Regulatory T cells (Tregs) are a subpopulation of CD4+ T cells which suppress immune responses of effector cells and are known to play a very important role in protection against autoimmune disease development, induction of transplantation tolerance and suppression of effective immune response against tumor cells. An effective in vivo Treg depletion agent would facilitate Treg-associated studies across many research areas. In this study, we have developed diphtheria toxin-based monovalent and bivalent murine IL-2 fusion toxins for depleting murine IL-2 receptor positive cells including CD25+ Treg in vivo. Their potencies were assessed by in vitro protein synthesis inhibition and cell proliferation inhibition assays using a murine CD25+ CTLL-2 cell line. Surprisingly, in contrast to our previously developed recombinant fusion toxins, the monovalent isoform (DT390-mIL-2) was approximately 4-fold more potent than its bivalent counterpart (DT390-bi-mIL-2). Binding analysis by flow cytometry demonstrated that the monovalent isoform bound stronger than the bivalent version. In vivo Treg depletion with the monovalent murine IL-2 fusion toxin was performed using C57BL/6J (B6) mice. Spleen Treg were significantly depleted with a maximum reduction of ~70% and detectable as early as 12 h after the last injection. The spleen Treg numbers were reduced until Day 3 and returned to control levels by Day 7. We believe that this monovalent murine IL-2 fusion toxin will be an effective in vivo murine Treg depleter.

Keywords: murine IL-2/fusion toxin/diphtheria toxin/Tregs/ Pichia Pastoris expression

Introduction
Regulatory T cells (Treg) are a specific subset of CD4+ T cells capable of inhibiting immune responses. Treg have been shown to be important for maintaining transplantation tolerance and for preventing autoimmune disease. Treg recruited to the local tumor microenvironment can suppress anti-tumor immune responses (Curiel et al., 2004; Knutson et al., 2006). An effective in vivo Treg-depletion reagent is needed for various mechanistic studies of transplantation tolerance and autoimmune disease as well as for facilitating combined cancer treatment.

Foxp3DTR knock-in mice are widely used for Treg-depletion models. All Foxp3+ cells in these mice express the human diphtheria-toxin receptor (DTR), thereby allowing efficient depletion of Treg following treatment with diphtheria toxin (DT) (Kim et al., 2007b; Lund et al., 2008). However, DT by nature is toxic and so may cause additional nonspecific inflammatory responses. Moreover, Foxp3DTR knock-in mice are not available for all murine disease models. Ontak® (denileukin difitox) is an FDA-approved pharmaceutical drug (recombinant monovalent human IL-2 fusion toxin, DAB389IL-2) produced in Escherichia coli by Eisai Medical Research, Inc. Ontak® has been investigated for in vivo murine Treg depletion (Knutson et al., 2006; Litzinger et al., 2007). However, the depletion was insufficient and Ontak® is currently discontinued.

Human and murine IL-2 amino acid sequences have 64% homology, which explains the observation of difference in cross-species reactivity between human IL-2 and murine IL-2 (Collins, 1989). Human IL-2 is 10 times less effective than murine IL-2 in stimulation of murine T cells (Collins, 1989). Therefore, we hypothesized that a species-specific murine IL-2 fusion toxin will deplete murine Treg in vivo more effectively.

Truncated diphtheria toxin (DT390) has been utilized to construct recombinant immunoconjugates/fusion toxins for targeting specific cell populations (Woo et al., 2002; Kim et al., 2007a; Wang et al., 2011; Peraino et al., 2013). The unique DT-resistant yeast strain Pichia Pastoris (Liu et al., 2003) facilitated this approach. In this study, we have developed both monovalent and bivalent murine IL-2 fusion toxins using this DT-resistant yeast P. Pastoris system. Binding affinity to murine CD25+ cells was analyzed by flow cytometry. Their potencies were assessed by in vitro protein synthesis inhibition and cell proliferation inhibition assays. In vivo murine Treg-depletion efficacy was assessed using C57BL/6J (B6) mice.

Materials and methods
Plasmid construction
As shown in Fig. 1, murine IL-2 fusion toxins were built to contain two moieties using the codon-optimized nucleotide sequences; the first is DT390 (Woo et al., 2002) and the second is murine IL-2 (Fig. 2). A strategy previously employed to construct A-dm-DT390biscFv (2-6-15) (Wang et al., 2011) was applied to build these murine IL-2 fusion toxins. The biscFv (2-6-15) moiety was replaced with the codon-optimized murine IL-2. DT390 and murine IL-2 portions were linked by a (G4S) linker made up of four glycine residues and a serine. The two murine IL-2 proteins were linked by a (G4S) linker made up of three tandem chains each containing four glycine residues and a
serine for building the bi-murine IL-2 fusion toxin. Six histidines (6x His tag) were added to the C-terminus of each construct to facilitate later purification. The codon-optimized murine IL-2 DNA (Fig. 2) was synthesized by GenScript (Piscataway, NJ). To construct DT390-mIL-2, the codon-optimized murine IL-2 DNA was amplified using polymerase chain reaction primers mIL2-X1 carrying XhoI and NcoI sites + mIL2-E1 carrying an EcoRI site, then cloned into pwPICZalpha (Peraino et al., 2012a) between XhoI and EcoRI sites for sequencing confirmation. The insert was then cut out with NcoI + EcoRI and cloned into pwPICZalpha-DT390 (Wang et al., 2011) between NcoI and EcoRI sites yielding the final construct DT390-mIL-2 in pwPICZalpha. To construct DT390-bi-mIL-2, the first murine IL-2 was amplified using PCR primers mIL2-X1 carrying XhoI and NcoI sites + mIL2-Bam1 carrying BamHI and EcoRI sites, then cloned into pwPICZalpha between XhoI and EcoRI sites for sequencing confirmation. The insert was then cut out with BamHI + EcoRI as insert I. The second murine IL-2 was PCR amplified using mIL2-Bam2 carrying XhoI and BamHI sites + mIL2-E1 carrying an EcoRI site then cloned into pwPICZalpha between XhoI and EcoRI sites for sequencing confirmation. The insert was then cut out with BamHI + EcoRI as insert II. The insert I carrying NcoI and BamHI sites + insert II carrying BamHI and EcoRI sites (NcoI-mIL2-Bam1/mIL2-Bam2-EcoRI) were together cloned into pwPICZalpha-DT390 between NcoI and EcoRI yielding the final construct DT390-bi-mIL-2 in pwPICZalpha. All PCR primers that were used are listed in Table I.

Protein expression and purification in P. pastoris were performed as previously described (Wang et al., 2011; Peraino et al., 2013). Western blot analysis, FACS binding analysis and $K_D$ determination were all performed as previously described (Peraino et al., 2012a) using a murine CD25+ CTLL-2 cell line. The DT390 alone, murine IL-2 alone were used as controls for our in vitro assays. These products were also expressed and purified in the yeast P. pastoris system.

**HPLC analysis**

Murine IL-2 fusion toxins were analyzed with Shimadzu HPLC system using a Superdex 200 size-exclusion column, 10/300 GL (GE healthcare, Cat#: 17-5175-01). The sample volume was 100 μl using a 100 μl loop. The flow rate was 0.35 ml/min. The running time was 120 min and the running buffer was 90 mM NaSO4, 10 mM NaPO4, pH 8.0, 1 mM EDTA.

**Protein synthesis inhibition**

Murine CTLL-2 cells were cultured in RPMI 1640 medium supplemented with 6% fetal bovine serum, 10 mM Heps (N-2-hydroxethylpiperazine-N-2-ethanesulfonic acid), 1× nonessential amino acids, 1 mM sodium pyruvate, 2 mM glutamine and 2.5×105 M 2-mercaptoethanol. Cells were washed twice with 50 ml of the above medium containing leucine-free RPMI by centrifugation at 1000 rpm, 20°C for 5 min. CTLL-2 cells were then diluted to 5.0×105 cells/mL and each murine IL-2 fusion toxin was serially diluted in the above culture medium with leucine-free RPMI. One hundred microliters of cells (5.0×10⁴ cells) were added to each well in a 96-well flat bottomed plate (Corning) with 10 μl of fusion toxin dilution and incubated at 37°C with 5% CO2 for 18 h. Each fusion toxin dilution was analyzed in triplicate. Plates were pulsed with 1 μCi/well of 3H-leucine for 1 h, then harvested onto filter mats (Perkin-Elmer) using a Harvester 96 Mach II cell harvester and allowed to dry at room temperature overnight. Beta emission was determined in counts per minute (cpm) read using a microbeta counter. The negative control for this assay was murine CTLL-2 cells plated without fusion toxin and the positive control was murine CTLL-2 cells plated with cycloheximide (1.25 mg/mL) for 15 min at 37°C with 5% CO2. Both controls were analyzed in triplicate for each assay.

The blocking assays follow the protocol above with a 1-h incubation of 10 μl of the murine IL-2 as inhibitor (10⁻⁹ M as the final concentration) with the murine CTLL-2 cells prior to Fig. 1. Diagrams of the murine IL-2 fusion toxins.

Fig. 2. Codon-optimized murine IL-2 DNA sequence.
addition of the fusion toxins. The inhibition assays were then pulsed, harvested and read as described above.

**Cellular proliferation inhibition**

Murine CD25^+ CTLL-2 cells were cultured in RPMI 1640 medium supplemented with 6% fetal bovine serum, 10 mM Hepes (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid), 1 x nonessential amino acids, 1 mM sodium pyruvate, 2 mM glutamine and 2.5 x 10⁻⁵ M 2-mercaptoethanol. Cells were washed twice with 50 ml of the above medium by centrifugation at 1000 rpm, 20°C for 5 min. Murine CTLL-2 cells were then diluted to 5.0 x 10⁴ cells/mL and each murine IL-2 fusion toxin was serially diluted in the above culture medium. One hundred microliters of cells (5.0 x 10⁴ cells) were added to each well in a 96-well flat bottomed plate (Corning) with 10 μl of fusion toxin dilution and incubated at 37°C with 5% CO₂ for 24 h. Each fusion toxin dilution was analyzed in triplicate. Plates were pulsed with 1 μCi/well of ³²H-thymidine for 24 h then harvested onto filter mats (Perkin-Elmer) using a Harvester 96® Mach II cell harvester and allowed to dry at room temperature overnight. Beta emission was determined in cpm read using a microbeta counter. The negative control for this assay was murine CTLL-2 cells plated without fusion toxin and the positive control was murine CTLL-2 cells plated with cycloheximide (1.25 mg/mL) for 1 h at 37°C with 5% CO₂. Both controls were analyzed in triplicate.

The inhibition assays follow the protocol above with a 1-h incubation of 10 μl of the murine IL-2 as inhibitor (10⁻¹⁹ M as the final concentration) with the murine CTLL-2 cells prior to addition of the fusion toxins. Inhibition assays were then pulsed, harvested and read as described above.

**In vivo Treg depletion**

Spleen cells were extracted and analyzed using the following antibodies: APC/Cy7-anti-mouse CD4 (RM4-5) purchased from BioLegend, PE/Cy7-anti-mouse CD8 (53-6.7) purchased from BioLegend, PE-anti-rat CD19 (1D3) purchased from BD Biosciences, FITC Rat anti-mouse CD25 (7D4), anti-mouse/rat Foxp3 (FJK-16 s) and PerCP/Cy5.5-anti-mouse NK1.1 (PK136). Flow cytometry was performed on a FACSverse and data were analyzed with FlowJo software.

**Statistical analysis**

All P values were calculated using two-way ANOVA of Prism or paired t-test. P < 0.05 was considered as significant.

### Table 1. PCR primers used in this study

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Restriction Enzymes</th>
</tr>
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<tbody>
<tr>
<td>mIL2-X1</td>
<td>5' CGG CTC GAG CGA TGG GTT GGT GTT GCT CCT TCT TCC TCT ACT 3'</td>
<td>EcoRI, NcoI</td>
</tr>
<tr>
<td>mIL2-Bam1</td>
<td>5' CGG GAA TTC CGG CGC GAA ACC AGA ACC ACC ACC ACC TTG TGG AGA ATG AGA GAT AAT AGA</td>
<td>EcoRI</td>
</tr>
<tr>
<td>mIL2-Bam2</td>
<td>5' CGG CTC GAG CGG CGC GGA TCC GGT GGT GGT GTT GCT CCT TCT TCC TCT ACT 3'</td>
<td>BamHI</td>
</tr>
<tr>
<td>mIL2-E1</td>
<td>5' CGG GAA TTC TTA GTG GTG GTG GTG GTG GTG TTG TGG AGA AGT AGA 3'</td>
<td>EcoRI</td>
</tr>
</tbody>
</table>

Results

**Expression and purification of murine IL-2 fusion toxins in yeast P. Pastoris**

As shown in Fig. 1, both monovalent and bivalent murine IL-2 fusion toxins were constructed so as to find the best isoform for *in vivo* murine Treg depletion. The codon-optimized murine IL-2 (Fig. 2) was cloned into a DT-390-containing yeast *P. Pastoris* expression vector pwPICZalpha-DT390 between NcoI and EcoRI (Wang et al., 2011). A GₛS linker was used to link the DT390 domain to the murine IL-2 domain. A (GₛS)₃ linker was used to connect between two murine IL-2 domains to generate the bivalent murine IL-2 fusion toxin. A 6xHis tag was added to the C-terminus of the murine IL-2 fusion toxins to facilitate later purification. The murine IL-2 fusion toxins were expressed using diphtheria toxin resistant yeast *P. pastoris* strain (Liu et al., 2003) in 11 Erlenmeyer flasks. The expressed murine IL-2 fusion toxins were secreted into the supernatant and subsequently captured using a Ni-Sepharose fast flow resin, then further purified using a strong anion-exchange resin Poros 50HQ. The final production level was ~4 mg/l of the original harvested supernatant for the monovalent and bivalent murine IL-2 fusion toxins. The purified murine IL-2 fusion toxins were analyzed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE, Fig. 3A), high-performance liquid chromatography (HPLC) (Fig. 3B and C) and western blot with an anti-6xHis tag mAb (data not shown) and anti-DT mAb (data not shown). SDS PAGE, HPLC and western blot analysis demonstrated that both monovalent and bivalent murine IL-2 fusion toxins were successfully expressed and purified with high purity and expected molecular weight (61.11 kDa for the monovalent version and 79.27 kDa for the bivalent version).

**Protein synthesis inhibition analysis of the murine IL-2 fusion toxins**

The potency of the murine IL-2 fusion toxins was assessed by an assay for *in vitro* protein synthesis inhibition using the murine CD25^+ CTLL-2 cell line through incorporation of the tritiated leucine. As shown in Fig. 4, the monovalent murine IL-2 fusion toxin (DT390-mIL-2, EC₅₀ = 1.55 x 10⁻¹³ M) is ~4-folds more potent than the bivalent murine IL-2 fusion toxin (DT390-bi-mIL-2, EC₅₀ = 6.40 x 10⁻¹³ M). DT390 alone and murine IL-2 alone were included as controls. We expected that the bivalent murine IL-2 fusion toxin would be more potent than the monovalent isoform as we previously
observed with other bivalent immunotoxins (Woo et al., 2002; Kim et al., 2007a; Wang et al., 2011). Unexpectedly, the monovalent murine IL-2 fusion toxin is more potent than its bivalent counterpart (P = 0.0001). This potency data were supported by binding affinity analysis in the later section.

Cell proliferation inhibition analysis of the murine IL-2 fusion toxins

To doubly assess the potency of the murine IL-2 fusion toxins, an in vitro cell proliferation inhibition assay was performed using the murine CD25⁺ CTLL-2 cell line through incorporation of tritiated thymidine. The cell proliferation inhibition analysis demonstrated that the monovalent murine IL-2 fusion toxin (EC₅₀ = 8.15 × 10⁻¹³ M) is more potent than the bivalent counterpart (EC₅₀ = 3.17 × 10⁻¹² M) (Fig. 5; P < 0.0001), which is consistent with the previously described protein synthesis inhibition analysis.

Flow cytometry binding analysis of the murine IL-2 fusion toxins

In order to inhibit the target cell protein synthesis, the fusion toxin must bind to the target cell receptor and then be internalized into the cytosol through endocytosis. Therefore, binding to the cell surface is the first critical step in inhibiting protein
The murine IL-2 fusion toxins were designed to bind to cells expressing the high affinity murine IL-2 receptor consisting of IL-2Rα (CD25), IL-2Rβ (CD122) and common cytokine receptor γc (CD132) subunits. The binding affinity of the murine IL-2 fusion toxins to the murine IL-2 receptor was analyzed by flow cytometry using a murine CD25⁺ CTLL-2 cell line. As shown in Fig. 6A, the monovalent fusion toxin (left panel) bound to the murine CD25⁺ IL-2 receptor stronger than the bivalent isomer (right panel) which correlated with the protein synthesis inhibition and cell proliferation inhibition analysis described previously. The monovalent murine IL-2 fusion toxin was found to have higher affinity, approximately eight times stronger, for murine CD25 (\(K_d = 0.32\) nM) (Fig. 6B) compared with the bivalent isomer (\(K_d = 2.52\) nM) (Fig. 6C).

**Fig. 6.** Flow cytometry binding analysis of the murine IL-2 fusion toxins to the murine CD25⁺ CTLL-2 cells. (A) Histograms of (i) DT390-mIL-2 (left panel); (ii) DT390-bi-mIL-2 (right panel). Only second staining control (PE-conjugated streptavidin), biotinylated rat anti-mouse IgG1 isotype control, biotinylated porcine CD3 eγ control (Peraino et al., 2012b) and biotinylated rat-anti-mouse CD25 mAb as positive control were also included. The data are representative of multiple individual experiments. (B and C) \(K_d\) determination by flow cytometry and nonlinear least-squares fit. MFI was plotted over a range of concentrations of biotinylated (B) DT390-mIL-2, (C) DT390-bi-mIL-2. The accompanying least-squares fit and parameters are shown based on the hyperbolic equation: 
\[ y = m_1 + \frac{m_2 \cdot M_0}{m_3 + m_0}, \]
where \(y\) = MFI at the given biotinylated murine IL-2 fusion toxin concentration, \(m_0\) = biotinylated murine IL-2 fusion toxin concentration, \(m_1\) = MFI of zero biotinylated murine IL-2 fusion toxin, \(m_2\) = MFI at saturation and \(m_3 = K_d\). The inset table in (B) shows a fitted \(K_d\) of 0.32 nM for DT390-mIL-2. The inset table in (C) shows a fitted \(K_d\) of 2.52 nM for DT390-bi-mIL-2.

**Binding specificity analysis of the murine IL-2 fusion toxins**

To examine the binding specificity of the murine IL-2 fusion toxins, murine IL-2 was used as an inhibitor to block protein synthesis inhibition and cell proliferation inhibition of the murine IL-2 fusion toxins. Murine IL-2 blocked the protein synthesis inhibition (Fig. 7A and B) and cell proliferation inhibition (data not shown) of both monovalent (\(P < 0.0001\)) and bivalent (\(P < 0.0001\)) murine IL-2 fusion toxins in a dose-dependent manner. These blocking assay data confirmed that the murine IL-2 fusion toxins bind specifically to the murine IL-2 receptor on the CTLL-2 cell surface. In addition, these blocking assays also demonstrated that the monovalent murine IL-2 fusion toxin is more potent than the bivalent isomer.
In vivo Treg depletion using the monovalent murine IL-2 fusion toxin

Mouse handling and experimental procedures were conducted in accordance with national and institutional guidelines for animal care and use. As shown in Fig. 8A, C57BL/6J (B6) mice were injected intraperitoneally with 5 μg/mouse/day of DT390-mIL-2 or control DT390 for four consecutive days. The percentage of CD25⁺FoxP3⁺ cells (Tregs) among CD4⁺ T cells within spleen cells was monitored by FACS at Days 0.5, 1, 2, 3 and 7 after the last injection of control DT390 or DT390-mIL2. We observed a significant decrease of Treg frequencies in the group treated with DT390-mIL-2 detectable as early as 12 h after last injection. Treg cell frequencies were reduced until Day 3 and returned to control levels by Day 7 (P < 0.05). In contrast, injection of control DT390 alone did not result in a decrease of percentages of Tregs among CD4⁺ T cells and even induced a slight and transient increase in Treg frequencies, presumably due to some pro-inflammatory effects of the toxin. On the other hand, the levels of other leukocyte populations, including CD4⁺, CD8⁺, CD19⁺ (B cells) and NK-1.1⁺ (NK cells) cells, remained unchanged upon administration of DT390 or DT390-mIL-2 (Fig. 8B).

Discussion

In this study, we have successfully developed both monovalent and bivalent murine IL-2 fusion toxins for depleting murine Treg in vivo. Surprisingly, in contrast to other developed

Fig. 7. Binding specificity analysis of the murine IL-2 fusion toxins to the target murine CD25⁺ CTLL-2 cells in the in vitro protein synthesis inhibition assay using murine IL-2 as inhibitor: (A) monovalent murine IL-2 fusion toxin (DT390-mIL-2) with or without inhibitor, murine IL-2; (B) bivalent murine IL-2 fusion toxin (DT390-bi-mIL-2) with or without inhibitor, murine IL-2. Y-axis: inhibition rate of the protein synthesis via the cpm value measuring incorporation of tritiated leucine. X-axis: plated murine IL-2 fusion toxin concentration. Wells containing the murine IL-2 as inhibitor were incubated for 1 h at 37°C before addition of fusion toxin. Cycloheximide (1.25 mg/ml) was used as a positive control. Cells without fusion toxin served as the negative control. P < 0.0001 by two-way ANOVA (n = 2). Error bars indicate ± SD.

Fig. 8. Kinetics of depletion of CD25⁺FoxP3⁺ cells (Tregs) among CD4⁺ T cells after administration of DT390-mIL-2. C57BL/6J (B6) mice were injected intraperitoneally with 5 μg/mouse/day of DT390-mIL-2 or control DT390 for four consecutive days. (A) After the last injection of DT390 or DT390-mIL-2, the frequencies of CD25⁺FoxP3⁺ cells (Tregs) among CD4⁺ T cells were monitored and compared with the untreated C57BL/6J female mice at 0.5, 1, 2, 3 and 7 days by FACS analysis (with each group having n = 3). The results are expressed as a percentage of Tregs among CD4⁺ T cells ± SD. (B) The frequencies of B cells (CD19⁺), T cells (CD3⁺, CD4⁺ or CD8⁺) and NK cells (NK-1.1⁺) were also monitored by FACS analysis (n = 3) at 0.5, 1, 2, 3 and 7 days after the last injection of DT390-mIL-2 or DT390. The results are expressed as percentage per total lymphocytes ± SD. No significant differences were observed between control (Day 0) levels and any of the post-treatment time points for any of the populations.
immunotoxins/fusion toxins (Woo et al., 2002; Kim et al., 2007a; Wang et al., 2011), the monovalent murine IL-2 fusion toxin is more potent than its bivalent counterpart. We speculate the following possibilities: (i) the conformation of the bivalent isoform is not optimal for binding to murine CD25; and (ii) the distribution of the murine IL-2 receptors on T cells is different from that of such as porcine or human IL-2 receptors. This surprising result will be investigated further.

We speculate that continuous depletion of Tregs might be achieved through repeated administration. We did not monitor the immunogenicity in this study; however, we would expect it to be similar to that reported for patients treated with Ontak, with which repeated dosing was approved by FDA (9 or 18 µg/kg/day by intravenous infusion over 30–60 min for five consecutive days every 21 days for eight cycles).

Ontak is a unique FDA-approved DT-based human IL-2 fusion toxin. This murine IL-2 fusion toxin provides a mouse species-specific version that can be used for Ontak related research using naïve and disease-specific mouse models.

This murine IL-2 fusion toxin can be used for preclinical studies to assess: (i) direct depletion of murine CD25+ tumor cells in vivo and (ii) combined cancer treatment as well as mechanism study of the transplantation tolerance and autoimmune diseases though depleting murine CD25+ Tregs. After repeated systemic application, the possible side effects might include: (i) triggering autoimmune response due to continued Treg depletion and (ii) depleting the activated CD25+ effect T cells and NK cells since NK cell also express the β and r subunits of the IL-2 receptor. All of these remaining questions need to be investigated further in naïve and disease mouse models.

In this study, murine NK cells were not deleted by murine IL-2 fusion toxin. Ontak as well as our bivalent human and porcine IL-2 fusion toxins significantly depleted NK cells in large animal models (Yamada et al., 2012 and our unpublished data). It remains unknown why murine NK cells were not depleted by murine IL-2 fusion toxin. The ability to deplete Tregs specifically while sparing NK cells will allow the murine version of IL-2 fusion toxin to be a better reagent for testing combined cancer treatment in murine models.

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**References**


