Journal of Immunological Methods 398–399 (2013) 33–43

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

Development of a diphtheria toxin-based recombinant porcine IL-2 fusion toxin for depleting porcine CD25$^+$ cells

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ARTICLE INFO

Article history:
Received 4 April 2013
Received in revised form 24 July 2013
Accepted 9 September 2013
Available online 18 September 2013

Keywords:
Porcine IL-2
Fusion toxin
Diphtheria toxin
Regulatory T cell
Pichia pastoris expression

ABSTRACT

Regulatory T cells (Tregs) have been widely recognized as crucial players in controlling immune responses. Because their major role is to ensure that the immune system is not over reactive, Tregs have been the focus of multiple research studies including those investigating transplantation tolerance, autoimmunity and cancer treatment. On their surface Tregs constitutively express CD25, a high affinity receptor for the cytokine interleukin-2 (IL-2). The reagents constructed in this study were generated by genetically linking porcine IL-2 to the truncated diphtheria toxin (DT390). This reagent functions by first binding to the cell surface via the porcine IL-2/porcine CD25 interaction then the DT390 domain facilitates internalization followed by inhibition of protein synthesis resulting in cell death. Four versions of the porcine IL-2 fusion toxin were designed in an interest to find the most effective isoform: 1) monovalent glycosylated porcine IL-2 fusion toxin (Gly); 2) monovalent non-*N*-glycosylated porcine IL-2 fusion toxin (NonGly); 3) bivalent glycosylated porcine IL-2 fusion toxin (Bi-Gly); 4) bivalent non-*N*-glycosylated porcine IL-2 fusion toxin (Bi-NonGly). Using a porcine CD25$^+$ B cell lymphoma cell line (LCL13271) in vitro analysis of the fusion toxins' ability to inhibit protein synthesis demonstrated that the Bi-NonGly fusion toxin is the most efficient reagent. These in vitro results are consistent with binding affinity as the Bi-NonGly fusion toxin binds strongest to CD25 on the same LCL13271 cells. The Bi-Gly fusion toxin significantly prolonged the survival ($p = 0.028$) of tumor-bearing NOD/SCID IL-2 receptor $\gamma^{-/-}$ (*NSG*) mice injected with LCL13271 cells compared with untreated controls. This recombinant protein has great potential to function as a useful tool for in vivo depletion of porcine CD25$^+$ cells for studying immune regulation.

1. Introduction

Antigen-specific immune responses such as those targeted against tumors are suppressed by Tregs characterized by CD4$^+$CD25^{high}FoxP3$^+$ expression. Treg depletion combined with tumor vaccination is a potentially promising approach to improve cancer treatment. The United States Federal Drug Administration approved truncated diphtheria toxin based human IL-2 fusion toxin (ONTAK) has been shown to deplete Tregs in both pre-clinical and clinical settings thereby facilitating improved cancer treatment (Morse et al., 2008; Mahnke et al., 2007; Litzinger et al., 2007; Gritzapis et al., 2012). While it is effective in depleting Tregs during cancer treatment, ONTAK also creates unwanted side effects as it has been shown to completely deplete NK cells for a prolonged period in a

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http://dx.doi.org/10.1016/j.jim.2013.09.006

cynomolgus monkey model (Yamada et al., 2012). Natural killer (NK) cells are a very important component of the innate immune system as their functions include fighting pathogenic infections and cancer (Salagianni et al., 2011).

IL-2 binds to its cell surface receptor with notably strong affinity. The IL-2 receptor is a trimer composed of three subunits, α–β–γ. The α-subunit of this receptor, also known as CD25, is constitutively expressed on Tregs and has very high affinity for IL-2. There are species differences between human and porcine IL-2 which affect CD25 binding and subsequent target cell proliferation and differentiation (Zhang et al., 2006; our unpublished data).

The truncated diphtheria toxin DT390 has been used to build recombinant immunotoxins (Woo et al., 2002; Kim et al., 2007; Wang et al., 2011). DT390 lacks the cell-surface binding domain and consists of the catalytic and translocation domains of the diphtheria toxin. In this study each of the glycosylated and non-*N*-glycosylated porcine IL-2 proteins was linked to DT390 through genetic engineering yielding porcine IL-2 fusion toxins. The ability of these reagents to deplete target cells was assessed using an in vitro assay which monitored the inhibition of protein synthesis. Binding specificity and affinity to the target cells were analyzed by flow cytometry. In vivo target cell depletion was assessed using a porcine $CD25^+$ B-cell lymphoma (LCL13271) NOD/SCID IL-2 receptor $\gamma^{-/-}$ (*NSG*) mouse model.

Massachusetts General Hospital (MGH) major histocompatibility complex (MHC)-defined miniature swine provide unique preclinical large animal model available for studying immune regulation and the induction of tolerance following transplantation. The purpose of this study was to build a DT390 based porcine IL-2 fusion toxin capable of effectively depleting porcine $CD25^+$ cells with the ultimate goal of studying transplantation tolerance induction using the MGH MHC-defined swine model.

2. Materials and methods

2.1. Plasmid construction

As shown in Fig. 1, porcine 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 porcine IL-2. A strategy previously employed to construct A-dm-DT390biscFv (2-6-15) (Wang et al., 2011) was applied to build these porcine IL-2 fusion toxins. The biscFv (2-6-15) moiety was replaced with the codon-optimized glycosylated or non-*N*-glycosylated porcine IL-2 (Fig. 2). A linker made up of three tandem chains each containing four glycine residues and a serine $(G_4S)_3$ was used to connect two porcine IL-2 proteins for building the bi-porcine IL-2 fusion toxins. Six histidines (6xHis tag) were added to the C-terminus of each construct to facilitate later purification. The codon-optimized glycosylated porcine IL-2 DNA was synthesized by GenScript (Piscataway, NJ) and the codon-optimized non-*N*-glycosylated porcine IL-2 DNA was generated by site-directed mutagenesis with sense PCR primer pIL2-N91A For and anti-sense PCR primer pIL2-N91A Rev (Agilent technologies). To construct DT390-pIL-2-Gly or DT390-pIL-2-Non-*N*-Gly, the codon-optimized glycosylated or non-*N*-glycosylated porcine IL-2 DNA (Fig. 2) was amplified using PCR primers pIL2-X1 carrying *Xho*I and *Nco*I site + pIL2-E1 carrying an *Eco*RI site then cloned into pwPICZalpha (J. Peraino et al., 2012a; J. Peraino et al., 2012b) 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-pIL-2-Gly or DT390-pIL-2-Non-*N*-Gly in pwPICZalpha. To construct DT390-bi-pIL-2-Gly or DT390-bi-pIL-2-Non-*N*-Gly, the first porcine IL-2-Gly or porcine IL-2-Non-*N*-Gly was amplified using PCR primers pIL2-X1 carrying *Xho*I and *Nco*I sites + pIL2-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 porcine IL-2-Gly or porcine IL-2-Non-*N*-Gly was PCR amplified using pIL2-Bam2 carrying *Xho*I and *Bam*HI sites + pIL-2-E1 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-pIL-2-*Bam*HI/*Bam*HI-pIL-2-*Eco*RI) were together cloned into pwPICZalpha-DT390 between *Nco*I and *Eco*RI yielding the final construct DT390-bi-pIL-2-Gly or DT390-bi-pIL-2-Non-*N*-Gly in pwPICZalpha. All PCR primers that were used are listed in Table 1.

Protein expression and purification in *Pichia pastoris* were performed as previously described with the following modifications (Wang et al., 2011; J. Peraino et al., 2012a; J. Peraino et al., 2012b). A Ni-Sepharose fast flow resin (GE healthcare) was used for the purification. Porcine IL-2 fusion toxins were eluted using 40 mM imidazole. Western blot analysis, FACS analysis, FACS competition/blocking analysis and K_D determination were all performed as previously described (J. Peraino et al., 2012a; J. Peraino et al., 2012b) using LCL13271 cells (Cho et al., 2007). The Ontak®-like monovalent human IL-2 fusion toxin (DT390-hIL-2) used as a control for our in vitro assay was constructed, expressed and purified exactly same as the monovalent porcine IL-2 fusion toxin. The DT390 alone and the non-*N*-glycosylated porcine IL-2 alone (pIL-2-Non-*N*-Gly) were used as controls for our in vitro assay. These products were also expressed and purified in the yeast *P. Pastoris* system.

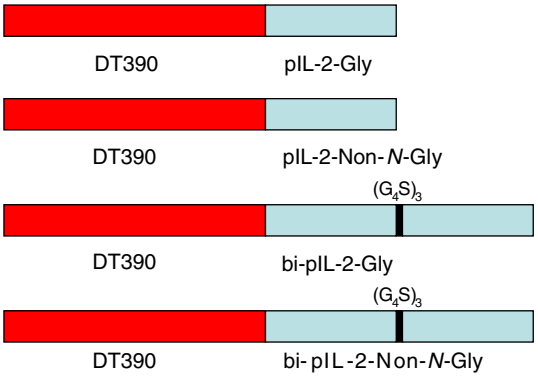

Fig. 1. Schematic representation of the four porcine IL-2 fusion toxins.