar to human MLR [7]. These results suggest that rhCTLA-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 inhibited the binding of rhCTLA-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 rhCTLA-4 to human DCs. These results suggest that rhCTLA-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 h 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 [³H]-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 rhCTLA-4-Ig, a 1000-fold difference. Because rhCTLA-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 rhCTLA-4-Ig and B7 is expected on DCs with significantly higher binding affinity than monomeric interaction that occurs between ERY2-4 and rhCTLA-4-Ig. This difference in affinity may require a significantly larger amount of ERY2-4 to completely inhibit the binding of rhCTLA-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.

ACKNOWLEDGMENT

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REFERENCES


**FIGURE LEGENDS**

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 *rhCTLA*-4 Ig or phosphate buffered saline (PBS) followed by incubation with fluorescein isothiocyanate (FITC)-labeled anti-*h* IgG 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 *rhCTLA*-4 Ig and FITC-labeled anti-*h* IgG 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 *rhCTLA*-4 Ig or hIgG. The proliferation of responder cells was evaluated by the incorporation of [³H]-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 *rhCTLA*-4 Ig or hIgG; the black columns indicate the responses in cultures with *rhCTLA*-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 *rhCTLA*-4 Ig or hIgG and vs. responses with hIgG at the same concentration. ***p < 0.001 vs. responses without *rhCTLA*-4 Ig or hIgG and vs. responses with hIgG at the same concentration.
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 rhCTLA-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.
Fig. 1

A

Events

Fluorescent intensity

PBS

rhCTLA-4 Ig (100 nM)

B

MFI

DC

T cell-fr

Concentration of rhCTLA-4-Ig (nM)
Fig. 2

Proliferation of responder (cpm)

Concentration (µg/ml)

0  10  30

0  500  1000  1500  2000  2500

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Fig. 3

A

Events vs. Fluorescent intensity for PBS, 30 nM rhCTLA-4 Ig, 30 nM rhCTLA-4 Ig + 30 μM ERY2-4, and 30 nM rhCTLA-4 Ig + 30 μM YT1-S.

B

Graph showing MFI vs. Concentration of peptide added (μM) for YT1-S and ERY2-4.
T cell-fr + DC + ERY2-4

T cell-fr + ERY2-4

PBS YT1-

ERY2-4

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Days of culture

Number of cluster

ERY2-4

PBS

YT1-S

0 2 4 6 8 10 12 14 16 18

1 2 3 4

Days of culture
Supplementary Fig. 1. The structure and amino acid sequence of an HLH peptide library and hCTLA-4 targeting peptide.

(Left) The structure and amino acid sequence of an HLH peptide library, ΔPTA-12RC-2. Randomized amino acid residues are indicated by X. (Middle) The structure and amino acid sequence of Y-2, which was selected from ΔPTA-12RC-2 by east-display and fluorescence-activated cell sorting. The Y-2-specific amino acids are shown in red. (Right) The structure and amino acid sequence of ERY2-4 generated from Y-2 by error-prone PCR. The amino acids different from Y-2 are highlighted in yellow.
Supplementary Fig. 2. Detection of hCTLA-4-targeting HLH peptides.

(A) Schematic illustration of the surface display of HLH peptides on EBY100. EBY100 was transformed using a pYD11-BxXN plasmid inserted with nucleotides encoding ΔPTA-12RC-2 and a FLAG tag peptide. The transformed EBY100 was incubated with rhCTLA-4 Ig and anti-FLAG tag mAb. (B) Isolation of the EBY100 displaying hCTLA-4-targeting peptides. To detect the EBY100 displaying hCTLA-4-targeting peptides, electronic windows were set to select the FLAG tag displaying yeast cells that were highly bound to CTLA-4 Ig (P4). Cells in the P4 gate were isolated by sorting.

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Supplementary Fig. 3. The binding activity of rhCTLA-4 to canine DCs with a higher amount of hlgG.

(A) Fluorescence intensity of DCs incubated with AF488-labeled rhCTLA-4 Ig (100 nM) with or without a larger amount of hlgG (300, 1000, and 3000 nM). Representative results are shown. (B) Median of fluorescence intensity of DCs incubated with AF488-labeled rhCTLA-4 Ig with or without a larger amount of hlgG. Experiments were independently carried out three times. Results were expressed as mean ± SD.
Supplementary Fig. 4. Effect of ERY2-4 treatment on canine MLRs.

Cells of the T cell fraction were incubated with allogeneic canine DCs in the presence of ERY2-4, YT1-S, or PBS for four days. The proliferation of responder cells was evaluated by incorporation of [\(^{3}\)H]-thymidine into DNA (cpm). Experiments were independently carried out three times. Results were expressed as mean ± SD. *p < 0.05.