The present invention is a DNA expression vector comprising: a toxP; a mutant toxO that blocks Fe-mediated regulation of gene expression; and a DNA sequence encoding a protein, wherein the toxP and the mutant toxO regulate expression of the DNA segment encoding the protein. It is preferred that DNA expression vectors of the present invention include DNA sequences encoding a signal peptide so that a protein expressed is attached to the signal peptide prior to processing. Novel proteins are produced off the DNA expression vector of the present invention.
METHODS OF PRODUCING AGGREGATE-FREE MONOMERIC DIPHTHERIA TOXIN FUSION PROTEINS AND THERAPEUTIC USES

REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Patent Application No. 62/306,281 filed on March 10, 2016, which is hereby incorporated by reference for all purposes as if fully set forth herein.

INCORPORATION-BY-REFERENCE OF MATERIAL SUBMITTED ELECTRONICALLY

The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on March 2, 2017, is named P13869-02_SL.txt and is 134,966 bytes in size.

STATEMENT OF GOVERNMENTAL INTEREST

This invention was made with government support under grant nos. A137856, A136973, A1097138, UC7A1095321-01, awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Ontak® (denileukin diftitox), is a 521 amino acid, recombinant, DNA-derived cytotoxic protein composed of the amino acid sequences for diphtheria toxin fragments A and a portion of fragment B (Meti-His38s) and the sequences for human interleukin-2 (IL-2; Alai-
It is currently produced in an *E. coli* expression system and has a molecular weight of 58 kD. Neomycin is used in the fermentation process but is undetectable in the final product. Ontak®, which is supplied in single use vials as a sterile, frozen solution intended for intravenous (IV) administration, was approved by the FDA in 1999 for the treatment of cutaneous T cell lymphoma (CTCL). The FDA placed Ontak® on clinical hold in June 2011 because of concerns regarding the presence of protein aggregates of heterogeneous molecular weight, excess residual DNA, and excess residual detergent in the final formulation. The production of Ontak® was achieved by expressing the recombinant protein in the *E. coli* cytoplasm, and this expression system resulted in the recombinant protein forming large insoluble aggregates or so-called inclusion bodies comprised of the Ontak® polypeptide. In the current process of production, which includes denaturation and refolding of the inclusion body forms, protein aggregates of heterogeneous molecular weight were still present in the final formulation. The presence of these aggregates in the purified form is a consequence of using *E. coli*-derived cytoplasmic inclusion bodies as the source of the polypeptide and because of the intrinsic hydrophobic nature of the toxin’s transmembrane domain even in the presence of Tween 20. Ontak® produced using this method will hereafter be referred to as classic-Ontak® or c-Ontak®.

In addition, like all of the bacterial and plant toxins, c-Ontak® carries amino acid motifs that induce vascular leak syndrome (VLS). Approximately 30% of patients treated with c-Ontak® develop VLS symptoms ranging from peripheral edema with rapid weight gain to hypoalbuminemia to pulmonary edema. What is needed are 1) a process enabling the production of Ontak-like proteins at high yields and purity, eliminating aggregates in the final
commercial product, and 2) modified Ontak-like proteins with minimal VLS side-effects to provide safer drugs to patients.

**SUMMARY OF THE INVENTION**

One embodiment of the present invention is a DNA expression vector comprising: a toxO that blocks Fe-mediated regulation of gene expression; and a DNA sequence encoding a protein, wherein the toxP and the mutant toxO regulate expression of the DNA segment encoding the protein. It is preferred that DNA expression vectors of the present invention include DNA sequences encoding a signal peptide so that a protein expressed off a DNA expression vector is attached to the signal peptide that is typically cleaved off to form a mature protein. The preferred mutant toxO is SEQ ID NO: 1 and the preferred signal peptide is SEQ ID NO: 5. The DNA expression vectors of the present invention may be used to produce many kinds of proteins such as CRM 197 and CRM 107, or a combination thereof. CRM protein sequences are illustrated in SEQ ID NOs: 18-21. It is preferred that the DNA expression vectors of the present invention encode a diphtheria toxin, or functional part thereof, attached to a receptor binding protein, or a functional part thereof to form a diphtheria toxin receptor fusion protein. The receptor binding protein portion of such fusion proteins may be selected from the group comprising IL-2, IL-3, IL-4, IL-6, IL-7, IL-15, EGF, FGF, substance P, CD4, aMSH, GRP, TT fragment C, GCSF, heregulin βI, a functional part thereof, or a combination thereof.

Examples of diphtheria toxin fusion proteins include the proteins illustrated in any one of SEQ ID NOs: 11-15.

Another embodiment of the present invention is a DNA expression vector comprising: a toxP; a mutant toxO that blocks Fe-mediated regulation of gene expression; a DNA sequence encoding a protein comprising a signal sequence; a diphtheria toxin, or a functional part thereof, that is free of a diphtheria receptor binding domain or has a non-functional diphtheria toxin receptor binding domain, and a target receptor binding domain selected from the group comprising IL-2, IL-3, IL-4, IL-6, IL-7, IL-15, EGF, FGF, substance P, CD4, aMSH, GRP, TT fragment C, GCSF, heregulin βI, a functional
part thereof, or a combination thereof, wherein the toxP and the mutant toxO regulate expression of the DNA sequence encoding the protein. Typically, a bacteria transformed with a DNA expression vector of the present invention produces a diphtheria toxin receptor binding fusion protein attached to a signal peptide that is directed to a periplasm, a culture medium, or both locations by the signal peptide. If the bacteria is *E. coli* then the signal peptide typically directs the diphtheria toxin receptor binding fusion protein to the periplasm. If the bacteria is *Corynebacterium diphtheria* then signal peptide directs the diphtheria toxin receptor binding fusion protein to the culture medium. It is preferred that a DNA expression vector of the present invention comprises SEQ ID NO: 3 and may comprise a DNA encoding a cleavable protein tag wherein the cleavable protein tag is attached to the diphtheria toxin receptor binding fusion protein. Example of diphtheria toxin receptor binding fusion protein produced from the DNA expression vectors of the present invention include anyone of SEQ ID Nos: 11 to 15.

Another embodiment of the present invention includes a method for producing aggregate-free monomeric diphtheria toxin fusion proteins comprising the following steps: transforming bacteria with a DNA expression vectors of the present invention; forming a transformant; incubating the transformant in a culture medium to allow expression of a protein that is secreted into the culture medium; and purifying the protein from the culture medium. The preferred bacteria used in this method is *Corynebacterium diphtheria*.

Another embodiment of the present invention includes a method for producing aggregate-free monomeric diphtheria toxin fusion proteins comprising the following steps: 1) transforming *Corynebacterium diphtheria* strain with a DNA vector comprising: a toxP; a mutant toxO that blocks Fe-mediated regulation of gene expression; a DNA sequence encoding a protein comprising: signal peptide; a diphtheria toxin, or a functional part thereof, that is free of a diphtheria receptor binding domain or has a non-functional diphtheria toxin receptor binding domain; and a target receptor binding domain selected from the group comprising IL-2, IL-3, IL-4, IL-6, IL-7, IL-15, EGF, FGF,
substance P, CD4, aMSH, GRP, TT fragment C, GCSF, heregulin β1, TNFa, TGFp, a functional part thereof, or a combination thereof, wherein the toxP and the mutant toxO regulate expression of the DNA sequence encoding the protein; 2) forming a transformant; 3) incubating the transformant in a culture medium to allow expression of the protein and that is secreted into the culture medium; and 4) purifying the diphtheria toxin fusion protein from the culture medium. Examples of diphtheria toxin receptor fusion protein produced by methods of the present invention include any one of SEQ ID NOs: 11 to 15. The preferred Corynebacterium diphtheria strain used in the methods of the present invention is Corynebacterium C7 beta (-), tox (-).

Another embodiment of the present invention includes a method of treating a patient with tuberculosis comprising the following steps: preparing a diphtheria toxin fusion protein as provided in this application; administering the diphtheria toxin fusion protein to a patient with tuberculosis.

Another embodiment of the present invention includes a DNA expression vector comprising a mutant toxO promoter.

Another embodiment of the present invention includes a Corynebacterium diphtheria strain containing a DNA expression vector of the present invention.

Another embodiment of the present invention is method of making a protein comprising the following steps: providing a DNA expression vector comprising a toxP, a mutant toxO that blocks Fe-mediated regulation of gene expression, a signal sequence, and a DNA sequence encoding a protein; transforming a bacteria strain with the DNA vector to form a transformant; incubating the transformant in a culture medium for a period of time to allow expression of a protein that is secreted into the culture medium; and purifying the protein from the culture medium.

Another embodiment of the present invention is a fusion protein selected from any one of SEQ ID NOs: 11-15.

Another embodiment of the present invention is a pharmaceutical composition comprising a fusion protein described above.
Another embodiment of the present invention is a pharmaceutical composition comprising a fusion protein described above, and at least one or more other chemotherapy agents. Examples of chemotherapy agents include isoniazid, rifampin, rifabutin, rifapentine, pyrazinamide, ethambutol, streptomycin, amikacin, kanamycin, ethionamide, protonamidine, terizidone, thiacetazone, cycloserine, capreomycin, para-amino salicylic acid (PAS), viomycin, ofloxacin, ciprofloxacin, levofloxacin, moxifloxacin, bedaquiline, or delamanid, linezolid, tedezolid, amoxicillin-clavulanic acid, meropenem, imipenem, clarithromycin or clofazimine.

A pharmaceutical composition comprising a fusion protein described above, and at least one or more other antimicrobial agents. Examples of antimicrobial agents include isoniazid, rifampin, rifabutin, rifapentine, pyrazinamide, ethambutol, streptomycin, amikacin, kanamycin, ethionamide, protonamidine, terizidone, thiacetazone, cycloserine, capreomycin, para-amino salicylic acid (PAS), viomycin, ofloxacin, ciprofloxacin, levofloxacin, moxifloxacin, bedaquiline, or delamanid, linezolid, tedezolid, amoxicillin-clavulanic acid, meropenem, imipenem, clarithromycin, or clofazimine.

Another embodiment of the present invention is a method of treating or preventing cancer in a subject comprising administering to the subject an effective amount of a pharmaceutical composition comprising a fusion protein selected from any one of SEQ ID NOs: 11-15.

Another embodiment of the present invention is a method of treating or preventing tuberculosis in a subject comprising administering to the subject an effective amount of a pharmaceutical composition comprising a fusion protein selected from any one of SEQ ID NOs: 11-15.

Another embodiment of the present invention is a prokaryotic cell line comprising a DNA expression vector of the present invention.

Another embodiment of the present invention is a kit comprising the DNA expression vector of the present invention.

Another embodiment of the present invention is a toxP comprising SEQ ID NO: 2.
Another embodiment of the present invention is a protein of any one of SEQ ID NOs: 11 to 15.

**BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1a-1b illustrates: a) a mutant toxO of the present invention (SEQ ID NO: 1), b) a wild type toxO (SEQ ID NO: 25), and c) aDtxR consensus binding sequence.

Figure 2a-2b illustrates: a) illustrates the classic denileukin diftitox (c-denileukin diftitox) expression vector used to manufacture Ontak® and b) illustrates the secreted denileukin diftitox (s-denileukin diftitox) expression vector including the tox promoter (toxP), and mutant toxO of the present invention. Figure 2a discloses SEQ ID NO: 26 and Figure 2b discloses SEQ ID NO: 27.

Figure 3 illustrates a vascular leak mutant (VLM) called c-denileukin diftitox-VLM has equivalent potency to c-denileukin diftitox in killing IL2-receptor-bearing cells.

Figure 4 illustrates c-denileukin diftitox-VLM does not cause vascular leak in vitro.

Figure 5 illustrates that c-denileukin diftitox-VLM has significantly less acute toxicity in vivo than c-Ontak® using a mouse survival model.

Figure 6 illustrates a diphtheria toxin-based fusion protein toxin platform technology of the present invention.

Figure 7 illustrates plasmid pKN2.6Z-LC127 with the tox promoter (toxP of SEQ ID NO: 2) and a mutant tox operator (toxO) (DNA SEQ ID NO: 1), a signal peptide (DNA SEQ ID NO: 4) attached to c-denileukin diftitox DNA sequences (DNA SEQ ID NO: 6).

Figure 8a-8b illustrates: a) the problems of the conventional process of manufacturing Ontak® as cytoplasmic inclusion bodies in E. coli and b) illustrates easy and clean manufacturing process of producing a secreted-denileukin diftitox having one less amino acid
than the Ontak® protein. Figure 8a discloses "fMGADD" as SEQ ID NO: 28 and Figure 8b
discloses "GADD" as SEQ ID NO: 29.

Figure 9 illustrates an immunoblot of s-denileukin diftitox prepared by the process of
the present invention where s-denileukin diftitox is expressed within a Corynebacterium
diphtheria strain C7 beta (-), tox (-) and is secreted into the culture medium.

Figure 10 illustrates how a denileukin diftitox of the present invention, is expected to
deplete IL-2R (CD25+) bearing T cells (Tregs) within a tuberculous granuloma. Tregs are
immunosuppressive by their inhibition of T eff cells.

Figure 11 illustrates diphtheria fusion proteins used in the in vivo treatment of
subjects (mice) with M. tuberculosis.

Figure 12 illustrates the results of treating subjects (mice) infected with M.
tuberculosis with diphtheria toxin-based fusion proteins.

Figure 13 illustrates a diphtheria toxin-based fusion protein regimen for treating
subjects (mice) infected with M. tuberculosis.

Figure 14 illustrates the use of a diphtheria toxin-based fusion protein to treat
subjects (humans) with malignant melanoma.

Figure 15 illustrates the three constructs for rapid production of VLM s-Ontak and
related proteins using His (histidine tags) ("His6/6x His" and "His9/9x His" disclosed as SEQ
ID NOS 23 and 48, respectively).

Figure 16 illustrates purified VLM s-Ontak at greater than 97% purity produced using
the C-terminal Hise VLM s-Ontak construct ("Hise" disclosed as SEQ ID NO: 23).
DEFINITIONS

Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which this invention belongs. The following references provide one of skill with a general definition of many of the terms used in this invention: Singleton et al, Dictionary of Microbiology and Molecular Biology (2nd ed. 1994); The Cambridge Dictionary of Science and Technology (Walker ed., 1988); The Glossary of Genetics, 5th Ed., R. Rieger et al. (eds.), Springer Verlag (1991); and Hale & Marham, The Harper Collins Dictionary of Biology (1991). As used herein, the following terms have the meanings ascribed to them below, unless specified otherwise.

The term "activity" refers to the ability of a gene to perform its function such as Indoleamine 2,3-dioxygenase (an oxidoreductase) catalyzing the degradation of the essential amino acid tryptophan (trp) to N-formyl-kynurenine.

By "agent" is meant any small molecule chemical compound, antibody, nucleic acid molecule, or polypeptide, or fragments thereof.

By "ameliorate" is meant decrease, suppress, attenuate, diminish, arrest, or stabilize the development or progression of a disease.

By "alteration" is meant a change (increase or decrease) in the expression levels or activity of a gene or polypeptide as detected by standard art known methods such as those described herein. As used herein, an alteration includes a 10% change in expression levels, preferably a 25% change, more preferably a 40% change, and most preferably a 50% or greater change in expression levels."

By "analog" is meant a molecule that is not identical, but has analogous functional or structural features. For example, a polypeptide analog retains the biological activity of a
corresponding naturally-occurring polypeptide, while having certain biochemical modifications that enhance the analog's function relative to a naturally occurring polypeptide. Such biochemical modifications could increase the analog's protease resistance, membrane permeability, or half-life, without altering, for example, ligand binding. An analog may include an unnatural amino acid.

c- means "classic" when attached to a term such as c-denileukin diftitox means Ontak® or that commercially available protein.

By "disease" is meant any condition or disorder that damages or interferes with the normal function of a cell, tissue, or organ. Examples of diseases include cancer and tuberculosis.

By "effective amount" is meant the amount of a required to ameliorate the symptoms of a disease relative to an untreated patient. The effective amount of active compound(s) used to practice the present invention for therapeutic treatment of a disease varies depending upon the manner of administration, the age, body weight, and general health of the subject. Ultimately, the attending physician or veterinarian will decide the appropriate amount and dosage regimen. Such amount is referred to as an "effective" amount.

The term "express" refers to the ability of a gene to express the gene product including for example its corresponding mRNA or protein sequence(s).

By "fragment" is meant a portion of a polypeptide or nucleic acid molecule. This portion contains, preferably, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the entire length of the reference nucleic acid molecule or polypeptide. A fragment may contain 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100, 200, 300, 400, 500, 600, 700, 800, 900, or 1000 nucleotides or amino acids.
is- means "immature secreted" when attached to a term such as is-denileukin diftitox means immature secreted denileukin diftitox that contains a signal peptide.

ms-means "mature secreted" when attached to a term such as ms-denileukin diftitox means mature secreted denileukin diftitox that has been processed and no longer contains a signal peptide.

n- means "new" when attached to a term such as n-denileukin diftitox means new denileukin diftitox.

As used herein, the terms "prevent," "preventing," "prevention," "prophylactic treatment" and the like refer to reducing the probability of developing a disorder or condition in a subject, who does not have, but is at risk of or susceptible to developing a disorder or condition.

The terms "polypeptide," "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an analog or mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers. Polypeptides can be modified, e.g., by the addition of carbohydrate residues to form glycoproteins. The terms "polypeptide," "peptide" and "protein" include glycoproteins, as well as non-glycoproteins.

By "reduces" is meant a negative alteration of at least 10%, 25%, 50%, 75%, or 100%.

A "reference" refers to a standard or control conditions such as a sample (human cells) or a subject that is a free, or substantially free, of an agent such as one or more
compositions of the present invention comprising a nucleic acid or protein sequence such as
anyone of SEQ ID NOs: 11-15, or fusion proteins thereof.

A "reference sequence" is a defined sequence used as a basis for sequence
comparison. A reference sequence may be a subset of or the entirety of a specified sequence;
for example, a segment of a full-length cDNA or gene sequence, or the complete cDNA or
gene sequence. For polypeptides, the length of the reference polypeptide sequence will
generally be at least about 16 amino acids, preferably at least about 20 amino acids, more
preferably at least about 25 amino acids, and even more preferably about 35 amino acids,
about 50 amino acids, or about 100 amino acids. For nucleic acids, the length of the
reference nucleic acid sequence will generally be at least about 50 nucleotides, preferably at
least about 60 nucleotides, more preferably at least about 75 nucleotides, and even more
preferably about 100 nucleotides or about 300 nucleotides or any integer thereabout or there
between.

s- means "secreted" when attached to a term such as s-denileukin diftitox means
secreted denileukin diftitox. Secreted denileukin diftitox includes is- and m- forms.

As used herein, the term "subject" is intended to refer to any individual or
patient to which the method described herein is performed. Generally the subject is
human, although as will be appreciated by those in the art, the subject may be an
animal. Thus other animals, including mammals such as rodents (including mice, rats,
hamsters and guinea pigs), cats, dogs, rabbits, farm animals including cows, horses,
 goats, sheep, pigs, etc., and primates (including monkeys, chimpanzees, orangutans and
gorillas) are included within the definition of subject.
By "substantially identical" is meant a polypeptide or nucleic acid molecule exhibiting at least 50% identity to a reference amino acid sequence (for example, any one of the amino acid sequences described herein) or nucleic acid sequence (for example, any one of the nucleic acid sequences described herein). Preferably, such a sequence is at least 60%, more preferably 80% or 85%, and more preferably 90%, 95% or even 99% identical at the amino acid level or nucleic acid to the sequence used for comparison.

Sequence identity is typically measured using sequence analysis software (for example, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, Wis. 53705, BLAST, BESTFIT, GAP, or PILEUP/PRETTYBOX programs). Such software matches identical or similar sequences by assigning degrees of homology to various substitutions, deletions, and/or other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. In an exemplary approach to determining the degree of identity, a BLAST program may be used, with a probability score between $e^{-9}$ and $e^{-100}$ indicating a closely related sequence.

Ranges provided herein are understood to be shorthand for all of the values within the range. For example, a range of 1 to 50 is understood to include any number, combination of numbers, or sub-range from the group consisting 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50.
As used herein, the terms "treat," treating," "treatment," and the like refer to reducing or ameliorating a disorder and/or symptoms associated therewith. It will be appreciated that, although not precluded, treating a disorder or condition does not require that the disorder, condition or symptoms associated therewith be completely eliminated.

Unless specifically stated or obvious from context, as used herein, the term "or" is understood to be inclusive. Unless specifically stated or obvious from context, as used herein, the terms "a", "an", and "the" are understood to be singular or plural.

Unless specifically stated or obvious from context, as used herein, the term "about" is understood as within a range of normal tolerance in the art, for example within 2 standard deviations of the mean. About can be understood as within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.05%, or 0.01% of the stated value. Unless otherwise clear from context, all numerical values provided herein are modified by the term about.

The recitation of a listing of chemical groups in any definition of a variable herein includes definitions of that variable as any single group or combination of listed groups. The recitation of an embodiment for a variable or aspect herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof.

Any compositions or methods provided herein can be combined with one or more of any of the other compositions and methods provided herein.

VLM- means "vascular leakage mutant" when attached to a term such as denileukin diftitox-VLM means denileukin diftitox vascular leakage mutant.

w- means "wild type" when attached to a term such as w-diphtheria toxin means wild type-diphtheria toxin.
DETAILED DESCRIPTION OF THE INVENTION

One embodiment of the present invention is the discovery of a process that produces aggregate-free, monomeric, diphtheria toxin fusion proteins having enhanced purity and quality. This process includes transforming bacteria including preferably, strains of Corynebacterium diphtheria with DNA expression vectors of the present invention. DNA expression vectors of the present invention are designed to include specific genetic elements comprising a tox promoter (toxP) and an overlapping novel, mutated tox operator (toxO), preferably a signal sequence, and a DNA sequence encoding a protein. The protein is preferably a fusion protein including a diphtheria toxin, or functional part thereof, and a target receptor binding domain or a functional part thereof. The term "functional part thereof" means a part of a diphtheria toxin protein that acts as a toxin or the part of a target receptor binding domain that binds to its receptor. DNA expression vectors of the present invention are designed so proteins are expressed from a tox promoter (toxP) and a mutant tox operator (toxO).

Mutant toxO

toxO, is a 19-bp operator region that is composed of two 9 bp imperfect palindromic arms interrupted by a central cytosine (C) base. The wild type toxO (FIG. 1b) and a mutant toxO (FIG. 1a) operator discovered by inventors are shown in Figure 1. SEQ ID NO: 1 illustrates one embodiment of the DNA sequence of a mutant toxO this invention. toxP is a promoter having a DNA sequence of SEQ ID NO: 2. SEQ ID NO: 2 illustrates the toxP DNA sequences include the toxO DNA sequences. SEQ ID NO: 3 is a DNA sequence including a toxP, a toxO, a signal peptide, and a DNA sequence encoding a protein. The asterisks in SEQ ID NO: 3 indicate the changes introduced to create the mutant toxO.
The toxO DNA operator sequence is bound by a protein known as the diphtheria toxin repressor, DtxR. DtxR is a global iron-activated regulatory protein that is able to control gene expression. In iron-replete conditions, Fe$^{2+}$ and Fe$^{3+}$ ions bind to apo-DtxR causing a conformational change that allows the formation of homodimers of the DtxR repressor, which bind to the tox operator (toxO) DNA sequence and repress tox gene expression. In low iron environments, Fe$^{2+}$ and Fe$^{3+}$ ions disassociate from DtxR causing it to lose its DNA binding capability and disassociate from the operator; this event thereby allows expression of tox gene products. Fig. 1b illustrates the wild type toxO DNA sequence.

To overcome the inhibitory effect of Fe$^{2+}$ and Fe$^{3+}$ ions on tox expression, a DNA expression vector was created replacing the wild type (WT) toxO with a mutant toxO DNA sequence. This change blocks Fe ion-mediated regulation of tox gene expression. Fig. 1a, SEQ ID NO: 1, and SEQ ID NO: 3 illustrate the mutant toxO DNA sequence of the present invention. Under this invention, bacteria such as *E. coli* and *C. diphtheria* harboring a recombinant plasmid encoding a diphtheria toxin fusion protein under the control of toxP and the mutant toxO may be grown in Fe-replete media, allowed to grow to high densities, and
will not require a shift to Fe-free media to induce expression. The constitutive expression of
*tox* gene products in iron replete medium represents a significant advance in the field. *C.
diphtheria*, specifically the C7 beta (-), tox (-) strain is the preferred host bacteria for the
production of all diphtheria-toxin related recombinant proteins using the DNA expression
vectors of the present invention. The DNA expression vectors of the present invention may
be used in other bacteria such as *E.coli*.

**DNA Expression Vectors**

The DNA expression vectors of the present invention includes a *toxP*, mutant *toxO*, a
DNA sequence encoding a protein, and preferably a signal sequence. SEQ ID NO: 3 is one
example of a DNA sequence containing these genetic elements that may be part of a DNA
expression vector of the present invention. As mentioned, the asterisks observed in SEQ ID
NO: 3 are placed above the base pair changes between the mutant and wild type *toxO*. SEQ
ID NO: 3 is numbered such that the *toxP* extends from base 1 to 30, and *toxO* begins at base
24 and ends at base 42 (prior to the underlined DNA sequence). The underlined DNA
sequence represents base 74 to base 148 and is a region of DNA encoding a 25 amino acid
signal sequence (also observe in SEQ ID NO:4, SEQ ID NO: 5, and Fig. 2). The DNA
expression vectors of the present invention are preferably constructed so one or more proteins
are expressed from *toxP*, mutant *toxO*, and are translated with an N-terminal signal sequence.

The N-terminal signal sequence targets the one or more proteins (expressed from the vector)
for secretion, and the N-terminal signal peptide is later cleaved to make mature active
proteins. SEQ ID NO: 3 includes DNA sequences encoding proteins such as a novel
denileukin diftitox called secreted-denileukin diftitox, or *s-denileukin diftitox*. The *s-
denileukin diftitox* has two forms called immature secreted-denileukin diftitox (*is-denileukin

diftitox) and mature secreted-denileukin diftitox (ms-denileukin diftitox). SEQ ID NO: 12 is of is-denileukin diftitox of the present invention and SEQ ID NO: 13 is of ms-denileukin diftitox of the present invention. The is-denileukin diftitox contains a signal sequence that during processing is cleaved off to form ms-denileukin diftitox. In addition, SEQ ID NO: 3 includes a DNA sequence beginning at base 149 to 1711 of SEQ ID that encodes a protein, specifically a fusion protein containing the functional parts of a diphtheria toxin and the functional parts of IL-2. A new denileukin diftitox fusion protein sequence is formed called ms-denileukin diftitox that is a 520 amino acid polypeptide and is composed of the amino acid sequences for diphtheria toxin fragments A and a portion of fragment B (Gly i-His_{387}) and the sequences for human interleukin-2 (IL-2; Ala_{1}-Thr_{133}). As a result of cleavage of the signal sequence, ms-denileukin diftitox of the present invention lacks the first methionine present in classic-denileukin diftitox (c-denileukin diftitox) and is thereby one amino acid shorter than the amino acid sequence of the classic-denileukin diftitox protein known as Ontak®. SEQ ID NO: 13 is the protein sequence of the new diftitox protein sequence ms-denileukin diftitox which may be compared to SEQ ID NO: 10 containing the protein sequence of the classic-denileukin diftitox (c-denileukin diftitox) known as Ontak®.

DNA expression vectors of the present invention include DNA sequences encoding one or more protein(s). A preferred protein of the present invention is a fusion protein comprising a diphtheria toxin (or a functional part thereof) and a target receptor binding protein (or a functional part thereof). An example of a diphtheria toxin that may be produced from a DNA expression is any functional part of a diphtheria toxin or any functional part of a diphtheria toxin vascular leakage mutant. Examples of proteins of target receptor binding domains produced from a DNA expression vector of the present invention include, IL-2, IL-
3, IL-4, IL-6, IL-7, IL-15, EGF, FGF, substance P, CD4, aMSH, GRP, TT fragment C, GCSF, heregulin β1, TNFa, TGFp, or a combination thereof. Other target receptor binding domains may be used depending upon the therapeutic application; however, SEQ. ID NO. 9 is a preferred DNA sequence encoding a functional part of IL2 receptor binding domain. For the purposes of the present invention, some of the DNA plasmids and the genetic elements thereof are illustrated in Fig. 1, Fig. 2, Fig. 6, and Fig. 7. Examples of fusion proteins encoded by DNA expression vectors of the present invention include SEQ ID NOS: 11, 12, 13, 14, 15, 19, and 21.

SEQ ID NO: 3 (DNA sequence encoding secreted-denileukin diftitox or s-denileukin diftitox). Sequence includes toxP, mutant toxO, signal sequence, a functional part of diphtheria toxin and a functional part of IL2. Bold font and asterisks indicate the changes introduces to create the mutant toxO)

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1  TTGATTTTCAGACACCCCTTTATATAATTAGGATAGCTAGTCATTTATATTATAT
51  GAGTCTCTGTAAGGGATACGTTGTGACAGAAACACTGTTTGCGTCAATCG
101  TTTAATAGGGGCCTACTGGGGATAGGGCCTACCTTCAGGCAATGAG
151  CGCTGATGATGTTGATATCTATACTTTTGTGATGAAAAACTTTT
201  ATTCGTACCCACCTCTGAAATATGTTGATTAGTCAATTTCA
251  AAAAAAGCCAAAATCTGGTAAGCAAAATAGGATAGCTAGTCATTTATATTATAT
301  AGGGTTTTATAGTGACGACATATATACGCTGCGGGATATCTGCTAG
351  ATATGAAACCGCTCTCCGAAAGCTGAGGGAGCGTGATCGAAAATGTAGC
401  ATATCAGGACTGGAAGTTCTCGCACTAAAAGTGGATAATGCCGAAAC
451  ATATGAAACCGCTCTCCGAAAGCTGAGGGAGCGTGATCGAAAATGTAGC
501  TCGGAAAGAAGAGTTTTATCAAAAGGTTCCGCTAGGATGCGTTCGCGTCTGTA
551  GTGCTACCCACCTCTGAAATATGTTGATTAGTCAATTTCA
601  TAAGGGAAACGGGAAACGGGAAAGGTTCCGCTGAGGAGTGCTGGATATATTAA
651  AAACCGGTGGAAACCGGAAACGGGAAAGGTTCCGCTGAGGAGTGCTGGATATATTAA
701  GCCTGTCAGGAAATTCGTCAGGCAATCAGGCTGAGGCTGCTACGTCATG
SEQ ID NO: 4 (Signal DNA Sequence)
74 GTGAGCAGAAAAACTGTGTTTGGCTCAATCTTAATAGGGGCGCTACTGGGGAT
124 AGGGGGCCCAACTTCAGCCCATGCA < 148

SEQ ID NO: 5 (Signal Protein Sequence)
-25 MSRKLFASILIGALLGIGAPPSAHA < -1

SEQ ID NO: 6 (classic-denileukin diftitox DNA sequence)
1 ATG
4 GGCCTGAT GATGTT GTTGATTCTTCTAAATCTTTTGTGATGGAAAACTT
35 TTCTCCTGACCCAGGACTAAACCTGGTTATGTAGATTCCATTCAAAAAG

Formation of Diphtheria Toxin Fusion Proteins Having Minimal, or no, Vascular Leakage
(denileukin diftitox-VLMs)

21
Like all of the bacterial and plant toxins, denileukin diftitox carries amino acid motifs that may induce vascular leak syndrome (VLS). Approximately 30% of patients treated with Ontak® develop VLS ranging from rapid weight gain with peripheral edema to hypoalbuminemia to pulmonary edema. Mutations were made to the DNA sequence of Ontak® as described in US Patent No. 8,865,866. It was discovered that DNA mutations made to the DNA sequence such that the valine (GTT) at the 7th residue of SEQ ID NO: 10 is replaced with an alanine as shown in SEQ ID NO: 16, resulted in the fusion toxin having little, or no, vascular leak syndrome side effects. These mutants are referred to as "vascular leak mutants" (VLM). The vascular leak mutants, or denileukin diftitox-VLMs are shown to have the same potency as c-denileukin diftitox in Fig. 3, not to cause vascular leak in Fig. 4, and to have significantly less acute toxicity in vivo than c-denileukin diftitox in Fig. 5. s-denileukin diftitox-VLM, has an alanine replacing the valine at the 6th residue shown in in SEQS: 14 and 15. s-denileukin diftitox-VLM protein should have a similar decrease in toxicity as that found with the c-denileukin diftitox-VLM protein.

Also, the sequences V29D30S and I290D291S292 shown in SEQ ID NO: 10 (amino acid sequence of c-denileukin diftitox), when mutated also will reduce VLS. A claim in this discovery is that introduction of substitutions in V29D30 S31 and/or I290D291 S292 such as V29A or I290A may be introduced into the corresponding positions of diphtheria toxin fusion proteins and that these substitutions will also have value in further reducing vascular leakage syndrome.

SEQ ID NO: 7 (denileukin diftitox-VLM underlined codon encodes for alanine, here shown as GCT, described in US Patent No. 8,865,866.)
1 ATG
4 GGCGCTGAT GATGTTGCTGATTTCTCTAAATCTTTTGTGATGGAAAACTT
5 4 ... GAGACCGCA
1 5 0 4 ACCATCGTAGAATTCCTGAACCGTTGGATCACCTTCTGTCAGTCTATCAT
1554 CTCTACCCTGACC < 1566
Alignment of DNA sequences comparing SEQ ID NO: 7 [denileukin diftitox-VLM described in US patent No. 8,865,866] with SEQ ID NO: 8 [is-denileukin diftitox-VLM of the present invention] demonstrates SEQ ID NO: 8 is missing a codon (three bases) in line 1381-1437.

Similarity: 1563/1638 (95.42 %)

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<tr>
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TGGATAATGCCGAAACTATTAAGAAAGAGTTAGGTTTAAGTCTCACTGAACCGTTGATG

CGAAAGCGTTAAGCGTAGAACTTGAGATTAATTTTGAAACCCGTGGAAAACGTGGCCAA

AGCTGAGGCGTGGTCAAAGTGACGTATCCAGGACTGACGAAGGTTCTCGCACTAAAA

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GTAGCTCATTGTCATGCATCAACCTGGATTGGGATGTTATCCGTGATAAAACTAAAACT

CGAAAGCGTTAAGCGTAGAACTTGAGATTAATTTTGAAACCCGTGGAAAACGTGGCCAA

AGAT C GAAT C T C T GAAAGAACAC G G T C C GAT C AAAAACAAAAT GAG C GAAAG C C C GAAC

ATGCGATGTATGAGTATATGGCTCAAGCCTGTGCAGGAAATCGTGTCAGGCGATCAGTA

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TCAGCCTTCCCTCCTGAGGGGAGTTCTAGCGTTGAATATATTAATAACTGGGAACAG

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CGAAAGCGTTAAGCGTAGAACTTGAGATTAATTTTGAAACCCGTGGAAAACGTGGCCAA

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CGAAAGCGTTAAGCGTAGAACTTGAGATTAATTTTGAAACCCGTGGAAAACGTGGCCAA

AGCTGAGGCGTGGTCAAAGTGACGTATCCAGGACTGACGAAGGTTCTCGCACTAAAA

TCAGCCTTCCCTCCTGAGGGGAGTTCTAGCGTTGAATATATTAATAACTGGGAACAG

TGGATAATGCCGAAACTATTAAGAAAGAGTTAGGTTTAAGTCTCACTGAACCGTTGATG

CGAAAGCGTTAAGCGTAGAACTTGAGATTAATTTTGAAACCCGTGGAAAACGTGGCCAA
Proteins Produced Using DNA Expression Vectors of the Present Invention

The first amino acid of a mature active diphtheria toxin related fusion protein of the present invention is a glycine as shown in bold (amino acid 1) in SEQ ID NO: 13 and 15.

The signal sequence within SEQ ID NO: 4 is labeled with negative numbers, counting back from the first glycine of the mature fusion protein and has the following amino acid sequence MSRKLFAS ILIGALLGIGAPPSAHA (SEQ ID NO: 22). The signal sequence is shown in SEQ ID NOS: 11 and 12 and is underlined. The mature secreted diphtheria toxin fusion protein includes a diphtheria toxin portion, such as Gly-His387, and a target receptor binding domain, such as an IL-2 protein from Ala388-Thrs20 in SEQ ID NO: 3. Other target receptor
binding domains used in the present invention that may be fused to a diphtheria toxin protein (or functional part thereof) include IL-3, IL-4, IL-6, IL-7, IL-15, EGF, FGF, substance P, CD4, aMSH, GRP, TT fragment C, GCSF, heregulin β1, TNFa, TGFp, among others, or a combination thereof.  

SEQ ID NO: 10 describes c-denileukin diftitox that is not secreted and is requires purification from inclusion bodies in E. coli. SEQ ID NO: 12 describes immature secreted is-denileukin diftitox with a signal sequence. SEQ ID NO: 13 describes MS-denileukin diftitox wherein the signal sequence has been cleaved off during the process of secretion to the extracellular space.

SEQ ID NO: 10 (Protein Sequence of c-denileukin diftitox known as Ontak®)

```
1 MGADDWDSSSFVMENFSSYHGTPK
27 GYVDSIQKGIQPSTQGNYDDWKGFYSTDNKYDAAGYSVDNENPLSG
77 KAGGWKVTYPGLTKVLKVDNAETIKKELGLSLETLEMQVGEFFIK
127 RFGDGASRWLSLPFAGSSSVYINWEQAKALSVELEINFETRGKRQ
177 DAMYEYMAQACAGNRVRSGSCLNCINLWDVIRDKTKTIJKSLKHF
227 IKNKMSESPNKTVESEKAKQYLEEFHQTALEHPESELKTVTGKNPVAF
277 ANYAAWAVNVAQIDSETADNVNLKTAALSILPIGSVGMIAGDGAHHINT
327 EEIVAQIALSSLMVAQAIPLVGELVDIGFAAYNFVESIINLFQWHNSY
377 NRPAYSQGHKTHAPSSSTKKTQLQHELHLLDLMQILNIGNYKNPKLTR
427 MLTFKPFYMPKKATLEKHLQCLEEELKLPEELVNLQASKNFHLPRLISN
477 INVIVLELKSETTMCEYADETATIVEFLNRWKIFQSIISTLT<521
```
301 ANYAAWAVNVAQVIDSETADNEKTAAALSILPGIGSVMGIADGAVHHNT
351 EEIVAQS IALSSLMVAQAIPLVGELVDIGFAAYNFVES IIINLFQWHNSY
401 NPRAYSPGHKTQPFPLHGDYZAVSWNTVEDSIIRTGFQGSEGHDIAKTAENT
451 PLPIAGVLLPTI PGKLDVNSKTISVNGRKRMRCAIDGDVTFCRPKS
501 PYYVONGVHANLHAFHRSSEKISNEISSDSIGVLGYQKTDHTKNS
551 KLSLFFEIKS <560

SEQ ID NO: 12 (is-denileukin diftitox)

10

-25 MSRKLFSALIGALLGIGAPPSAHAGADDWDSKSFVMENFSSYHGTPK
26 GYVDSIQKGIQKPKSGTQGNYDDDKGFTSTDNYDAAGYSVJNENPLSG
76 KAGGWKT YPGTLKVLKVDNAETIKKEGLSLEPLME QVTGEFIFK
126 RFGDGASRWLSLPFAGSSSESVEYINNWQAKALSVELEINFETRGKRGQ
176 DAMYEYMAQACAGNRRVRSVGSSLSCINLDWDVIRDKTKKIESLKEHGP
226 IKNKMSESPNKTVSEEKAKQYLEEHHQTALEHPSELKVTGTPNVFAG
276 ANYAAWAVNVAQVIDSETADNEKTAAALS ILPGIGSVMGIADGAVHHNT
326 EEIVAQS IALSSLMVAQAIPLVGELVDIGFAAYNFVES IIINLFQWHNSY
376 NPRAYSPGHKTQPFPLHGDYZAVSWNTVEDSIIRTGFQGSEGHDIAKTAENT
426 INVIVLEKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLT <52 0

SEQ ID NO: 13 (ms-denileukin diftitox)

25

1 GADDWDS KSFVMENFSSYHGTPK
26 GYVDSIQKGIQKPKSGTQGNYDDDKGFTSTDNYDAAGYSVJNENPLSG
76 KAGGWKT YPGTLKVLKVDNAETIKKEGLSLEPLME QVTGEFIFK
126 RFGDGASRWLSLPFAGSSSESVEYINNWQAKALSVELEINFETRGKRGQ
176 DAMYEYMAQACAGNRRVRSVGSSLSCINLDWDVIRDKTKKIESLKEHGP
226 IKNKMSESPNKTVSEEKAKQYLEEHHQTALEHPSELKVTGTPNVFAG
276 ANYAAWAVNVAQVIDSETADNEKTAAALS ILPGIGSVMGIADGAVHHNT
326 EEIVAQS IALSSLMVAQAIPLVGELVDIGFAAYNFVES IIINLFQWHNSY
376 NPRAYSPGHKTQPFPLHGDYZAVSWNTVEDSIIRTGFQGSEGHDIAKTAENT
426 INVIVLEKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLT <52 0

30
SEQ ID NO: 14 (Protein sequence of is-denileukin diftitox-VLM)

```
5  -25 MSPKLFASILIGALLGIGAPP SHAHAGADDVADSSKFVMENFSSYHGTKP
26  GYVDSIQKGIQPKSQTGNYDDWKGFYSTD N KYAAGY SVD N ENPLSG
76  KAGGKWKT YPLTKVLALKVDNAETIKKELGLS L TEPLME QVTGE E PIK
126 RFGDGASR W L PFAEGSSSVEYIN W EQA KALSVE LEINFETRGKRGQ
176 DACMEYMAQACGRVR VRSVGSSLSCINLDWDVIRDKKT KIESLKEHGP
10 226 IKNKMS E S P NKTVSEEKAKQ YLEEFHQT A LE H PSELKTVGT N PVFAG
276 ANYA AAWNVQAQ V IDSETADNLEKTAALS ILPGIGSVMGIA DGAVHHNT
326 EEI A QSIAL L S SMVAQ AI PV GELVDIGFAAYNFVES IINLFQWHNS Y
376 NRPAYS PHKTHAP TSSTK QTQLQ LEHHLL DLQMLINGIN NNYKPKL TR
426 M LTFKFYM P K K ATE KHLQCLEELKPLEEV LNA QSKNHF LRP DLISN
15 476 INVIVLELKGSSETTFM CEYADETATIVEFLNRWITFCQSI ISTLT <52 0
```

SEQ ID NO: 15 (Protein sequence of ms-denileukin diftitox-VLM)

```
1  GADDVADSSKFVMENFSSYHGTKP
20  GYVDSIQKGIQPKSQTGNYDDWKGFYSTDNYDAAGYSVDNE PLSG
26  KAGGKWKT YPLTKVLALKVDNAETIKKELGLS LTEPLME QVTGEE PIK
76  RFGDGASRWLSLPFAEGSSSVEYINWEQAKALSVELEINFETRGKRGQ
126 DACMEYMAQACGRVRVRSVGSSLSCINLDWDVIRDKKT KIESLKEHGP
226 IKNKMSESPNTVSEEKAKQYLEEHQTALEHPSELKTVGTNPVFAG
276 ANYA AAWNVQAQVIDSETADNLEKTAALS ILPGIGSVMG IADGA VHHNT
326 EEI AQSIALSSMLVAQAI PVLGE LDIGFAAYNFVES IINLFQWHNS Y
376 NRPAYS PHKTHAPTSSTKQTQLQLEHLDDLQMLINGINNYKNPKLTR
426 M LTFKFYMPPKATEKHLQCLEELKPLEEVLNAQSKNFHLRPDLISN
476 INVIVLELKGSSETTFMCEYADETATIVEFLNRWITFCQSIISTLT <52 0
```

SEQ ID NO: 16 (Protein sequence of denileukin diftitox-VLM described in US Patent No. 8,865,866)

```
1  MGADDVADSSKFVMENFSSYHGTKP
35  GYVDSIQKGIQPKSQTGNYDDWKGFYSTDNYDAAGY SVD NENPLSG
```

31
Protein Alignment of SEQ ID NO: 16 is denileukin difitox-VLM described in US Patent No. 8,865,866 that has an extra amino acid (L) at position 445 when compared with SEQ ID NO: 14 is-denileukin difitox-VLM of the present invention. Similarity : 521/522 (99.81 %)

NO:16 1 M---------------GADDVADSSKSFVMENFSYHGKPGYVDSIQKGI 36
NO:14 1 MSRKLFAI LIGALLGAPPSSAHAGADOSSKSFVMENFYHTKPGYVDSIQKGI 60

NO:16 37 QPKSGTQNYDDWKGYSSTDNYDAAGYSVDSENPLSGKAGGWKTYPGLTVLALK 96
NO:14 61 QPKSGTQNYDDWKGYSSTDNYDAAGYSVDSENPLSGKAGGWKTYPGLTVLALK 120

NO:16 97 VDNAETIKKELGLSLEPLMGEQTEEPKRFGDGASRWSLPFAEGSSSYEINNEQ 156
NO:14 121 VDNAETIKKELGLSLEPLMGEQTEEPKRFGDGASRWSLPFAEGSSSYEINNEQ 180

NO:16 157 AKALSVELEINFETRGKRGQDAMYEYMAQACGNVRRSGSLSNLDWVIRKT 216
Use of DNA Expression Vectors to Manufacture Proteins.
The method using Fe-independent, secreted expression of proteins related to diphtheria toxin described above has several commercial applications in addition to the use of the method to express s-denileukin diftitox. The method can be used to improve (enhance) expression (yield) of:

5 **WT diphtheria toxin:**

The wild type Diphtheria toxin (SEQ ID NO: 11) used to make diphtheria toxoid, a vaccine for diphtheria which is present in DTP, TDaP, and other combination vaccines may be made using the DNA expression vector of the present invention. The DNA segment encoding SEQ ID NO: 11 may be placed in the DNA expression vector of the present invention and located downstream of the 7\text{\textit{x}P}/mutant ToxO.

**Cross-reacting material-197 (CRM197) and Cross-reacting material-107 (CRM107):**

CRM197 and CR107 are mutant proteins of full-length diphtheria toxin which are highly immunogenic but are completely devoid of toxin activity. They are used as carriers for several polysaccharide conjugate vaccines. For example, Wyeth and Pfizer took advantage of this immunogenicity in the 1990s when it conjugated seven polysaccharides from Streptococcus pneumoniae to CRM197 to create the original Prevnar vaccine which was FDA approved in February 2000. A 13-polysaccharides Prevnar was FDA-approved in 2010. The meningococcal vaccine Menveo, from Novartis, is four Neisseria meningitidis polysaccharides plus CRM197. This vaccine gained FDA approval in 2010. The cancer immunotherapy company Imugene (ASX: IMU) reported dramatic improvements in antibody titers from its B cell peptide cancer immunotherapy targeting HER2 when it used CRM197 as a carrier protein. CRM197 is also being evaluated as a potential drug delivery protein. The Swiss-based Turing Pharmaceuticals is working on CRM197 fusion constructs with
therapeutic proteins of up to 1,000 amino acids in length. The DNA expression vectors of the present invention maybe used to produce CRM 197 and CRM 107. One or more of the DNA segment(s) encoding SEQ ID NOs: 18-21 may be placed in the DNA expression vector of the present invention and located downstream of the 7bxB mutants ToxO.

Diphtheria toxin based fusion proteins with cleavable peptide or protein tags used to enhance purification.

Cleavable peptide tags (such as Hise (SEQ ID NO: 23) or FLAG [DYKDDDDK] (SEQ ID NO: 24)) or protein tags (such as GST [glutathione S-transferase] or SUMO [Small Ubiquitin-like Modifier protein]) may be fused with specific protease cleavage sites to diphtheria toxin based fusion proteins. Affinity chromatography methods using antibodies or ligands which bind to the tag may be used for rapid purification of the tagged protein.

Following purification, the specific cleavage site enables separation of the tag from the desired diphtheria toxin related proteins. Such fusions may enhance purification of diphtheria toxin based fusion proteins of the present invention.

SEQ ID NO: 17 (Protein sequence of ms-CRM197)

1 GADDWDSSKS FVMENFSSYHTKPGVDS IQKGIQPKSHTQGNYDDDW
51 KEFYSTDNYAAGYSVDENPLSGKAGWKVTYPGLTKLALKVDNAE
101 TIKKELGSLTEPLMEQVGTEEFIKRFGDGASRWLSLPSAEGERSSVEYI
151 NNWEOFKALSVELEINFETRKGQDAMYEYMAQACAGNRRVRSVGSSL
201 CINLWDVIRDNTKTKIESLKEHGPIKNKMSIPNKTVEEAKQYLEEF
251 HQTALEHPESELKVTGTNPVFAGANYAWAVNVAQVIDSETADNLEKT
301 TAAALPGVGSMGIADGAVHHHTEEIAVAQSMALSLMVQAIPLVGL
351 VDIGFAAYNVESI INLFQWHNSYNRPAYSPGHTQTPFLHGDYAVSWNT
401 VEDSIIRTGFQGESHDIKITAENTPLPIAGVLLPTIPGKLVDNVKSKTHI
451 SVNGRKIRMCRAL DGDVFRCRPKS PVYVNGVHANLHVAFHRS SSEKIH
501 SNEISSDSIGVLYQKTVDHTKNSKLSSLFEIKS <535
SEQ ID NO: 18 (Protein sequence of is-CRM197)

1 MSRKLFASILIGAPPASAHAAGADDWDSKSFVMENGSSYHGTPK
5 GYVDSIQKGIGQPKPSTQGNYDDNKFYSTDNYDAAGSVDNENPLSG
10 KAGGKWVTPGTLKVLKVDNAETIKKELGLSLTEPLMEQVGGTEEFIK
15 RFGDGASRWLSLPPAEQSSVEYINNWQAALVELEINFETRKGQ
20 DAMYEYMAQACAGNRRRSVGSSLCINLDWDVIRDKTNTKIESLKEHP
25 IKKMSESPNNTVSEEKAKQYLEFHQTALHPSELKTVTGNPVEAG
30 ANYAAAWVNAVIDSETADNLKTAALILPGISVMDAGAVHHNT
35 EEIVAQSIALLMVAQAIPLVGFVDDIGFAYNFSVEINLQWHSNY
40 NRPAYSPGKHQTQFFHGGAVSWNTVEDSIURTGFQGESGHDIKITAENT
45 PPLPIAVL丕PTIPGKLVNSKTHISVNGRKIRMRCRAIDGDVTFCRPRS
50 PYYVNGVHANLHFAFRSSEKISNEISSDSIGVGLGQKTVDHTKVF
55 KLSLFFEEIKS <560

SEQ ID NO: 19 (Protein sequence of ms-CRM107)

GADDWDSKSFVMENFSSYHGTPK
5 GYVDSIQKGIGQPKPSTQGNYDDNKFYSTDNYDAAGSVDNENPLSG
10 KAGGKWVTPGTLKVLKVDNAETIKKELGLSLTEPLMEQVGGTEEFIK
15 RFGDGASRWLSLPPAEQSSVEYINNWQAALVELEINFETRKGQ
20 DAMYEYMAQACAGNRRRSVGSSLCINLDWDVIRDKTNTKIESLKEHP
25 IKKMSESPNNTVSEEKAKQYLEFHQTALHPSELKTVTGNPVEAG
30 ANYAAAWVNAVIDSETADNLKTAALILPGISVMDAGAVHHNT
35 EEIVAQSIALLMVAQAIPLVGFVDDIGFAYNFSVEINLQWHSNY
40 NRPAYSPGKHQTQFFHGGAVSWNTVEDSIURTGFQGESGHDIKITAENT
45 PPLPIAVL丕PTIPGKLVNSKTHISVNGRKIRMRCRAIDGDVTFCRPRS
50 PYYVNGVHANLHFAFRSSEKISNEISSDSIGVGLGQKTVDHTKVF
55 KLSLFFEEIKS <560

SEQ ID NO: 20 (Protein sequence of is-CRM107;
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<td>Protein sequence of N terminal His tag to VLM s-Ontak</td>
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<td>Protein sequence of N terminal His tag to VLM s-Ontak after signal sequence is cleaved</td>
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<td>SEQ ID NO: 40</td>
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<td>SEQ ID NO: 44</td>
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</tr>
<tr>
<td>SEQ ID NO: 31</td>
<td>DNA sequence of C terminal His tag to VLM s-Ontak</td>
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Table 1
Purification of VLM s-Ontak using His-tagged versions of the polypeptide

In some preparations of VLM s-Ontak produced in *Corynebacterium diphtheriae* C7 slow proteolytic cleavage of the mature 520 amino acid polypeptide occurs. This is probably due to secreted proteases made by *Corynebacterium diphtheriae* C7. This proteolytic cleavage occurs at approximately amino acid 390 of the mature 520 amino acid VLM s-Ontak.

Histidine-tagged (His-tagged) versions of VLM s-Ontak have been constructed for the purpose of accelerating the purification of the desired protein away from the secreted proteases present in the culture supernatant. Tobacco Etch Virus (TEV) nuclear-inclusion-a endopeptidase (EC 3.4.22.44) recognition sites have also been engineered into these His-tagged versions of VLM s-Ontak. The purpose of the TEV cleavage sites is to enable the removal of the poly-His sequences in the final preparation of VLM s-Ontak. TEV is a highly specific endopeptidase which recognizes the amino acid sequence ENLYFQVX where ‘V denotes the cleaved peptide bond, and X represents any small hydrophobic or polar amino acid such as glycine (G).
N-terminal His-tagged VLM s-Ontak with TEV cleavage site. As shown in SEQ ID: 38
(Protein sequence of N terminal His tag to VLM s-Ontak) it is possible to add the amino
sequence HHHHHHENLYFQ to the immature protein sequence of VLM s-Ontak near its N-
terminus. In this version, the sequence HHHHHHENLYFQ appears immediately after the 26
amino acid signal sequence and immediately before the mature sequence of VLM s-Ontak
(GADDVA. . .). The first glycine of VLM s-Ontak comprises the final recognition residue
for the TEV protease which recognizes ENLYFQ\X with X being any small amino acid. The
mature, secreted protein sequence of this N-terminal His-tagged VLM s-Ontak is shown in
SEQ ID: 39 (Protein sequence of N terminal His tag to VLM s-Ontak after signal sequence is
cleaved) which is a good candidate for Nickel-column affinity purification with its His6 tag.
The affinity purified VLM s-Ontak may then be exposed to small amounts of pure TEV
protease, leading to enzymatic proteolysis that removes the 13 N-terminal residues
MHHHHHHENLYFQ and releases mature, untagged VLM s-Ontak as is shown in SEQ ID:
40 (Protein sequence of N terminal His tag to VLM s-Ontak after signal sequence is cleaved
and TEV site is cleaved).

Because the secreted protease(s) of Corynebacterium diphtheriae C7 cleave at
approximately amino acid 390, N-terminal His-tagging can lead to two species: full length
desired VLM s-Ontak (520 amino acids) and a 390-amino acid N-terminal breakdown
fragment. These two polypeptides, being relatively close in size (as well as molecular
composition) are difficult to separate by size exclusion chromatography. Hence we have also
developed C-terminal His-tagged version of VLM s-Ontak.

C-terminal His-tagged VLM s-Ontak without TEV cleavage site. As shown in SEQ ID: 42
(Protein sequence of C terminal His tag to VLM s-Ontak) it is possible to add the amino
sequence HHHHHH to the immature protein sequence of VLM s-Ontak at its C-terminus. In this version, the sequence HHHHHH appears immediately after the C-terminal threonine of VLM s-Ontak (…IIISTLT). The mature, secreted protein sequence of this C-terminal His-tagged VLM s-Ontak is shown in SEQ ID: 43 (Protein sequence of C terminal His tag to VLM s-Ontak after signal sequence is cleaved) which is a good candidate for Nickel-column affinity purification with its His6 tag.

C-terminal His-tagged VLM s-Ontak with TEV cleavage site. In order to avoid having the His6 sequence in the final polypeptide sequence of the above version of VLM s-Ontak made by C-terminal His-tagging (SEQ ID: 43), it is possible to insert a TEV recognition sequence at the C-terminus to enable removal of the His-tag sequence. In this version, the sequence ENLYFQGHHPHHHHH appears immediately after the C-terminal threonine of VLM s-Ontak (…IIISTLT). Since nickel affinity binding is enhance by poly-His sequences even longer than six amino acids, it is possible to include nine His residues. The amino acid sequence of this C-terminal His-tagged VLM s-Ontak with TEV cleavage site is shown in SEQ ID: 45 (Protein sequence of C terminal TEV His9 tag to VLM s-Ontak). The mature, secreted protein sequence of this C-terminal His-tagged VLM s-Ontak with TEV cleavage site is shown in SEQ ID: 46 (Protein sequence of C terminal TEV His9 tag to VLM s-Ontak after signal sequence is cleaved) and is a good candidate for Nickel-column affinity purification with its His9 tag. The affinity purified VLM s-Ontak may then be exposed to small amounts of pure TEV protease, leading to enzymatic proteolysis that removes the 10 C-terminal residues GHHHHHHHHH, and releases mature, untagged VLM s-Ontak as is shown in SEQ ID: 30. Of note, this version of purified VLM s-Ontak (SEQ ID: 30) is 526 amino acids in length rather than 520 amino acids (SEQ ID 15) because it contains six
additional amino acids of the TEV protease recognition sequence (ENLYFQ fused to the usual C-terminus threonine of VLM s-Ontak (….IISTLT). The end result of this version of C-terminal His-tagged VLM s-Ontak with TEV cleavage site (SEQ ID: 30) is a C-terminal sequence …IISTLTENLYFQ.

Manufacturing method for VLM s-Ontak which include His-tags and TEV protease sites. The above three His-tag versions of VLM s-Ontak (N-terminal His\(_6\) tag with TEV protease site, C-terminal His\(_6\) tag without TEV protease site, and C-terminal His\(_9\) tag with TEV protease site) are examples of methods to use His-tag/Nickel column affinity chromatography in the manufacturing method of VLM s-Ontak. Because of secreted proteases from Corynebacterium diphtheriae C7 that are present in the culture supernatant, it is important to purify VLM s-Ontak away from other proteins in the culture supernatant rapidly in order to avoid significant loss of the desired product. The inclusion of His-tags and TEV protease sites represents a significant improvement and may enable a rapid, streamlined manufacturing process for VLM s-Ontak.

The allelic exchange substrates to knock out protease 1 and protease 2 are shown in SEQ ID: 34 and SEQ ID: 37, respectively. These sequences when inserted into pkI8mobsacB, a conjugative, mating plasmid with sacB counterselection (Schafer A, Tauch A, Jager W, Kalinowski J, Thierbach G, Piihler A (1994) Small mobilizable multi-purpose cloning vectors derived from the Escherichia coli plasmids pK18 and pK19: selection of defined deletions in the chromosome of Corynebacterium glutamicum. Gene 145:69-73. PMID: 8045426), lead to constructs which will knockout each protease. A recombinant Corynebacterium diphtheriae strain lacking both protease 1 and protease 2 will be a valuable production strain for future manufacturing methods to generate VLM s-Ontak.

**Protein Manufacturing Process of Diphtheria Toxin-based Fusion Proteins**

Using the DNA plasmids and expression vectors of the present invention, a novel process was discovered eliminating the problems associated with the conventional method of manufacturing Ontak®. Ontak® is currently expressed using a DNA vector in an E. coli expression system, c-denileukin diftitox or Ontak® is 521 amino acids in length and has a molecular weight of 58 kD. The conventional Ontak® manufacturing process results in the formation of Ontak® aggregates of heterogeneous molecular weight, residual DNA, and excessive residual detergent in the final formulation resulting in the FDA placing classic-Ontak® on clinical hold in June 2011. As observed in Fig. 8a, Ontak® is expressed from a plasmid in E. coli and results in insoluble, cytosolic Ontak® (protein) accumulations known as inclusion body forms. Using the process of the present invention, Fig. 8b illustrates the expression of s-denileukin diftitox as an extracellular mature secreted protein in a cell free supernatant that can be easily purified and results in higher protein yields as illustrated in Fig.
9. Fig. 9 shows both a Coomassie Blue stain for total protein and an anti-IL2 immunoblot of s-denileukin diftitox generated using the process of the present invention probed with anti-IL-2.

The novel process of the present invention comprises: 1) transforming bacteria, preferably a Corynebacterium diphtheria strain, with a DNA expression vector of the present invention, 2) forming a transformant; 3) incubating the transformant in a culture medium for a period of time to allow growth and expression of a protein (such as a diphtheria toxin-based fusion protein and CRM typically containing a signal peptide), 4) secretion of the protein into the culture medium (due to a signal peptide attached to the protein); and (8) purifying the diphtheria toxin-based fusion protein from the culture medium. The DNA expression vectors include a ToxP and mutant ToxO that regulate the expression of at least one protein, such as a diphtheria toxin fusion protein, CRM protein, or other protein that may be attached to a signal peptide of the present invention.

**Therapeutic Applications of Diphtheria Toxin-based Fusion Proteins of the Present Invention**

Clinical efficacy of Ontak® has been demonstrated in cutaneous T cell lymphoma, peripheral T cell lymphoma, steroid-refractory graft versus host disease, methotrexate-refractory psoriasis, and methotrexate-refractory rheumatoid arthritis. Clinical efficacy has also been demonstrated in malignant melanoma and ovarian carcinoma as shown in Fig. 14. The diphtheria toxin-based fusion proteins of the present invention (including s-denileukin diftitox, ms-denileuken diftitox, is-denileukin diftitox-VLM, ms-denileukin diftitox-VLM) produced by the methods of the present invention will perform similarly, or better, than Ontak® that is commercially available with regard to clinical efficacies of treating or preventing disease.
Treatment for tuberculosis.

As illustrated in Fig. 10, inventors of the present invention believe diphtheria toxin fusion proteins of the present invention will be active against tuberculosis. Denileukin diftitox is known to deplete IL-2-receptor (CD25+)-bearing cells including T regulatory (Tregs) cells. Tregs cells express CD25 as well as FoxP3 and are immunosuppressive by their inhibition of Teffector (Teff) cells. Teff cells such as CD4+ Helper (Th) cells and CD8+ cytotoxic T lymphocytes (CTLs) are needed within a tuberculous granuloma to contain the M. tuberculosis bacterial infection. During tuberculous infection, cellular lesions called granulomas form to contain the infection but are unable to fully eradicate the bacilli.

Regulatory T cells (Tregs) are recruited to granulomas, leading to suppression of effector T cell function, potentially contributing to a permissive environment for M. tuberculosis persistence and growth. The diphtheria toxin fusion proteins of the present invention are used to deplete Tregs, which express IL-2 receptor, in order to ameliorate immune suppression by these cells during TB infection. Figure 11 illustrates diphtheria fusion proteins used in the in vivo treatment of subjects (mice) with M. tuberculosis. Mice were infected with M. tb. strain H37Rv by aerosol infection giving an initial implantation of -2.8 logio CFU counts in lungs on day 0. The groups of mice were treated with 750 ng of c-Ontak® intraperitoneally (IP) or intravenously (IV) as one treatment cycle (lx, dosed at week 2 post-infection) or two treatment cycles (2x, dosed at -day 3 pre-infection and week 2 post-infection). A treatment cycle of denileukin diftitox is defined as 35 mg/kg (750 ng for a typical mouse) given two times, two days apart. RHZ daily treatment by oral gavage was started at week 2. R is rifampin and was given to mice at 10 mg/kg. H is isoniazid and was
given to mice at 10 mg/kg. Z is pyrazinamide and was given to mice at 150 mg/kg. The outcome of this study is illustrated in Figs. 12 and 13.

**Treatment for Cancer**

Tregs have also been shown to inhibit anti-tumor immunity, and the cellular expansion of Tregs in tumors generally correlates with poor prognosis in patients.

Denileukin diftitox treatment in melanoma patients resulted in transient depletion of Tregs and increased 1 year median overall survival. s-denileukin diftitox and s-denileukin diftitox-VLM of the present invention will be used to deplete Tregs in patients with tumors heavily infiltrated with Tregs as a cancer immunotherapy.

All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

The use of the terms "a" and "an" and "the" and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms "comprising," "having," "including," and "containing" are to be construed as open-ended terms (i.e., meaning "including, but not limited to," ) unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or
otherwise clearly contradicted by context. The use of any and all examples, or exemplary
language (e.g., "such as") provided herein, is intended merely to better illuminate the
invention and does not pose a limitation on the scope of the invention unless otherwise
claimed. No language in the specification should be construed as indicating any non-claimed
element as essential to the practice of the invention.

Preferred embodiments of this invention are described herein, including the best mode
for carrying out the invention. Variations of those preferred embodiments may become
apparent to those of ordinary skill in the art upon reading the foregoing description. The
inventors expect skilled artisans to employ such variations as appropriate, and the inventors
intend for the invention to be practiced otherwise than as specifically described herein.

Accordingly, this invention includes all modifications and equivalents of the subject matter
recited in the claims appended hereto as permitted by applicable law. Moreover, any
combination of the described elements of the invention in all possible variations thereof is
encompassed by the invention unless otherwise indicated herein or otherwise clearly
contradicted by context.

Embodiments of the disclosure concern methods and/or compositions for treating
and/or preventing disorders such as cancer and tuberculosis in which a subject is
administered a composition of the present invention comprising a nucleic acid or protein
sequence such as anyone of SEQ ID NOs: 11-15, or fusion proteins thereof.

An individual known to having disease such as cancer and/or tuberculosis, suspected
of having such a disease, or at risk for having such a disease may be provided an effective
amount of a composition of the present invention comprising a nucleic acid or protein
sequence such as anyone of SEQ ID NOs: 11-15, or fusion proteins thereof. Those at risk for
cancer or tuberculosis may be those individuals having one or more genetic factors, may be of advancing age, and/or may have a family history, for example.

In particular embodiments of the disclosure, an individual is given an agent for cancer and/or tuberculosis therapy in addition to a composition of the present invention comprising a nucleic acid or protein sequence such as anyone of SEQ ID NOs: 11-15, or fusion proteins thereof. Such additional therapy may include chemotherapy or antimicrobial agents, for example. When combination therapy is employed with a composition of the present invention comprising a nucleic acid or protein sequence such as anyone of SEQ ID NOs: 11-15, or fusion proteins thereof, the additional therapy may be given prior to, at the same time as, and/or subsequent to a composition of the present invention comprising a nucleic acid or protein sequence such as anyone of SEQ ID NOs: 11-15, or fusion proteins thereof.

Pharmaceutical Preparations

Pharmaceutical compositions of the present invention comprise an effective amount of one or more composition of the present invention comprising a nucleic acid or protein sequence such as anyone of SEQ ID NOs: 11-15, or fusion proteins thereof, dissolved or dispersed in a pharmaceutically acceptable carrier. The phrases "pharmaceutical or pharmacologically acceptable" refers to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, such as, for example, a human, as appropriate. The preparation of a pharmaceutical composition that comprises at least one composition of the present invention comprising a nucleic acid or protein sequence such as anyone of SEQ ID NOs: 11-15, or fusion proteins thereof, or additional active ingredient will be known to those of skill in the art in light of the present disclosure, as exemplified by Remington: The Science and Practice of Pharmacy, 21st Ed.
Lippincott Williams and Wilkins, 2005, incorporated herein by reference. Moreover, for animal (e.g., human) administration, it will be understood that preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biological Standards.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, surfactants, antioxidants, preservatives (e.g., antibacterial agents, antifungal agents), isotonic agents, absorption delaying agents, salts, preservatives, drugs, drug stabilizers, gels, binders, excipients, disintegration agents, lubricants, sweetening agents, flavoring agents, dyes, such like materials and combinations thereof, as would be known to one of ordinary skill in the art (see, for example, Remington's Pharmaceutical Sciences, 18th Ed. Mack Printing Company, 1990, pp. 1289-1329, incorporated herein by reference).

Except insofar as any conventional carrier is incompatible with the active ingredient, its use in the pharmaceutical compositions is contemplated.

The one or more compositions of the present invention comprising a nucleic acid or protein sequence such as anyone of SEQ ID NOs: 11-15, or fusion proteins thereof, may comprise different types of carriers depending on whether it is to be administered in solid, liquid or aerosol form, and whether it need to be sterile for such routes of administration as injection. The present compositions can be administered intravenously, intradermally, transdermally, intrathecally, intraarterially, intraperitoneally, intranasally, intravaginally, intrarectally, topically, intramuscularly, subcutaneously, mucosally, orally, topically, locally, inhalation (e.g., aerosol inhalation), injection, infusion, continuous infusion, localized perfusion bathing target cells directly, via a catheter, via a lavage, in cremes, in lipid compositions (e.g., liposomes), or by other method or any combination of the forgoing as
would be known to one of ordinary skill in the art (see, for example, Remington's Pharmaceutical Sciences, 18th Ed. Mack Printing Company, 1990, incorporated herein by reference).

The one or more compositions of the present invention comprising a nucleic acid or protein sequence such as anyone of SEQ ID NOs: 11-15, or fusion proteins thereof, may be formulated into a composition in a free base, neutral or salt form. Pharmaceutically acceptable salts, include the acid addition salts, e.g., those formed with the free amino groups of a proteinaceous composition, or which are formed with inorganic acids such as for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric or mandelic acid. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as for example, sodium, potassium, ammonium, calcium or ferric hydroxides; or such organic bases as isopropylamine, trimethylamine, histidine or procaine. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as formulated for parenteral administrations such as injectable solutions, or aerosols for delivery to the lungs, or formulated for alimentary administrations such as drug release capsules and the like.

Further in accordance with the present disclosure, the composition of the present invention suitable for administration is provided in a pharmaceutically acceptable carrier with or without an inert diluent. The carrier should be assimilable and includes liquid, semi-solid, i.e., pastes, or solid carriers. Except insofar as any conventional media, agent, diluent or carrier is detrimental to the recipient or to the therapeutic effectiveness of a composition contained therein, its use in administrable composition for use in practicing the methods of
the present invention is appropriate. Examples of carriers or diluents include fats, oils, water, saline solutions, lipids, liposomes, resins, binders, fillers and the like, or combinations thereof. The composition may also comprise various antioxidants to retard oxidation of one or more component. Additionally, the prevention of the action of microorganisms can be brought about by preservatives such as various antibacterial and antifungal agents, including but not limited to parabens (e.g., methylparabens, propylparabens), chlorobutanol, phenol, sorbic acid, thimerosal or combinations thereof.

In accordance with the present invention, the composition is combined with the carrier in any convenient and practical manner, i.e., by solution, suspension, emulsification, admixture, encapsulation, absorption and the like. Such procedures are routine for those skilled in the art.

In a specific embodiment of the present invention, the composition is combined or mixed thoroughly with a semi-solid or solid carrier. The mixing can be carried out in any convenient manner such as grinding. Stabilizing agents can be also added in the mixing process in order to protect the composition from loss of therapeutic activity, i.e., denaturation in the stomach. Examples of stabilizers for use in an the composition include buffers, amino acids such as glycine and lysine, carbohydrates such as dextrose, mannose, galactose, fructose, lactose, sucrose, maltose, sorbitol, mannitol, etc.

In further embodiments, the present invention may concern the use of a pharmaceutical lipid vehicle compositions that include one or more composition of the present invention comprising a nucleic acid or protein sequence such as anyone of SEQ ID NOs: 11-15, or fusion proteins thereof, one or more lipids, and an aqueous solvent. As used herein, the term "lipid" will be defined to include any of a broad range of substances that is
characteristically insoluble in water and extractable with an organic solvent. This broad class of compounds are well known to those of skill in the art, and as the term "lipid" is used herein, it is not limited to any particular structure. Examples include compounds which contain long-chain aliphatic hydrocarbons and their derivatives. A lipid may be naturally occurring or synthetic (i.e., designed or produced by man). However, a lipid is usually a biological substance. Biological lipids are well known in the art, and include for example, neutral fats, phospholipids, phosphoglycerides, steroids, terpenes, lysolipids, glycosphingolipids, glycolipids, sulphatides, lipids with ether and ester-linked fatty acids and polymerizable lipids, and combinations thereof. Of course, compounds other than those specifically described herein that are understood by one of skill in the art as lipids are also encompassed by the compositions and methods of the present invention.

One of ordinary skill in the art would be familiar with the range of techniques that can be employed for dispersing a composition in a lipid vehicle. For example, the one or more compositions of the present invention comprising a nucleic acid or protein sequence such as anyone of SEQ ID NOs: 11-15, or fusion proteins thereof may be dispersed in a solution containing a lipid, dissolved with a lipid, emulsified with a lipid, mixed with a lipid, combined with a lipid, covalently bonded to a lipid, contained as a suspension in a lipid, contained or complexed with a micelle or liposome, or otherwise associated with a lipid or lipid structure by any means known to those of ordinary skill in the art. The dispersion may or may not result in the formation of liposomes.

The actual dosage amount of a composition of the present invention administered to an animal patient can be determined by physical and physiological factors such as body weight, severity of condition, the type of disease being treated, previous or concurrent
therapeutic interventions, idiopathy of the patient and on the route of administration. Depending upon the dosage and the route of administration, the number of administrations of a preferred dosage and/or an effective amount may vary according to the response of the subject. The practitioner responsible for administration will, in any event, determine the concentration of active ingredient(s) in a composition and appropriate dose(s) for the individual subject.

In certain embodiments, pharmaceutical compositions may comprise, for example, at least about 0.1% of an active compound. In other embodiments, an active compound may comprise between about 2% to about 75% of the weight of the unit, or between about 25% to about 60%, for example, and any range derivable therein. Naturally, the amount of active compound(s) in each therapeutically useful composition may be prepared is such a way that a suitable dosage will be obtained in any given unit dose of the compound. Factors such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological considerations will be contemplated by one skilled in the art of preparing such pharmaceutical formulations, and as such, a variety of dosages and treatment regimens may be desirable.

In other non-limiting examples, a dose may also comprise from about 1 microgram/kg/body weight, about 5 microgram/kg/body weight, about 10 microgram/kg/body weight, about 50 microgram/kg/body weight, about 100 microgram/kg/body weight, about 200 microgram/kg/body weight, about 350 microgram/kg/body weight, about 500 microgram/kg/body weight, about 1 milligram/kg/body weight, about 5 milligram/kg/body weight, about 10 milligram/kg/body weight, about 100 milligram/kg/body weight, about 200
milligram/kg/body weight, about 350 milligram/kg/body weight, about 500 milligram/kg/body weight, to about 1000 mg/kg/body weight or more per administration, and any range derivable therein. In non-limiting examples of a derivable range from the numbers listed herein, a range of about 5 mg/kg/body weight to about 100 mg/kg/body weight, about 5 microgram/kg/body weight to about 500 milligram/kg/body weight, etc., can be administered, based on the numbers described above.

**Alimentary Compositions and Formulations**

In one embodiment of the present disclosure, the one or more compositions of the present invention comprising a nucleic acid or protein sequence such as anyone of SEQ ID NOs: 11-15, or fusion proteins thereof, are formulated to be administered *via* an alimentary route. Alimentary routes include all possible routes of administration in which the composition is in direct contact with the alimentary tract. Specifically, the pharmaceutical compositions disclosed herein may be administered orally, buccally, rectally, or sublingually. As such, these compositions may be formulated with an inert diluent or with an assimilable edible carrier, or they may be enclosed in hard- or soft- shell gelatin capsule, or they may be compressed into tablets, or they may be incorporated directly with the food of the diet.

In certain embodiments, the active compounds may be incorporated with excipients and used in the form of ingestible tablets, buccal tables, troches, capsules, elixirs, suspensions, syrups, wafers, and the like (Mathiowitz et al, 1997; Hwang et al, 1998; U.S. Pat. Nos. 5,641,515; 5,580,579 and 5,792,451, each specifically incorporated herein by reference in its entirety). The tablets, troches, pills, capsules and the like may also contain the following: a binder, such as, for example, gum tragacanth, acacia, cornstarch, gelatin or combinations thereof; an excipient, such as, for example, dicalcium phosphate, mannitol,
lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate or combinations thereof; a disintegrating agent, such as, for example, corn starch, potato starch, alginic acid or combinations thereof; a lubricant, such as, for example, magnesium stearate; a sweetening agent, such as, for example, sucrose, lactose, saccharin or combinations thereof; a flavoring agent, such as, for example peppermint, oil of wintergreen, cherry flavoring, orange flavoring, etc. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar, or both. When the dosage form is a capsule, it may contain, in addition to materials of the above type, carriers such as a liquid carrier. Gelatin capsules, tablets, or pills may be enterically coated. Enteric coatings prevent denaturation of the composition in the stomach or upper bowel where the pH is acidic. See, e.g., U.S. Pat. No. 5,629,001. Upon reaching the small intestines, the basic pH therein dissolves the coating and permits the composition to be released and absorbed by specialized cells, e.g., epithelial enterocytes and Peyer's patch M cells. A syrup of elixir may contain the active compound sucrose as a sweetening agent methyl and propylparabens as preservatives, a dye and flavoring, such as cherry or orange flavor. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compounds may be incorporated into sustained-release preparation and formulations.

For oral administration the compositions of the present disclosure may alternatively be incorporated with one or more excipients in the form of a mouthwash, dentifrice, buccal tablet, oral spray, or sublingual orally- administered formulation. For example, a mouthwash
may be prepared incorporating the active ingredient in the required amount in an appropriate solvent, such as a sodium borate solution (Dobell's Solution). Alternatively, the active ingredient may be incorporated into an oral solution such as one containing sodium borate, glycerin and potassium bicarbonate, or dispersed in a dentifrice, or added in a therapeutically-effective amount to a composition that may include water, binders, abrasives, flavoring agents, foaming agents, and humectants. Alternatively the compositions may be fashioned into a tablet or solution form that may be placed under the tongue or otherwise dissolved in the mouth.

Additional formulations which are suitable for other modes of alimentary administration include suppositories. Suppositories are solid dosage forms of various weights and shapes, usually medicated, for insertion into the rectum. After insertion, suppositories soften, melt or dissolve in the cavity fluids. In general, for suppositories, traditional carriers may include, for example, polyalkylene glycols, triglycerides or combinations thereof. In certain embodiments, suppositories may be formed from mixtures containing, for example, the active ingredient in the range of about 0.5% to about 10%, and preferably about 1% to about 2%.

**Parenteral Compositions and Formulations**

In further embodiments, one or more composition of the present invention comprising a nucleic acid or protein sequence such as anyone of SEQ ID NOs: 11-15, or fusion proteins thereof, may be administered via a parenteral route. As used herein, the term "parenteral" includes routes that bypass the alimentary tract. Specifically, the pharmaceutical compositions disclosed herein may be administered for example, but not limited to intravenously, intradermally, intramuscularly, intraarterially, intrathecally, subcutaneous, or
intraperitoneally U.S. Pat. Nos. 6,753,751, 6,613,308, 5,466,468, 5,543,158; 5,641,515; and 5,399,363 (each specifically incorporated herein by reference in its entirety).

Solutions of the active compounds as free base or pharmacologically acceptable salts may be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions may also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms. The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions (U.S. Patent 5,466,468, specifically incorporated herein by reference in its entirety). In all cases the form must be sterile and must be fluid to the extent that easy injectability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (i.e., glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and/or vegetable oils. Proper fluidity may be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.
For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous, and intraperitoneal administration. In this connection, sterile aqueous media that can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage may be dissolved in isotonic NaCl solution and either added hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologies standards.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. A powdered composition is combined with a liquid carrier such as, e.g., water or a saline solution, with or without a stabilizing agent.
Miscellaneous Pharmaceutical Compositions and Formulations

In other preferred embodiments of the invention, the one or more compositions of the present invention comprising a nucleic acid or protein sequence such as anyone of SEQ ID NOs: 11-15, or fusion proteins thereof, may be formulated for administration via various miscellaneous routes, for example, topical (i.e., transdermal) administration, mucosal administration (intranasal, vaginal, etc.) and/or inhalation.

Pharmaceutical compositions for topical administration may include the active compound formulated for a medicated application such as an ointment, paste, cream or powder. Ointments include all oleaginous, adsorption, emulsion and water-soluble based compositions for topical application, while creams and lotions are those compositions that include an emulsion base only. Topically administered medications may contain a penetration enhancer to facilitate adsorption of the active ingredients through the skin. Suitable penetration enhancers include glycerin, alcohols, alkyl methyl sulfoxides, pyrrolidones and luarocapram. Possible bases for compositions for topical application include polyethylene glycol, lanolin, cold cream and petrolatum as well as any other suitable absorption, emulsion or water-soluble ointment base. Topical preparations may also include emulsifiers, gelling agents, and antimicrobial preservatives as necessary to preserve the active ingredient and provide for a homogenous mixture. Transdermal administration of the present invention may also comprise the use of a "patch". For example, the patch may supply one or more active substances at a predetermined rate and in a continuous manner over a fixed period of time.

In certain embodiments, the pharmaceutical compositions may be delivered by eye drops, intranasal sprays, inhalation, and/or other aerosol delivery vehicles. Methods for
delivering compositions directly to the lungs via nasal aerosol sprays has been described e.g., in U.S. Pat. Nos. 5,756,353 and 5,804,212 (each specifically incorporated herein by reference in its entirety). Likewise, the delivery of drugs using intranasal microparticle resins (Takenaga et al, 1998) and lysophosphatidyl-glycerol compounds (U.S. Pat. No. 5,725,871, specifically incorporated herein by reference in its entirety) are also well-known in the pharmaceutical arts. Likewise, transmucosal drug delivery in the form of a polytetrafluoroethylene support matrix is described in U.S. Pat. No. 5,780,045 (specifically incorporated herein by reference in its entirety).

The term aerosol refers to a colloidal system of finely divided solid of liquid particles dispersed in a liquefied or pressurized gas propellant. The typical aerosol of the present invention for inhalation will consist of a suspension of active ingredients in liquid propellant or a mixture of liquid propellant and a suitable solvent. Suitable propellants include hydrocarbons and hydrocarbon ethers. Suitable containers will vary according to the pressure requirements of the propellant. Administration of the aerosol will vary according to subject's age, weight and the severity and response of the symptoms.

Kits of the Disclosure

Any of the compositions described herein may be comprised in a kit. In a non-limiting example, one or more composition of the present invention comprising a nucleic acid or protein sequence such as anyone of SEQ ID NOs: 11-15, or fusion proteins thereof, may be comprised in a kit.

The kits may comprise a suitably aliquoted of one or more compositions of the present invention comprising a nucleic acid or protein sequence such as anyone of SEQ ID NOs: 11-15, or fusion proteins thereof, and in some cases, one or more additional agents.
The component(s) of the kits may be packaged either in aqueous media or in lyophilized form. The container means of the kits will generally include at least one vial, test tube, flask, bottle, syringe or other container means, into which a component may be placed, and preferably, suitably aliquoted. Where there are more than one component in the kit, the kit also will generally contain a second, third or other additional container into which the additional components may be separately placed. However, various combinations of components may be comprised in a vial. The kits of the present invention also will typically include a means for containing the one or more compositions of the present invention comprising a nucleic acid or protein sequence such as anyone of SEQ ID NOs: 11-15, or fusion proteins thereof, and any other reagent containers in close confinement for commercial sale. Such containers may include injection or blow-molded plastic containers into which the desired vials are retained.

When the components of the kit are provided in one and/or more liquid solutions, the liquid solution is an aqueous solution, with a sterile aqueous solution being particularly preferred. The one or more compositions of the present invention comprising a nucleic acid or protein sequence such as anyone of SEQ ID NOs: 11-15, or fusion proteins thereof, may be formulated into a syringeable composition. In which case, the container means may itself be a syringe, pipette, and/or other such like apparatus, from which the formulation may be applied to an infected area of the body, injected into an animal, and/or even applied to and/or mixed with the other components of the kit.

However, the components of the kit may be provided as dried powder(s). When reagents and/or components are provided as a dry powder, the powder can be reconstituted by
the addition of a suitable solvent. It is envisioned that the solvent may also be provided in another container means.
Claims:

1. A DNA expression vector comprising:
   a. a toxP;
   b. a mutant toxO that blocks Fe-mediated regulation of gene expression; and
   c. a DNA sequence encoding a protein,

   wherein the toxP and the mutant toxO regulate expression of the DNA segment encoding the protein.

2. The DNA expression vector of claim 1 further comprising a DNA sequence encoding a signal peptide.

3. The DNA expression vector of claim 2 wherein the protein is attached to the signal peptide.

4. The DNA expression vector of claim 1 wherein the mutant toxO is SEQ ID NO: 1.

5. The DNA expression vector of claim 2 wherein the signal peptide is SEQ ID NO: 5.

6. The DNA expression vector of claim 1 wherein the protein is selected from the group consisting of CRM 197, CRM 107, or a combination thereof.

7. The DNA expression vector of claim 6 wherein the protein is selected from any one of SEQ ID NOs: 11, 7 to 20.

8. The DNA expression vector of claim 1 wherein the protein comprises a diphtheria toxin, or functional part thereof, attached to a receptor binding protein, or a functional part thereof.
9. The DNA expression vector of claim 8 wherein the receptor binding protein is selected from the group comprising IL-2, IL-3, IL-4, IL-6, IL-7, IL-15, EGF, FGF, substance P, CD4, aMSH, GRP, TT fragment C, GCSF, heregulin βÎ, a functional part thereof, or a combination thereof.

10. The DNA expression vector of claim 8 wherein the protein is selected from any one of SEQ ID NOs: 12-15.

11. A DNA expression vector comprising:
   a. a toxP;
   b. a mutant toxO that blocks Fe-mediated regulation of gene expression;
   c. a DNA sequence encoding a protein comprising a
      i. signal sequence;
      ii. a diphtheria toxin, or a functional part thereof, that is free of a diphtheria receptor binding domain or has a non-functional diphtheria toxin receptor binding domain, and
      iii. a target receptor binding domain selected from the group comprising IL-2, IL-3, IL-4, IL-6, IL-7, IL-15, EGF, FGF, substance P, CD4, aMSH, GRP, TT fragment C, GCSF, heregulin βÎ, a functional part thereof, or a combination thereof,

wherein the toxP and the mutant toxO regulate expression of the DNA sequence encoding the protein.

12. The DNA expression vector of claim 11 wherein a bacteria transformed with the DNA expression vector produces a diphtheria toxin receptor binding fusion protein attached to a signal peptide that is directed to a periplasm, a culture medium, or both locations by the signal peptide.
13. The DNA expression vector of claim 12 where the bacteria is *E. coli* and the
signal peptide directs the diphtheria toxin receptor binding fusion protein to the
periplasm.

14. The DNA expression vector of claim 12 where the bacteria is *Corynebacterium
diphtheria* and the signal peptide directs the diphtheria toxin receptor binding
fusion protein to the culture medium.

15. The DNA expression vector of claim 11 comprising SEQ ID NO: 3

16. The DNA expression vector of claim 12 further comprising a DNA encoding a
cleavable protein tag.

17. The DNA expression vector of claim 12 wherein the cleavable protein tag is
attached to the diphtheria toxin receptor binding fusion protein.

18. The DNA vector of claim 11 wherein the diphtheria toxin receptor binding fusion
protein is selected from anyone of SEQ ID Nos: 12 to 15.

19. A method for producing aggregate-free monomeric diphtheria toxin fusion
proteins comprising the following steps:

   a. transforming bacteria with a DNA expression of claims 1-18 vector;
   b. forming a transformant;
   c. incubating the transformant in a culture medium to allow expression of a
      protein that is secreted into the culture medium; and
   d. purifying the protein from the culture medium.

20. The method of claim 19 wherein the bacteria is *Corynebacterium diphtheria*.

21. A method for producing aggregate-free monomeric diphtheria toxin fusion
proteins comprising the following steps:
a. transforming *Corynebacterium diphtheria* strain with a DNA vector comprising:

i. a toxP;

ii. a mutant toxO that blocks Fe-mediated regulation of gene expression;

iii. a DNA sequence encoding a protein comprising:

a. signal peptide;

b. a diphtheria toxin, or a functional part thereof, that is free of a diphtheria receptor binding domain or has a non-functional diphtheria toxin receptor binding domain; and

c. a target receptor binding domain selected from the group comprising IL-2, IL-3, IL-4, IL-6, IL-7, IL-15, EGF, FGF, substance P, CD4, aMSH, GRP, TT fragment C, GCSF, heregulin β1, TNFa, TGFp, a functional part thereof, or a combination thereof, wherein the toxP and the mutant toxO regulate expression of the DNA sequence encoding the protein;

b. forming a transformant;

c. incubating the transformant in a culture medium to allow expression of the protein and that is secreted into the culture medium; and

d. purifying the diphtheria toxin fusion protein from the culture medium.

22. The method of claim 21 wherein the diphtheria toxin receptor fusion protein is selected from the any one of SEQ ID NOs: 12 to 15.
23. The method of claim 21 wherein the *Corynebacterium diphtheria* strain is *Corynebacterium C7* beta (-), tox (-).

24. A method of treating a patient with *tuberculosis* comprising the following steps:
   a. preparing a diphtheria toxin fusion protein as provided in claim 21;
   b. administering the diphtheria toxin fusion protein to a patient with tuberculosis.

25. A DNA expression vector comprising a mutant toxO promoter.

26. A *Corynebacterium diphtheria* strain containing a DNA expression vector of claims 1 through 18.

27. A method of making a protein comprising the following steps:
   a. providing a DNA expression vector comprising a toxP, a mutant toxO that blocks Fe-mediated regulation of gene expression, a signal sequence, and a DNA sequence encoding a protein;
   b. transforming a bacteria strain with the DNA vector to form a transformant;
   c. incubating the transformant in a culture medium for a period of time to allow expression of a protein that is secreted into the culture medium; and
   d. purifying the protein from the culture medium.

28. A fusion protein selected from any one of SEQ ID NOs: 12-15.


30. A pharmaceutical composition comprising a fusion protein of Claim 28, and at least one or more other chemotherapy agents.

31. The pharmaceutical composition of claim 30, wherein the other chemotherapy agents is selected from the group consisting from isoniazid, rifampin, rifabutin,
rifapentine, pyrazinamide, ethambutol, streptomycin, amikacin, kanamycin,
ethionamide, protonamide, terizidone, thiacetzone, cycloserine, capreomycin,
para-amino salicylic acid (PAS), viomycin, ofloxacin, ciprofloxacin, levofloxacin,
moxifloxacin, bedaquiline, or delamanid, linezolid, tezolid, amoxicillin-
clavulanic acid, meropenem, imipenem, clarithromycin or clofazimine.

32. A pharmaceutical composition of Claim 28, and at least one or more other
antimicrobial agents.

33. The pharmaceutical composition of claim 33, wherein the antimicrobial agents are
selected from the group consisting of isoniazid, rifampin, rifabutin, rifapentine,
pyrazinamide, ethambutol, streptomycin, amikacin, kanamycin, ethionamide,
protonamide, terizidone, thiacetzone, cycloserine, capreomycin, para-amino
salicylic acid (PAS), viomycin, ofloxacin, ciprofloxacin, levofloxacin,
moxifloxacin, bedaquiline, or delamanid, linezolid, tezolid, amoxicillin-
clavulanic acid, meropenem, imipenem, clarithromycin, or clofazimine.

34. A method of treating or preventing cancer in a subject comprising adminstering
to the subject an effective amount of a pharmaceutical composition comprising a
fusion protein selected from any one of SEQ ID NOs: 12-15.

35. A method of treating or preventing tuberculosis in a subject comprising
administering to the subject an effective amount of a pharmaceutical composition
comprising a fusion protein selected from any one of SEQ ID NOs: 11-15.

36. A prokaryotic cell line comprising a DNA expression vector of any one of claims
1-18.

37. A kit comprising the DNA expression vector of any of claims 1 through 18.

38. A tox P comprising SEQ ID NO: 2.

39. A protein of any one of SEQ ID NOs: 12 to 15
FIG. 1  *toxO* sequence and mutations

a) Mutant *toxO* in *this invention report*:

TTAGGATAGCTAAGTCCAT  (altered bases shown in red)

b) Wild type *toxO*

TTAGGATAGCTTTACCTAA  19 bp imperfect palindrome around the large C
FIG. 2 Addition of the tox promoter, mutant tox operator, and signal sequence in pKN2.6Z-LC127

a) Classic denileukin difftitox (c-denileukin difftitox) expression vector

T7 promoter


Classic Ontak® (c-Ontak®): Ontak® expressed in inclusion body form in the cytosol of recombinant E. coli. Must be denatured and refolded in the presence of Tween 20 to be active.

b) Secreted denileukin difftitox (s-denileukin difftitox) expression vector


gsignal sequence

Secreted Ontak® (s-Ontak®): expressed as a secreted protein (i) into the periplasm of recombinant E. coli, or (ii) into the spent culture medium of recombinant C. diphtheriae, C7s(-)tox-, as a fully folded biologically active protein. The red slash indicates that the toxO operator sequence has been mutated to prevent DtxR repressor binding. The mutant toxO enables constitutive, iron-independent expression of the downstream gene.
FIG. 3 c-denileukin diftitox-VLM shows similar activity to c-denileukin diftitox

c-denileukin diftitox-VLM has a potency equivalent to c-denileukin diftitox for killing IL-2R bearing cells

Cell Toxicity Assay

$IC_{50}$s are within experimental error

Fusion Protein Toxin Concentration (M)

% control $[^{14}C]$-leucine incorporation

1.0 x 10^{-13} 1.0 x 10^{-12} 1.0 x 10^{-11} 1.0 x 10^{-10} 1.0 x 10^{-9} 1.0 x 10^{-8} 1.0 x 10^{-7}

- c-denileukin diftitox
- c-denileukin diftitox
FIG. 4 c-denileukin diftitox-VLM: decreased vascular leak

c-denileukin diftitox-VLM does not cause vascular leakage in vitro

In Vitro Vascular Leak Assay

How it works:

Measure the ability of the fluorescent marker to move from inner to outer chamber in the presence of drug.
FIG. 5 c-denileukin diftitox-VLM: Safety and Tolerability

c-denileukin diftitox-VLM has significantly less acute toxicity in vivo than c-denileukin diftitox

Mouse Toxicity Assay

Survival

Day 1  Day 2  Day 3  Day 4  Day 5  Day 6  Day 7  Day 8

DOSING

c-Ontak® (10 μg/d i.p.)
c-Ontak - VLM (10 μg/d i.p.)
c-Ontak - VLM (20 μg/d i.p.)

100% 90% 80% 70% 60% 50% 40% 30% 20% 10% 0%

c-denileukin diftitox-VLM 100% survival
FIG. 6 Diphtheria toxin-based fusion protein toxin platform technology

DNA encoding native diphtheria toxin

Catalytic domain

Transmembrane domain

Receptor binding domain

DNA encoding a surrogate receptor binding domain

-IL-2
-IL-3
-IL-4
-IL-7
-IL-15

-EGF
-FGF
-substance P
-CD40

Gene encoding a diphtheria toxin-related fusion protein toxin
FIG. 7 Construct to express s-denileukin difftox from *Corynebacterium diphtheriae* as a secreted protein into the culture medium in an iron-independent manner

---

**Novel synthetic, mutant toxO operator (iron independent)**

**WT toxP promoter from *C. diphtheriae***

**Recombinant gene encoding secreted form of (s-denileukin difftox)**
FIG. 8: Addressing the problems of inclusion body formation, denaturation, and refolding required for c-denileukin diftitox production

Ontak® is expressed in the *E. coli* cytoplasm and forms inclusion bodies; these must be denatured and refolded. Residual DNA, detergent, and protein aggregates are a limitation. Ontak® is 521 amino acids in length. Blue ovals indicate the gram-negative envelope of *E. coli*. fMGADD represent the first 5 amino acids of the N-terminus of Ontak®.

s-denileukin diftitox is expressed by *C. diphtheriae* as a secreted protein into the cell free culture supernatant fluid in biologically active form. Mature s-denileukin diftitox is 520 amino acids in length. Red oval indicates the gram-positive envelop of *C. diphtheriae*. GADD represent the first 4 amino acids of the N-terminus of s-denileukin diftitox of the present invention.
FIG. 9 s-denilekin diftitox: expressed as a secreted protein into the culture medium of Corynebacterium diphtheriae strain C7(-) tox-

1: Protein size ladder
2: Coomassie blue stain
3. anti-IL-2 Western blot

supernatant was concentrated 10x and 23 microliters of concentrate was loaded onto the gel
FIG. 10 Ontak® (DAB_{389} IL-2) is expected to deplete IL-2R-bearing (CD25+) T cells (T_{reg}) within the tuberculous granuloma. T_{reg} cells are immunosuppressive by their inhibition of T_{eff} cells.

A
Foamy cell

DC

B

Cytokines
Chemokines

Cytokines
Chemokines

NOS2/ARG1

MDSC

D

Lymphocytes

IL-4R

IL-2R

IL-2R

IL-2R
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<th>Week 0</th>
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<td>Grp 2. Ont-2x IP</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
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<tr>
<td>Grp 3. Ont-2x IV</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
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<td>5</td>
</tr>
<tr>
<td>Grp 4. Ont-1x IP</td>
<td>5</td>
<td>5</td>
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<tr>
<td>Grp 5. RHZ</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Grp 6. RHZ + Ont-1x IP</td>
<td>5</td>
<td>5</td>
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</table>
FIG. 12 Ontak to treat TB. Mouse TB Model
Lung CFUs during treatment with different ONTAK regimens

Ontak 1x dosing:
Ontak 2x dosing:

Grp 1. No treatment
Grp 2. Ont-2x IP
Grp 3. Ont-2x IV
Grp 4. Ont-1x IP
Grp 5. RHZ
Grp 6. RHZ + Ont-1x IP

Start of daily oral RHZ treatment

Day 0  Week 2  Week 5

Treatment Duration

Lung CFUs
FIG 13. Ontak to treat TB. Mouse TB Model
Lung CFUs during treatment with different c-denileukin diftitox regimens

Data from Figure 12 with just Group 1 (no treatment) and Group 4 (c-denileukin diftitox 1x or one treatment cycle as monotherapy IP at week 2 post-infection)

Fig. 3 Ontak activity in mouse model of tuberculosis. Mice were infected with M. tb on Day 0. Ontak treated group was given 2 IP injections of 35 µg/kg at day 11 and 13. Mice were sacrificed on day 14 and 35 and colony forming units from the lungs were enumerated.
FIG. 14 c-denileukin diftitox as an immunotherapeutic agent: Malignant Melanoma

1 cycle = 12 μg/kg QD x 4; rest 21 days
FIG. 15 Ontak production: His tag

N-terminal: His₆-TEV-VLM s-Ontak

C-terminal: His₆-VLM s-Ontak

CTerminal TEV-His₉-VLM s-Ontak
FIG 16. VLM s-Ontak:

Purification to >97% with C-terminal His6-tagged VLM s-Ontak

Coomassie-Blue stained SDS-PAGE

A. Molecular Weight Standards
B. purified VLM s-Ontak
### Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item Ic of the first sheet)

1. **With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:**
   
   a. **X** forming part of the international application as filed:
      
      - **X** in the form of an Annex C/ST.25 text file,
      - **X** on paper or in the form of an image file.
   
   b. **X** furnished together with the international application under PCX Rule 1(a) for purposes of international search only in the form of an Annex C/ST.25 text file.
   
   c. **☐** furnished subsequent to the international filing date for the purposes of international search only:
      
      - **☐** in the form of an Annex C/ST.25 text file (Rule 13(a)).
      - **☐** on paper or in the form of an image file (Rule 13(b) and Administrative Instructions, Section 73).

2. **☐** In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. **Additional comments:**
**INTERNATIONAL SEARCH REPORT**

**International application No.**
PCT/US 2017/021715

### A. CLASSIFICATION OF SUBJECT MATTER

(see extra sheet)

According to International Patent Classification (IPC) or to both national classification and IPC

### B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N 15/63, 1/21, 15/11, 15/62, 15/31, 15/77, A61K 38/00, C12P 21/00, C12R 1/16, 1/19

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

E-LIBRARY, EAPATIS, ESPACENET, RUPTO, USPTO, WIPO, PATSEARCH, Google Scholar, PubMed, NCBI, BLAST

### C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<tr>
<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<td>X</td>
<td>LEE JH. et al. Characterization of specific nucleotide substitutions in DtxR-specific operators of <em>Corynebacterium diphtheriae</em> that dramatically affect DtxR binding, operator function, and promoter strength. Journal of bacteriology, 2000, Vol. 182, no.2, p.432-438, especially abstract, p.432-434, Table 1</td>
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<td>BISHAI WR. et al. High-level expression of a proteolytic ally sensitive diphtheria toxin fragment in <em>Escherichia coli</em>. Journal of bacteriology, 1987, Vol. 169, no.11, p.5 140-5 151, especially abstract, p.5 140-5 141, Table 2</td>
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* Special categories of cited documents:
  - "X" document defining the general state of the art which is not considered to be of particular relevance
  - "Y" earlier document but published on or after the international filing date
  - "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  - "O" document referring to an oral disclosure, use, exhibition or other means
  - "P" document published prior to the international filing date but later than the priority date claimed
  - "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
  - "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
  - "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
  - "&" document member of the same patent family

**Date of the actual completion of the international search**
15 May 2017 (15.05.2017)

**Date of mailing of the international search report**
29 June 2017 (29.06.2017)

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**Telephone No.** 495 531 65 15

Form PCT/ISA/210 (second sheet) (January 2015)
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<td>Y</td>
<td>KOHANSKI MA. et al. How antibiotics kill bacteria: from targets to networks. Nature Reviews Microbiology, 2010, Vol.8, no.6, p.423-435, especially Table 1</td>
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