ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Production of Anti-Cancer Immunotoxins in Algae: Ribosome Inactivating Proteins as Fusion Partners

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ABSTRACT: The eukaryotic green algae, *Chlamydomonas reinhardtii* has been shown to be capable of producing a variety of recombinant proteins, but the true potential of this platform remains largely unexplored. To assess the potential of algae for the production of novel recombinant proteins, we generated a series of chimeric proteins containing a single chain antibody (scFv) targeting the B-cell surface antigen CD22, genetically fused to the eukaryotic ribosome inactivating protein, gelonin, from *Gelonium multiflorm*. These unique molecules, termed immunotoxins, are encoded as a single gene that produces an antibody—toxin chimeric protein capable of delivering a cytotoxic molecule to targeted B-cells. We show that the addition of an Fc domain of a human IgG1 to these fusion proteins results in the production of assembled dimeric immunotoxins, containing two cell binding scFvs and two gelonin molecules. Additionally, we demonstrate that these algal expressed proteins are capable of binding and reducing the viability of B-cell lymphomas, while treatment of T-cells, that lack the CD22 antigen, had no impact on cell viability. Since other protein expression platforms are incapable of folding and accumulating these complex immunotoxins as soluble and enzymatically active proteins, our studies document a novel and efficient method for immunotoxin production.

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Introduction

The development of recombinant DNA technologies has revolutionized the treatment of a number of diseases. We are now able to transform microorganisms or homogenous cell cultures with synthetic DNA (Andersen and Krummen, 2002) and use those cellular systems to produce recombinant proteins ranging from insulin (Goeddel et al., 1979) to monoclonal antibodies (Nakamura, 1983). These recombinant therapeutic proteins can mimic the function of naturally occurring proteins to treat such diseases as diabetes, or can be used to target specific cells like cancers, as a means to reduce cell proliferation. While these first generation therapeutics have been successful at treating many conditions and illnesses, there are many diseases and large patient populations that remain untreatable by these therapies. To address the insufficiencies of existing protein therapeutics, new synthetic fusion proteins that take advantage of characteristics from different molecules have been designed, produced, and administered to patients. Such therapies include immunotoxins (Madhumathi and Verma, 2012) and antibody drug conjugates (Goldenberg and Sharkey, 2012), which combine the specificity of antibodies with the potency of drug or enzyme molecules (Sievers and Senter, 2013). However, a major hurdle to the production of these synthetic antibody fusions has been their cost, resulting from both the lack of an affordable expression system for their production, and the cost of clinical trials.

Green algae are capable of converting solar energy into biomass at efficiencies much greater than terrestrial crops, making them a promising source of renewable fuels and other bioproducts (Georgianna and Mayfield, 2012; Hannon et al., 2010). While energy is certainly important, the ability to accumulate biomass with minimal inputs makes algae promising for the production of inexpensive proteins as well. To demonstrate the robustness of chloroplasts as a protein production platform, eukaryotic green algae have been engineered to produce a wide range of recombinant proteins. The green algae *Chlamydomonas reinhardtii* has been

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engineered to produce industrial enzymes such as xylanase (Rasala, 2012), and a variety of therapeutically relevant proteins ranging from vaccines (Gregory et al., 2012; Jones et al., 2012), to hormones (Rasala et al., 2010), to antibody fragments, and even full length human antibodies (Tran et al., 2009). The secretion of recombinant proteins has also been shown in *C. reinhardtii*, demonstrating the versatility of this green alga as a recombinant protein expression system (Rasala et al., 2012). More recently, the chloroplast of *C. reinhardtii*, which constitutes 40–70% of the volume algal cell volume, was used to produce immunotoxins as soluble enzymatically active proteins consisting of an antibody binding domain genetically conjugated to exotoxin A from *Pseudomonas aeruginosa* (Tran et al., 2013).

The protein translation apparatus of chloroplasts resemble that found in prokaryotes, with 70S-like ribosomes (Manuell et al., 2005) and elongation factors that are homologs of those found in bacteria (Beligni et al., 2004). Bacteria however lack the ability to fold complex multi-domain proteins because of the compartmentalization of the protein folding machinery. The periplasmic space of bacteria contain protein disulfide isomerases (PDI) (Denoncin et al., 2010) that are required to form correct disulfide bonds and assemble proteins with multiple subunits but lack the chaperones and chaperonins that are essential for preventing protein aggregation. These chaperones are present in the cytosol and attempts have been made to co-express these chaperones in the periplasmic space with recombinant proteins of interest with limited success (Outchkourov et al., 2008). When proteins are expressed and secreted from mammalian cell culture they are assembled in the endoplasmic reticulum where BiP, an HSP70 chaperone (Haas and Wabl, 1983), works with PDI to allow proteins sufficient time to fold and assemble into functional complexes (Braakman and Bulleid, 2011). Unlike bacteria, the chloroplast houses both a complex system of chaperones (Schroda, 2004) and PDIs in a single compartment, the stroma (Danon and Mayfield, 1994). This compartmentalization allows complex human antibodies to be folded and assembled into functional molecules capable of binding to their target antigen (Tran et al., 2009). In addition to the ability to fold and assemble complex proteins like antibodies, the chloroplast allows toxins that target the eukaryotic translational apparatus to be produced, where they are sequestered from the cytosol where they would otherwise be lethal to the host cell (Tran et al., 2013).

One of the major advances in cancer treatment in recent years has been the advent of chemotherapy conjugated antibodies that elicit cell cycle arrest and apoptosis in target cells (van de Donk and Dhimolea, 2012), and such drugs are currently being administered to cancer patients (Pro et al., 2012). A major drawback of chemically conjugating antibodies with a toxic molecule is the high cost that is associated with these therapies arising from the need to first purify the monoclonal antibody, chemically coupling it with a toxic molecule, and subsequently purifying the conjugated molecule (Selyukh, 2011). Another approach to making antibody conjugates has been to produce single chain (scFv) antibodies as genetic fusions with a eukaryotic toxin. The scFv antibody is able to specifically target cancer cells just like a full-length antibody and deliver toxin molecules which then cause the cell to undergo apoptosis (Weldon and Pastan, 2011). Previously, the only systems able to produce and accumulate these molecules were prokaryotic, due to the ability of these toxins to inhibit the proliferation of any eukaryotic host cell. Bacterial expression systems currently used to produce these fusion proteins are hampered by their inability to produce molecules that are more complex than scFv-toxin fusions. Additionally, scFv-toxin fusions produced in bacteria accumulate as insoluble aggregates, complicating the purification process by requiring that these proteins be denatured and re-folded ex vivo (Reiter et al., 1996). These limitations make the bacterial expression route less than ideal. Recently, *C. reinhardtii* was shown to be capable of expressing and accumulating complex PE40 based immunotoxin proteins (Tran et al., 2013). These immunotoxin proteins were capable of binding to target cancer cells and the PE40 molecule delivered to target cancer cells inhibited their proliferation both in a cell based assay and mice tumor model.

To determine if *C. reinhardtii* chloroplasts are capable of accumulating a functional immunotoxin protein containing a ribosome inactivating protein capable of *N*-glycosidase activity, we genetically fused a recombinant gene encoding a scFv antibody that recognizes CD22, a B-cell surface epitope that once bound by an antibody is rapidly endocytosed into target cells (Du et al., 2008; Fig. 1A), to gelonin, a ribosome inactivating protein (Fig. 1B). This fusion molecule was termed αCD22Gel. Gelonin is natively produced and stored in the seeds of *Gelonium multiflorum* and inhibits translation by cleaving the 28srRNA of eukaryotic ribosomes. Gelonin has also been shown to exhibit moderate DNase activity (Nicolas et al., 1997). Fusions of gelonin to scFv antibodies that are produced in bacteria have been shown to be highly effective at reducing the viability of targeted cancer cells (Pirie et al., 2011). In addition to this scFv fusion, we also placed the Fc domain of a human IgG1 between the scFv antibody and gelonin (Fig. 1C). Since chloroplasts have PDIs that allow disulfide bridges to form, placing the Fc domain in the protein allows chloroplasts to assemble the protein molecule into a dimer with two protein binding domains and two toxin molecules. This protein was termed αCD22CH23Gel. One of the major problems with scFv-toxin molecules is that their small size greatly reduces their serum half-life. By adding an Fc domain to dimerize the immunotoxin the protein half-life is dramatically increased (Saito et al., 1994). In addition to the increased protein half-life, delivering more toxin molecules potentially make gelonin immunotoxins more effective as there is a threshold of gelonin molecules that need to enter a cell before gelonin will enter the cytosol and cause the targeted cell to undergo apoptosis (Goldmacher et al., 1989). The scFv antibody was produced as a control molecule and termed αCD22 (Fig. 1A).

We developed transgenic strains of algae that produced either the monovalent or divalent gelonin based immunotoxins. Herein, we demonstrate that gelonin based immunotoxins

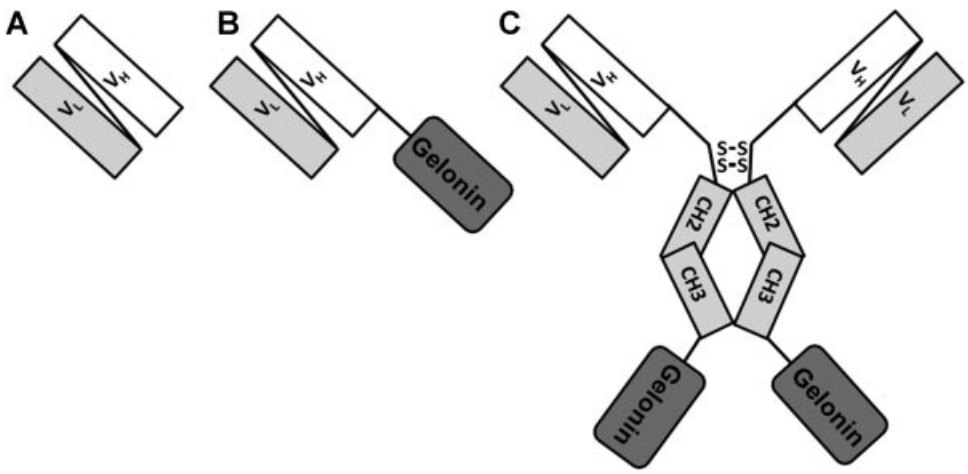


Figure 1. Diagram of algal expressed immunotoxin proteins. **A**: Single chain antibody (scFv) directed against the CD22 cell surface antigen made by linking the variable domains of the heavy and light chain antibodies with a glycine–serine linker. **B**: CD22-scFv genetically linked to gelonin, a ribosome inactivating protein from *G. multiflorum*. **C**: CD22-scFv genetically fused to the hinge and constant domains of an IgG1, and to gelonin, to create a chimeric protein homodimer, through disulfide bonds formed between hinge regions. This fusion allows the molecule to have two binding domains as well as two toxin molecules.

are produced as soluble proteins by *C. reinhardtii* chloroplasts that are enzymatically active and capable of binding to target cancer B-cells and reducing their viability.

Materials and Methods

Plasmid Construction

Algal transformation vectors were first constructed in *Escherichia coli* as previously described. Three individual vectors were constructed for each heterologous gene that was to be tested. The DNA sequence of the heavy and the light chain genes of an antibody RFB4 (Wayne et al., 2010) were synthesized in *C. reinhardtii* chloroplast codon bias (DNA 2.0, Gene Designer) with a codon adaption index (CAI) (Sharp and Li, 1987) above 70.0 and an effective number of condons (Nc) (Puigbo et al., 2008) >30 previously described (Tran et al., 2013). These individual sequences were genetically fused with a DNA sequence coding for a 4× (G_4S) linker and ligated downstream of a DNA sequence coding for a 1× flag peptide (DYKDDDDK) and a TEV protease recognition site (GENLYFQG) that were designed in *C. reinhardtii* chloroplast codon bias and yielding a 834-bp single chain antibody (scFv) termed αCD22. The αCD22 gene was ligated upstream of a 2× (G_4S) linker, a chloroplast codon optimized gene coding for gelonin and a DNA sequence coding for a KDEL protein targeting sequence. The final gene product was 1626-bp and was termed αCD22GEL (Genbank accession number: KC999470). To produce divalent immunotoxins the hinge and constant domains 2 and 3 of a human IgG1 (CH23) were ligated between the αCD22 and gelonin genes, and separated by a 2× (G_4S) linker on both sides of CH23. The final gene product was 2,340-bp and termed αCD22CH23GEL (Genbank accession number: KC999471). Each gene was placed downstream of the psbA chloroplast transformation cassette that contains a kanamycin resistance gene, aphA6 (Tran et al., 2013).

Particle Bombardment Transformations of Algal Chloroplasts

Transformation of algal chloroplasts proceeded as previously described (Tran et al., 2013). In short, DNA was first precipitated onto gold particles (Seashell Technology, San Diego, CA) and transferred to a 3 inch Tefzel tubing (Bio-rad laboratories, Hercules, CA), which was then cut with a tubing cuter (Bio-rad) which is designed to cut tefzel tubing into the appropriate cartridge size.

The cartridges containing the DNA-gold mixtures were loaded into a cartridge holder and placed into a helios gene gun (Bio-rad). The helios gene gun was connected to a helium tank by a helios hose assembly and through the use of a regulator, the helios gene gun was primed by opening the helium tank to 375-psi. Once primed, the gun was held 4.0 cm above plated algae and fired. This process was repeated four times for each transformation construct. The non-photosynthetic strain w1.1, which is deficient for the psbA which was replaced by a M-SAA gene was used as a transformation host (Manuell et al., 2007). The M-SAA strain of algae is deficient for the psbA gene thereby eliminating the difficulty of selecting against photosynthesis.

PCR Analysis of Transgenic Algae

PCR analysis was performed to determine if transformed strains of *C. reinhardtii* contained the transgenic genes

of interest. PCR screening protocols for chloroplast transformations were performed as previously described (Tran et al., 2009). To determine if transformants were positive for the αCD22 gene a forward primer made against the psbA 5′-UTR (5′-GTGCTAGGTAACTAACGTTTGATTTTT-3′) and a reverse primer made against the αCD22 (5′-TGGAGGTGGAGG-TAGTGGTGGTGG-3′) gene were used. To determine if transformants were positive for gelonin forward (5′-GGTTTA-GATACAGTTTCATTTTCAA-3′) and reverse primers (5′-CAAGTTTTTTGATGCCGATGCGAAG-3′) against the gelonin gene product were used in the PCR analysis. To determine if strains were homoplasmic for the gene of interest two sets of primers were used. First, a psbA 5′-UTR forward primer (5′-GTGCTAGGTAACTAACGTTTGATTTTT-3′) and M-SAA reverse primer (5′-GATACCTTGAATAGTTTCTCTAGCG-3′) were used to determine if the M-SAA gene product was no longer present. Also in this PCR mix was a control set of primers including a forward primer (5′-CCGAACTGAGGTTGGG TTTA-3′) and reverse primer (5′-GGGGGAGCGAATAGGAT-TAG-3′) against the DNA coding for the 16srRNA.

Immunotoxin Protein Accumulation and Biochemical Characterization

Accumulation of gelonin immunotoxins from homplasmic strains of *C. reinhardtii* was determined by Western blot analysis. Homoplasmic strains of *C. reinhardtii* were grown in 20 L carboys (VWR). Samples were grown for 96 h in continuous light (5,000 Lux) and harvested using a continuous flow centrifuge (Lavin Model #L2). Once harvested, cells were lysed (50 mM Tris–HCl, pH 8.0, 500 mM NaCL, 0.5% tween 20, one roche protease inhibitor tablet) using sonication (Sonic Dismembrator, Fisher Scientific, Waltham, MA). Soluble lysate was then mixed with 1 mL of anti-M2-flag agarose resin (Sigma, St. Louis, MO) and tumbled at 4°C for 1 h. Samples were washed and then eluted with elution buffer (100 mM glycine–HCl, pH 3.5, 500 mM NaCl). Samples were eluted into 1 M Tris–HCl, pH 8.0 to neutralize acidic elution buffer. Samples were then buffer exchanged into phosphate buffered saline. Proteins were separated on reducing and non-reducing PAGE gels. Samples were transferred onto a nitrocellulose membrane. Nitrocellulose membranes were probed with an alkaline phosphatase conjugated anti-M2-flag antibody. To determine the levels of protein accumulation in *C. reinhardtii* purified αCD22, αCD22Gel, or αCD22HCH23PE40 proteins were each titrated into crude algal lysate and compared to total soluble lysates from recombinant strains of *C. reihardtii*. One microgram of each lysate was coated on maxisorp plates (Nunc) in triplicate. Wells were then blocked with ELISA blocking buffer (Pierce) for 1 h. Following blocking, samples were washed and probed with an anti-flag antibody that was conjugated with horseradish peroxidase. After subsequent binding and washing steps, samples were developed with a TMB substrate (Pierce). The reaction was stopped with 2 M sulfuric acid and absorbance measured at 450 nm. Total soluble lysate of strains expressing αCD22Gel or αCD22CH23Gel were compared to total soluble lysate from a wt strain with the purified αCD22Gel or αCD22CH23Gel titrated into the lysate.

Gelonin Immunotoxin Cell Binding Assay

CA-46 B-cells, Ramos B-cells, and Jurkat T-cells were incubated in the presence of algal produced αCD22, αCD22Gel, and αCD22CH23Gel in a buffer containing PBS and 0.01% sodium azide and subsequently incubated with an α-Gelonin antibody produced in rabbit (MD Anderson, Dr. Michael Rosenblum). Finally, cells were then incubated with an anti-Rabbit dylight 488 conjugated antibody (Sigma). Cells were then analyzed by flow cytometry using a BD influx (Becton Dickinson, Franklin Lakes, NJ). FlowJo software was used to analyze data.

Gelonin Activity Assay

To determine if algal chloroplast has produced an enzymatically active protein, a DNAse activity assay was done (Melchior and Tolleson, 2010). Puc19b plasmid (1 μg) was incubated in a RIP buffer (50 mM Tris–HCl, pH 7.5, 50 mM KCl, and 10 mM $MgCl_2$) and 0.01, 0.05, 0.1, 0.5, and 1.0 μg of αCD22, αCD22Gel, or αCD22CH23Gel purified using anti-M2 flag resin from algal lysate for 1 h at 37°C. Samples were separated on a 1% agarose gel and stained with 0.05% gel red. DNA with no immunotoxin protein was used as a control along with plasmid digested with HindIII restriction enzyme.

Gelonin Cell Viability Assay

Using a 96-well tissue culture plate, 100 μL of Ramos B-cells, CA-46 B-cells and Jurkat T-cells were seeded into each well at concentration of 5×10^4 cells/mL. Cells were incubated for 24 h at 37°C in a humidified incubator with 10% CO_2. Following the incubation, αCD22, αCD22Gel, and αCD22CH23Gel diluted in PBS with 0.2% human serum albumin (HSA) were added to the cells in increasing concentrations. As a positive control for cell death, cycloheximide was added to untreated cells at a concentration of 10 μM. As a negative control for 100% cell survival, PBS + 0.2%HSA was added to untreated cells. Cells were then incubated for 72 h after the addition of immunotoxin samples and controls. Following the incubation, 10 μL of wst-8 reagent (Dojindo, Santa Clara, CA) was added to each well to determine cell viability (Kawa et al., 2011). Cell viability curves were determined from the absorbance measurements and the IC_{50} for each protein was calculated using Grafit software (Erithacus, Surrey, UK).

Results

Expression Vector Construction and Engineering of Immunotoxin Genes

All DNA manipulations were performed as previously described (Cohen et al., 1998; Sambrook et al., 1989). The

variable domains of a human antibody against the B-cell surface antigen were genetically linked to produce a gene product called αCD22scFv. The αCD22scFv was ligated downstream of a sequence coding for a 1× flag peptide (DYKDDDDKS) and separated by a sequence that codes for a TEV protease cleavage site (ENLYFQG) (Fig. 1A). This gene has a codon adaption index (CAI) = 0.722 and Nc = 30.5). To produce a monovalent immunotoxin gene the codon optimized gene sequence for αCD22scFv was placed upstream of a codon optimized gelonin gene, separated by a sequence coding for a 2× (4GlySer) linker. This gene has a CAI = 0.748 and Nc = 33.4. The resulting protein product is αCD22Gel (Fig. 1B). Finally, to produce a divalent immunotoxin, a codon optimized hinge region, and CH2 and CH3 domains of a human IgG1 (CH23) was placed between the αCD22 and gelonin sequences, and separated by a sequence coding for a 2× (4GlySer) on either side. This chimeric protein is called αCD22CH23Gel, and is diagrammed in Figure 1C. αCD22CH23Gel has a CAI = 0.760 and Nc = 33.4. These three immunotoxin genes were then placed into a *C. reinhardtii* chloroplast psbA transformation vectors that contain a kanamycin resistance gene, aphA6 (Fig. 2A).

Integration of Recombinant Genes into Chloroplast Plastid Genome

PCR of whole cell lysates was used to verify the presence of the immunotoxin genes in transformed *C. reinhardtii*. The presence of αCD22 was confirmed in transformants of αCD22, αCD22Gel, and αCD22CH23Gel (Fig. 2B). Transformed strains of *C. reinhardtii* were also screened for the presence of the gelonin gene, present in αCD22Gel and αCD22CH23Gel (Fig. 2C). Finally, and for the presence of the CH23 domain, present in only αCD22CH23Gel (Fig. 2D). Gene positive colonies were subsequently screened for homoplasmicity to ensure that all copies of the chloroplast plastid genome no longer contained the replaced (M-SAA) gene. All transgenic strains were identified as homoplasmic (Fig. 2E).

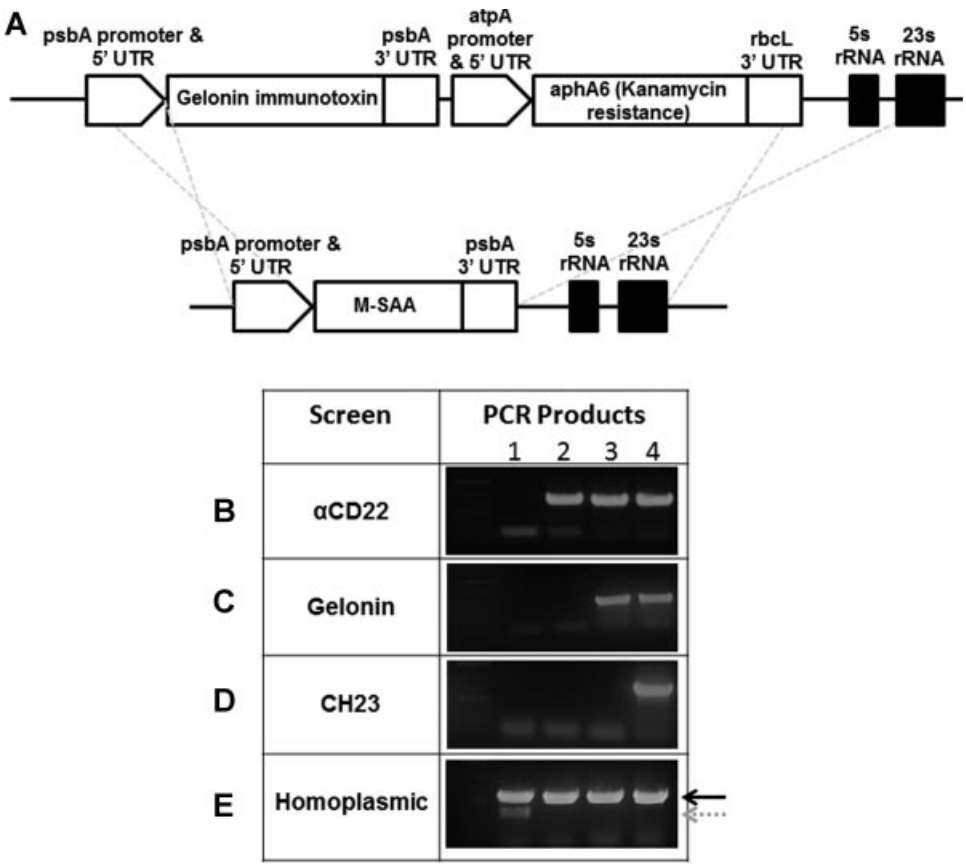


Figure 2. Transformation and selection vector, and identification of *C. reinhardtii* transformants. **A**: Immunotoxin genes were ligated downstream of the *psbA* promoter and 5′-UTR and upstream of the *psbA* 3′-UTR. This gene is placed upstream of an aphA6 gene that confers kanamycin resistance to transformed cells of algae. Regions of chloroplast genome are placed at either end of the transformation vector to allow for homologous integration of the entire transformation cassette into the chloroplast genome. PCR analysis was done on the following strains: (Lane 1) w1.1, (Lane 2) αCD22, (Lane 3) αCD22Gel, (Lane4) αCD22CH23Gel using primers specific to the (**B**) αCD22 scFv gene, (**C**) gelonin gene, (**D**) CH23 gene, or (**E**) genomic primers used to identify homoplasmic strains with black arrow indicating amplified control PCR product and dashed arrow indicating amplified M-SAA PCR gene product.

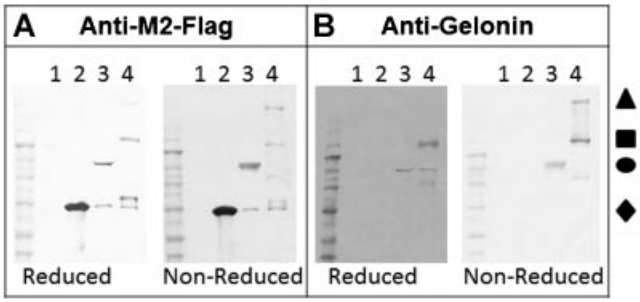


Figure 3. Western blot analysis of immunotoxin accumulation in transgenic algae. **A**: Samples were separated on a SDS–PAGE gel under reducing or non-reducing conditions, transferred to a nitrocellulose membrane and probed with either an anti-flag (A) or anti-gelonin (**B**) antibodies, conjugated with alkaline phosphatase. The lanes contain the following samples: (Lane 1) wt soluble lysate, (Lane 2) αCD22, (Lane 3) αCD22Gel, (Lane 4) αCD22CH23Gel. Black triangle indicated dimeric assembled αCD22CH23Gel, black square indicates monomeric αCD22CH23gel, black circle indicates αCD22gel, and black diamond indicate αCD22.

Heterologous Protein Accumulation in Transgenic Algal Strains

Western blot analysis was used to determine if transgeneic strains of *C. reinhardtii* accumulated gelonin containing immunotoxins. Western blot analysis was done with anti-M2 flag antibodies and anti-Gelonin antibodies. Non-reducing protein gels and Western blots were used to determine in the algal cells accumulated αCD22CH23Gel as a dimeric complex. Detection with an anti-M2 flag antibody, conjugated with alkaline phosphatase, demonstrates that *C. reinhardtii* chloroplasts accumulate the 29 kDa αCD22, the 58 kDa αCD22Gel, and the 85 kDa αCD22CH23Gel under reducing conditions (Fig. 3A). Furthermore, non-reducing Western blots, also using an anti-M2 flag antibody, demonstrate the chloroplasts of *C. reinhardtii* are capable of assembling two subunits of αCD22CH23Gel into a dimeric 170 kDa complex (Fig. 3A, black arrow). Western blots using an anti-gelonin antibody confirms the ability of chloroplasts to accumulate both αCD22Gel and αCD22CH23Gel immunotoxins as well as their ability to assemble two subunits of αCD22CH23Gel into a molecule that contains two antigen binding domains as well as two gelonin toxin domains (Fig. 3B). The presence of an αCD22 band in lanes 3 and 4 are the result of a protein degradation. ELISA assays demonstrate that αCD22 accumulates at approximately 0.6–0.7% total soluble protein (TSP), αCD22Gel accumulates at 0.2–0.3% TSP and αCD22CH23Gel accumulates at 0.1–0.2% TSP.

DNAse Activity of Algal Expressed Gelonin Immunotoxins

Previously it was demonstrated that bioactive ribosome-inactivating proteins also have a DNAse activity, which is capable of nicking supercoiled plasmids resulting in a linear molecule that can be detected on DNA gels. To determine if the algal produced ribosome inactivating protein, gelonin, has this activity, 1.0 μg of puc19b supercoiled plasmid was incubated in the presence of either 1 μg of αCD22, increasing concentration of αCD22Gel, or increasing concentrations of αCD22CH23Gel. To serve as a control 1.0 μg of puc19b plasmid linearized with HindIII was used. All three proteins were M2-flag purified to ensure the results of the assay were not from purification contaminants. The results of the DNAse activity assay demonstrate that increasing concentrations of both αCD22Gel (Fig. 4A), and αCD22CH23Gel (Fig. 4B) increases the proportion of DNA that is either nicked or linearized, while the ScFv alone had no activity. These results confirm that the gelonin containing immunotoxins are correctly folded and have DNase enzymatic activity.

Binding of Algal Immunotoxins to CD22 Positive B-Cells

To verify that the αCD22 scFv portion of the immunotoxins are capable of binding to target cells, human Ramos B-cells and CA-46 B-cells were incubated with both αCD22Gel and αCD22CH23Gel algal produced immunotoxins, and then incubated with a rabbit α-gelonin antibody (MD Anderson), and finally incubated with a goat-anti-rabbit dylight 488 antibody. Both the monovalent αCD22Gel and the divalent αCD22CH23Gel were capable of binding to Ramos cells, as shown by the fluorescent shift in FACS based assays (Fig. 5A). Additionally, both algal produced gelonin immunotoxins were able to bind to CA-46 B-cells (Fig. 5B). This data demonstrates that algal expressed immunotoxins have

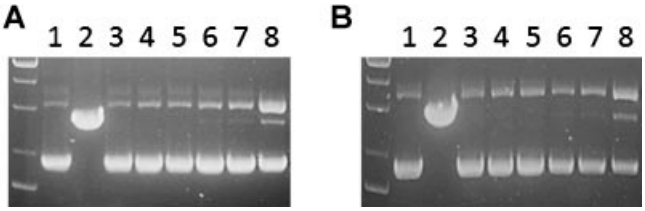


Figure 4. DNAase activity assay. **A**: Lane 1: αCD22 incubated with puc19b plasmid, Lane 2: puc19b plasmid digested with HindIII, Lanes 3–8: increasing concentrations of αCD22Gel. **B**: Lane 1: αCD22 incubated with puc19 plasmid, Lane 2: puc19b plasmid digested with HindIII, Lanes 3–8: increasing concentrations of αCD22CH23Gel.

functional antigen binding domains that allows them to bind to target cells.

Cytotoxicity of Algal Produced Immunotoxins Towards B-Cell Lymphoma Cell Lines

Algal expressed immunotoxins were tested for their ability to target and prevent the proliferation of CD22 antigen containing B-cell lymphomas. Jurkat T-cells lacking CD22 were used to demonstrate the specificity of immunotoxins. Monovalent αCD22 by itself was incapable of inhibiting growth of CA46 cells, Ramos Cells, or Jurkat Cells (Fig. 6A–C). Monovalent αCD22gel was capable of inhibiting CA46 cell growth with an IC_{50} of 2.316 ± 0.471 nM, and Ramos cells with an IC_{50} of 21.051 ± 9595 nM. The monovalent αCD22gel immunotoxin did inhibit Jurkat T-cell growth (Fig. 6C). Divalent αCD22CH23Gel was able to inhibit growth of CA46 cells with an IC_{50} of 0.093 ± 0.024 nM, and Ramos cells with an IC_{50} of 1.421 ± 0.298 nM (Fig. 6A–C). The divalent αCD22CH23Gel did not reduce Jurkat T-cell viability (Fig. 6C).

Discussion

Over the last few decades, genetic tools have been developed that allow molecular engineering of algae for use as a protein expression platform (Specht et al., 2010), or for engineering of secondary metabolism (Blatti et al., 2012). The unicellular green algae *C. reinhardtii* has been used to produce complex human antibodies capable of binding to their target antigen with avidities similar to that of genetically identical antibodies expressed in mammalian cells (Tran et al., 2009), and more recently, chloroplasts of *C. reinhardtii* were used to accumulate PE40-based immunotoxin proteins (Tran et al., 2013). Here, we demonstrate that *C. reinhardtii* can accumulate immunotoxins that contain ribosome-inactivating proteins that can target and reduce the viability of human cancer cells. Additionally, by producing gelonin immunotoxins in the chloroplast, we further demonstrate that no protein molecules leave the chloroplast, as a single gelonin molecule leaving the chloroplast and entering the cytosol would be sufficient to inhibit the translation of the algal cell and cause the cell to die.

The accumulation of gelonin based immunotoxins helps to increase our understanding of the protein folding machinery in *C. reinhardtii* chloroplasts. By showing that algal chloroplasts are capable of folding multi-domain proteins such as αCD22Gel, and assembling two subunits of αCD22CH23Gel into a dimeric molecule, with two CD22 antibody binding domains and two gelonin molecules, we further demonstrate the capacity of chloroplasts to express, fold, and assemble complex multi-domain mammalian proteins. The protein

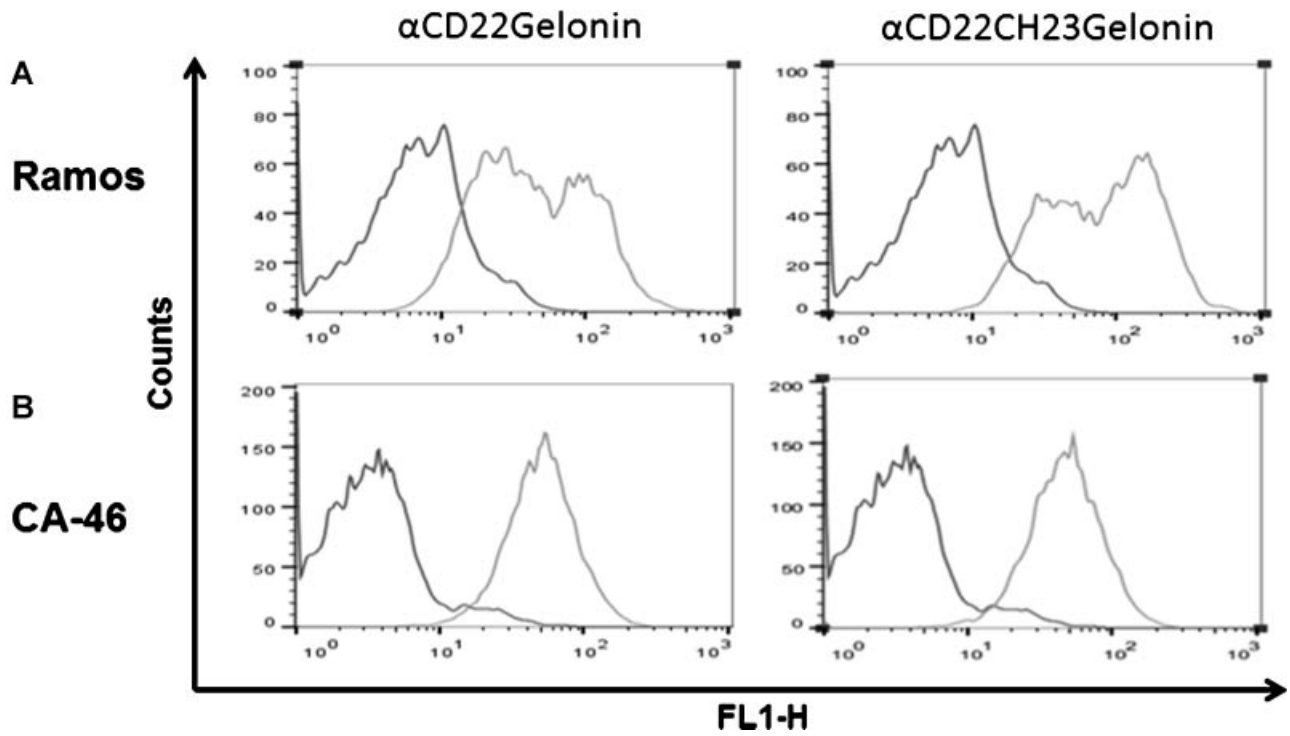
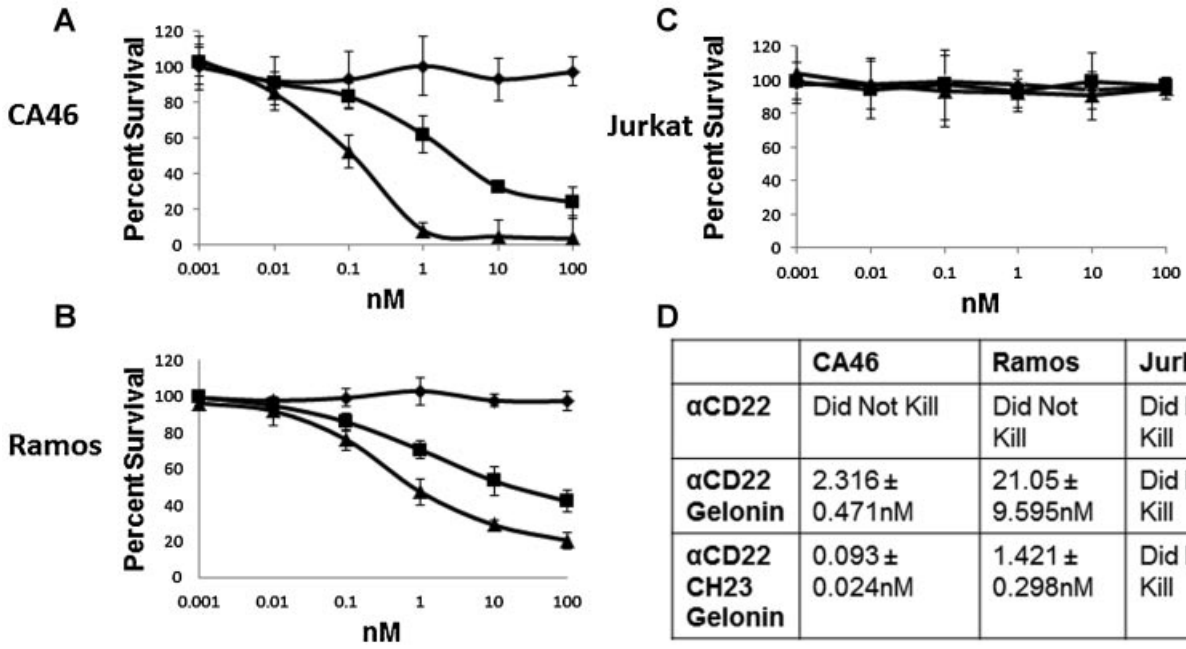


Figure 5. Flow cytometry analysis of binding of immunotoxins to human B-cells. αCD22Gel and αCD22CH23Gel were incubated with CA-46 B-cells or Ramos B-cells. After primary incubation cells were subsequently incubated with rabbit anti-gelonin antibody, and finally with anti-rabbit-dylight 488. After incubation cells were analyzed by flow cytometry. **A**: A shift in the fluorescence spectra (light gray) when compared to untreated cells (dark gray) demonstrates that αCD22Gel and αCD22CH23Gel binds to Ramos Cells. **B**: Fluorescence analysis also demonstrates that αCD22Gel and αCD22CH23Gel bind to CA-46 cells.



	CA46	Ramos	Jurkat
αCD22	Did Not Kill	Did Not Kill	Did Not Kill
αCD22 Gelonin	2.316 ± 0.471nM	21.05 ± 9.595nM	Did Not Kill
αCD22 CH23 Gelonin	0.093 ± 0.024nM	1.421 ± 0.298nM	Did Not Kill

Figure 6. Cell based analysis of algal expressed immunotoxin activity on human cancer cells. Effect of αCD22 (Triangle), αCD22Gel (Square), and αCD22CH23Gel (Diamond), incubated with CA-46 B-Cells, Ramos B-cells, or Jurkat T-cells for 72 h, on cell proliferation. **A**: αCD22Gel and αCD22CH23Gel were effective at reducing CA-46 B-cell viability, while αCD22 alone did not impact CA-46 cell growth. **B**: The αCD22Gel and αCD22CH23Gel were also effective in reducing Ramos Cell viability, while αCD22 was unable to inhibit Ramos cell proliferation. **C**: αCD22, αCD22Gel, and αCD22CH23Gel did not reduce the viability of Jurkat T-cells. **D**: The IC_{50} of each immunotoxin against the three cell lines was calculated to determine how effective each was at reducing cancer cell viability. Both immunotoxins were capable of reducing B-cell viability but dimeric αCD22CH23Gel was more effective at preventing the growth of targeted cells in vitro when compared to αCD22Gel.

folding machinery consists of protein disulfide isomerases (Danon and Mayfield, 1994), chaperones (Schroda, 2004), chaperonins (Suzuki et al., 2009), and PPIases (Breiman et al., 1992). While it is known that this machinery exists in chloroplasts, little is known about the ability of these components to work cohesively to fold and accumulate complex heterologous proteins. Studies with immunotoxin proteins produced in *E. coli* have demonstrated that these molecules have the potential to be used as highly effective treatments against cancer. However the method of production of these molecules leaves much to be desired. Currently, gelonin based immunotoxins are produced in *E. coli* as insoluble aggregates which are then denatured and refolded (Pirie et al., 2011). Showing that these complex immunotoxins can accumulate in chloroplasts as soluble enzymatically active proteins demonstrates the ability of the protein folding complexes in the chloroplast to work in concert to ensure that complex recombinant proteins do not misfold or aggregate, as they do in bacteria. The inability of existing expression systems to accumulate immunotoxin proteins as soluble molecules uniquely positions *C. reinhardtii* as a system of choice for their production.

To demonstrate that the soluble algal produced immunotoxins are effective at binding to cell surface antigens present on cancer cells, we chose two B-cell lymphoma cell lines, Ramos, and CA-46, and demonstrated by flow cytometry that immunotoxins purified from chloroplasts are capable of binding specifically to B-cells that contain the CD22 epitope. More importantly, we demonstrated that in an in vitro cell viability assay, algal expressed gelonin immunotoxins are able to specifically prevent Ramos and CA-46 B-cell proliferation, while leaving Jurkat T-cells unharmed. When directly compared, the divalent αCD22CH23Gel is able to inhibit CA-46 and Ramos B cell growth nearly 25- and 15-fold more effectively than the monovalent αCD22Gel respectively. This suggests that the divalent forms of these immunotoxins could play a vital role in decreasing the effective dosage of this form of therapy. Lower dosages can reduce the side effects of these types of molecules that might otherwise cause vascular leak syndrome (Liu et al., 2012). The observed increase in call growth inhibition may be due to the increased valency that has been shown to increase binding avidity. It is also possible that the increased efficacy of the divalent immunotoxins is due to the increase in toxin molecules delivered to the cell, thereby increasing the toxic load of each molecule that is endocytosed. Additionally, the larger size of αCD22CH23Gel can potentially increase the proteins serum half-life, giving the molecule more time to be effective whereas smaller immunotoxins similar to αCD22Gel often times suffer from extremely short serum half-lives (Ribbert et al., 2010).

Antibody-drug conjugates appear poised to play a major role in cancer treatment in the decades to come, but their cost of production remains high. By producing these antibody conjugates as genetic fusions in a system that has greatly

reduced media and capital expense costs, we can potentially decrease the cost of production dramatically. Algal chloroplast appears uniquely positioned as a protein production platform to produce such fusion proteins. The ability of algal chloroplasts to fold and assemble complex multi-subunit proteins allows them to fold immunotoxins into soluble molecules, however other more complex proteins may also be produced in this system, and we are just beginning to explore such options. There are still improvements to the algal expression system that can be made such as the development of protease deficient strains of algae similar to those made for *E. coli* expression to remove a limitation of protein accumulation (Grodberg and Dunn, 1988) and the development of synthetic promoters and UTRs that overcome endogenous regulator elements (Specht and Mayfield, 2013). Still, the beneficial traits of algal chloroplast and their ability to produce complex proteins make it a truly unique expression platform.

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