Published in final edited form as:

J Immunol Methods. 2014 March ; 405: 57–66. doi:10.1016/j.jim.2014.01.008.

Diphtheria toxin-based bivalent human IL-2 fusion toxin with improved efficacy for targeting human CD25⁺ cells

Jaclyn Stromp Peraino^{a,b}, Huiping Zhang^{a,b}, Priyani V. Rajasekera^{a,b}, Min Wei^{a,b}, Joren C. Madsen^a, David H. Sachs^{a,b}, Christene A. Huang^{a,b}, and Zhirui Wang^{a,b,*}

Zhirui Wang: zhirui.wang@tbrc.mgh.harvard.edu

^aTransplantation Biology Research Center, Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA

^bMGH-DF/HCC Recombinant Protein Expression and Purification Core, Boston, MA, USA

Abstract

Regulatory T cells (Treg) constitute a major inhibitory cell population which suppresses immune responses. Thus, Treg have proven to be key players in the induction of transplantation tolerance, protection from autoimmune disease and prevention of the development of effective anti-tumor immune reactions. Treg express high levels of the high affinity interleukin-2 receptor (IL-2R) consisting of IL-2R α (CD25) together with IL-2R β (CD122) and the common γ -chain (CD132). An effective reagent capable of depleting Treg *in vivo* would facilitate better cancer treatment and allow mechanistic studies of the role of Treg in transplantation tolerance and the development of autoimmune disease. In this study, we have developed a novel bivalent human IL-2 fusion toxin along with an Ontak®-like monovalent human IL-2 fusion toxin and compared the functional ability of these reagents *in vitro*. Here we show that genetically linking two human IL-2 domains in tandem, thereby generating a bivalent fusion toxin, results in significantly improved capacity in targeting human CD25⁺ cells *in vitro*. Binding analysis by flow cytometry showed that the bivalent human IL-2 fusion toxin has notably increased affinity for human CD25⁺ cells. *In vitro* functional analysis demonstrated that the bivalent isoform has an increased potency of approximately 2 logs in inhibiting cellular proliferation and protein synthesis in human CD25⁺ cells compared to the monovalent human IL-2 fusion toxin. Additionally, we performed two inhibition assays in order to verify that the fusion toxins target the cells specifically through binding of the human IL-2 domain of the fusion toxin to the human IL-2 receptor on the cell surface. These results demonstrated that 1) both monovalent and bivalent human IL-2 fusion toxins are capable of blocking the binding of biotinylated human IL-2 to human CD25 by flow cytometry; and 2) human IL-2 blocked the fusion toxins from inhibiting protein synthesis and cellular proliferation *in vitro*, thus confirming that the human IL-2 fusion toxins target the cells specifically through binding to the human IL-2 receptor. We believe that the bivalent human IL-2 fusion toxin will be a more potent, and therefore, more optimal agent than the current clinically-used monovalent fusion toxin (denileukin diftitox, Ontak®) for *in vivo* depletion of Treg.

Keywords

Human IL-2; Diphtheria toxin; Fusion toxin; Regulatory T cell; *Pichia pastoris* expression

1. Introduction

The immune system regulates its response so that foreign pathogens are recognized and eliminated without damaging the host cells. Regulatory T cells (Treg) are a major player for suppressing the immune response and preventing effector T cells from targeting the body's own cells. Experimental and clinical data demonstrated that Treg, characterized as CD4⁺CD25⁺Foxp3⁺, have significantly reduced suppression function in animal models and patients with autoimmune diseases such as rheumatoid arthritis, multiple sclerosis and type I diabetes (Viglietta et al., 2004; Lindley et al., 2005; Ehrenstein et al., 2004; Sakaguchi et al., 2008). A reagent capable of depleting Treg *in vivo* could offer a useful tool for researchers studying autoimmune diseases in animal models.

Treg are also extensively studied in transplantation in an effort to understand the immunological mechanisms behind tolerance and rejection of allogeneic and xenogeneic organs. Increased levels of CD4⁺CD25^{hi}Foxp3⁺ Treg have been detected in donor kidneys of tolerant recipients in experimental animal models and clinical patients (Miyajima et al., 2011). It is unclear, however, what role Treg play in the induction and maintenance of tolerance of these allografts. Efficient targeting and depletion of Treg *in vivo* may aid in determining the mechanisms of how Treg facilitate the initiation of and subsequently sustain tolerance to transplanted organs.

While Treg function advantageously in development of transplantation tolerance and prevention of autoimmunity, their down regulation of immune responses may impede the body's ability to clear tumorigenic cell populations. Tumor progression induces proliferation of two T cell populations: those that target cancer cells; and those that down-regulate the targeting population, allowing the cancer to progress. The immune modulating cell populations are a major obstruction to treatments designed to activate and expand cells capable of targeting tumor cells. Treg suppress immune responses to tumors, therefore, methods that target and deplete this cell population *in vivo* could prove to be useful in improving cancer immunotherapy.

Ontak® (denileukin diftitox, DAB389IL-2, Eisai Medical Research, Inc.) is a monovalent human IL-2 fusion toxin expressed in *Escherichia coli* which has been approved by the Food and Drug Administration (FDA) for clinical treatment of human CD25⁺ cutaneous T cell lymphoma. It was also tested on CD25⁻ tumors in an effort to indirectly improve cancer treatment by depleting Treg and thereby, allowing immune responses to run their course unimpeded. Ontak® shows some efficacy in depleting CD25⁺ Treg and improving tumor immunotherapy in some studies (Morse et al., 2008; Barnett et al., 2005; Telang et al., 2011), but no significant Treg depletion in other studies (Attia et al., 2005; Yamada et al., 2012). Currently, denileukin diftitox is clinically discontinued.

The truncated diphtheria toxin DT390 has been successfully used to build several functional recombinant immunotoxins/fusion toxins (Woo et al., 2002; Kim et al., 2007; Wang et al., 2011; Peraino et al., 2013a) for depleting specific cell populations *in vivo*. In this study we expressed both monovalent and bivalent human IL-2 fusion toxins using a yeast *Pichia pastoris* expression system and assessed their functions *in vitro* using protein synthesis inhibition and cellular proliferation inhibition assays. The binding affinity of these recombinant fusion toxins to human CD25 was analyzed using flow cytometry. Binding specificity was determined using the human IL-2 fusion toxins as inhibitors to block the binding of biotinylated human IL-2 to human CD25⁺ cells by flow cytometry and utilizing human IL-2 as an inhibitor to block the ability of the human IL-2 fusion toxins to inhibit protein synthesis and cellular proliferation in human CD25⁺ cells *in vitro*.

2. Materials and methods

2.1. Plasmid construction

As shown in Fig. 1, human IL-2 fusion toxins were constructed using the codon-optimized nucleotide sequences and contain two moieties; DT390 (Woo et al., 2002) and human IL-2 (Fig. 2). A strategy previously employed to generate A-dm-DT390biscFv (2-6-15) (Wang et al., 2011) was applied to construct these human IL-2 fusion toxins, substituting codon-optimized human IL-2 for the biscFv (2-6-15) moiety. DT390 and human IL-2 domains are connected by a linker consisting of four glycines and a serine residue (G₄S). The two human IL-2 domains of the bivalent fusion toxin are joined by three tandem G₄S linkers (G₄S)₃. Six histidines (6× His tag) were added to the C-terminus of each construct to facilitate protein purification. The codon-optimized human IL-2 DNA (Fig. 2) was synthesized by PCR amplification as described previously (Hermanrud et al., 2011). To construct DT390-hIL-2, the codon-optimized human IL-2 DNA was amplified using PCR primers hIL2-X1 carrying *Xho*I and *Nco*I site + hIL2 Rev carrying an *Eco*RI site then cloned into pwPICZalpha (Peraino et al., 2012a) between *Xho*I and *Eco*RI sites for sequencing confirmation. The insert was then cut out with *Nco*I + *Eco*RI and cloned into pwPICZalpha-DT390 (Wang et al., 2011) between *Nco*I and *Eco*RI sites yielding the final construct DT390-hIL-2 in pwPICZalpha. To construct DT390-bi-hIL-2, the first human IL-2 was amplified using PCR primers hIL2-X1 carrying *Xho*I and *Nco*I sites + hIL2-Bam1 carrying *Bam*HI and *Eco*RI sites then cloned into pwPICZalpha between *Xho*I and *Eco*RI sites for sequencing confirmation. The insert was subsequently cut out with *Nco*I + *Bam*HI as insert I. The second human IL-2 was PCR amplified using hIL2-Bam2 carrying *Xho*I and *Bam*HI sites + hIL-2 Rev carrying an *Eco*RI site then cloned into pwPICZalpha between *Xho*I and *Eco*RI sites for sequencing confirmation. The insert was then cut out with *Bam*HI + *Eco*RI as insert II. The insert I carrying *Nco*I and *Bam*HI sites + insert II carrying *Bam*HI and *Eco*RI sites (*Nco*I– hIL-2-*Bam*HI/*Bam*HI-hIL-2–*Eco*RI) were together cloned into pwPICZalpha-DT390 between *Nco*I and *Eco*RI yielding the final construct DT390-bi-hIL-2 in pwPICZalpha. All PCR primers that were used are listed in Table 1.

Protein expression in *P. pastoris* and subsequent purifications were performed as previously described (Wang et al., 2011; Peraino et al., 2013a). Western blot analysis, binding affinity and specificity analysis by flow cytometry and K_d determination were all performed as

previously described (Peraino et al., 2013a) using a human CD25⁺ T-cell lymphoma cell line HUT 102/6TG (Williams et al., 1990) (kindly provided by Dr. Robert Harrison, Anjin Group, Inc., Boston, MA). Isolation of human PBMC as well as *in vitro* binding analysis of the human IL-2 fusion toxin to human CD25 on PBMC using flow cytometry was performed as previously described (Peraino et al., 2013b). DT390 and human IL-2 were used as controls for all *in vitro* functional analysis. These products were also expressed in the yeast *P. pastoris* system.

2.2. Protein synthesis inhibition

Human CD25⁺ T-cell lymphoma cells HUT 102/6TG were cultured in RPMI 1640 media supplemented with 12% fetal bovine serum, 10 mM hepes (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid), 1× nonessential amino acids, 1 mM sodium pyruvate, 2 mM glutamine, and 2.5×10^{-5} M 2-mercaptoethanol. Cells were washed twice with 50 mL of the above media containing leucine-free RPMI by centrifugation at 1000 rpm, 20 °C for 5 min. HUT 102/6TG cells were then diluted to 5.0×10^5 cells/mL and each human IL-2 fusion toxin was serially diluted in the above culture media with leucine-free RPMI. One hundred microliters of cells (5.0×10^4 cells) was added to each well in a 96-well flat bottom 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 and then centrifuged at 170 ×g for 5 min at 4 °C. The supernatant was discarded and the cells were lysed by adding 50 µL of potassium hydroxide (4 M) to each well for 10 min at room temperature. Proteins were precipitated by adding 150 µL of trichloroacetic acid (10% w/v) then plates were 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 million (cpm) read using a microbeta counter. The negative control for this assay was HUT 102/6TG cells plated without fusion toxin and the positive control was HUT 102/6TG cells plated with cycloheximide (Sigma) (1:8) for 15 min at 37 °C with 5% CO₂. Both controls were analyzed in triplicate for each assay. The inhibition assays follow the protocol above with a 1-h incubation of 10 µL of the human IL-2 inhibitor (10^{-6} M as the final concentration) with the HUT 102/6TG cells prior to addition of the fusion toxins. Inhibition assays were then pulsed, harvested and read as described above.

Cellular proliferation inhibition analysis was performed exactly as the protein synthesis inhibition assays described previously except for the following two differences: 1) HUT 102/6TG cells were cultured, washed, diluted and incubated using RPMI 1640 media supplemented with 12% fetal bovine serum, 10 mM hepes (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid), 1× nonessential amino acids, 1 mM sodium pyruvate, 2 mM glutamine, and 2.5×10^{-5} M 2-mercaptoethanol. Cells were incubated with fusion toxin dilutions for 24 h. 2) Plates were pulsed with 1 µCi/well of ³H-thymidine for a 24 h incubation.

3. Results

3.1. Expression and purification of the human IL-2 fusion toxins in yeast *P. pastoris*

Based on our experience in developing porcine IL-2 fusion toxins (Peraino et al., 2013b), we hypothesized that the bivalent human IL-2 fusion toxin would prove to be a more potent *in vivo* depletion agent of human CD25⁺ cells than the clinically-used monovalent human IL-2 fusion toxin (denileukin diftitox, Ontak®). Fig. 1 presents a schematic representation of the monovalent and bivalent human IL-2 fusion toxins that we constructed in this study.

The human IL-2 fusion toxins were expressed in a yeast *P. pastoris* expression system using one liter Erlenmeyer flasks. The human IL-2 fusion toxins were secreted into the extracellular supernatant then captured using a Ni-sepharose fast flow resin and further purified using a strong anion exchange resin. The final purification yield was ~5 mg per liter of the original harvested supernatant for both monovalent and bivalent human IL-2 fusion toxins. The purified human IL-2 fusion toxins were analyzed by SDS-PAGE (Fig. 3A) and Western blot using a mouse anti-His monoclonal antibody (Fig. 3B) and a mouse anti-diphtheria toxin monoclonal antibody (Fig. 3C).

3.2. Binding affinity analysis of the human IL-2 fusion toxins for human CD25

The diphtheria toxin-based human IL-2 fusion toxins target cells expressing the human IL-2 receptor *via* binding of the human IL-2 domain of the fusion toxins. Following cellular internalization, the DT390 domain functions to inhibit protein synthesis resulting in cell death (Murphy, 2011). Therefore, the first critical step in determining the functionality of the human IL-2 fusion toxins was to analyze their binding affinity for human CD25. Both bivalent and monovalent human IL-2 fusion toxins were labeled with sulfo-EZ-link NHS biotin (Thermo Scientific) for binding analysis using flow cytometry (Fig. 4A). Binding affinity was quantified by calculating the dissociation constant (K_d) for each human IL-2 fusion toxin from mean fluorescence intensity (MFI) (Peraino et al., 2013a). Consistent with previously developed recombinant fusion toxins/immunotoxins (Woo et al., 2002; Kim et al., 2007; Wang et al., 2011), the bivalent human IL-2 fusion toxin was found to have notably higher affinity, approximately two logs stronger, for human CD25 ($K_d = 0.21$ nM) (Fig. 4C) compared to the monovalent isoform ($K_d = 15.9$ nM) (Fig. 4B). Human IL-2 was also included as a control. All of the binding and potency assay results in this study were confirmed using a second human CD25⁺ lymphoma cell line, SR (ATCC CRL-2262) (Table 2).

To confirm the ability of the human IL-2 fusion toxin to bind to human CD25⁺ PBMC, isolated human PBMC were stained with biotinylated bivalent human IL-2 fusion toxin and analyzed by flow cytometry. As shown in Fig. 5A, biotinylated bivalent human IL-2 fusion toxin bound to the human CD4⁺ gated human PBMC in a dose dependent manner. Biotinylated bivalent human IL-2 fusion toxin bound to 91% of the CD4⁺CD25⁺ human PBMC (Fig. 5B).

3.3. Binding specificity of the human IL-2 fusion toxins for the human IL-2 receptor

In order to prove that the human IL-2 fusion toxins bind specifically to the human IL-2 receptor we analyzed the ability of the fusion toxins to block the binding of human IL-2 to its receptor on HUT 102/6TG cells. Both monovalent and bivalent human IL-2 fusion toxins proved capable of inhibiting the binding of biotinylated human IL-2 to the cells suggesting that the human IL-2 fusion toxins are binding specifically to the human IL-2 receptor (Fig. 6). Moreover, the same trend was observed with strength in inhibition as seen in the above binding analysis in that the bivalent isoform demonstrated stronger hindrance of human IL-2 binding compared to the monovalent human IL-2 fusion toxin. Human IL-2 was also included as positive control.

3.4. In vitro potency of the human IL-2 fusion toxins

Following successful binding of the human IL-2 domain(s) of the fusion toxins to the cell surface human IL-2 receptor, the fusion toxin is internalized via endocytosis. Once inside the cell, the catalytic domain (A chain) of the DT390 is cleaved and deactivates the elongation factor 2 (EF-2) thereby hindering the cell's ability to synthesize new proteins. The potency of protein synthesis inhibition of the human IL-2 fusion toxins was assessed *in vitro* and quantified by measuring the target cells' incorporation of tritiated leucine into newly synthesized proteins. While both monovalent and bivalent human IL-2 fusion toxins proved capable of impeding protein synthesis in HUT 102/6TG cells, the bivalent isoform displayed an increased potency ($IC_{50} = 2 \times 10^{-13}$ M) of approximately 2 logs when compared with the Ontak®-like monovalent version ($IC_{50} = 2 \times 10^{-11}$ M) (Fig. 7A). The degree of protein synthesis inhibition was comparable between clinically used Ontak® and our Ontak®-like monovalent human IL-2 fusion toxin (data not shown). Human IL-2 and DT390 served as controls in this assay.

In an effort to demonstrate that the HUT 102/6TG cells are being targeted specifically through the interaction of the human IL-2 domain on the fusion toxin and the IL-2 receptor on the cell surface, we assessed the fusion toxins' ability to halt protein synthesis in the presence of human IL-2. Target cells that were incubated with fusion toxin in the presence of human IL-2 showed a marked increase in protein synthesis compared to cells which were cultured with the corresponding concentration of fusion toxin only. Human IL-2 acted as an inhibitor of fusion toxin as it prevented both the monovalent (Fig. 7B) and bivalent (Fig. 7C) fusion toxins from targeting the human CD25⁺ cells. Additionally, the bivalent isoform proved to be more potent than the Ontak®-like monovalent fusion toxin in inhibiting protein synthesis.

In order to confirm that the hindrance of protein synthesis in the human CD25⁺ target cells was specifically due to the human IL-2 fusion toxins and not because of the consequence of culturing the cells in leucine-free media, we assessed the functional ability of these fusion toxins to inhibit cellular proliferation in the same target cells using complete culture media. Cellular proliferation was quantified by measuring the incorporation of tritiated thymidine into newly synthesized DNA in target human CD25⁺ cells following incubation with the human IL-2 fusion toxins. The result was consistent with that of protein synthesis inhibition assays in that both fusion toxins obstructed cellular proliferation in the target cells. The

bivalent isoform consistently demonstrated a potency of more than one log greater than the monovalent human IL-2 fusion toxin (Fig. 8A). As for the protein synthesis inhibition assays, human IL-2 and DT390 were included as controls.

To confirm that the fusion toxins bound to the target cells *via* interaction of the cell surface human IL-2 receptor with the human IL-2 domain of the fusion toxins in this cell proliferation inhibition assay, we observed the ability of the human IL-2 fusion toxins to inhibit cellular proliferation in the presence of an inhibitor, human IL-2. Consistently, human IL-2 drastically affected the ability of both fusion toxins to obstruct cellular proliferation in target cells, and the bivalent isoform (Fig. 8C), again, yielded a higher potency than the monovalent fusion toxin (Fig. 8B).

4. Discussion

Depleting Treg in animal models could help illuminate the mechanisms of immunological processes such as the development of transplantation tolerance and autoimmune disease. Treg depletion has also been widely utilized in an effort to improve cancer treatment. The clinical and research-based demand for effective Treg depletion *in vivo* led investigators to explore the possibility to utilize the FDA-approved Ontak® for this purpose. However, this *E. coli* expressed, monovalent human IL-2 fusion toxin was unable to achieve the optimal levels of Treg depletion (Morse et al., 2008; Barnett et al., 2005; Telang et al., 2011; Attia et al., 2005; Yamada et al., 2012) and its production has been discontinued since 2011. Endotoxin is another common concern when using the *E. coli* expression system. In this study we utilized a diphtheria toxin-resistant yeast *P. pastoris* expression system (Liu et al., 2003), which offers greatly enhanced protein expression levels, purification and yield. Moreover, we genetically linked two human IL-2 domains to generate a bivalent fusion toxin with increased affinity for the IL-2 receptor. This bivalent human IL-2 fusion toxin showed significantly higher efficacy for human CD25⁺ cells compared to the Ontak®-like monovalent isoform. We speculate that linking two human IL-2 domains in tandem increases the fusion toxins' affinity for the human IL-2 receptor, subsequently facilitating a more efficient internalization, and causing a notable increase in potency. We believe that producing the recombinant IL-2 fusion toxins in yeast rather than *E. coli* and generating a bivalent, more potent isoform augments the potential for clinical application of this reagent. In addition, this bivalent human IL-2 fusion toxin could serve as an ideal replacement for the clinically used and discontinued Ontak® for the direct treatment of human CD25⁺ tumors such as cutaneous lymphoma. It may also prove valuable to investigators for depleting CD25⁺ cells found to contribute to post transplantation lymphoproliferative disorder (PTLD). Murine, porcine and human specific bivalent IL-2 fusion toxin reagents are available through our self-managed MGH-DF/HCC Recombinant Protein Expression and Purification Core facility for preclinical development and translational research.

Acknowledgments

The work was supported in part by a Dana Farber/Harvard Cancer Center Core development grant. We would like to thank Angimmune LLC for kindly providing the diphtheria toxin resistant yeast *Pichia pastoris* strain and the codon-optimized DT390 DNA. We would like to thank Dr. Robert Harrison for generously providing the HUT 102/6TG cell line. We would like to thank Edward Harrington for technical assistance, and Dr. Bob Hawley and Sharon Germana for their intensive manuscript review.

References

- Attia P, Maker AV, Haworth LR, Rogers-Freezer L, Rosenberg SA. Inability of a fusion protein of IL-2 and diphtheria toxin (Denileukin Diftitox, DAB389IL-2, ONTAK) to eliminate regulatory T lymphocytes in patients with melanoma. *J Immunother.* 2005; 28:582. [PubMed: 16224276]
- Barnett B, Kryczek I, Cheng P, Zou W, Curiel TJ. Regulatory T cells in ovarian cancer: biology and therapeutic potential. *Am J Reprod Immunol.* 2005; 54:369. [PubMed: 16305662]
- Ehrenstein MR, Evans JG, Singh A, Moore S, Warnes G, Isenberg DA, Mauri C. Compromised function of regulatory T cells in rheumatoid arthritis and reversal by antiTNFalpha therapy. *J Exp Med.* 2004; 200:277. [PubMed: 15280421]
- Hermanrud CE, Lucas CL, Sykes M, Huang CA, Wang Z. Expression and purification of soluble murine CD40L monomers and polymers in yeast *Pichia pastoris*. *Protein Expr Purif.* 2011; 76:115. [PubMed: 21074618]
- Kim GB, Wang Z, Liu YY, Stavrou S, Mathias A, Goodwin KJ, Thomas JM, Neville DM Jr. A fold-back single-chain diabody format enhances the bioactivity of an anti-monkey CD3 recombinant diphtheria toxin-based immunotoxin. *Protein Eng Des Sel.* 2007; 20:425. [PubMed: 17693455]
- Lindley S, Dayan CM, Bishop A, Roep BO, Peakman M, Tree TI. Defective suppressor function in CD4(+)CD25(+) T-cells from patients with type 1 diabetes. *Diabetes.* 2005; 54:92. [PubMed: 15616015]
- Liu YY, Woo JH, Neville DM Jr. Targeted introduction of a diphtheria toxin resistant mutation into the chromosomal EF-2 locus of *Pichia pastoris* and expression of immunotoxin in the EF-2 mutants. *Protein Expr Purif.* 2003; 30:262. [PubMed: 12880776]
- Miyajima M, Chase CM, Alessandrini A, Farkash EA, Della Pelle P, Benichou G, Graham JA, Madsen JC, Russell PS, Colvin RB. Early acceptance of renal allografts in mice is dependent on foxp3(+) cells. *Am J Pathol.* 2011; 178:1635. [PubMed: 21435448]
- Morse MA, Hobeika AC, Osada T, Serra D, Niedzwiecki D, Lysterly HK, Clay TM. Depletion of human regulatory T cells specifically enhances antigen-specific immune responses to cancer vaccines. *Blood.* 2008; 112:610. [PubMed: 18519811]
- Murphy JR. Mechanism of diphtheria toxin catalytic domain delivery to the eukaryotic cell cytosol and the cellular factors that directly participate in the process. *Toxins.* 2011; 3:294. [PubMed: 22069710]
- Peraïno J, Zhang H, Hermanrud CE, Li G, Sachs DH, Huang CA, Wang Z. Expression and purification of soluble porcine CTLA-4 in yeast *Pichia pastoris*. *Protein Expr Purif.* 2012a; 82:270. [PubMed: 22326797]
- Peraïno JS, Hermanrud CE, Springett L, Zhang H, Li G, Srinivasan S, Gusha A, Sachs DH, Huang CA, Wang Z. Expression and characterization of recombinant soluble porcine CD3 ectodomain molecules: mapping the epitope of an anti-porcine CD3 monoclonal antibody 898H2-6-15. *Cell Immunol.* 2012b; 276:162. [PubMed: 22672968]
- Peraïno JS, Schenk M, Zhang H, Li G, Hermanrud CE, Neville DM Jr, Sachs DH, Huang CA, Duran-Struuck R, Wang Z. A truncated diphtheria toxin based recombinant porcine CTLA-4 fusion toxin. *J Immunol Methods.* 2013a; 391:103. [PubMed: 23470981]
- Peraïno JS, Schenk M, Li G, Zhang H, Farkash EA, Sachs DH, Huang CA, Duran-Struuck R, Wang Z. Development of a diphtheria toxin-based recombinant porcine IL-2 fusion toxin for depleting porcine CD25+ cells. *J Immunol Methods.* 2013b:398–399. 33.
- Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. *Cell.* 2008; 133:775. [PubMed: 18510923]
- Telang S, Rasku MA, Clem AL, Carter K, Klarer AC, Badger WR, Milam RA, Rai SN, Pan J, Gragg H, Clem BF, McMasters KM, Miller DM, Chesney J. Phase II trial of the regulatory T cell-depleting agent, denileukin diftitox, in patients with unresectable stage IV melanoma. *BMC Cancer.* 2011; 11:515. [PubMed: 22165955]
- Viglietta V, Baecher-Allan C, Weiner HL, Hafler DA. Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. *J Exp Med.* 2004; 199:971. [PubMed: 15067033]

- Wang Z, Duran-Struuck R, Crepeau R, Matar A, Hanekamp I, Srinivasan S, Neville DM, Sachs DH, Huang CA. Development of a diphtheria toxin based antiporcine CD3 recombinant immunotoxin. *Bioconjug Chem*. 2011; 22:2014. [PubMed: 21866954]
- Williams DP, Wen Z, Watson RS, Boyd J, Strom TB, Murphy JR. Cellular processing of the interleukin-2 fusion toxin DAB₄₈₆-IL-2 and efficient delivery of diphtheria fragment A to the cytosol of target cells requires Arg¹⁹⁴. *J Biol Chem*. 1990; 265:20673. [PubMed: 2243114]
- Woo JH, Liu YY, Mathias A, Stavrou S, Wang Z, Thompson J, Neville DM Jr. Gene optimization is necessary to express a bivalent anti-human anti-T cell immunotoxin in *Pichia pastoris*. *Protein Expr Purif*. 2002; 25:270. [PubMed: 12135560]
- Yamada Y, Aoyama A, Tocco G, Boskovic S, Nadazdin O, Alessandrini A, Madsen JC, Cosimi AB, Benichou G, Kawai T. Differential effects of denileukin diftitox IL-2 immunotoxin on NK and regulatory T cells in nonhuman primates. *J Immunol*. 2012; 188:6063. [PubMed: 22586034]

Fig. 1.
Schematic representation of the monovalent and bivalent human IL-2 fusion toxins.

```

gctccaacttcttcttctactaagaagactcaattgcaattggagcacttggtgttgac
A P T S S S T K K T Q L Q L E H L L L D
ttgcaaatgattttgaacggtattaacaactacaagaacccaaagttgactagaatgttg
L Q M I L N G I N N Y K N P K L T R M L
actttcaagttctacatgccaaagaaggctactgagttgaagcacttgcaatggttgag
T F K F Y M P K K A T E L K H L Q C L E
gaggaattgaagccattggaggaagttttgaacttggtcaatctaagaacttccacttg
E E L K P L E E V L N L A Q S K N F H L
agaccaagagacttgatttctaacattaacgttattgttttgagttgaagggttctgag
R P R D L I S N I N V I V L E L K G S E
actactttcatgtgtgagtacgctgacgagactgctactattgttgagttcttgaacaga
T T F M C E Y A D E T A T I V E F L N R
tggattactttctgtcaatctattatctctactttgact
W I T F C Q S I I S T L T

```

Fig. 2.
Codon-optimized human IL-2 DNA sequence.

Fig. 3. SDS PAGE and Western blot of the human IL-2 fusion toxins. **A)** SDS PAGE analysis (4–12% NuPAGE, Invitrogen); **B)** Western blot analysis using a mouse anti-His mAb (clone#: 4A12E4, Invitrogen). **C)** Western blot analysis using a mouse anti-diphtheria toxin mAb (clone# 3B6, Meridian). Lane 1: Protein marker; Lanes 2–3: DT390-hIL-2 (59.3 kDa); Lanes 4–5: DT390-bi-hIL (75.6 kDa).

Fig. 4. Binding of the human IL-2 fusion toxins to human CD25⁺ HUT 102/6TG cells. **A)** Histograms of the DT390-hIL-2 (middle panel), DT390-bi-hIL-2 (right panel) as well as positive control human IL-2 (left panel). Cells with only the secondary staining (PE-conjugated streptavidin) served as the negative control and PE-conjugated mouse anti-human CD25 mAb (clone# M-A251, BD Pharmingen, Cat# 555432) was used for the positive control. Biotin-labeled porcine CD3- $\epsilon\gamma$ (Peraïno et al., 2012b) was included as a negative control for background due to protein biotinylation. The data are representative of

multiple individual experiments. **B–C**) K_d determination using flow cytometry and nonlinear least squares fit. MFI was plotted over a range of concentrations of biotinylated **B**) DT390-hIL-2 and **C**) DT390-bi-hIL-2. The accompanying least-squares fit and parameters are shown based on the hyperbolic equation $y = m1 + m2 * m0 / (m3 + m0)$ where y = MFI at the given biotinylated human IL-2 fusion toxin concentration, $m0$ = biotinylated or human IL-2 fusion toxin concentration, $m1$ = MFI of zero biotinylated human IL-2 fusion toxin, $m2$ = MFI at saturation and $m3 = K_d$. The inset table in **B**) shows a fitted K_d of 15.9 nM for DT390-hIL-2. The inset table in **C**) shows a fitted K_d of 0.21 nM for DT390-bi-hIL-2.

Fig. 5.

In vitro binding analysis of the bivalent human IL-2 fusion toxin to human PBMC. **A)** Human PBMC were stained with biotinylated bivalent human IL-2 fusion toxin and analyzed by flow cytometry. The concentration of the biotinylated bivalent human IL-2 fusion toxin is indicated on the top of each panel. The X axis indicates the FITC-conjugated anti-human CD4 mAb (clone# SK3, Biolegend, Cat# 344604) as the only stain. The Y axis indicates the biotinylated bivalent human IL-2 fusion toxin as the primary stain and the PE-conjugated streptavidin as the secondary stain. The first panel (labeled as “0 nM” of the biotinylated bivalent human IL-2 fusion toxin) is the negative control stained with the biotinylated porcine CD3 $\epsilon\gamma$ (20 nM) (Perraino et al., 2012b) for any background as a result of protein biotinylation. Data are representative of two individual assays. **B)** Biotinylated bivalent human IL-2 fusion toxin bound to 91% of the human CD4⁺CD25⁺ PBMC. In the left panels, the X axis indicates the stain with FITC-conjugated anti-human CD4 mAb. The Y axis indicates the stain with PE-conjugated anti-human CD25 mAb (clone# M-A251, BD, Cat# 555432). Biotinylated bivalent human IL-2 fusion toxin was used as primary stain and the PE-conjugated streptavidin as the secondary stain (right panels). Data are representative of two individual assays.

Fig. 6.

Binding specificity of the human IL-2 fusion toxins for the human IL-2 receptor on human CD25⁺ HUT 102/6TG cells. Unlabeled human IL-2 fusion toxins as well as the positive control human IL-2 were each incubated with HUT 102/6TG cells at a range of concentrations for 5 min at 4 °C in the dark. Subsequently, without washing the cells, biotin-labeled human IL-2 was added to each tube containing cells in the presence of the unlabeled fusion toxin or human IL-2. Binding specificity of the human IL-2 fusion toxin or human IL-2 to the IL-2 receptor on HUT 102/6TG cells was measured by a decrease in biotin-labeled human IL-2 staining in the presence of increasing concentrations of the unlabeled proteins. Biotin-labeled porcine CD3- $\epsilon\gamma$ was included as a negative control for background due to protein biotinylation.

Fig. 7.

A) Human IL-2 fusion toxin-mediated protein synthesis inhibition in human CD25⁺ HUT 102/6TG cells *in vitro*: 1) Ontak®-like monovalent human IL-2 fusion toxin (DT390-hIL-2, red line); 2) DT390-bi-hIL-2 (green line); 3) human IL-2 alone (blue line); 4) DT390 alone (black line). Y-axis: cpm value measuring the incorporation of tritiated leucine. X-axis: plated IL-2 fusion toxin concentration. Cycloheximide (1:8) was used as a positive control. The negative control contained cells without fusion toxin. Data are representative of multiple assays. **B–C**) Binding specificity analysis of the human IL-2 fusion toxins to the target human CD25⁺ HUT 102/6TG cells in this *in vitro* protein synthesis inhibition assay

using human IL-2 as inhibitor: **B**) Monovalent human IL-2 fusion toxin (DT390-hIL-2) with (blue) and without (pink) human IL-2 inhibitor; **C**) bivalent human IL-2 fusion toxin (DT390-bi-hIL-2) with (green) and without (orange) human IL-2 inhibitor. Y-axis: cpm value measuring the incorporation of tritiated leucine. X-axis: plated human IL-2 fusion toxin concentration. Wells containing the human IL-2 inhibitor were incubated for 1 h at 37 °C before addition of fusion toxin. Cycloheximide (1:8) was used as a positive control. Cells without fusion toxin served as the negative control. Data are representative of multiple assays.

Fig. 8.

A) Human IL-2 fusion toxin-mediated cellular proliferation inhibition in human CD25⁺ HUT 102/6TG cells *in vitro*: 1) Ontak®-like monovalent human IL-2 fusion toxin (DT390-hIL-2, red line); 2) DT390-bi-hIL-2 (green line); 3) human IL-2 (blue line); 4) DT390 (black line). Y-axis: cpm value measuring incorporation of tritiated thymidine. X-axis: plated IL-2 fusion toxin concentration. Cycloheximide (1:8) was used as a positive control. The negative control contained cells without fusion toxin. Data are representative of multiple assays. **B–C)** Binding specificity analysis of the human IL-2 fusion toxins to the target human CD25⁺ HUT 102/6TG cells during the *in vitro* cellular proliferation inhibition assay using human

IL-2 as inhibitor: **B**) Ontak®-like monovalent human IL-2 fusion (DT390-hIL-2) toxin with (blue) and without (pink) human IL-2 inhibitor; **C**) bivalent human IL-2 fusion toxin (DT390-bi-hIL-2) with (green) and without (orange) human IL-2 inhibitor. Y-axis: cpm value measuring cellular incorporation of tritiated thymidine. X-axis: plated human IL-2 fusion toxin concentration. Wells containing the human IL-2 inhibitor were incubated for 1 h at 37 °C before addition of the fusion toxin. Cycloheximide (1:8) was used as a positive control. Cells without fusion toxin served as the negative control. Data are representative of multiple assays.

Table 1

PCR primers used in this study.

hIL2-X1
5' CCG <u>CTC GAG CCA TGG</u> GGT GGT GGT GGT TCT GCT CCA ACT TCT TCT TCT ACT 3'
<i>XhoI</i> <i>NcoI</i>
hIL2-Bam1
5' CCG <u>GAA TTC</u> CGC GCG <u>GGA TCC</u> ACC ACC ACC AGA ACC ACC ACC AGT CAA AGT AGA GAT AAT AGA TTG 3'
<i>EcoRI</i> <i>BamHI</i>
hIL2-Bam2
5' CCG <u>CTC GAG</u> CGG GCG <u>GGA TCC</u> GGT GGT GGT GGT TCT GCT CCA ACT TCT TCT TCT ACT 3'
<i>XhoI</i> <i>BamHI</i>
hIL2 Rev
5' CCG <u>GAA TTC</u> TTA GTG GTG GTG GTG GTG GTG AGT CAA AGT AGA GAT AAT AGA TTG 3'
<i>EcoRI</i>

Table 2

Kd values of the human IL-2 fusion toxins in two human CD25⁺ tumor cell lines.

Tumor cell line	Kd (nM)	
	DT390-hIL-2	DT390-bi-hIL-2
HUT 102/6TG	15.9	0.21
SR	8.25	0.065