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Diphtheria toxin-based anti-human CD19 immunotoxin for targeting human CD19+ tumors

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Keywords
CD19+ tumor; diphtheria toxin; human CD19; immunotoxin; Pichia Pastoris

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CD19+ tumor; diphtheria toxin; human CD19; immunotoxin; Pichia Pastoris

1 Introduction
CD19 is expressed on normal and neoplastic B cells and is a promising target for immunotherapy. However, there is still an unmet need to further develop novel therapeutic drugs for the treatment of the refractory/relapsing human CD19+ tumors. We have developed a diphtheria toxin-based anti-human CD19 immunotoxin for targeting human CD19+ tumors. We have constructed three isoforms of the CD19 immunotoxin: monovalent, bivalent, and foldback diabody. In vitro binding affinity and efficacy analysis demonstrated that the bivalent isoform had the highest binding affinity and in vitro efficacy. The in vivo efficacy of the CD19 immunotoxins was assessed using human CD19+ JeKo-1 tumor-bearing NOD/SCID IL-2 receptor γ−/− (NSG) mouse model. In these animals, CD19 immunotoxins significantly prolonged the median survival from 31 days in controls to 34, 36, and 40 days in animals receiving the monovalent isoform, foldback diabody isoform, and bivalent isoform, respectively. The bivalent CD19 immunotoxin is a promising therapeutic drug candidate for targeting relapsing/refractory human CD19+ tumors.

Abbreviations
7-AAD, 7-aminoactinomycin D; ATP, adenosine triphosphate; B-ALL, B-cell acute lymphoblastic leukemia; BID, bis in die; BiscFv, bivalent single-chain variable fragment; CLL, chronic lymphatic lymphoma; CSC, China Scholarship Council; DT, diphtheria toxin; G4S, four glycine residues and one serine residue; His, histidine; IACUC, Institutional Animal Care and Use Committee; IC50, half maximal inhibitory concentration; IP, intraperitoneal injection; IV, intravenous injection; kDa, kilodalton; KD, dissociation constant; mAb, monoclonal antibody; MFI, mean fluorescence intensity; MGH, Massachusetts General Hospital; NHL, non-Hodgkin’s B-cell lymphoma; Ni, nickel; nM, nanomolar; NOD/SCID, nonobese diabetic/severe combined immunodeficiency; NSG, NOD/SCID IL-2 receptor γ−/−; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; SA, streptavidin; scFv, single-chain variable fragment; SDS/PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SD, standard deviation; VH, heavy chain variable domain; VL, light chain variable domain.

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2 Materials and methods

2.1 Cell lines and antibodies

Human CD19\(^+\) mantle cell lymphoma cell line JeKo-1 (cat# CRL-3006) and human CD19\(^-\) cell line CCRF-CEM (cat# CCL-119) were purchased from ATCC (Manassas, VA, USA). Human CD19\(^-\) cell line M14 and MD-MBA-231 were generously provided by Soldano Ferrone (Massachusetts General Hospital).

mAbs used in this study are listed in Table 1.

2.2 DNA construction

As shown in Fig. 1, anti-human CD19 immunotoxins were constructed using DT390 (Woo et al., 2002) and the codon-optimized scFv (FM63) nucleotide sequences (Figure 2, Nicholson et al., 1997). A strategy previously used to generate the anti-human CCR4 immunotoxin (Wang et al., 2015) was applied to construct the anti-human CD19 immunotoxins. DT390 and scFv (FM63) or bi-scFv (FM63) are connected by a G\(_4\)S (four glycines and one serine residue) linker. The two scFv (FM63) of the bivalent immunotoxin are joined by (G\(_4\)S)\(_3\) linker. Six histidines (6x His tag) were added to the C terminus of each construct to facilitate protein purification. To construct DT390-scFv (FM63), the codon-optimized scFv (FM63) was synthesized by GenScript and cloned into pwPICZalpha. To construct DT390-bi-scFv (FM63), the first scFv (FM63), the codon-optimized scFv (FM63) or bi-scFv (FM63) are connected by a G\(_4\)S (four glycines and one serine residue) linker. The two scFv (FM63) of the bivalent immunotoxin are joined by (G\(_4\)S)\(_3\) linker. Six histidines (6x His tag) were added to the C terminus of each construct to facilitate protein purification. To construct DT390-scFv (FM63), the codon-optimized scFv (FM63) was synthesized by GenScript and cloned into pwPICZalpha. To construct DT390-bi-scFv (FM63), the first scFv (FM63) was amplified using PCR primers FMC-Nco and FMC-Bam1 carrying BamHI and EcoRI sites and then cloned into pwPICZalpha between XhoI and EcoRI sites for sequencing confirmation. The insert was subsequently cut out with NeoI + BamHI as insert I. The second scFv (FM63) was PCR-amplified using FMC-Bam2 carrying XhoI and BamHI sites + FMC-Eco carrying an EcoRI site and then cloned into pwPICZalpha between XhoI and EcoRI sites for sequencing confirmation.

Table 1. Antibodies used in this study.

| Antibody Name                  | Clone# | Source      | Cat#    |
|-------------------------------|--------|-------------|---------|
| PE-mouse anti-human CD19      | FM63   | Millipore   | MAB1794H|
| APC-mouse anti-human CD4      | L200   | BD          | 551980  |
| FITC-mouse anti-human CD38    | SP-34-2| BD          | 556611  |
| PE-mouse anti-human CD19      | SP-34-2| BD          | 556612  |
| PE-mouse anti-human CD19      | HIB19  | BioLegend   | 302208  |
| APC-mouse anti-human CD8      | RPA-T8 | BioLegend   | 301014  |
| FITC-anti-human CD14          | M5E2   | BioLegend   | 301803  |
| PE-mouse anti-human CD16      | 3G8    | BioLegend   | 302008  |
| PE-mouse anti-human CD194     | L291H4 | BioLegend   | 359412  |
| PE-streptavidin               |        | BioLegend   | 405204  |
| FITC-Annexin V                |        | BioLegend   | 640906  |
| 7-Aminoactinomycin (7-AAD)    |        | Sigma       | A9400   |

Fig. 1. Schematic diagrams of the monovalent, bivalent, and single-chain foldback diabody anti-human CD19 immunotoxins.
and digested using BamHI. The VH portion was amplified using PCR primers BgL-FMC carrying BglII site + FMC-Bam1 and digested using BglII. The BamHI-digested VL portion and BglII-digested VH were ligated together for 4 h at room temperature as PCR template to amplify the scFv (FMC63) with short linker (one G4S) for constructing the single-chain foldback diabody isofrm following the construction procedure for DT390-BiscFv (FMC63). All PCR primers that were used are listed in Table 2.

CD19 immunotoxin expression in the diphtheria toxin-resistant yeast Pichia Pastoris expression system and purifications were performed as previously described (Peraino et al., 2013a; Wang et al., 2011). Western blot analysis, binding and blocking analysis by flow cytometry were all performed as previously described (Peraino et al., 2013a) using a human CD19⁺ mantle cell lymphoma cell line JeKo-1. DT390 was used as negative control for in vitro functional analysis. C21 immunotoxin (a nonrelated DT390-based immunotoxin) was used as negative control for in vivo efficacy characterization. Both DT390 and C21 immunotoxins were also expressed using yeast Pichia Pastoris expression system in our laboratory. Isolation of human PBMC, in vitro binding and depletion analysis of the CD19 immunotoxins to human blood, or PBMC with flow cytometry was performed as previously described (Wang et al., 2015).

2.3 In vitro efficacy analysis

In vitro efficacy of the CD19 immunotoxins was assessed using CellTiter-Glo® Luminescent Cell Viability Assay (Promega, cat# G7571) to human CD19⁺ mantle cell lymphoma cell line JeKo-1. Briefly, the JeKo-1 cells were added to the wells of the opaque-walled 96-well plate at 10⁴ cells/well. The CD19 immunotoxin was diluted in the tissue culture medium and added to the well at final concentration of 10⁻⁶, 10⁻⁷, 10⁻⁸, 10⁻⁹, 10⁻¹⁰, 10⁻¹¹, and 10⁻¹² M. The final volume is 100 μL per well for both JeKo-1 cells and the CD19 immunotoxin. The control wells containing only the tissue culture medium were included to obtain the background luminescence value. Cycloheximide (1.25 mg·mL⁻¹) was added to the positive control wells. The plates were incubated for 24 h at 37 °C, 5% CO₂. The plates were equilibrated at room temperature for approximately 30 min. 100 μL of the CellTiter-Glo® reagent was added and mixed for 2 min on an orbital shaker to induce cell lysis. The plate was then incubated at room temperature for 10 min to stabilize the luminescent signal, and the luminescence signals were recorded using Wallac Victor2 1420 multilabel counter (Perkin Elmer, Waltham, MA, USA).

2.4 In vivo efficacy analysis

The breeding pairs of NSG mice were purchased from Jackson laboratories (Bar Harbor, Maine) and bred in our rodent barrier facilities. All animal experiments were approved by Massachusetts General Hospital (MGH) Institutional Animal Care and Use Committee (IACUC). The NSG mice were divided into following groups: (a) C21 immunotoxin control group (a nonrelated diphtheria toxin-based immunotoxin as negative control) (n = 7); (b) monovalent CD19 immunotoxin group (n = 7); (c) bivalent CD19 immunotoxin group (n = 7); and (d) single-chain foldback diabody CD19 immunotoxin group (n = 7). All animals were injected

Table 2. PCR primers used in this study.

| PCR primers          | Sequence                           |
|----------------------|------------------------------------|
| FMC-Nco 5’ CCG      | Xhol Neol                              |
| NcoI                 |                                     |
| FMC-Bam1 5’ CCG GAA | EcoRI BamHI                           |
| GTC GAG CCA TGG      |                                       |
| GGT GGT GGT TCT GAC | ACT CAA ATG ACT CAA ACT ACT 3’        |
| FMC-Bam2 5’ CCG     | BamHI                                |
| GTC GAG GGA TCC     |                                       |
| GGT GGT GGT TCT GAC | ATT CAA ATG ACT CAA ACT ACT 3’        |
| FMC-Eco 5’ CCG      | EcoRI                                |
| GAA TTC TTA         |                                       |
| GTG GTG GTG GTG GTG | GGA AGA AAC AGT AAC AAC AAC 3’        |
| GGA AGA AGA AGT AAC | ACT AC 3’                             |
| AGT AC 3’           |                                       |
| Bam-FMC 5’ CCG      | BamHI                                |
| GGA TCC ACC ACC AGT |                                       |
| AAT CTC CAA CTTC    | AGT ACC AC 3’                         |
| AGT AC 3’           |                                       |
| Bgl-FMC 5’ GGA      | BglII                                |
| AGA TCT GAG GTT     |                                       |
| AAG TTG CAA GAG TCT | 3’                                   |

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IV with 10 million of human CD19+ mantle cell lymphoma cells (JeKo-1) via the tail vein. The immunotoxin was injected IP from day 0 on at 100 µg·kg⁻¹, BID for four consecutive days as one course, four courses in total, and three-day break between any two courses. The injected animals were observed daily for signs and symptoms of illness and scored twice weekly based on the parameters as previously reported by our laboratory (Peraino et al., 2013b and Wang et al., 2015). In addition, to assess whether the CD19 immunotoxin is toxic to the animals, NSG mice (n = 2) injected with the bivalent CD19 immunotoxin only were also included as control.

2.5 Statistical analysis

All p values were calculated using log-rank (Mantel–Cox) test of Prism. p < 0.05 was considered as significant. IC₅₀ was determined using nonlinear regression (curve fit) of Prism.

3 Results

3.1. Expression and purification of the CD19 immunotoxin

As shown in Fig. 1, monovalent, bivalent, and single-chain foldback diabody versions of the anti-human CD19 immunotoxins were constructed to find the isoform with the highest binding affinity and greatest ability to deplete human CD19+ cells. The codon-optimized anti-human CD19 scFv (FMC63) DNA (Fig. 2) (Nicholson et al., 1997) was cloned into the C terminus of the DT390-containing yeast Pichia Pastoris expression vector pPICZalpha-DT390 between the Neol and EcoRI sites (Wang et al., 2015). A 6xHis tag was added to the C terminus of each immunotoxin isoform to facilitate the purification. The DT390 domain was genetically linked to the scFv (FMC63) domain by a linker containing four glycine residues and a serine residue (G₄S). For the monovalent and bivalent versions, the VL and VH were linked together by a long linker (G₄S)₃ to generate the scFv (FMC63). For the single-chain foldback diabody isoform, the VL and VH were linked by a short linker G₄S. The two scFv (FMC63) of the bivalent and the single-chain foldback diabody isoforms were also joined by a long linker (G₄S)₃.

The CD19 immunotoxins were expressed using unique diphtheria toxin-resistant yeast Pichia Pastoris (Liu et al., 2003) expression system in 1-L Erlenmeyer flasks. The CD19 immunotoxins were captured using a Ni-Sepharose fast flow resin and further purified using strong anion-exchange resin. The final purification yield was ~15 mg per liter for the monovalent and ~6 mg per liter for the bivalent and ~2 mg per liter for the single-chain foldback diabody CD19 immunotoxins of the original harvested supernatant. The purified anti-human CD19 immunotoxins were analyzed by SDS/PAGE (Fig. 3A) and western blot using a mouse anti-6xHis mAb (Fig. 3B) and a mouse anti-CD19 mAb (Fig. 3C).
antidiphtheria toxin mAb (Fig. 3C). SDS/PAGE and western blot analysis demonstrated that the three versions of the CD19 immunotoxins were expressed and purified with expected molecular weight of 70.09 kDa for the monovalent isoform, 96.95 kDa for the bivalent isoform, and 95.69 kDa for the single-chain foldback diabody isoform.

3.2 Binding affinity analysis of the CD19 immunotoxins using flow cytometry

CD19 immunotoxins were designed to deplete human CD19+ cells via binding of the anti-human CD19 scFv (FMC63) domain of the immunotoxins on the CD19 receptor of the CD19+ cells. Following the internalization, the released DT390 domain functions to inhibit protein synthesis resulting in the cell death (Murphy, 2011); therefore, the first step to characterize the CD19 immunotoxins was to analyze their binding affinity to human CD19 receptor. The anti-human CD19 immunotoxins were biotin-labeled for the binding analysis to human CD19+ JeKo-1 cells using flow cytometry. As shown in Fig. 4A, monovalent (left panel), bivalent (middle panel), and single-chain foldback diabody (right panel) CD19 immunotoxins bound to the human CD19 in a dose-dependent manner. The binding affinity was quantified by the dissociation constant ($K_D$) for each CD19 immunotoxin isoform using mean fluorescence intensity (MFI) (Perraino et al., 2013a). The foldback diabody isoform ($K_D = 11.22$ nm, Fig. 4D) bound stronger than the monovalent isoform ($K_D = 47.42$ nm, Fig. 4B). However, the bivalent isoform exhibited the highest binding affinity ($K_D = 4.59$ nm, Fig. 4C).

The binding specificity of the CD19 immunotoxins was further analyzed by blocking the binding of the parent anti-human CD19 mAb (FMC63) to its receptor on human CD19+ JeKo-1 tumor cells. As shown in Fig. 5, monovalent (left panel), bivalent (middle panel), and single-chain foldback diabody (right panel) CD19 immunotoxins blocked the binding of anti-human CD19 mAb (FMC63) to the JeKo-1 cells in a dose-dependent fashion. The data strongly suggest that the CD19 immunotoxins bind specifically to the human CD19 receptor.

3.3 In vitro efficacy analysis of the CD19 immunotoxin

In vitro efficacy of the CD19 immunotoxins was assessed using CellTiter-Glo® Luminescent Cell Viability Assay (Promega) in a human CD19+ JeKo-1...
tumor cell line. As shown in Fig. 6, all of the three CD19 immunotoxin isoforms (monovalent, bivalent, and foldback diabody) were effective. The foldback diabody isoform (IC_{50} = 1.7 \times 10^{-11} \text{ M}) was better than the monovalent isoform (IC_{50} = 2 \times 10^{-10} \text{ M}), while the bivalent isoform (IC_{50} = 2 \times 10^{-12} \text{ M}) was greatest. This findings correlate well with the corresponding binding affinity analysis.
3.4 *In vitro* binding and depletion analysis of the CD19 immunotoxins to human blood or PBMC

CD19 is expressed on normal human B cells. To further characterize the CD19 immunotoxins, we performed *in vitro* binding and depletion analyses of the CD19 immunotoxins in human blood or PBMC. As shown in Fig. 7A, all three isoforms of the biotinylated CD19 immunotoxins bound to CD19+ human blood in a dose-dependent fashion. The foldback diabody isoform bound more strongly than the monovalent isoform, while the bivalent isoform was the most effective, which is consistent with the previous binding analysis using the human CD19+ JeKo-1 cell line. We then further performed *in vitro* depletion analysis in CD19+ human PBMC using the CD19 immunotoxins. As shown in Fig. 7B, CD19+ human PBMC was depleted in a dose-dependent manner following incubation of the CD19 immunotoxin with the human PBMC for 48 h. The bivalent and foldback diabody isoforms were better than the monovalent isoform, while the bivalent isoform was the most effective.

3.5 Off-target analysis of the CD19 immunotoxin

To rule out the off-target effect of the CD19 immunotoxin, three human CD19+ tumor cell lines were analyzed with biotinylated bivalent human CD19 immunotoxin using flow cytometry: (a) CCRF-CEM, (b) M14, and (c) MD-MBA-231. The results demonstrated that there was no binding activity with the three human CD19+ tumor cell lines (Fig. 8A). The viability of the three human CD19+ tumor cell lines was also analyzed by flow cytometry using 7-aminoactinomycin D and Annexin V staining following
**Fig. 7.** *In vitro* binding and depletion analysis of the anti-human CD19 immunotoxins to human CD19⁺ blood or PBMC. (A) Flow cytometry binding analysis of the anti-human CD19 immunotoxins to the CD19⁺ cells within human blood. Monovalent: monovalent anti-human CD19 immunotoxin [DT390-scFv (FMC63)]; bivalent: bivalent anti-human CD19 immunotoxin [DT390-BiscFv (FMC63)]; foldback: single-chain foldback diabody anti-human CD19 immunotoxin. PE-SA: PE-conjugated streptavidin; bio-pCD3: biotin-labeled porcine CD3 (Peraino et al., 2012). (B) *In vitro* depletion of the CD19⁺ cells within human PBMC using the anti-human CD19 immunotoxins. C21 immunotoxin and DT390 alone were included as negative controls. Data of both (A) and (B) are representative of three individual experiments.
**Fig. 8.** Off-target analysis of the CD19 immunotoxin. (A) Flow cytometry binding analysis of the biotinylated bivalent anti-human CD19 immunotoxin to three human CD19−/CD19+ tumor cells (CCRF-CEM, M14, and MD-MBA-231). PE-mouse anti-human CD19 mAb was included as a positive antibody control, and biotinylated porcine CD3-εγ control (Peraino et al., 2012) as a negative control for background due to protein biotinylation. (B) Cell viability analysis of the three human CD19−/CD19+ tumor cell lines by staining with 7-AAD and Annexin V following incubation with the bivalent anti-human CD19 immunotoxin for 18 h. (C) Depletion analysis by flow cytometry for the human CD19− subpopulations within human PBMC following incubation with the bivalent anti-human CD19 immunotoxin for 48 h. The data are representative of three individual experiments for all of the data in figure.
incubation with the bivalent CD19 immunotoxin for 18 h. Their viability was not affected by this treatment (Fig. 8B).

To further rule out the off-target effect of the CD19 immunotoxin, we also analyzed the human CD19+ PBMC populations following incubation with the bivalent CD19 immunotoxin for 48 h: CD4 T cell (CD3+CD4+), CD8 T cell (CD3+CD8+), NK cells (CD16+CD56+), and monocyte (CD14+CD16+). The results demonstrated that there was no effect on human CD19+ PBMC populations (Fig. 8C).

**3.6 In vivo efficacy analysis of the CD19 immunotoxins**

*In vivo* efficacy of the CD19 immunotoxins was assessed using human CD19+ JeKo-1 mantle cell lymphoma-bearing immunodeficient NSG mouse model. Human CD19+ JeKo-1 cells (1 × 10⁷) were injected IV and the CD19 immunotoxins were injected IP at 100 μg·kg⁻¹, BID, for four consecutive days as one course, four courses in total, and three-day break between any two courses. As shown in Fig. 9, the C21 immunotoxin was included as a negative control (n = 7). These data demonstrated that all three CD19 immunotoxin isoforms prolonged animal survival significantly from a median of 31 days in the negative control group (C21 immunotoxin) to 34 days in the monovalent isomorf treatment group (n = 7, p < 0.0001), to 36 days in the foldback diabody isomorf treatment group (n = 7, p < 0.0001), to 40 days in the bivalent isomorf treatment group (n = 7, p < 0.0001) (Fig. 9). Mice receiving the CD19 immunotoxin only did not show any toxicity (data not shown). All animals injected with the human CD19+ JeKo-1 cells succumbed to tumors (data not shown).

**4 Discussion**

Several attempts to develop recombinant anti-human CD19 immunotoxins have been unsuccessful (Du et al., 2008). We speculate that the use of an inappropriate expression system was part of the problem. Diphtheria toxin-resistant yeast *Pichia Pastoris* expression system is suitable for DT390-based immunotoxin development. We speculate that eukaryotic expression systems such as yeast *Pichia Pastoris* expression system might be better than prokaryotic expression system such as *E. coli*.

Based on our previous experience, the foldback diabody immunotoxin isomorf is superior to both bivalent and monovalent isoforms in terms of the *in vitro* binding affinity and efficacy (Kim et al., 2007; Wang et al., 2015). However, the foldback diabody isomorf in this study did not improve the *in vitro* binding affinity and efficacy comparing with the bivalent isomorf. Therefore, it is case by case for the immunotoxin development to find the best isomorf (Wei et al., 2014).

The *in vivo* dosing schedule (Fig. 9) in this study has not been optimized. Our focus was simply to assess whether the CD19 immunotoxin functions *in vivo*. There is still room to increase the dose and duration to improve the animal survival. It is also necessary to characterize the *in vivo* efficacy further using established xenograft tumor-bearing *NSG* mouse model. Furthermore, analyzing the residue tumor cells *in vitro* to determine whether they are still sensitive to the CD19 immunotoxin is warranted. If they remain sensitive, higher doses and multiple course of treatment could improve animal survival further.

Conventional chemotherapy and targeted mAb therapy have contributed significantly to the treatment for CD19+ B-cell leukemia/lymphoma. However, there is still an unmet need to develop novel targeted therapies against the relapsing/refractory patients with CD19+
leukemia/lymphoma following the chemotherapy and targeted mAb therapy. We believe that CD19 immuno- toxin will provide a novel alternative therapy for the refractory/relapsing CD19⁺ tumor patients.

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Author contributions

QZ and Zhaohui Wang primarily performed the experiments and data analysis as well as participated in writing the manuscript; HZ participated in the experiments; JCM, DHS, and CAH participated in the project design, data analysis, and writing the manuscript; Zhirui Wang primarily designed the project, analyzed the data, and wrote the manuscript.

References

Bachanova V, Frankel AE, Cao Q, Lewis D, Grzywacz B, Verneris MR, Ustun C, Lazaryan A, McClune B, Warlick ED et al. (2015) Phase I study of a bispecific ligand-directed toxin targeting CD22 and CD19 (DT2219) for refractory B-cell malignancies. *Clin Cancer Res* 21, 1267–1272.

Du X, Beers R, Fitzgerald DJ and Pastan I (2008) Differential cellular internalization of anti-CD19 and CD22 immunotoxins results in different cytotoxic activity. *Cancer Res* 68, 6300–6305.

Kim GB, Wang Z, Liu YY, Stavrou S, Mathias A, Goodwin KJ, Thomas JM and Neville DM Jr (2007) A fold-back single-chain diabody format enhances the bioactivity of an anti-monkey anti-CD3 recombinant diphtheria toxin-based immunotoxin. *Protein Eng Des Sel* 20, 425–432.

Liu YY, Woo JH and Neville DM Jr (2003) 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* 30, 262.

Murphy JR (2011) Mechanism of diphtheria toxin catalytic domain delivery to the eukaryotic cell cytosol and the cellular factors that directly participate in the process. *Toxins* 3, 294.

Nicholson IC, Lenton KA, Little DJ, Decorsor T, Lee FT, Scott AM, Zola H and Hohmann AW (1997)

Construction and characterization of a functional CD19 specific single chain Fv fragment for immunotherapy of B lineage leukemia and lymphoma. *Mol Immunol* 34, 1157–1165.

Peraino JS, Hermanrud CE, Springett L, Zhang H, Li G, Srinivasan S, Gusha A, Sachs DH, Huang CA and Wang Z (2012) 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* 276, 162.

Peraino JS, Schenk M, Li G, Zhang H, Farkash EA, Sachs DH, Huang CA, Duran-Struuck R and Wang Z (2013b) Development of a diphtheria toxin-based recombinant porcine IL-2 fusion toxin for depleting porcine CD25⁺ cells. *J Immunol Methods* 398–399, 33.

Peraino JS, Schenk M, Zhang H, Li G, Hermanrud CE, Neville DM Jr, Sachs DH, Huang CA, Duran-Struuck R and Wang Z (2013a) A truncated diphtheria toxin based recombinant porcine CTLA-4 fusion toxin. *J Immunol Methods* 391, 103.

Turtle CJ, Riddell SR and Maloney DG (2016) CD19-Targeted chimeric antigen receptor-modified T-cell immunotherapy for B-cell malignancies. *Clin Pharmacol Ther* 100, 252–258.

Vallera DA, Todhunter DA, Kuroki DW, Shu Y, Sicheneder A and Chen H (2005) A bispecific recombinant immunotoxin, DT2219, targeting human CD19 and CD22 receptors in a mouse xenograft model of B-cell leukemia/lymphoma. *Clin Cancer Res* 11, 3879–3888.

Wang Z, Duran-Struuck R, Crepeau R, Matar A, Hanekamp I, Srinivasan S, Neville DM, Sachs DH and Huang CA (2011) Development of a diphtheria toxin based antiporcine CD3 recombinant immunotoxin. *Bioconjug Chem* 22, 2014–2020.

Wang K, Wei G and Liu D (2012) CD19: a biomarker for B cell development, lymphoma diagnosis and therapy. *Exp Hematol Oncol* 1, 36.

Wang Z, Wei M, Zhang H, Chen H, Germana S, Huang CA, Madsen JC, Sachs DH and Wang Z (2015) Diphtheria-toxin based anti-human CCR4 immunotoxin for targeting human CCR4⁺ cells *in vivo*. *Mol Oncol* 9, 1458–1470.

Wayne AS, Fitzgerald DJ, Kreitman RJ and Pastan I (2014) Immunotoxins for leukemia. *Blood* 123, 2470–2477.

Wei M, Marino J, Trowell A, Zhang H, Peraino JS, Rajasekera PV, Madsen JC, Sachs DH, Huang CA, Benichou G et al. (2014) Diphtheria toxin-based recombinant murine IL-2 fusion toxin for depleting murine regulatory T cells *in vivo*. *Protein Eng Des Sel* 27, 289–295.

Woo JH, Liu YY, Mathias A, Stavrou S, Wang Z, Thompson J and Neville DM Jr (2002) Gene optimization is necessary to express a bivalent anti-human anti-T cell immunotoxin in *Pichia pastoris*. *Protein Expr Purif* 25, 270.