



FULL PAPER

Immunology

# Characterization of bovine interleukin-2 stably expressed in HEK-293 cells

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**ABSTRACT.** Interleukin 2 (IL-2) is a pleotropic cytokine and well-known as a T cell growth factor in immunology. It is now known to exert both immunostimulatory and immunosuppressive effects, optimizing immunological microenvironments for effector and regulatory T cell responses. The immunomodulatory role of IL-2 is critical for deciding whether or not T cell responses against specific antigens result in protection. We have established a mammalian cell line (HEK-293) stably expressing bovine IL-2 (boIL-2) (designated as HEK-293/boll-2), using the piggyBac transposon system. The concentration of recombinant bovine IL-2 (rboll-2) in the culture supernatant of HEK-293/boll-2 reached 100 ng/ml on day 7 and showed similar proliferative activity to recombinant human IL-2 (rhIL-2) for bovine peripheral mononuclear blood cells. Although rhIL-2 has been often used to activate bovine T cells, our results indicate that characteristics of the T cell activation through rboll-2 and hll-2 appear slightly but significantly different. Interestingly, the rboll-2/anti-boIL-2 monoclonal antibody (C5) (rboll-2/C5) complex strongly induced proliferation of bovine NKp46<sup>+</sup> cells, natural killer (NK) cells, *in vitro*. This indicates that the rboll-2/C5 complex could function as an IL-2 agonist specifically to increase the NK cell population, which in turn could enhance the activity of NK cells leading to protective immunity.

**KEY WORDS:** bovine interleukin-2, bovine interleukin-2 monoclonal antibody, stable expression

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Interleukin-2 (IL-2) is the first cytokine cloned and biologically produced as a recombinant cytokine [1]. It has long been known as a T cell growth factor mainly secreted by CD4<sup>+</sup> T cells that influence activities of CD4<sup>+</sup> T, CD8<sup>+</sup> T, γδ T, NK, and other cells such as innate lymphoid cells (ILCs) in autocrine and paracrine manners [2, 15, 29]. IL-2 is also used for cancer treatment, vaccine adjuvant, anti-viral therapy or T cell epitope mapping in human and mice [5, 15, 26, 28, 32]. The adjuvant effect of the bovine IL-2 (boIL-2) was also reported for *Brucella abortus* vaccines in cattle [34]. It was recently discovered that IL-2 can induce not only effector immune cells but also immune suppressive cells, such as regulatory T (Treg) cells. These contradictory functions depend on quantity and quality of interaction with its counterpart receptor, the IL-2 receptor (IL-2R), which consists of three chains: IL-2R $\alpha$  (CD25), IL-2R $\beta$  (CD122), and common ( $\gamma$ ) (CD132) chains [29]. Although IL-2R with high affinity consists of all three chains, the one with intermediate affinity is a heterodimer of IL-2R $\beta$  and  $\gamma$  chains. The functional intermediate-affinity receptors are expressed primarily on resting NK cells and CD8<sup>+</sup> T cells, while the high-affinity receptors are constitutively expressed on Treg cells. Both IL-2R $\beta$  and  $\gamma$  chains have activation signal motifs in their cytoplasmic domains, while the  $\alpha$  chain does not have cytoplasmic activating nor inhibitory motifs and therefore does not mediate for signaling [23, 25].

Biologically active bovine IL-2 (boIL-2) was first purified from bovine peripheral blood mononuclear cells (PBMC) stimulated with the T cell mitogen concanavalin A (ConA) by Namen *et al.* and Brown *et al.* [9, 24]. Since IL-2 was immediately recognized to play an important role in protective immunity, production of biologically active recombinant bovine IL-2 (rbIL-2) was attempted for studying bovine immune systems. Since human IL-2 (huIL-2) became known to stimulate bovine lymphocytes, it has long been used for activating bovine PBMC or T cells, while several researchers also produced rbIL-2 using different expression systems. The rbIL-2 was first expressed in *Escherichia coli* and found biologically active for a bovine T cell line [9]. The

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rboIL-2 production in other systems includes yeast, baculovirus, and bovine herpes virus-1 expression systems [4, 19, 20, 27, 33]. Mammalian cell lines, such as 293T or COS cells, have also been used to transiently express boIL-2 and stimulate bovine NKp46<sup>+</sup> cells [8, 30]. These transient mammalian expression systems appeared superior over other systems because they have a high yield of rboIL-2 and, more importantly, can reserve original biological properties and stabilities by maintaining the native form of post-translational modification, *o*-glycosylation [13, 21, 31]. In addition, the culture supernatant can be readily used to assess the activities without generating toxic byproducts and/or recombinant vector virus contamination. The selective cell stimulation using the bioactivity of IL-2/anti-IL-2 antibody complex (IL-2C) is also known from the recent progress of IL-2 study [7]. Therefore, a recombinant IL-2 is extremely attractive for a variety of therapeutic applications.

In this study, we established a mammalian cell line, the HEK-293 cell line, that stably secretes boIL-2 in the culture supernatant. The supernatant containing boIL-2 can be readily used to stimulate IL-2-responsive immune cells. In fact, we have successfully used this rboIL-2 to propagate CD4<sup>+</sup> T cells and identified several CD4<sup>+</sup> T cell epitopes (data not shown). In addition, the rboIL-2 can selectively stimulate NKp46<sup>+</sup> cell lineages when added as a complex with a monoclonal antibody (mAb) against boIL-2. These characteristics of the boIL-2 were presented in this work.

## MATERIALS AND METHODS

### Animals

Female Japanese Black cattle, aged 16 to 17 years old, maintained at the facility in the University of Miyazaki were used in this study. The results were derived from experiments on a 16-year old animal. All animals were confirmed free from bovine viral diarrhea virus and bovine leukemia virus. The experimental procedures and care of animals were in accordance with the guidelines approved by the Institutional Animal Care and Use Committee of the University of Miyazaki, Miyazaki, Japan.

### Cell preparation

Fresh blood were collected from the Japanese Black cattle and the peripheral blood mononuclear cells (PBMCs) were isolated using Histopaque 1077 (Sigma Aldrich, St. Louis, MO, USA). These PBMCs were used for RNA extraction, carboxyfluorescein diacetate succinimidyl ester (CFSE) proliferation assay, or frozen at -80°C in 10% DMSO/FBS for any additional experiments.

### RNA extraction and cloning of *boIL-2* gene in the piggyBac vector

To amplify the *boIL-2* gene, total RNA was extracted from bovine PBMCs using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) and synthesized the first strand cDNA using iScript<sup>TM</sup> cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer instruction. The full length of *boIL-2* cDNA was amplified using TaKaRa Ex Taq Hot Start Version (Takara Bio, Kusatsu, Japan). The primers used were as follows: *boIL-2F*, 5'-AAGGATCCACAATGTACAAGATACTCT-3' (forward) and *boIL-2R*, 5'-AAGCGGCCGCTCAAGTCATTGTTGAGTAGATG-3' (reverse). These primers were designed to include *Bam*H1 and *Not*I recognition sites, respectively, to clone the *boIL-2* gene into the piggyBac vector, PB-CMV-MCS-EF1 $\alpha$ -GreenPuro PiggyBac expression vector (System Biosciences, Palo Alto, CA, USA.), in correct direction for expression. The PCR condition was 94°C for 2 min, 35 cycles of 94°C for 30 sec, 57°C for 15 sec, and 72°C for 30 sec, with final extension of 72°C for 7 min. The PCR amplicon was digested with *Bam*H1 and *Not*I (TOYOBO, Ltd., Osaka, Japan), electrophoresed, and purified with QIAquick gel extraction kit (QIAGEN, Hilden, Germany). The purified PCR amplicon was ligated into piggyBac vector plasmid DNA digested with the same restriction enzymes. The ligated product was introduced to One Shot TOP10 Chemically Competent *E.coli* (Life Technologies) by heat shock at 42°C. After extraction of the plasmid DNA, the direction and sequence of the *boIL-2* gene was confirmed by sequencing with BigDye terminator v3.1 (Applied Biosystems, Forster City, CA, USA).

### Establishment of HEK-293/*boIL-2* cell line

The constructed piggyBac expression vector (*piggyBac/boIL-2*) plasmid DNA was transfected to HEK-293 cells (Invitrogen) using Xfect<sup>TM</sup> Transfection Reagent (Takara Bio) according to the manufacturer's instruction. Briefly, 0.5 µg of *piggyBac/boIL-2* plasmid DNA and 0.5 µg of Super PiggyBac Transposase Expression Vector (System Biosciences) were co-transfected into 50% confluent HEK-293 cells in a 24-wells plate using 0.3 µl of Xfect polymer. Four hours after transfection, culture medium was exchanged to fresh medium. Two days later, cells culture condition set up as the presence of 3 µg/ml puromycin and keep the presence of 3 µg/ml of puromycin for 13 passages to select the *boIL-2* expression gene-transposed cells. The culture condition of *boIL-2*-transfected HEK-293 cells was in 10% FBS (HyClone, South Logan, UT, USA) containing Dulbecco's Modified Eagle Medium (Sigma-Aldrich) with penicillin and streptomycin, and it was maintained by passage every 5–6 days. To establish a *boIL-2*-expressing clonal cell line, the cells were plated on the 96-wells flat plate in 0.5 cell/well (as a calculated number). After confirming a single cell growing in a single well, grown clonal cells were transferred to a 25 cm<sup>2</sup> flask after 2 passages. The cells were also confirmed for their expression of GFP co-expressed with the *boIL-2* using fluorescent microscope.

### Western blotting of *rboIL-2* in the culture supernatant

To confirm *rboIL-2* expression in the culture supernatant, Western blotting was performed. The fifth day culture supernatant of HEK-293 or HEK-293/*boIL-2* was loaded after sample buffer treatment, electrophoresed, and transferred to PVDF-membrane (GE healthcare Life Sciences, Marlborough, MA, USA). Two µg/ml of anti-*boIL-2* mAb (C5, IgG2a; Cloud-Clone Corp., Katy, TX, USA) was used as a primary antibody and 0.2 µg/ml of goat anti-mouse IgG HRP-conjugated polyclonal antibody (Abcam,

Cambridge, UK) was used as a secondary antibody. Subsequently, the positive bands were visualized with ChemiDoc™ Touch Imaging System (Bio-Rad) after adding the SuperSignal™ West Dura Extended Duration Substrate (Thermo Scientific, Rockford, IL, USA).

#### *Quantitation of rboIL-2 in culture medium by ELISA*

To quantitate the rboIL-2 expressed in the culture supernatant of the HEK-293/boIL-2 and HEK-293 cells (as a negative control) were seeded in  $3 \times 10^6$  cells/ 21.5 ml/75 cm<sup>2</sup> flask and 0.5 ml of the culture supernatant were taken every day and quantity of the rboIL-2 was measured using IL-2 bovine ELISA Kit (Life Technologies) according to manufacturer protocol. When higher concentration was desired, the rboIL-2 was concentrated using Amicon® Ultra-15 Centrifugal Filter Units (Merck Millipore Ltd., Co., Cork, Ireland). The R software (version 3.2.2, Vienna, Austria) was used for analyzing and visualizing quantitative data.

#### *CFSE proliferation assay*

The PBMC culture medium was composed as follow: the RPMI1640 medium (Gibco, Carlsbad, CA, USA) with 10% FBS (HyClone), 50 µM 2-mercaptoethanol (Gibco), 50 µg/ml Gentamycin (Sigma-Aldrich), 10 mM HEPES (Sigma-Aldrich) and 2 mM L-glutamine (Gibco). For CFSE proliferation assay, bovine PBMCs were stained with 10 ml of 2.5 µM CFSE (Invitrogen) for 10 min at 37°C and washed with the same volume of cold medium twice, and were served for the following assay. The CFSE-stained PBMCs were stimulated with 3-fold serial dilutions of rboIL-2 starting at 10 ng/ml, or with recombinant huIL-2 (rhuIL-2) (PeproTech, Cranbury, NJ, USA) starting at 100 ng/ml, in  $4 \times 10^6$  cells /well on a 24-wells plate for 5 days. The CFSE-stained PBMCs were similarly stimulated with 2 µg/ml of ConA for 5 days. After stimulation, the CFSE-stained PBMCs were incubated with anti-bovine CD3 monoclonal antibody (mAb) (MM1A, IgG1; Bio-Rad), anti-bovine CD4 mAb (CC8, IgG2a; Bio-Rad), anti-bovine CD8 mAb (CC63, IgG2a; Bio-Rad), anti-bovine WC1 mAb (CC15, IgG2a; Bio-Rad), or anti-bovine CD335 (NKp46) mAb (AKS1, IgG1; Bio-Rad) on ice and washed. Subsequently, the cells were stained with goat anti-mouse IgG PE-conjugated polyclonal antibody (abcam). After staining, the cells were further stained with 7-Aminoactinomycin D (Invitrogen) at 1 µg/ml for 10 min on ice to exclude dead cells and fixed with 1% PFA/PBS solution until FACS analysis. The percentage of proliferated population (CFSE<sup>low</sup>) in each cell surface marker was calculated as cell number of CFSE<sup>low</sup>surface-marker<sup>+</sup>/total cell number within a lymphocyte gate. More than 30,000 cells in the lymphocyte population were analyzed for the CFSE-proliferation assay with a FACSCaliber flow cytometer and CELLQuest Software (BD Biosciences, San Jose, CA, USA).

#### *Responsiveness of a huIL-2 dependent human T-cell line to the rboIL-2*

The IL-2 dependent acute T-cell leukemia (ATL) cell line, ILT-Mat, was purchased from RIKEN cell bank and maintained in complete RPMI medium supplemented with 20 ng/ml of rhuIL-2. To examine whether or not rboIL-2 can replace rhuIL-2 for stimulating ILT-Mat cells, ILT-Mat cells were washed twice with the medium and cultured in complete RPMI medium supplemented with rboIL-2 (approximately 100 ng/ml).

#### *Proliferation assay with rboIL-2/anti-boIL-2 mAb (rboIL-2/C5) complex*

The rboIL-2/C5 complex was generated by incubating rboIL-2 and anti-boIL-2 mAb (C5) (1:100 in mole ratio) for 10 min at room temperature. To examine the proliferative responses of PBMC to rboIL-2/C5 complex, the CFSE proliferation assay was performed as described above. The concentration of the rboIL-2/C5 complex, boIL-2, or anti-boIL-2 mAb (C5) were adjusted by diluting with HEK-293 culture supernatant.

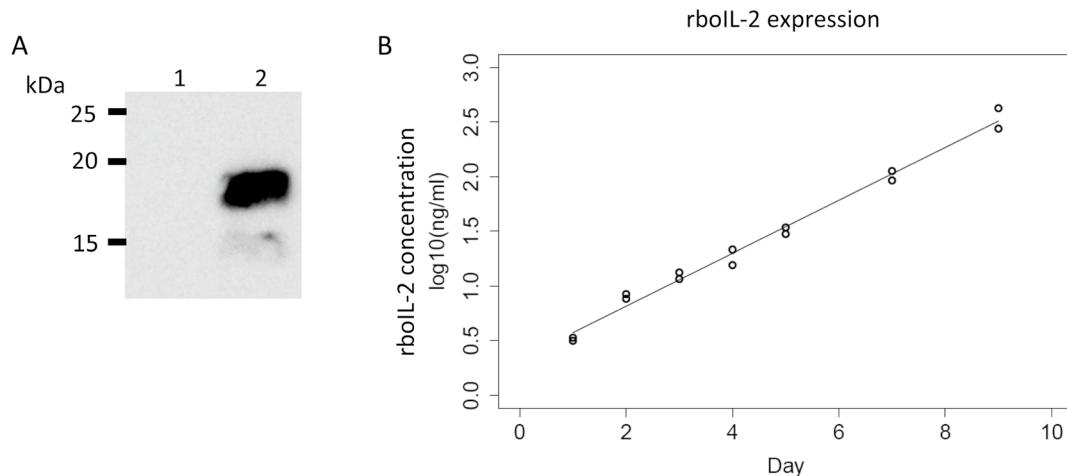
## RESULTS

#### *Stable expression of rboIL-2*

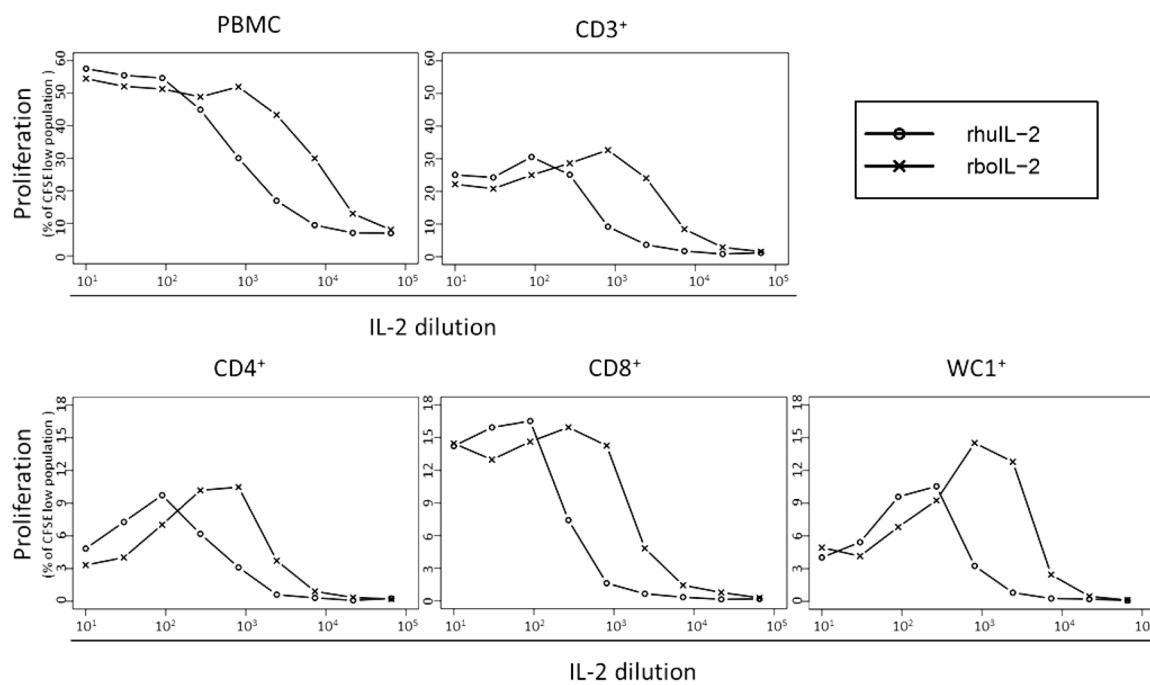
The boIL-2/piggyBac plasmid DNA was transfected into HEK-293 cells and puromycin (3 µg/ml) was added from 2 days after transfection. The expression of rboIL-2 was confirmed by both GFP expression (data not shown) and Western blotting. For the Western blotting, the rboIL-2 band was located approximately at 17–20 kDa as predicted from the encoded amino acid sequence and previous reports [9, 24] (Fig. 1A). It appeared close to the size of the predicted native form with post-translational modification [13]. The rboIL-2 concentration in the culture supernatants gradually increased day by day and accumulated up to 330 ng/ml for 9 days (Fig. 1B). The culture supernatant on day 7 was routinely collected and stored until use, which generally contain about 100 ng/ml of rboIL-2. After more than 24 passages, similar concentration of the rboIL-2 was maintained.

#### *Biological activity of the rboIL-2*

To assess biological activity of the rboIL-2, bovine PBMCs were stimulated with the rboIL-2 or rhuIL-2. The bovine PBMCs were stimulated with 9 serial dilutions of rboIL-2 from 10 ng/ml to 1.52 pg/ml or with rhuIL-2 from 100 ng/ml to 15.2 pg/ml. The proliferative responses to each stimulation were analyzed using CFSE proliferation assay and presented as the percentages of the CFSE<sup>low</sup> population in Fig. 2. The PBMCs proliferated with both rboIL-2 and rhuIL-2 in a comparatively dose-dependent manner, though rboIL-2 had higher activity than rhuIL-2 did even at lower concentrations. To understand what subsets of the PBMCs were responding to each stimulation, CD3<sup>+</sup> T cells including CD4<sup>+</sup>, CD8<sup>+</sup>, and WC1<sup>+</sup> T cell populations were analyzed (Fig. 2). Among the CD3<sup>+</sup> T cell population, the CD8<sup>+</sup> T cell population responded more strongly than the CD4<sup>+</sup> T cell population did. It was obvious that the CD4<sup>+</sup> and WC1<sup>+</sup> T cell populations were lower responders compared with the CD8<sup>+</sup> T cell population at the higher



**Fig. 1.** The recombinant bovine interleukin 2 (rboIL-2) was expressed in the boIL-2/piggyBac transposed- and cloned- cell line. A: Western blotting with mouse anti-boIL-2 mAb (clone C5). Lane 1: Culture supernatant from HEK-293 cells and lane 2: Culture supernatant from the HEK-293/rboIL-2 clone. The rboIL-2 band was located in 17 kDa-20kDa. B: The rboIL-2 concentration (Y-axis: log<sub>10</sub>) in the HEK-293/rboIL-2 clone cell culture supernatant were calculated using boIL-2 sandwich ELISA and plotted as concentration (ng/ml) of each day. Data shown is a representative of 2 independent experiments.

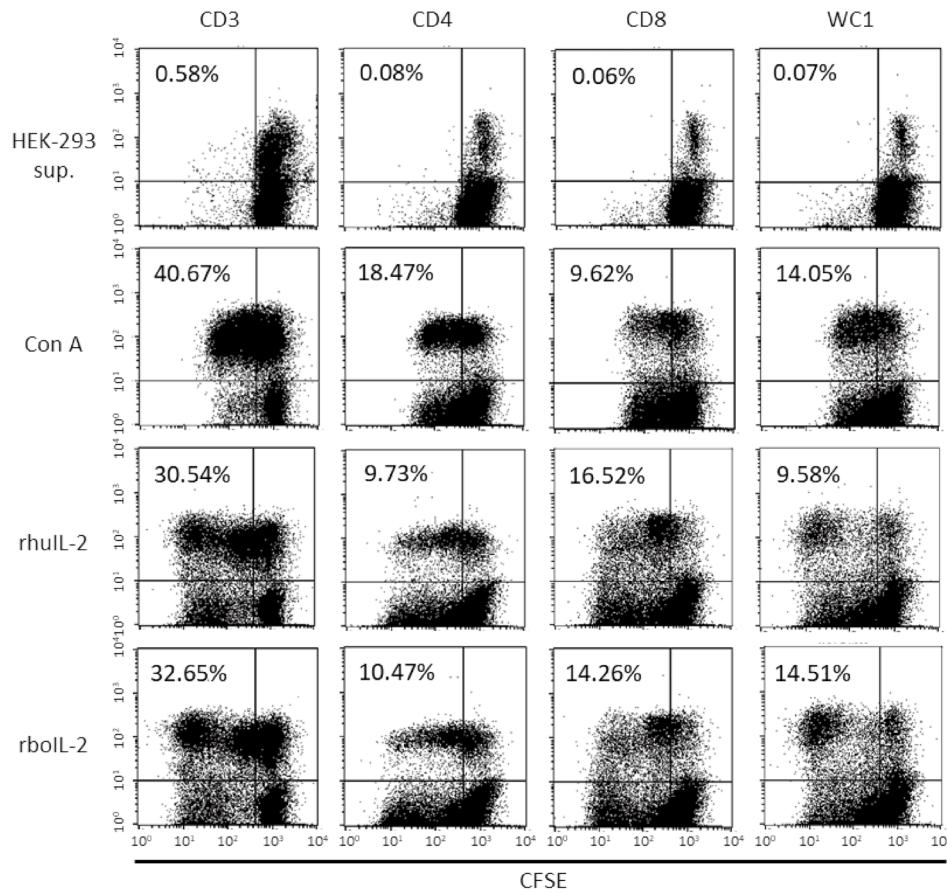


**Fig. 2.** Interleukin 2 (IL-2) titration on each subset in bovine peripheral blood mononuclear cells (PBMC). X-axis represent the fold of IL-2 dilution. 10<sup>1</sup>-fold dilution of recombinant human IL-2 (rhuIL-2) means 100 ng/ml of rhuIL-2, 10<sup>1</sup>-fold dilution of rboIL-2 means 10 ng/ml of rboIL-2. The marks (rhuIL-2: empty circle; rboIL-2: cross) indicate the proliferated cells of each CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup> or WC1<sup>+</sup> cell population. Data shown is a representative of 3 independent experiments.

concentrations (0.37–10 ng/ml of rboIL-2, 11.1–100 ng/ml of rhuIL-2). The WC1<sup>+</sup> T cells that define a subpopulation of  $\gamma\delta$  T cell well responded to rboIL-2 similar to the CD8<sup>+</sup> T cell population at 123 pg/ml but not to rhuIL-2. Overall, all cell populations examined proliferated strongly around 123 pg/ml for rboIL-2 and 11.1 ng/ml for rhuIL-2, though rhuIL-2 quickly reduced its activity after the peak.

#### Proliferative responses to rboIL-2 and rhuIL-2

To clarify whether bovine T cell populations had different proliferative responses with rboIL-2 and rhuIL-2, proliferation of each T cell population at the best stimulatory dose of each recombinant IL-2 (123 pg/ml for rboIL-2 and 11.1 ng/ml for rhuIL-2) were



**Fig. 3.** Each T cell subset proliferation after culture with HEK-293 supernatant, 2 µg/ml of ConA, 11.1 ng/ml of recombinant human IL-2 (rhuIL-2) or 123 pg/ml of recombinant bovine interleukin 2 (rboIL-2). The populations of proliferated each cell (carboxyfluorescein diacetate succinimidyl ester (CFSE)<sup>low</sup> of CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup> or WC1<sup>+</sup> cells) were shown upper left of individual dot plots. X-axis indicates CFSE staining. Y-axis represents the expression of individual T cell subsets. The percentage on the individual plots represent proliferated the CFSE<sup>low</sup> subsets. Data shown is a representative of more than 3 independent experiments.

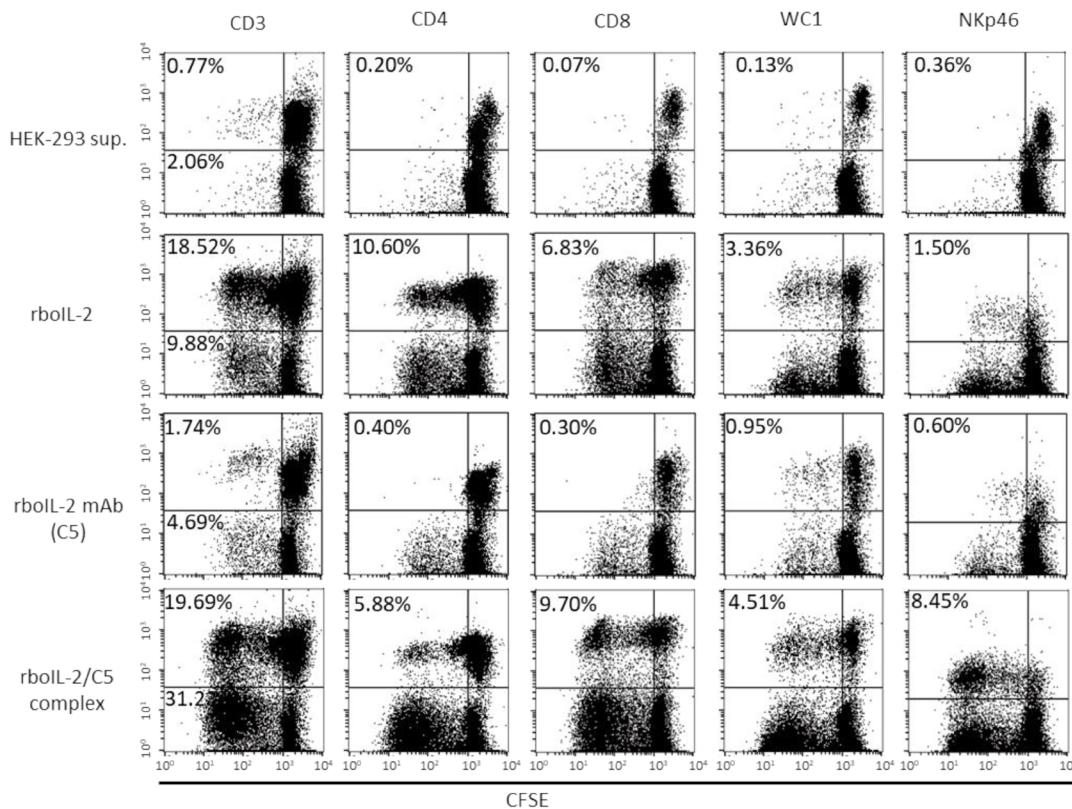
compared as shown in Fig. 3. As expected, a T cell mitogen, ConA (Invitrogen), stimulated CD4<sup>+</sup> T cell population better than CD8<sup>+</sup> T cell population. Interestingly, rhuIL-2 stimulated more CD8<sup>+</sup> T cell population than CD4<sup>+</sup> or WC1<sup>+</sup> T cell population.

#### Proliferative effect of rboIL-2 on a huIL-2-dependent T cell line

The ILT-Mat cell line was used to examine whether or not the rboIL-2 could maintain a huIL-2 dependent human T-cell line. Although lower concentration of rboIL-2 could not maintain the ILT-Mat cell line, 100 ng/ml of rboIL-2 could maintain the cell line up to 12 passages (further passages have not been tested).

#### Biological activity of rboIL-2/C5 complex

Because anti-IL-2 antibodies can selectively expand a certain population of IL-2-responsive cells, biological activity of a rboIL-2/C5 complex was examined using CFSE proliferation assay. As shown in Fig. 4, when rboIL-2 (123 pg/ml) was added, CD3<sup>+</sup> T cell population was well proliferated to 18.52% as compared with HEK-293 supernatant alone to 0.77%. Since the same amount of HEK-293 supernatant in rboIL-2 was added, possible inhibitory effect of the supernatant was excluded. By adding rboIL-2/C5 complex, CD3<sup>+</sup> T cell population increased to 19.69% similarly in response to rboIL-2. Regarding cell populations in response to rboIL-2/C5 complex, it was noticeable that there was increased CD3<sup>-</sup> cell population. When CD4<sup>+</sup>, CD8<sup>+</sup>, and WC1<sup>+</sup> T cell population were examined, these populations responded to both rboIL-2 and rboIL-2/C5 complex similarly except CD4<sup>+</sup> T cell population which responded more to rboIL-2 and rboIL-2/C5 complex. To examine CD3<sup>-</sup> cell population, the bovine NKp46 molecule representing NK cell surface markers was added to the CFSE proliferation assay analysis. As shown Fig. 4, the NKp46<sup>+</sup> cell population strongly responded to rboIL-2/C5 complex, increasing to 8.45% as compared to 1.5% with rboIL-2 alone. Overall, CD8<sup>+</sup> T and NKp46<sup>+</sup> cell populations were good responders for rboIL-2/C5 complex.



**Fig. 4.** The recombinant bovine interleukin 2 (rboIL-2)/C5 complex induces CD8<sup>+</sup> T cell and NKp46<sup>+</sup> cell proliferation. Carboxyfluorescein diacetate succinimidyl ester (CFSE) proliferation assay on the results of peripheral blood mononuclear cells (PBMC) cultivation for 5 days with HEK-293 culture supernatant, 0.25 ng/ml of rboIL-2, 100 ng/ml of anti-boIL-2 mAb (clone C5), or rboIL-2/C5 complex. X-axis represent intensity of CFSE. Y-axis means individual PE-labeled lymphocyte subsets (CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, WC1<sup>+</sup> or NKp46<sup>+</sup> cells). The percentages on the individual panels represent the CFSE<sup>low</sup> subsets. Data shown is a representative of 2 independent experiments.

## DISCUSSION

IL-2 is an important modulator for balancing activation of effector immune cells and Treg cells. Studies of boIL-2 began in 1980's starting with purification of biologically active boIL-2 from ConA-stimulated PBMC [9, 24]. The mature boIL-2 consists of 135 amino acids and has 65% identity with huIL-2 [10]. It has been known that huIL-2 could stimulate and maintain bovine T cells, though it didn't stimulate NK cells [14]. Therefore, commercially available huIL-2 was often used for immunological experiments for bovine systems.

We have established the boIL-2-expressing mammalian cell line, HEK-293/boIL-2, and verified the stimulatory activity of its supernatant for PBMCs and CD3<sup>+</sup> T cell populations. The rboIL-2 also strongly stimulated NKp46<sup>+</sup> cell population when added as the rboIL-2/C5 complex. The recombinant IL-2 is a useful immunological tool and allow us to study its activities and to maintain T cells for an extended period. The rboIL-2 was first produced in *E. coli* and yeast [4, 10, 27, 33]. Further, rboIL-2 was generated by baculovirus expression system and shown to enhance bovine PBMC proliferation [11, 19]. Transient mammalian expression systems were also often used to express rboIL-2 and successfully applied to many immunological assays in bovine system [8, 13]. Although all these rboIL-2s have shown some stimulatory activities, the structures that reflect activity of boIL-2 are slightly different depending on whether or not the expressed protein is glycosylated [21, 31]. To maintain native activity of boIL-2, correct post-translational modification should be also taken account. In this regard, it is reasonable to use mammalian cell lines to produce boIL-2 since human IL-2 generated in BHK, HeLa, or CHO cells was *O*-glycosylated, similar to the protein secreted from natural T cells [13]. For a continual need of such a boIL-2, a stable expression of boIL-2 in mammalian cells is therefore advantageous. In this study, the piggyBac transposon system was used to introduce *boIL-2* gene to HEK-293 cells. The sustainable cell line with stable expression of boIL-2 was established after cloning by limiting dilution. Because it is secreted, harvesting and storing the culture supernatant at the time of passage can provide sufficient amount of active boIL-2.

Stimulatory activities of the rboIL-2 were varied among CD3<sup>+</sup> T cells including CD4<sup>+</sup>, CD8<sup>+</sup>, WC1<sup>+</sup> T cells. The differences of activities observed among these cells were most likely due to the level of expression of each IL-2R subunit on each cell population. The IL-2R consists of three subunits: IL-2R $\alpha$  (CD25), IL-2R $\beta$  (CD122), and  $\gamma$  (CD132) chains [27]. The IL-2R with high affinity requires all three chains (trimer), while the one with intermediate affinity is a heterodimer of IL-2R $\beta$  and  $\gamma$  chains. Although both IL-2R $\beta$  and  $\gamma$  chains have activation signal motifs in their cytoplasmic domains, the IL-2R $\alpha$  chain has a short cytoplasmic

tail and lacks such a signal motif, and therefore does not deliver signals [23, 25]. It is known that the balance in expression of each chain can determine how the target cells respond. The most of CD3<sup>+</sup> T cell population constitutively express all three IL-2R subunits but at different level. For example, Treg cells in the CD4<sup>+</sup> T cell population highly express its signature molecule, CD25, on the cell surface and respond to a small amount of IL-2, suppressing effector T cells. On the other hand, most of CD8<sup>+</sup> T cells preferentially express the IL-2R $\beta/\gamma$  heterodimer with intermediate affinity and require higher dose of IL-2 for their activation. In the present study, the bovine CD4<sup>+</sup> T cell population well responded to ConA stimulation as expected but less responded to both rboIL-2 and rhUIL-2 compared to other cell populations. This may not be surprising since this population may contain Treg cells that could suppress effector T cells. Treg cells is sensitive to a small amount of IL-2 and demonstrate inhibitory effects by the competitive removal of IL-2 from CD8<sup>+</sup> T cells [22]. Since WC1<sup>+</sup> cells also play a regulatory role for effector cells [16], explanation of stimulatory effects using PBMC *in vitro* became more complicated in cattle. However, we speculate that adding rboIL-2 above threshold may overcome the Treg-cell inhibitory effects. The bovine CD8<sup>+</sup> T cell population proliferated with both rboIL-2 and rhUIL-2 in higher dose. The rhUIL-2 appeared active only in higher concentration for bovine CD8<sup>+</sup> T cell population, suggesting that rhUIL-2 has lower affinity to bovine IL-2R even if to the trimer and thus requires higher dose compared with rboIL-2 to stimulate bovine cells. And vice versa, the rboIL-2 was able to maintain huIL-2-dependent human T cell line, ILT-Mat, but only in a high concentration (100 ng/ml), indicating that the rboIL-2 has lower affinity to human IL-2R. As described earlier, the identity of amino acid sequence between boIL-2 and huIL-2 is 65% and they are interchangeable for T cell activation to some extent. Similarly, huIL-2 has 57% identity with mouse IL-2, yet stimulates mouse IL-2Rs whereas mouse IL-2 binds rather weakly to huIL-2Rs [2]. Although 3D structure of the quaternary IL-2/IL-2R complex has been defined, it is difficult to address how those differences in their amino acid sequences and structures influence the biological activities [3]. The WC1<sup>+</sup> T cell population, a major subset of  $\gamma\delta$  T cells, appears to have an intermediate responsiveness in between CD4<sup>+</sup> and CD8<sup>+</sup> T cell populations to stimuli with ConA, rhUIL-2, and rboIL-2.

Subtle changes of the structure in the IL-2 molecule alter interaction with the IL-2R, delivering different signals and thus selectively differentiating target subsets [6]. In mice, two different mAbs, JES6-1 and S4B6, specific to mouse IL-2 could selectively expand opposing cell types, Treg cells and effector T cells respectively by complexing with mouse IL-2 [6]. The C5 is a boIL-2-specific mAb and confirmed its specificity to the rboIL-2 produced in this study. After validating bioactivity of the rboIL-2, we examined what subset of T cells could expand with the rboIL-2/C5 complex. Among CD3<sup>+</sup> T cells, CD8<sup>+</sup> T cells expanded better with rboIL-2/C5 complex than rboIL-2, while CD4<sup>+</sup> T cells expanded better with rboIL-2 than rboIL-2/C5 complex. This may suggest that mAb C5 binds an epitope where the boIL-2 has contact with boIL-2R $\alpha$  chain and therefore blocking their interaction, leading to a preferential stimulation of the boIL-2R $\beta/\gamma$  heterodimer-bearing T cells such as CD8<sup>+</sup> T cells. However, IL-2 antibody binding may not simply block the specific site but also alter IL-2 half-life or could induce conformational change that altered IL-2 binding to the IL-2R [6]. In this experiment, it was noticeable that CD3<sup>-</sup> cells population obviously expanded better with rboIL-2/C5 complex (31.23%) than with rboIL-2 (9.88%). This prompted us to examine bovine NK cells, NKp46<sup>+</sup> cells, for their expansion because bovine NK cells reportedly propagate with boIL-2 [8, 30]. The NKp46<sup>+</sup> cell expanded with rboIL-2/C5 complex better than rboIL-2 alone, as shown 8.45% and 1.5% in expanded population respectively. It is reported that the CD25<sup>low</sup> NK cells were the predominant subset of NK cells within the peripheral blood [17]. We also speculate that the CD3<sup>-</sup> cell population may also include NKp46<sup>+</sup> and NKp46<sup>-</sup> ILCs [18]. Considering NKp46<sup>+</sup>CD3<sup>+</sup> cells [12], significant number of NKp46<sup>+</sup> cells appears to respond to rboIL-2/C5 complex.

In this study, the mammalian cell line stably expressing boIL-2 was established and its activity was evaluated. We have recently used this rboIL-2 for bovine CD4<sup>+</sup> T cell propagation *in vitro* and successfully identified CD4<sup>+</sup> T cell epitopes on structural proteins on bovine leukemia virus (data not shown) using tetramer-guided epitope mapping methods [26]. The rboIL-2 is a useful immunological tool not just as a T cell growth factor but also capable of expand a variety of immune cells. Further, the rboIL-2/C5 complex could function as an IL-2 agonist that selectively increases the NK cell population or ILCs, which in turn could clinically be used to enhance the activity of NK cells. More anti-boIL-2 mAbs need to be investigated. Modification of boIL-2 is a unique way of creating a biological molecule to either stimulate or regulate immune cells by targeting a specific cell. Detailed expression profiles of boIL-2R subunits on each boIL-2-responsive cell need to be clarified to understand how modification of boIL-2 affects cell signaling and selection of immune cells.

CONFLICT OF INTEREST. The authors declare no competing interests exist.

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