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Protein particles comprising disulfide crosslinkers and uses related thereto

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ABSTRACT
This disclosure relates to particles comprising recombinant proteins, pharmaceutical composition comprising the particles, and therapeutic uses related thereto. In certain embodiments, the particles are made by the process of producing recombinant proteins and conjugating the recombinant proteins to form nanoparticles with a linking reagent comprising disulfide bonds. Typically, the recombinant protein has a polypeptide of viral, fungal, or bacterial origin such as secreted effector proteins AvrA and YopJ. In certain embodiments, the disclosure relates to treating or preventing autoimmune diseases, cancer, or inflammatory diseases, or conditions such as inflammatory bowel disease (IBD).

9 Claims, 5 Drawing Sheets
Specification includes a Sequence Listing.
FIG. 1

A

Size Distribution by Volume

B

C

% GFP fluorescence loss

FIG. 2
FIG. 3
FIG. 4

FIG. 5
FIG. 6

Degradation in reducing environment

FIG. 7
PROTEIN PARTICLES COMPRISING DISULFIDE CROSSLINKERS AND USES RELATED THERETO

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a division of U.S. application Ser. No. 14/774,764 filed Sep. 11, 2015, which is the National Stage of International Application No. PCT/US2014/023175 filed Mar. 11, 2014, which claims the benefit of priority to U.S. Provisional Application No. 61/778,920 filed Mar. 13, 2013. The entirety of each of these applications is hereby incorporated by reference for all purposes.

INCORPORATION-BY-REFERENCE OF MATERIAL SUBMITTED AS A TEXT FILE VIA THE OFFICE ELECTRONIC FILING SYSTEM (EFS-WEB)

The Sequence Listing associated with this application is provided in text format in lieu of a paper copy, and is hereby incorporated by reference into the specification. The name of the text file containing the Sequence Listing is 12090USDIV_ST25.txt. The text file is 46 KB, was created on Apr. 5, 2018, and is being submitted electronically via EFS-Web.

BACKGROUND

Crohn’s disease and ulcerative colitis (Inflammatory bowel disease or IBD) are chronic relapsing disorders of the intestinal tract that may also have systemic manifestations. Symptoms can be debilitating and include abdominal pain and bloody diarrhea. These disorders are generally accepted to result from aberrant immune recognition of the normal commensal microbiota, often associated with an underlying genetic predisposition. They typically manifest with acute and chronic inflammation (granulomatous in the case of Crohn’s disease), tissue injury, scarring and predisposition to adenocarcinoma. Additionally, the intestine is the site of other inflammatory conditions including celiac disease, enteric infection and others. Current IBD therapy involves inflammatory suppression with local 5-aminosalicylates, systemic corticosteroids and/or immunosuppressants, or use of endogenous biologicals (anti-TNF monoclonal antibodies (Infliximab) and others). These approaches present complications of systemic immunosuppression and other toxicities. Thus, there is a need to identify improved therapeutic approaches for these diseases.

Traditionally, delivery of most IBD therapeutics has been accomplished by local enema, oral ingestion or systemic infusion. For biologics in particular, injection and oral administration are not ideal because they cannot survive serum proteases, the harsh environment of the gastrointestinal tract, or clearance processes in blood and tissue. Encapsulation of drugs into polymeric carriers can mitigate these limitations.

Nanoparticles have been investigated for a variety of gut applications including vaccination, diabetes, and IBD. Wilson et al., report orally delivered thiolated nanoparticles loaded with TNF-alpha siRNA target inflammation and inhibit gene expression in the intestines. See Nature Materials, 2010, 9(11): 923-928. Specific biological molecules have been immobilized on nanoparticle surfaces to direct uptake. Russell-Jones reports the use of targeting agents to increase uptake and localization of drugs to the intestinal epithelium. See Journal of Drug Targeting, 2004, 12(2):113-123.


References cited herein are not an admission of prior art.

SUMMARY

This disclosure relates to particles comprising recombinant proteins, pharmaceutical composition comprising the particles, and therapeutic uses related thereto. In certain embodiments, the particles are made by the process of producing recombinant proteins and conjugating the recombinant proteins to form nanoparticles with a linking reagent comprising disulfide bonds. In certain embodiments, the recombinant protein has a wild-type polypeptide sequence of a viral, fungal, or bacterial origin such as secreted effector proteins AvrA and YopJ. In certain embodiments, the disclosure relates to treating or preventing autoimmune diseases, cancer, or inflammatory diseases or conditions such as inflammatory bowel disease (IBD).

In certain embodiments, the disclosure relates to particles comprising a) recombinant proteins; and b) linking groups comprising disulfide bonds, wherein the linking groups conjugate the recombinant proteins to form a particle. In certain embodiments, the particles comprise a diameter of about between 50 and 100 nm or 25 and 200 nm or 10 and 500 nm. In certain embodiments, the recombinant protein comprises a viral or bacterial protein, fragment, or combination thereof, such as a secreted effector protein and a flagellin. In certain embodiments, the secreted effector protein is selected from AvrA, mAvrA(C186A), YopJ, VopA, or AopP. In certain embodiments, the secreted effector protein has greater than 10% sequence identity to SEQ ID NO: 1.

In certain embodiments, the recombinant secreted effector protein is a fusion comprising the secreted effector protein and an N-terminal or C-terminal polypeptide or amino acid with a primary amino group (e.g., —CH₂—NH₂). The free amino group may be the side chain of a lysine. Within a polypeptide the lysine may be separated from the secreted effector protein sequence by a linker such as a glycine or polyglycine. The N-terminal or C-terminal polypeptide may be a polypeptide of greater than two amino acids comprised of glycine, poly-glycine, proline, poly-proline, lysine, poly-lysine and combinations thereof.

In certain embodiments, the disclosure relates to producing particles disclosed herein by mixing recombinant proteins disclosed herein with a linking agent containing a disulfide that reacts with primary amine groups under conditions to form particles. Typically the linking reagent is 3,3-dithiobis(sulfosuccinimidyl propionate)(DTSSP). DTSSP contains two sulfo-N-hydroxysuccinimide esters that react with the primary amines in the side chain of lysines and the protein N-terminus.
In certain embodiments, the crosslinking reagent may be any containing a sulfide bond that reacts with primary amine, such as provided in the following formula:

\[ \text{L} \overset{(X)}{\text{O}} \text{S} \overset{(X)}{\text{S}} \overset{(X)}{\text{O}} \text{L} \]

or salts thereof wherein \( n \) is 1 to 100, typically \( n \) is 1 to 10, or 1 to 6 or 1 to 4. \( L \) is a leaving group which reacts with amines to form amides such as an acid anhydride ester, pentafluorophenol ester, thioester, N-hydroxy heterocyclyl ester, N-hydroxy succinimidy ester, N-hydroxy sulfosuccinimidy ester, sulfonic ester, or halogen; and \( X \) is at each occurrence individually and independently selected from \( O, \text{CH}_2, \text{OCH}_2, \text{CH}_2\text{O}, \text{NHCH}_2, \text{CH}_2\text{NH}, \text{OCH}_2\text{CH}_2, \text{CH}_2\text{CH}_2\text{O}, \text{NHCH}_2\text{CH}_2\text{O}, \) or \( \text{CH}_2\text{CH}_2\text{NH} \). Typically \( X = \text{CH}_3 \) wherein \( n \) is 1 to 4.

In certain embodiments, the recombinant protein comprises bovine serum albumin (BSA), green fluorescent protein (GFP), glutathione S-transferase (GST), polyhistidine, polylysine, or combinations thereof. In the case of a fusion protein with a secreted effector protein, the bovine serum albumin (BSA), green fluorescent protein (GFP), glutathione S-transferase (GST), polyhistidine, poly-lysine, or combinations may be on the N-terminal or C-terminal end. In such a case, the opposite end may contain polypeptide or amino acid with a primary amino group (e.g., \(-\text{CH}_3\text{NH}_2\)) or it may contain another protein such as a cell penetrating peptide.

In certain embodiments, the recombinant protein is a fusion comprising a protein of interest, e.g., secreted effector protein, and polypeptide or amino acid with a primary amino group (e.g., \(-\text{CH}_3\text{NH}_2\)) on the N-terminal and/or C-terminal end and a cell penetrating peptide on the opposite end, e.g., the polypeptide or amino acid with a primary amino group (e.g., \(-\text{CH}_3\text{NH}_2\)) is on the C-terminal end, and the cell penetrating peptide is on the N-terminal end. In any of these configurations, arbitrary linking polyptides or amino acids may be inserted between the protein of interest/secreted effector protein and the cell penetrating peptide, or linking polyptides/amino acids may be inserted between the protein of interest/secreted effector protein and the primary amino acid with a primary amino group (e.g., \(-\text{CH}_3\text{NH}_2\)). Contemplated linking polyptides or amino acids may be, but are not limited to, glycine, polypeptide, proline, poly-proline, or combinations thereof. These recombinant fusion proteins optionally contain a targeting polypeptide. Typically the targeting peptides are configured to be exposed on the surface of a particle formed by crosslinking methods disclosed herein, e.g., on the opposite end compared to the polypeptide or amino acid with a primary amino group (e.g., \(-\text{CH}_3\text{NH}_2\)) as the purpose of the primary amino group is to allow for crosslinking.

In certain embodiments, it is contemplated that particles herein may be formed from two or more recombinant proteins. For example, a first recombinant protein contains a protein of interest/secreted effector protein and polypeptide or amino acid with a primary amino group, and a second recombinant protein contains a cell penetrating peptide and polypeptide or amino acid with a primary amino group. Particles are formed by mixing the first and second recombinant proteins with a crosslinking reagent that reacts with the primary amino acid groups on both recombinant proteins under conditions to form particles comprising the protein of interest/secreted effector protein and the cell penetrating peptide. In certain embodiments, a third recombinant protein contains a targeting peptide and polypeptide or amino acid with a primary amino group, and particles are formed by mixing first, second, and third recombinant proteins with the crosslinking reagent under conditions such that the desired particles are formed.

In certain embodiments, it is contemplated that additional polypeptide or amino acid with a primary amino group are not included in the recombinant protein, e.g., in the situation where there are sufficient lysine residues at appropriate locations within the wild-type sequence to form the particles by the methods disclosed herein.

In certain embodiments, recombinant proteins disclosed herein comprise a cell penetrating peptide. In certain embodiments, the cell penetrating peptide is oligoarginine peptide (7-12), octa-arginine (R8), the peptide segment (SEQ ID NO: 7) FFLPKG a penetration accelerating sequence (Pas), Pas-octa-arginine (SEQ ID NO: 8) (FFLIPKG-RRRRRRRRRR), segment (SEQ ID NO: 9) (GKP-PLIF) of cathepsin D-cleavable sequence, segment (R-cathlD, (SEQ ID NO: 10) RRRRRRRR-GKPIFF), or Tat peptide of HIV-1.

In certain embodiments, recombinant proteins and particles disclosed herein comprise a polypeptide with affinity for a cell surface receptor, i.e., targeting polypeptide. In certain embodiments, the targeting polypeptide is a transferrin, EGFR antibody, HEK-2 antibody, or ICAM-1 antibody or appropriately binding segments thereof.

In certain embodiments, the disclosure relates to nucleic acids encoding recombinant proteins disclosed herein. In certain embodiments, the disclosure relates to vectors comprising nucleic acids encoding recombinant proteins disclosed herein. In certain embodiments, the disclosure relates to expression systems producing recombinant proteins disclosed herein by mixing with vectors comprising nucleic acids encoding recombinant proteins disclosed herein.

In certain embodiments, the disclosure relates to pharmaceutical compositions comprising particles disclosed herein and a pharmaceutically acceptable excipient, e.g., in the form of a pill, tablet, capsule, or aqueous phosphate buffer solution.

In certain embodiments, the disclosure contemplates methods of treating or preventing a disease or condition disclosed herein comprising administering an effective amount of a pharmaceutical composition comprising particles disclosed herein to a subject in need thereof.

In certain embodiments, the disclosure contemplates methods of treating or preventing inflammatory bowel disease, Crohn's disease, ulcerative colitis, proctitis, fulminant colitis, proctosigmoiditis, left-sided colitis, celiac disease, or enteric infection. In certain embodiments, the subject is in need of treatment as the subject is at risk of, exhibiting symptoms, or diagnosed with an inflammatory bowel disease. In certain embodiments, the subject is exhibiting symptoms of abdominal pain or bloody diarrhea or combination thereof. In certain embodiments, the composition is administered in combination with a second anti-inflammatory agent such as 5-aminosalicylic acid, sulfasalazine, balsalazide, olsalazine, non-steroidal anti-inflammatory drugs (NSAIDs), aspirin, ibuprofen, cortisone, prednisone, hydrocortisone, methylprednisolone, budesonide, immunosuppressants, mercaptopurine, methotrexate, azathioprine, anti-
TNF monoclonal antibodies, infliximab, adalimumab, certolizumab pegol, and golimumab, and etanercept.

In certain embodiments, the disclosure contemplates methods of preparing particles disclosed herein comprising mixing recombinant proteins disclosed herein with crosslinking agents disclosed herein under conditions that particles disclosed herein are formed.

**BRIEF DESCRIPTION OF THE DRAWINGS**

**FIG. 1** illustrates schematic of a proposed AvrA nanoparticle therapy for IBD.

**FIG. 2** shows Zetasizer data (A, peak 108 nm) and scanning electron micrograph (B, scale bar 300 nm) of AvrA/BSA nanoparticles. Percent fluorescence loss of soluble GFP (dark gray) or nanoparticle GFP (light gray) after 2 hours in intestinal simulation fluid with pancreatin compared to simulation fluid without pancreatin (C).

**FIG. 3** shows data for recombinant nanoparticles with GFP delivered to HeLa cells (A) soluble protein (light gray), or GFP nanoparticles (dark gray). Confocal images of HeLa cells incubated with GFP nanoparticles for 1 (B), 3 (C), and 6 (D) hours. Images are overlays of GFP fluorescence, Hoechst dye, and bright field at a slice in the center of cells (scale bar 10 μm).

**FIG. 4** shows western blots with indicated antisera (A) and IL-8 ELISA data (B) in polarized THP1 monolayers. AvrA nanoparticles were applied for 3 hrs prior to TNF-α stimulation.

**FIG. 5** shows data on the quantification of neutrophils isolated by peritoneal lavage from mice (5 per indicated condition).

**FIG. 6** shows data: (A) representative microendoscopic image of colon from mouse treated as indicated. (C) Quantification of clinical and endoscopic score from 5 five mice treated as indicated. (B) Representative colon histology from mouse treated as indicated. (D) Quantification of histological score from 5 five mice treated as indicated.

**FIG. 7** illustrates crosslinking of recombinant proteins.

**FIG. 8** illustrates nanoparticle delivery barriers. (A) Internalization controlled via particle size, receptor ligands and cell penetrating peptides (CPPs). (B) Particle escape from endo/lysosomes and disassembly will be controlled via endolytic peptides and crosslinking density. (C) Hydrophilic coatings aid particle transport across mucus layer.

**FIG. 9** shows data indicating a therapeutic effect of AvrA nanoparticles in a colitis model. Nanoparticles, including active (AvrA:anano), mutant inactive (mAvrA) and inert control (gFEP) were administered transrectally at two indicated time points to groups of 5 mice. Phosphate buffered saline (PBS) was administered as a negative control. Model colitis was induced by the addition of dextran sodium sulfate in drinking water at day 0 to day 7. No treatments were given to control (ctrl) mice. All animals were followed daily and weight, presence of fecal blood, and diarrhea were used to derive a disease activity index (DAI).

**DETAILED DISCUSSION**

Before the present disclosure is described in greater detail, it is to be understood that this disclosure is not limited to particular embodiments described, and as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present disclosure will be limited only by the appended claims.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. Although any methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present disclosure, the preferred methods and materials are now described.

All publications and patents cited in this specification are herein incorporated by reference as if each individual publication or patent were specifically and individually indicated to be incorporated by reference and are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. The citation of any publication is for its disclosure prior to the filing date and should not be construed as an admission that the present disclosure is not entitled to antedate such publication by virtue of prior disclosure.

Further, the dates of publication provided could be different from the actual publication dates that may need to be independently confirmed.

As will be apparent to those of skill in the art upon reading this disclosure, each of the individual embodiments described and illustrated herein has discrete components and features which may be readily separated from or combined with the features of any of the other several embodiments without departing from the scope or spirit of the present disclosure. Any recited method can be carried out in the order of events recited or in any other order that is logically possible.

Embodiments of the present disclosure will employ, unless otherwise indicated, techniques of medicine, organic chemistry, biochemistry, molecular biology, pharmacology, and the like, which are within the skill of the art. Such techniques are explained fully in the literature.

It must be noted that, as used in the specification and the appended claims, the singular forms a, an, and the include plural referents unless the context clearly dictates otherwise. In this specification and in the claims that follow, reference will be made to a number of terms that shall be defined to have the following meanings unless a contrary intention is apparent.

Prior to describing the various embodiments, the following definitions are provided and should be used unless otherwise indicated.

"Subject" refers any animal, preferably a human patient, livestock, rodent, monkey or domestic pet.

As used herein, the terms "prevent" and "preventing" include the prevention of the recurrence, spread or onset. It is not intended that the present disclosure be limited to complete prevention. In some embodiments, the onset is delayed, or the severity of the disease is reduced.

As used herein, the terms "treat" and "treating" are not limited to the case where the subject (e.g., patient) is cured and the disease is eradicated. Rather, embodiments, of the present disclosure also contemplate treatment that merely reduces symptoms, and/or delays disease progression.

As used herein, the term "combination with" when used to describe administration with an additional treatment means that the agent may be administered prior to, together with, or after the additional treatment, or a combination thereof.

The term "nucleic acid sequence encoding" a specified polypeptide refers to a nucleic acid sequence comprising the coding region of a gene or in other words the nucleic acid sequence which encodes a gene product. The coding region may be present in either a cDNA, genomic DNA or RNA form. When present in a DNA form, the oligonucleotide,
polynucleotide, or nucleic acid may be single-stranded (i.e., the sense strand) or double-stranded. Suitable control elements such as enhancers/promoters, splice junctions, polyadenylation signals, etc. may be placed in close proximity to the coding region of the gene if needed to permit proper initiation of transcription and/or processing of the primary RNA transcript. Alternatively, the coding region utilized in the expression vectors of the present invention may contain endogenous enhancers/promoters, splice junctions, intervening sequences, polyadenylation signals, etc. or a combination of both endogenous and exogenous control elements.

The terms “vector” or “expression vector” refer to a recombinant nucleic acid containing a desired coding sequence and appropriate nucleic acid sequences necessary for the expression of the operably linked coding sequence in a particular host organism or expression system, e.g., cellular or cell-free. Nucleic acid sequences necessary for expression in prokaryotes usually include a promoter, an operator (optional), and a ribosome binding site, often along with other sequences. Eukaryotic cells are known to utilize promoters, enhancers, and termination and polyadenylation signals.

Protein “expression systems” refer to in vivo and in vitro (cell free) systems. Systems for recombinant protein expression typically utilize cells transfected with a DNA expression vector that contains the template. The cells are cultured under conditions such that they translate the desired protein. Expressed proteins are extracted for subsequent purification. In vivo protein expression systems using prokaryotic and eukaryotic cells are well known. Also, some proteins are recovered using denaturants and protein-refolding procedures. In vitro (cell-free) protein expression systems typically use translation-compatible extracts of whole cells or compositions that contain components sufficient for transcription, translation and optionally post-translational modifications such as RNA polymerase, regulatory protein factors, transcription factors, ribosomes, tRNA cofactors, amino acids and nucleotides. In the presence of an expression vectors, these extracts and components can synthesize proteins of interest. Cell-free systems typically do not contain proteases and enable labeling of the protein with modified amino acids. Some cell free systems incorporated encoded components for translation into the expression vector. See, e.g., Shimizu et al., Cell-free translation reconstituted with purified components, 2001, Nat. Biotechnol., 19, 751-755 and Asahama & Chong, Nucleic Acids Research, 2010, 38(13): e141, both hereby incorporated by reference in their entirety.

The term “recombinant” when made in reference to a nucleic acid molecule refers to a nucleic acid molecule which is comprised of segments of nucleic acid joined together by means of molecular biological techniques. The term “recombinant” when made in reference to a protein or a polypeptide refers to a protein molecule which is expressed using a recombinant nucleic acid molecule.

The terms “in operable combination”, “in operable order” and “operably linked” refer to the linkage of nucleic acid sequences in such a manner that a nucleic acid molecule capable of directing the transcription of a given gene and/or the synthesis of a desired protein molecule is produced. The term also refers to the linkage of amino acid sequences in such a manner so that a functional protein is produced.

The term “regulatory element” refers to a genetic element which controls some aspect of the expression of nucleic acid sequences. For example, a promoter is a regulatory element which facilitates the initiation of transcription of an operably linked coding region. Other regulatory elements are splicing signals, polyadenylation signals, termination signals, etc.

Transcriptional control signals in eukaryotes comprise “promoter” and “enhancer” elements. Promoters and enhancers consist of short arrays of DNA sequences that interact specifically with cellular proteins involved in transcription (Maniatis, et al., Science 236:1237, 1987). Promoter and enhancer elements have been isolated from a variety of eukaryotic sources including genes in yeast, insect, mammalian and plant cells. Promoter and enhancer elements have also been isolated from viruses and analogous control elements, such as promoters, are also found in prokaryotes. The selection of a particular promoter and enhancer depends on the cell type used to express the protein of interest. Some eukaryotic promoters and enhancers have a broad host range while others are functional in a limited subset of cell types (for review, see Voss, et al., Trends Biochem. Sci., 11:287, 1986; and Maniatis, et al., supra 1987).

The terms “promoter element,” “promoter,” or “promoter sequence” as used herein, refer to a DNA sequence that is located at the 5’ end (i.e. precedes) the protein coding region of a DNA polymer. The location of most promoters known in nature precedes the transcribed region. The promoter functions as a switch, activating the expression of a gene. If the gene is activated, it is said to be transcribed, or participating in transcription. Transcription involves the synthesis of mRNA from the gene. The promoter, therefore, serves as a transcriptional regulatory element and also provides a site for initiation of transcription of the gene into mRNA. The term “cell type specific” as applied to a promoter refers to a promoter which is capable of directing selective expression of a nucleotide sequence of interest in a specific type of cell in the relative absence of expression of the same nucleotide sequence of interest in a different type of cell within the same tissue. Promoters may be constitutive or regulatable. The term “constitutive” when made in reference to a promoter means that the promoter is capable of directing transcription of an operably linked nucleic acid sequence in the absence of a stimulus (e.g., heat shock, chemicals, light, etc.). Typically, constitutive promoters are capable of directing expression of a transgene in substantially any cell and any tissue. In contrast, a “regulatable” or “inducible” promoter is one which is capable of directing a level of transcription of an operably linked nucleic acid sequence in the presence of a stimulus (e.g., heat shock, chemicals, light, etc.) which is different from the level of transcription of the operably linked nucleic acid sequence in the absence of the stimulus.

The enhancer and/or promoter may be “endogenous” or “exogenous” or “heterologous.” An “endogenous” enhancer or promoter is one that is naturally linked with a given gene in the genome. An “exogenous” or “heterologous” enhancer or promoter is one that is placed in juxtaposition to a gene by means of genetic manipulation (i.e., molecular biological techniques) such that transcription of the gene is directed by the linked enhancer or promoter. For example, an endogenous promoter in operable combination with a first gene can be isolated, removed, and placed in operable combination with a second gene, thereby making it a “heterologous promoter” in operable combination with the second gene.

Efficient expression of recombinant nucleic acid sequences in eukaryotic cells requires expression of signals directing the efficient termination and polyadenylation of the resulting transcript. Transcription termination signals are generally found downstream of the polyadenylation signal and are typically a few hundred nucleotides in length. The
Role of Commensal and Pathogenic Bacteria in Regulation of Host Inflammatory Pathways

The gut exerts defense mechanisms that are tailored to efficiently counter microbial challenges. However, microbes, both pathogens and commensals, have in turn evolved highly effective mechanisms to undermine or modulate the immune system through active manipulation of regulators of the inflammatory response. Enteric pathogens influence eukaryotic cell pathways through soluble effector proteins translocated into the target cells via a "type III secretion apparatus" that usurp host cellular functions for the benefit of the invading organism.

AvrA, a *Salmonella* protein, that is translocated into host cells during initial epithelial cellular invasion. Significantly, homologs of this protein are found in a variety of bacteria, both pathogens and symbionts, which are intimately associated with animal and plant eukaryotic hosts. The protein family members are acetyltransferases that covalently modify and inactivate members of the MAPK superfamily, and thus have potent and diverse effects on a wide variety of eukaryotic growth, survival and immune pathways. AvrA overexpressed in transfected cells or in a *Drosophila* transgenic model blocks activation of NF-κB, MAPK, JNK, and transcriptional activation of a range of inflammatory effector genes. In yeast, flies, humans and murine intestinal epithelia, immune blockade occurs without induction of the apoptotic cell death characteristicly seen during inhibition of host stress signaling pathways. The biochemical profile of JNK/NF-κB suppression combined with ERK activation results in inflammatory suppression without stimulating apoptosis or other types of cellular death.

Secreted Effector Proteins of Pathogens

In certain embodiments, the disclosure relates to nanoparticles comprising recombinant proteins wherein the recombinant protein comprises a viral, fungal, or bacterial protein sequence such as a secreted effector protein selected from AvrA, mAvrA(1C186A), YopJ, YopA, or AopP.

AvrA has a YopJ domain, i.e., AvrA sequence is minus amino acids 1-14. Yoon et al., report *Yersinia* effector YopJ inhibits yeast MAPK signaling pathways by an evolutionarily conserved mechanism. See *J Biol Chem*, 2003, 278 (4):2131-5. *Salmonella enterica* YopJ has the following sequence (SEQ ID NO: 2) MLSPTPSMPG ASLSPQPPDS VENLGETALTC IVERLESEEI DISWHSIYEE TDLEMPFPL VAQANKKYPE LNLKFVMSVH ELVSSKTEIK MEGVESARFI YNMGGSGHII SVYDV FRVMGD KTVLIEPEA ACSAEFPALL AURKRAAER EQLDCYFM VELDIQRSSS ECGFSLAL A KKLQELFMMNVLK IHEDNICE RLCGEEPELP SDRADYLVP SFYKHTQGQ RLNEYVQANP AAGSSIVNKK NETLYERDFDN NAVMLNDK KKL QI ISAHK KIAE FYKSLKP.
In certain embodiments, the term “AvrA” refers to proteins of any bacteria that have substantial homology to SEQ ID NO:1 and variants. In certain embodiments, nanoparticles are comprised of recombinant proteins having greater than 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 95% sequence identity or similarity to SEQ ID NO:1. In certain embodiments, the disclosure contemplates variants that are substitutions, deletions, or insertions of about, less than, or more than one, two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, fifteen, or sixteen amino acids. In certain embodiments, the substitutions are conserved substitutions. In certain embodiments, the substitutions, deletions, or insertions are not within the YopJ domain. In certain embodiments, one, two, three, or four substitutions, deletions, or insertions are within the YopJ domain. In certain embodiments, nanoparticles disclosed herein comprise a polypeptide that has greater than 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 95% sequence identity or similarity to SEQ ID NO:2. In certain embodiments, the disclosure contemplates variants that are substitutions, deletions, or insertions of one, two, three, four, five, six, seven, eight, nine, or ten amino acids. In certain embodiments, the substitutions are conserved substitutions.

In certain embodiments, the nanoparticles comprise a polypeptide with a YopJ domain or any member of the Yop superfamily, i.e., NCBI superfamily c107849 available at http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?sid=c107849. One example is Vibrio outer protein J (VopJ) of the following sequence (SEQ ID NO: 3)

MKVNIQONHY YEQRQGSSDL EQLSSYIDLM DDAAIQRQGL PKDTAANA DLMDDFLAIQ NQKEGLNAIF FRSPJDVMN YVKSLESPE TADARV NYMK SQGICLAVD CAIQKNGCIES IEIEPVTMNS LGASMLAIRL QSVKCRELPE TSLVIMETDIM QRQSOGECML MFLLYVKKMHK ECEDEFYQLHD KNINRELPFL QGLIIVSKVDA DSSLPPSLMK HTQSPNRILQK YEMRLPEAMN CVCNKKEGETI KTQRQIRHTT IELGEKTVSY SNSIEQKRIK EAKGLLNNI.

Another contemplated example is Vibrio outer protein A (VopA) comprising the following sequence (SEQ ID NO:4)

ndialfp diannkaklal ntaicpkevklk saurinf nrgeggihcm avcdlvsdkg eilgiqveg mssqapll iralskcre jpeanlal tmqrsygge amfsslkk hhkenaflg lkhnlidnl pksggigv sanflnlps linhkvapk lerylkse aadvvnnkkg etllrrqay iatien.

Another contemplated example is Aeromonas outer protein P (AopP) comprising the following sequence (SEQ ID NO:5) npampikd dinagekke iqtavekqel illliemelase asfgelhse nyasidkeg pmiaamuk bglhvnflk rysvamqg ihiaindek vdklckllm epamhmgv amamlvsvs hkehieipk phciahviq gmsnnsvsf msvlagkms empsdail elerlrpgk ndvclveq leddyplfy kvrsqrdq giriapdpgq dvstvngkel lldkrlmiv pvedelisi iklqrmens aisidkgk.

In certain embodiments, nanoparticles disclosed herein comprise a polypeptide of greater than 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 95% sequence identity or similarity to a YopJ superfamily protein. In certain embodiments, the disclosure contemplates variants that are substitutions, deletions, or insertions of one, two, three, four, five, six, seven, eight, nine, or ten amino acids. In certain embodiments, the substitutions are conserved substitutions.

Cell penetrating peptides (CPPs)

In certain embodiments, the disclosure relates to particles comprising recombinant proteins which are a chimeric or fusion protein comprising a cell penetrating peptide (CPP) in combination with a bacteria secreted effector protein. CPPs typically have an amino acid composition that either contains a high relative abundance of positively charged amino acids such as lysine or arginine or has sequences that contain an alternating pattern of polar/charged amino acids and non-polar, hydrophobic amino acids referred to as polycationic or amphipathic, respectively.

In one example, the CPP is trans-activating transcriptional activator (Tat) from Human Immunodeficiency Virus 1 (HIV-1) (SEQ ID NO:6) MFPYDVRLPF WKHPQSFQPKT PCTKCYCCKC CLHCQVCFMNT KGLGQISYGRK KRRQRRRAPO DKNKQVGLS EQKPTSRAGD PTGQFESKEK VEKETVDPY T and variants thereof. In certain embodiments, nanoparticles disclosed herein comprise a polypeptide of greater than 70%, 80%, 90%, or 95% sequence identity or similarity to Tat peptide of HIV-1. In certain embodiments, the disclosure contemplates variants that are substitutions, deletions, or insertions of one, two, three, four, five, six, seven, eight, nine, or ten amino acids. In certain embodiments, the substitutions are conserved substitutions.

Additional examples of contemplated CPPs are selected from oligouridine peptide (7-12), octa-arginine (R8), the peptide segment (SEQ ID NO: 7) FFLIPKG a penetration accelerating sequence (Pas), Pas-octa-arginine (SEQ ID NO: 8) FFLIPKG-RRRR, segment with (SEQ ID NO: 9) GPKLFF of capthelin D-cleavable sequence, and a segment R8-cath(D, (SEQ ID NO: 10) RRRR-GPKLFF. Additional examples of CPPs are (SEQ ID NO: 11) GALFLFGAL10,AGSTMAGAWSQ20,PKKKRKV and GALFLFAA10,ALSLMGWSAQ20,PKKKRKV (SEQ ID NO: 12) as disclosed in Deshayes et al., Biochemistry, 2004, 43 (24), pp 7688-7706; (SEQ ID NO: 13) RIVRWFQKN-KRCDKK and (SEQ ID NO: 14) RQIKIWQNNRKRK-WKK and (SEQ ID NO: 15) GWTILNASYGGLKIN-KALLAALKIL as disclosed in Magzoub et al., Biosci Biophys Acta, 2001, 1512(1):77-89.

In one example, CCP is a transferrin (Tf). The wild type human Tf is a 679 amino acid protein of approximately 75 kDa (not accounting for glycosylation), with two main domains or lobes, N (about 330 amino acids) and C (about 340 amino acids). See GenBank accession numbers NM_001063, XM_002793, M12530, XM_039845, XM_039847 and 595936, all of which are here incorporated by reference in their entirety, as well as SEQ ID NO: 16 (SEQ ID NO: 16 comprises the additional 19 amino acid sequence of the human transferrin leader sequence).

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Recombinant proteins disclosed herein may be made with any TF protein, fragment, domain, or engineered domain. For instance, recombinant proteins may be produced using the full-length TF sequence, with or without the native TF signal sequence. TF recombinant proteins may also be made using a single TF domain, such as an individual N or C domain or a modified form of TF comprising 2N or 2C domains (see U.S. Pat. Appl. Publ. No. US 2006/0130158).

Other contemplated transferrin variants are provided in U.S. Pat. No. 8,158,579 (modified TF). In certain embodiments, this disclosure contemplates particles comprising recombinant proteins disclosed herein comprising a transferrin variant of the following sequence (SEQ ID NO: 17):

Val Pro Asp Lys Thr Val Arg Trp Cys Ala Val Ser Glu His Glu Ala Thr Lys Cys Gln Ser Phe Arg Asp His Met Lys Ser Val

Ile Pro Ser Asp Gly Pro Ser Val Ala Cys Val Lys Ala Ser Tyr

Leu Asp Cys Ile Arg Ala Ile Ala Asn Glu Ala Asp Ala Val Thr

Leu Asp Ala Gly Leu Val Tyr Asp Ala Tyr Leu Ala Pro

Asn Asn Leu

Lys Pro Val Ala Glu Phe Tyr Gly Ser Lys Gln Asp Pro

Gln Thr

Phe Tyr Tyr Ala Val Ala Val Val Lys Lys Asp Ser Gly Phe

Gln Met

Asn Gln Leu Arg Gly Lys Ser Cys His Thr Gly Leu Gly Arg Ser

Ala Gly Trp Asn Ile Pro Ile Gly Leu Tyr Cys Asp Leu Pro Glu

Pro Arg Lys Pro Leu Gln Lys Ala Val Ala Asn Phe Pro Ser

Gly Ser

Cys Ala Pro Cys Ala Asp Gln Arg Thr Asp Phe Pro Glu

Cys Gln Leu

Cys Pro Gly Gln Cys Gly Gln Ser Thr Leu Asn Gln Tyr Phe

Gly Tyr Ser

Gly Ala Phe Lys Cys Leu Lys Asp Gly Ala Gly Asp Val

Ala Phe Val

Lys His Ser Thr Ile Phe Glu Asn Ala Asn Lys Ala Asp

Arg Asp

Gln Tyr Gly Leu Leu Cys Leu Asp Thr Arg Lys Pro

Val Asp Gln

Tyr Lys Asp Cys His Leu Ala Gln Val Pro Ser His Thr Val

Val Ala

Arg Ser Met Gly Gly Lys Gln Asp Leu Ile Trp Gln Leu

Leu Asn Gln

Ala Gln Glu His Phe Gly Lys Asp Lys Ser Lys Glu Phe

Glu Ser Pro His Gly Lys Asp Leu Phe Lys Asp Ser Ala

His Gly

Phe Leu Lys Val Pro Pro Arg Met Asp Ala Lys Met Tyr

Leu Gly Tyr

Glu Tyr Val Thr Ala Ile Arg Asn Leu Arg Glu Gly Thr Cys

Pro Glu

Ala Pro Thr Asp Glu Cys Lys Pro Val Lys Trp Cys Ala Leu

Ser His

His Glu Arg Leu Lys Cys Asp Glu Trp Ser Val Asn Ser Val

Gly Lys

Ile Glu Cys Val Ser Ala Glu Thr Thr Glu Asp Cys Ile Ala

Lys Ile

Met Asn Gly Glu Ala Asp Ala Met Ser Leu Asp Gly Gly

Phe Val Tyr

Ile Ala Gly Lys Cys Gly Leu Val Pro Val Leu Ala Glu Asn

Tyr Asn

Lys Ala Asn Asp Cys Glu Asp Thr Pro Glu Ala Gly Tyr

Phe Ala Val

Ala Val Val Lys Ser Ala Ser Asp Leu Thr Trp Asp Asn

Leu Lys

Gly Lys Ser Cys His Thr Ala Val Gly Arg Thr Ala Gly

Trp Asn

Ile Pro Met Gly Leu Tyr Asn Lys Ile Asn His Cys Arg

Phe Asp

Glu Phe Phe Ser Glu Gly Cys Ala Pro Gly Ser Lys

Asp Ser Ser

Leu Cys Lys Leu Cys Met Gly Ser Lys Leu Asn Lys Cys

Pro Asn

Asn Lys Glu Gly Tyr Gly Tyr Gly Thr Gly Ala Phe Arg

Cys Leu Val

Glu Lys Gly Asp Val Ala Phe Val Lys His Gln Thr Val Pro

Gln Asn

Thr Gly Gly Asn Pro Asp Pro Trp Ala Lys Asn Leu

Asn Glu Lys

Asp Tyr Glu Leu Lys Cys Leu Cys Lys Gly Asp Tyr Thr Arg

Lys Pro

Val Glu Glu

Tyr Ala Asn Cys His Leu Ala Arg Ala Pro Asn His Ala Val

Thr Val Thr

Arg Lys Asp Gly Lys Ala Cys His Val His Lys Ile Leu Arg

Gln Glu

His Leu Phe Gly Ser Val Ala Cys Ser Gly Asn

Phe Cys Leu

Phe Arg Ser Glu Thr Lys Asp Leu Phe Arg Asp Asp

Thr Val Cys

Leu Ala Lys Leu His Arg Asp Arg Thr Gly Tyr Lys

Tyr Leu Gly Glu

Glu Tyr Val Lys Ala Val Gly Asn Leu Arg Lys Cys Ser Thr

Ser Ser

Leu Glu Ala Cys Thr Pro Arg Arg Pro or variants

thereof.

In certain embodiments, nanoparticles disclosed herein comprise a polypeptide of greater than 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 95% sequence identity or similarity to SEQ ID NOs 16 or 17.

Engineered Nanoparticle Components for Intracellular Delivery

Traditionally, delivery of most IBD therapeutics has been accomplished by local enema, oral ingestion or systemic infusion. For biologies in particular, injection and oral administration are not ideal because they cannot survive serum proteases, the harsh environment of the gastrointestinal tract, or clearance processes in blood and tissue. Encapsulation of drugs into polymeric carriers can mitigate these limitations. Prior to release, the polymer protects drugs from degradation or premature metabolism and particles can deliver a concentrated dose of drug. Targeting ligands can also be attached to the particle surface to direct accumulation of particles at a particular site, thereby increasing the drug concentration locally while minimizing systemic side effects. These benefits have typically been realized by particles carrying small molecule drugs. In the case of protein drugs, however, this delivery strategy still has limitations. Protein loading is extremely low, the particles are often too
large to be internalized by cells (≥50 μm), and harsh fabrication or degradation conditions can damage the protein. Hydrophilic polymers, biopolymers, or albumin may be used as the encapsulating material.

Contemplated embodiments nanoparticles of this disclosure are illustrated in FIGS. 1, 7, and 8. Nanoparticle surface chemistry is important in dictating initial interactions with mucus and the cell membrane, as well as subsequent internalization routes (FIGS. 8A-C). Surface chemistry can refer to the general surface charge or hydrophobicity of a particle. For the nanoparticles disclosed herein, these two features are a function of the core material, AvrA. One of the benefits of nanoparticle delivery systems is the ability to modify particle surfaces independently of the cargo. Within certain embodiments of the disclosure nanoparticles targeting cell surface receptors and utilizing cell penetrating peptides (CPPs) inspired by viral pathogens are contemplated. In one embodiment, the transferrin receptor is attached to the surface of nanoparticles with the same cross-linking strategy used to form AvrA nanoparticles. CPPs are short peptide sequences that enhance intracellular internalization. In certain embodiments, the CPP octa-arginine, modeled on the TAT peptide used by HIV-142, is inserted into the AvrA DNA sequence and incorporated during in vivo recombinant protein expression. CPPs significantly increase endocytic uptake.

Nanoparticles are taken up into endocytic pathway. AvrA reaches cytosol in order to achieve bioactivity (FIG. 8B. In certain embodiments, particles disclosed herein comprises a protein with the following sequence (SEQ ID NO: 18) WGI(Pal)VKKKKPGI16 wherein Pal is a palmitoyl group anchored to a synthetic diaminopropanoic acid (Dap) residue. This is an amphiphilic palmitoylated peptide which promotes endosomal escape. See Delecluse et al., Integrative Biology, 2010, 2(5-6):265-277. These peptides may be synthesized on solid supports and either physically adsorbed on the nanoparticle surface (due to their amphiphilic nature) or covalently bound. If covalent chemistry is necessary the carbonyl terminus of peptides may be activated by the carbodiimide EDC and then linked to free amines on the particle surface.


Nanoparticle design incorporates a variety of factors including size, surface chemistry, endosomal escape mechanisms, and crosslinking type and density. Each feature has the capability to influence internalization and trafficking processes shown in FIG. 8. Additionally, they can also impact nanoparticle interaction with mucus and general stability in vivo. In certain embodiment, the disclosure contemplates particles of a size typically of less than 200 nm in diameter and greater than 50 nm in diameter. In certain embodiments the disclosure contemplates providing a hydrophilic or neutral coating such as polyethylene glycol.

The polymer PEG may be adsorb on the particle surface or a covalent approach may be used.

Enhanced targeting to injured tissue may be achieved by altering the particle binding specificity. For example, intracellular adhesion molecule 1 (ICAM-1) is upregulated on intestinal epithelial cells in areas of inflammation and after exposure to invasive bacteria. In certain embodiments, the disclosure contemplates recombinant protein particles disclosed herein with anti-ICAM-1 antibodies, e.g., conjugated or adsorbed to the surface as with PEG. While targeting strategies or inherent mucus differences can bias nanoparticles toward inflamed tissue, there may be advantages to nanoparticles interacting with healthy epithelium as well. This could be useful for protective treatments prior to injury/collitis induction. Additional targeting agents include EGFR and HER-2 antibodies such as cetuximab, erlotinib, and gefitinib particularly for uses in the treatment of breast or prostate cancer.

In certain embodiments, the disclosure contemplates that the particles disclosed herein may comprise an enteric coating. The enteric coating may comprise traditional polymolecular coating such as methyl acrylate-methacrylic acid copolymers, cellulose acetate succinate, hydroxy propyl methyl cellulose phthalate, hydroxy propyl methyl cellulose acetate succinate (hypromellose acetate succinate), polyvinyl acetate phthalate (PVAP), methyl methacrylate-methacrylic acid copolymers, sodium alginate, and stearic acid. The nanoparticles can be encapsulated into microparticles of a chitosan and/or alginate composition by forming an emulsion and calcium crosslinking.

While a specific Salmonella protein, AvrA, has been chosen as a model immunosuppressive protein for packing into nanoparticles for IBD therapy, this approach is actually very general. There are a variety of bacterial proteins that have been shown to suppress or enhance immune signaling and likely more that have not yet been discovered or characterized. For example, AvrA family members (YopL, YopA, AopP) that have variable effects on MAPK and are more potent immunosuppressive, but also pro-apoptotic. Another candidate immune-modulatory effectors include OspG/F from Shigella that inhibit the ubiquitination step of NF-kB.

Flagellin

Also contemplated by this disclosure is the use of TLR ligands such as flagellin, peptidoglycans, and other immunostimulatory molecules. Packaged TLR ligands could serve as immunostimulatory vaccine adjuvants or cytoprotective molecules. These ligands are also amenable for nanoparticle fabrication.

As used herein, “flagellin” refers to the monomer subunit in flagella, e.g., flagellin gene product of FliC and FliB in S. typhimurium and FlaA in L. pneumophila, or variants, homologs, derivatives, fragments or combination thereof, such as a domain or polypeptide sequence in the domain. Typically, the flagellin monomer contains D0, D1, D2, and D3 domains. An alignment of the amino acid sequences from different Gram-negative species shows a high degree of similarity in the amino and carboxy terminal domains. The central regions of these proteins may be quite divergent. It is believed that flagellin responsible for interaction with TLR5 is found in the D1 domain. Smith, K. D., et al, Nature Immunol. (2003) 4:1274-1275 disclose that TLR5 recognizes a site on the flagellin of Salmonella typhimurium (FliC) composed of N-terminal residues 78-129 and 135-173 and C-terminal residues 395-444. The term “flagellin” is not intended to be limited to any particular amino acid sequence provided that it has some
homology to known flagellin sequences and the molecule retains the ability to stimulate innate immune responses. The innate immune responses of flagellin are known to include cytokine production in response to TLR (including TLR5) activation and activation of Caspase-1 and IL-1β secretion in response to certain NLRs (including Ipaf). In certain embodiments, a flagellin is contemplated to include additional amino acids within the sequence, such as in the case of fusion or chimeric proteins, provided that these proteins continue to affect an innate immune response that comprises a TLR5-mediated immune response, an Ipf-mediated immune response or both. Also specifically contemplated are fragments, variants, analogs, homologs, or derivatives of said flagellin, and combinations thereof provided these molecules continue to affect an innate immune response that comprises a TLR5-mediated immune response, an Ipf-mediated immune response or both. A flagellin may be isolated from natural sources, through recombinant or combinatorial technologies or combinations thereof.

Individual salmonella serotypes usually alternate between the production of two forms of flagellin, termed phase 1 and phase 2, each specified by separate structural genes Flc and Flj. The amino acid sequences of phase-1 flagella protein of salmonella typhimurium (Flc) is set forth in SEQ ID NO: 20: 20 MAQVINTNLS SLITQNNLNK SQASALTIAE RLSSGLRINS AKDDAAGQAA ANRFTANIKL LTQASRNAD CNISIAQTEQ ALNINQNLQ RVRELAVQSA NSTSNSQSLDL QSIIEAQRTL NEIRDVSQGTF QNFNGVS QVLAQ DNTLUTIQG A N D G T D I D D KG Q N S L Q V A A Q N A Q P Q N V L S L L R .

In certain embodiments, nanoparticles disclosed herein comprise a polypeptide of greater than 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 95% sequence identity or similarity to SEQ ID NO: 20.

The amino acid sequences of F41 fragment of flagellin of salmonella typhimurium is set forth in SEQ ID NO: 21: FTANIKLQETO ASRNANDIGIS LAQTTEGAEL INEINQLQRVR EIAAQSNST SQDSQDSQG AEIQRGAEI DRVSQGTFQGN GVKFLAQDNT LTQVIQGAND ETI DIQDKI NSQTLGDLTD NVQKYKVDSD TDAATVTG YADF TIAALDNSTF KASATLGGTG DQKDIDGKLF DDTGGYKAY VTVTGTTGGKD GYEVSDVKT NGEVTAGGG TSLTGLGPA TATEDVKNVQ VANDLTEAK AALTAAGTVYG TAVSVKSMTY DNNNGKTDD LLGAGDKTEV IIGQTGAK SKEAGHNFKA QPDLAEAAAT TETENLQKID APAQVQDTRL SLDAVQNRF NSAITLNGTNVLNLSAR.

The amino acid sequences of a flagellin fusion protein is set forth in SEQ ID NO: 22: MALTVNTNLSLINTQRNLSNN INASALTSLQK KSTGTSRINS ARNLTDSQVNG LNVTANAND QSIATQAE IALQSRSTLQ RMRLSDLQSA NGNSDSDLN ALENKVQO KELDRISNT FGGRKILDD SGFVSD FQVG SAANEMISVG IGGGKLMIKL KFGVFFTVLSS年薪APQ NITDLCAEVY NTQHTLNDK IFSYTE

SLAG KREMAITFTK NGATFQVEVP GSQHDSQKK AIERMKDTLR IAYTLEAKVE KLVVWNKTPHAIAA ISMAN.

Polypeptide fragments of flagellin include SEQ ID NO: 23: GALNINQNLQ RVRELAVQSA NSTSNSQSLDL QSIIEAQRTL NEIRDVSQGTF QNFNGVS QVLAQ DNTLUTIQGA N D G T D I D D KG Q N S L Q V A A Q N A Q P Q N V L S L L R .

SEQ ID NO: 24: TQSFSGVKVLQDNTLTQGV GANDGET IDIDLQKINS OTLGDLTD.


SEQ ID NO: 26: MAQVINTNLS SLITQNNLNK SQASALTIAE RLSSGLRINS AKDDAAGQAA ANRFTANIKL LTQASRNAD CNISIAQTEQ ALNINQNLQ RVRELAVQSA NSTSNSQSLDL QSIIEAQRTL NEIRDVSQGTF QNFNGVS QVLAQ DNTLUTIQGA N D G T D I D D KG Q N S L Q V A A Q N A Q P Q N V L S L L R .

SEQ ID NO: 27: IQKIDAALLAQDTRL SRLAQUALVQNRNSAITLNGTNVLNLSAR.

SEQ ID NO: 28: TLRSDLAVQNRNSAITLNGTNVLNLSAR.

SEQ ID NO: 29: EQAATENPLQKIDAALLAQDTRL.

SEQ ID NO: 30: TLRSDLAVQNRNSAITLNGTNVLNLSAR.

Combination of fragments of flagelin include SEQ ID NO: 30: Met Arg Gly Ser His His His His His Gly Met A Val Ser Met Thr Gly Gin Met Gly Arg Asp Leu Tyr Asp Asp Asp Lys Asp Pro Met Ala Gin Val Ile Asn Thr Asn Ser Leu Ser Leu Thr Gin Asn Asn Leu Asn Lys Ser Gin Ser Leu Ser Ser Ser Ser Ala Ile Glu Arg Leu Ser Ser Gly Leu Arg Ile Asn Ser Ala Asn Lys Asp Ala Ala Gly Gin Ala Ile Asn Arg Phc Thr Ser Ser Asn Ile Lys Gly Lys Leu Thr Gin Ala Ser Asn Asn Asn Asn Arg Asp Lys Ile Ala Gin Thr Lys Gin Gin Lys Leu Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gia
ampoule or in any other suitable single-dose or multi-dose holder or container (which may be properly labeled); optionally with one or more leaflets containing product information and/or instructions for use. Generally, such unit dosage will contain between 1 and 1000 mg, and usually between 5 and 500 mg, of the particles of the disclosure, e.g., about 10, 25, 50, 100, 200, 300 or 400 mg per unit dosage.

For an oral administration form, the particles can be mixed with suitable additives, such as excipients, stabilizers or inert diluents, and brought by means of the customary methods into the suitable administration forms, such as tablets, coated tablets, hard capsules, aqueous, alcoholic, or oily solutions. Examples of suitable inert carriers are gum arabic, magnesium, magnesium carbonate, potassium phosphate, lactose, glucose, or starch, in particular, corn starch. In this case, the preparation can be carried out both as dry and as moist granules. Suitable oily excipients or solvents are vegetable or animal oils, such as sunflower oil or cod liver oil. Suitable solvents for aqueous or alcoholic solutions are water, ethanol, sugar solutions, or mixtures thereof. Polyethylene glycols and polypropylene glycols are also useful as further auxiliaries for other administration forms. As immediate release tablets, these compositions may contain microcrystalline cellulose, dicalcium phosphate, starch, magnesium stearate and lactose and/or other excipients, binders, extenders, disintegrants, diluents and lubricants known in the art.

When administered by nasal aerosol or inhalation, the compositions may be prepared according to techniques well-known in the art of pharmaceutical formulation and may be prepared as solutions in saline, employing benzyl alcohol or other suitable preservatives, absorption promoters to enhance bioavailability, fluorocarbons, and/or other solubilizing or dispersing agents known in the art. Suitable pharmaceutical formulations for administration in the form of aerosols or sprays are, for example, solutions, suspensions or emulsions of the compounds of the disclosure or their physiologically tolerable salts in a pharmaceutically acceptable solvent, such as ethanol or water, or a mixture of such solvents. If required, the formulation may additionally contain other pharmaceutical auxiliaries such as surfactants, emulsifiers and stabilizers as well as a propellant.

For subcutaneous or intravenous administration, the particles, if desired with the substances customary therefore such as solubilizers, emulsifiers or further auxiliaries are brought into solution, suspension, or emulsion. The compounds may also be lyophilized and the lyophilizates obtained used, for example, for the production of injection or infusion preparations. Suitable solvents are, for example, water, physiological saline solution or alcohols, e.g. ethanol, propanol, glycerol, sugar solutions such as glucose or mannitol solutions, or mixtures of the various solvents mentioned. The injectable solutions or suspensions may be formulated according to known art, using suitable non-toxic, parenterally-acceptable diluents or solvents, such as mannitol, 1,3-butane-diol, water, Ringer's solution or isotonic sodium chloride solution, or suitable dispersing or wetting and suspending agents, such as sterile, bland, fixed oils, including synthetic mono- or diglycerides, and fatty acids, including oleic acid.

When rectally administered in the form of suppositories, the formulations may be prepared by mixing the particles with a suitable non-irritating excipient, such as cocoa butter, synthetic glyceride esters or polyethylene glycols, which are solid at ordinary temperatures, but liquefy and/or dissolve in the rectal cavity.

In certain embodiments, it is contemplated that these compositions can be extended release formulations. Typical extended release formations utilize an enteric coating. Typically, a barrier is applied to oral medication that controls the location in the digestive system where it is absorbed. Enteric coatings prevent release of medication before it reaches the small intestine. Enteric coatings may contain polymers of polysaccharides, such as maltodextrin, xanthan, scleroglu- can dextran, starch, alginates, pullulan, hyaluronic acid, chitin, chitosan and the like; other natural polymers, such as proteins (albumin, gelatin etc.), poly-L-lysine; sodium poly(acrylic acid); poly(hydroxyalkylmethacrylates) (for example poly(hydroxyethyl methacrylate)); carboxypolymethylene (for example Carbopol™); carboxer; polyvinyl pyrrolidone; gums, such as guar gum, gum arabic, gum karaya, gum ghatti, locust bean gum, tamarind gum, gellan gum, gum tragacanth, agar, pectin, gluten and the like; poly(vinyl alcohol); ethylene vinyl alcohol; polyethylene glycol (PEG); and cellulose ethers, such as hydroxymethyl cellulose (HMC); hydroxypropylcellulose (HPC); hydroxylcellulose (HEC); hydroxypropylcellulose (HPC); methylcellulose (MC); ethylcellulose (EC), carboxymethylcellulose (CMC); ethylhydroxyethylcellulose (EHESC); carboxymethylhydroxyethylcellulose (CMHEC); hydroxypropylmethylcellulose (HPMC); hydroxypropylethylcellulose (HPEC) and sodium carboxymethylcellulose (Na CMC); as well as copolymers and/or (simple) mixtures of any of the above polymers. Certain of the above-mentioned polymers may further be crosslinked by way of standard techniques.

The choice of polymer will be determined by the nature of the active ingredient/drug that is employed in the composition of the disclosure as well as the desired rate of release. In particular, it will be appreciated by the skilled person, for example in the case of HPMC, that a higher molecular weight will, in general, provide a slower rate of release of drug from the composition. Furthermore, in the case of HPMC, different degrees of substitution of methoxyl groups and hydroxypropoxy groups will give rise to changes in the rate of release of particles from the composition. In this respect, and as stated above, it may be desirable to provide compositions of the disclosure in the form of coatings in which the polymer carrier is provided by way of a blend of two or more polymers of, for example, different molecular weights in order to produce a particular required or desired release profile.

Microspheres of polylactide, polyglycolide, and their copolymers poly(lactide-co-glycolide) may be used to form sustained-release protein delivery systems. Particles can be entrapped in the poly(lactide-co-glycolide) microsphere depot by a number of methods, including formation of a water-in-oil emulsion with waterborne particles and organic solventborne polymer (emulsion method), formation of a solid-in-oil suspension with solid protein dispersed in a solvent-based polymer solution (suspension method), or by dissolving the protein in a solvent-based polymer solution (dissolution method). One can attach poly(ethylene glycol) to the particles (PEGylation) to increase the in vivo half-life of circulating therapeutic proteins and decrease the chance of an immune response.

Methods of Use

In certain embodiments, the disclosure relates to methods of treating or preventing an inflammatory disease or condition, auto-immune disease, allergy, or cancer comprising administering an effective amount of a pharmaceutical composition disclosed herein to a subject in need thereof.

In certain embodiments, the inflammatory disease is inflammatory bowel disease, Crohn's disease, ulcerative
clositis, proctitis, fulminant colitis, proctosigmoiditis, left-sided colitis, celiac disease, or enteric infection.

In certain embodiments, the inflammatory disease or condition is selected from arthritis, heart disease, infective or non-infective endocarditis, cardiomyopathy, myocarditis, chronic, or persistent, inflammatory myopathy, polymyositis, dermatomyositis, inclusion body myositis, inflammatory respiratory disease, asthma, cystic fibrosis, emphysema, chronic obstructive pulmonary disorder or acute respiratory distress syndrome.

In certain embodiments, the autoimmune diseases is selected from diabetes mellitus type I, Rheumatoid arthritis, temporal arteritis, eencephalomyelitis, autoimmune cardiomyopathy, eczema, Lupus erythematosus, or Sjögren’s syndrome.

In certain embodiments, the subject is in need of treatment as the subject is at risk of, exhibiting symptoms, or diagnosed with the disease or condition.

In certain embodiments, pharmaceutical comprising particles disclosed herein are administered in combination with an anti-inflammatory agent such as glucocorticoids, COX-2 inhibitors, non-steroidal anti-inflammatory drugs. Contemplated anti-inflammatory agents include, but are not limited to, aspirin, acetylsalicylic acid, advil, diclofenac, amfenac, amino-phenazone, ampicillin, amphetamine, amitriptyline, analgesia, anitrazafens, azapropazone, bendazac, benzylamine, bromfenac, bumadizone, carprofen, celecoxib, cimcicic, clofenzine, clonixin, copper ibuprofen, daranofen, dexibuprofen, diketoprofen, diclofenac, dihydrobenz, dihydrochloride, disulfiram, dextroxyzep, epirizole, ethansalicylate, etodolac, etofenamate, etoricoxib, famprofazone, felnbin, feunicam, acid, fenbuzen, fenofenac, fenofibrate, fenoprofen, fenzacina, fenoxoic acid, flaxofenine, flumizone, flunixin, fluroquinolone, ibuprofen, indomethacin, indoprofen, ketoprofen, ketorolac, licofelone, lonazol, loroxin, cloprofen, lornoxicam, magnesium salicylate, mavacon, mefenamic acid, meloxicam, meselaconzine, nifopropen, mofetibutuzone, norazone, nabumetone, naproxen, naproxin, nefapenac, nimesulide, oxaprozin, oxicam, oxypentobutazone, parecoxib, phenacetin, phenylbutazone, piroxicam, pirprofen, propanrofen, proglumetacin, pulvino, robenacoxib, rofecoxib, salicylic acid, salicylate, salinac, salac, salpane, tarenflurbil, tendap, tenoxicam, tepoxalin, tiaprofenic acid, trimexacin, tolfenamic acid, tolmetin, valdecoxib, vedaprolen, zomepirac, alclometasona, beclometasone dipropionate, betamethasone dipropionate, budesonide, chloroprednisone, ciclesonide, cortisporin, cortisporin, dexamethasone, fluoroxyacetic acid, fluviodide, fluconoxolone, fluvotretinene, fluvoruton, flumetholone, fluticasone, hydrocortamate, megastrol acetate, meprednisone, methylprednisolone, mometasone furoate, otoptic, paramethasone, predisolone, prednisolone, prednylidene, prednisediene, prednortriene, pregrenone, proctosedyl, rimexolone, tetarylhydrocortosterone, tobramycin/dexamethasone, trimacinolone, ububesol, and combinations thereof.

In certain embodiments, this disclosure relates to methods of treating cancer comprising administering an effective amount of a pharmaceutical composition comprising particles disclosed herein to a subject in need thereof. In certain embodiments, the cancer is selected from breast, pancreatic, colon, metastatic lung cancers, bladder cancer, lung cancer, breast cancer, melanoma, colon and rectal cancer, non-Hodgkin lymphoma, endometrial cancer, pancreatic cancer, kidney cancer, prostate cancer, leukemia, thyroid cancer, glioblastoma (GBM), and brain cancer. In certain embodiments, the particles are administered in combination with a second anti-cancer agent such as an agent selected from tamoxifen, gefitinib, erlotinib, docetaxel, cis-platin, 5-fluorouracil, gemcitabine, tegafur, raltitrexed, methotrexate, cytosine arabinoside, hydroxyurea, adriamycin, bleomycin, doxorubicin, daunomycin, epirubicin, idarubicin, mitomy cin-C, daunomycin, and vinblastine, vindesine, vincristine, taxol, temozolomide, bevacizumab, procarbazine, lomustine, vincristine, taxotere, etoposide, teniposide, amrascan, topotecan, camptothecin, bortezomib, anagrelide, streptol, raloxifene, droloxifene, idoxylene, fulvestrant, bicatulamide, flutamide, nilutamide, cyproterone, goserin, leuprolin, buserelin, megestrol, anastrozole, letrozole, vorozole, exemestane, finasteride, marinestat, trastuzumab, cetuximab, dasatinib, imatinib, combretastatin, thalidomide, and/or lenalidomide or combinations thereof.

The particles can be administered by a variety of routes including the oral, ocular, rectal, transdermal, subcutaneous, intravenous, intramuscular or intratotal routes, depending mainly on the specific preparation used. The particles will generally be administered in an “effective amount”, by which is meant any amount of particles that, upon suitable administration, is sufficient to achieve the desired therapeutic or prophylactic effect in the subject to which it is administered. Usually, depending on the condition to be prevented or treated and the route of administration, such an effective amount will usually be between 0.01 to 1000 mg per kilogram body weight of the patient per day, more often between 0.1 and 500 mg such as between 1 and 250 mg, for example about 5, 10, 20, 50, 100, 150, 200 or 250 mg, per kilogram body weight of the patient per day, which may be administered as a single daily dose, divided over one or more daily doses. The amount(s) to be administered, the route of administration and the further treatment regimen may be determined by the treating physician, depending on factors such as the age, gender and general condition of the patient and the nature and severity of the disease/symptoms to be treated. Reference is made to U.S. Pat. No. 6,372,715, U.S. Pat. No. 6,369,086, U.S. Pat. No. 6,250,152 and U.S. Pat. No. 6,372,733 and the further references mentioned above, as well as to the standard handbooks, such as the latest edition of Remington’s Pharmaceutical Sciences.

Examples

Nanoparticle Production and Characterization

The genes of AvrA and mutant form mAvrA were cloned into pGEX expression plasmids containing glutathione S-transferase (GST) and 6xhis tags using standard recombinant techniques. The mutant form contains a single cysteine substitution (C186A) that renders the acetyltransferase inactive and eliminates JNK inhibition. Following expression in E. coli, AvrA and mAvrA were column purified with GST resin under native conditions.

Protein nanoparticles were fabricated by ethanol solvation with several proteins, including AvrA, mAvrA, bovine serum albumin (BSA), green fluorescent protein (GFP), flagellin, and mixtures of these proteins. 3.5'-Dithiobis[sa-lufosucinimidylpropionate] (DTSSP) was used to crosslink the particles for stability during delivery to cells. DTSSP contains a central disulfide bond that can be reduced inside cells to release individual proteins. Solvent volume, cross-linker concentration, reaction time, and use of a reaction quencher were explored to produce protein nanoparticles with diameters of ~100 nm, shown in FIG. 2A, B. Particles containing GFP were fluorescent, implying that protein structure was not significantly affected by the crosslinking.
GFP particles crosslinked with DTSSP were tested for their ability to disassemble in a reducing environment. Particles were incubated in PBS containing 0 mM, 1 mM or 10 mM glutathione at 37°C to mimic intracellular reducing conditions. Soluble protein concentration was measured by fluorimetry. In the presence of either 1 mM or 10 mM glutathione, after 30 minutes the particles suddenly and completely disassembled, releasing soluble protein. In order to estimate the stability of the particles and proteins in the in vivo intestinal environment, soluble GFP and GFP nanoparticles were incubated in fasted intestinal simulation media (pH 6.5) with or without pancreatin for 2 hours. Fluorescence was measured to observe loss of model protein function. Soluble GFP lost twice as much fluorescence as nanoparticle GFP, with pancreatin as compared to without pancreatin. This data demonstrates the stabilizing or protective effect of the nanoparticle formulation (FIG. 2C).

Cellular Uptake and Trafficking of Nanoparticles

Nanoparticles were incubated with HeLa cells for preliminary quantification of cellular uptake. GFP was included in the nanoparticle formulation to enable assessment by fluorimetry, flow cytometry, confocal microscopy, and to compare with soluble proteins not formulated as nanoparticles. FIG. 3A shows the increase in total GFP delivered to cells by nanoparticles, as compared to free GFP molecules, measured by fluorimetry over a 5 hour incubation period. After uptake, it is important that the particles disassemble and free proteins make their way to the cytosol to bind their targets. Confocal microscopy in FIGS. 3B-D illustrate the uptake of intact particles, identified as punctate fluorescent spots, into cells and that individual (GFP) proteins are being released from the particles and diffusing into the cytosol, identified as diffuse fluorescence in the cell. Very little uptake is seen after 1 hour, while after 3 hours nanoparticles appear in cells with some diffusion, and after 6 hours most particles seem to have disassembled and diffused into the cytosol.

Effects on Pro-Inflammatory Signaling and Effectors In Vivo

AvrA inhibits JNK phosphorylation and IkB degradation in transfection and transgenic approaches. Experiments with AvrA nanoparticle preparations applied to the apical surface of polarized T84 monolayers (for 3 hours to allow particle internalization and dissociation) were successful in partly suppressing both TNF-α induced JNK activation and IkB degradation (FIG. 4A). This partial suppression may reflect the amount of AvrA that occurs during natural infection. Real-time imaging of a Salmonella model infection has shown that a different TTSS-secreted effector, SipA, mediates biochemical functions within minutes of infection at a concentration of 1000 molecules/cell. See Schlumberger et al., Proc Natl Acad Sci USA, 2005, 102(35):12548-12553. This is the target dose to achieve with modified AvrA nanoparticles. T84 monolayers incubated with AvrA nanoparticles and stimulated with TNF-α were also assayed for IL-8 secretion at 6 hours by ELISA (FIG. 4B). Secreted IL-8 was markedly reduced in particle treated cells. Collectively, the data in FIG. 4 demonstrate that the degree of INK suppression is sufficiently strong to block transcriptional upregulation of key inflammatory mediators, and confirms the bioactivity of recombinant AvrA nanoparticles.

In Vivo Models of Inflammation

The murine peritonitis model of acute inflammation was employed. Mice were pretreated with an intraperitoneal (IP) dose of active AvrA nanoparticles, mAvrA particles, inert BSA particles or PBS control for one hour before IP instillation of 10 mg zymosan, a fungal cell wall component commonly used as an inducer of acute inflammation. After four additional hours, mice were sacrificed and inflammatory exudates in the peritoneal cavity collected by lavage. Neutrophils were marked with relevant antibodies and populations quantified by flow cytometry.

As shown in FIG. 5, while essentially no inflammatory cells were detected in control (PBS treated, no zymosan) mice, positive control (PBS pretreated, zymosan) mice showed the expected accumulation of neutrophils. Strikingly, pretreatment with AvrA nanoparticles resulted in a significant reduction of neutrophil influx into the peritoneal cavity. Inert BSA particles showed no effects while mutant AvrA particles showed a reduced, statistically insignificant reduction in inflammation.

AvrA particles were tested in an in vivo model of colitis. TNBS-colitis is a rapid transmural colitis model induced by the rectal administration of the hapten reagent TNBS associated with a TH1 T cell response. Inflammatory responses can be observed within 24 hours. Whether luminal (transrectal) instillation of particles four hours prior to TNBS administration could modify this colitis model was tested. As shown in FIG. 6, at 48 hours TNBS results in marked colitis as measured by clinical parameters using a small animal veterinary endoscope and standard histological indices. Remarkably, these parameters were significantly improved in mice pretreated with the AvrA particles, while control BSA particles had no effect. Mutant AvrA particles showed significant activity suggesting the therapeutic enzymatic activity of AvrA is not abolished with a single mutation in the acetyltransferase active site. AvrA has also been shown to exhibit deubiquitinating activity that is not entirely abolished by the mutation.

SEQUENCE LISTING

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 35  40
Leu Glu Ser Glu Ile Ile Asp Gly Ser Trp Ile His Ile Ser Tyr Glu
 50  55  60
Glu Thr Asp Leu Glu Met Met Pro Phe Leu Val Ala Gln Ala Asn Lys
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Lys Tyr Pro Glu Leu Asn Leu Lys Phe Val Met Ser Val His Glu Leu
 85   90  95
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Phe Leu Val Asn Met Gly Ser Ser Gly Ile His Ile Ser Val Val Asp
115 120 125
Phe Arg Val Met Asp Gly Lys Thr Ser Val Ile Leu Phe Glu Pro Ala
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145 150 155 160
Leu Glu Arg Glu Gln Leu Pro Asp Cys Tyr Phe Ala Met Val Glu Leu
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Ala Lys Lys Leu Gln Leu Glu Phe Met Asn Leu Val Lys Ile His Glu
195 200 205
Asp Asn Ile Cys Glu Arg Leu Cys Gly Glu Pro Phe Leu Pro Ser
210 215 220
Asp Lys Ala Asp Arg Tyr Leu Pro Val Ser Phe Tyr Lys His Thr Glu
225 230 235 240
Gly Ala Gln Arg Leu Asn Glu Tyr Val Glu Ala Asn Pro Ala Ala Gly
245 250 255
Ser Ser Ile Val Asn Lys Asn Glu Thr Leu Tyr Glu Arg Phe Asp
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Glu Arg Glu Leu Ser Glu Ile Ile Asp Gly Ser Trp Ile His Ile Ser
 35  40  45
Tyr Glu Glu Thr Asp Leu Glu Met Met Pro Phe Leu Val Ala Gln Ala
 50  55  60
Asn Lys Lys Tyr Pro Glu Leu Asn Leu Lys Phe Val Met Ser Val His
 65  70  75  80
Glu Leu Val Ser Ser Ile Lys Glu Thr Arg Met Glu Gly Val Glu Ser
 85  90  95
Ala Arg Phe Ile Val Asn Met Gly Ser Ser Gly Ile His Ile Ser Val
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Lys Ala Ala Leu Glu Arg Glu Gln Leu Pro Asp Cys Tyr Phe Ala Met 145 150 155 160
Val Glu Leu Asp Ile Gln Arg Ser Ser Ser Glu Cys Gly Ile Phe Ser 165 170 175
Leu Ala Leu Ala Lys Lys Leu Gln Leu Glu Phe Met Asn Leu Val Lys 180 185 190
Ile His Glu Asp Arg Ile Cys Glu Arg Leu Cys Gly Glu Glu Pro Phe 195 200 205
Leu Pro Ser Asp Lys Ala Asp Arg Tyr Leu Pro Val Ser Phe Tyr Lys 210 215 220
His Thr Gln Gly Val Gln Arg Leu Asn Glu Tyr Val Gln Ala Asn Pro 225 230 235 240
 Ala Ala Gly Ser Ser Ile Val Asn Lys Lys Asn Glu Thr Leu Tyr Glu 245 250 255
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Asp Ile Ala Leu Met Asp Asp Phe Ile Ala Ala Ala Asn Gln Lys Lys 50 55 60
Glu Gly Leu Asn Ala His Phe Phe Arg Ser Pro Leu Asp Met Val Asn 65 70 75 80
Tyr Val Lys Ser Leu Ile Pro Ser Glu Arg Thr Thr Ala Arg Phe Val 85 90 95
Val Asn Met Gly Ser Gly Gly Ile His Cys Ile Ala Val Asp Cys Ala 100 105 110
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Lys Arg Glu Leu Pro Glu Thr Ser Leu Val Ile Met Glu Thr Asp Met 145 150 155 160
Gln Arg Ser Gln Gly Glu Cys Leu Met Phe Ser Leu Phe Leu Val Lys 165 170 175
Lys Met His Lys Asp Gly Leu Phe Gly Tyr Leu His Arg Lys Asn 190 195 190
Ile Asn Arg Glu Leu Pro Leu Thr Gln Gly Leu Ile Val Ser Val Lys
195 200 205
Asp Ala Asp Ser Leu Leu Pro Pro Ser Leu Met Lys His Thr Gln Ser
210 215 220
Pro Asn Arg Leu Gln Lys Tyr Leu Glu Met Arg Pro Glu Ala Met Asn
225 230 235 240
Cys Val Val Asn Lys Gly Glu Thr Leu Lys Thr Arg Gln Gln Arg
245 250 255
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LENGTH: 7
TYPE: PRT
ORGANISM: Artificial
FEATURE:
OTHER INFORMATION: Synthetic

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SEQ ID NO: 8
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ORGANISM: Artificial
FEATURE:
OTHER INFORMATION: Synthetic

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5
10
15

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FEATURE:
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SEQ ID NO: 10
LENGTH: 15
TYPE: PRT
ORGANISM: Artificial
FEATURE:
OTHER INFORMATION: Synthetic

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10
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OTHER INFORMATION: Synthetic

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1
5
10
15

Ala    Ala    Ala    Gly    Ser    Thr    Met    Gly    Ala    Trp    Ser    Gln    Gln    Gln    Gln
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FEATURE:
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SEQ ID NO 14
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ORGANISM: Artificial
FEATURE:
OTHER INFORMATION: Synthetic

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SEQ ID NO 15
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FEATURE:
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SEQ ID NO 16
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| Leu Asp Cys Ile Arg Ala Ile Ala Ala Asp Ala Asp Ala Val Thr |
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| Phe Leu Lys Val Pro Pro Arg Met Asp Ala Lys Met Tyr Leu Gly Tyr |
| 325 | 330 | 335 |  |
| Glu Tyr Val Thr Ala Ile Arg Asn Leu Arg Glu Gly Thr Cys Pro Glu |
| 340 | 345 | 350 |  |
| Ala Pro Thr Asp Glu Cys Lys Pro Val Lys Thr Cys Ala Leu Ser His |
| 355 | 360 | 365 |  |
| His Gin Leu Phe Cys Gin Ser Phe Val Gin Thr Val Ser Val Arg Ser |
| 370 | 375 | 380 |  |
| Ile Gin Cys Gin Ser Gin Gin Thr Gin Thr Gin Gin Gin Gin |
| 385 | 390 | 395 | 400 |  |
| Met Asn Gin Glu Ala Asp Ala Met Ser Leu Asp Gin Gin Gin Gin |

-continued
Val Pro Asp Lys Val Arg Trp Cys Ala Val Ser Glu His Glu Ala
1  5  10  15
Thr Lys Cys Gln Ser Phe Arg Asp His Met Lys Ser Val Ile Pro Ser
20 25  30
Asp Gly Pro Ser Val Ala Cys Val Lys Ala Ser Tyr Leu Asp Cys
35 40  45
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50 55  60
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<211> LENGTH: 679
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic

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545 550 555 560
Leu Leu Cys Leu Asp Gly Thr Arg Lys Pro Val Glu Glu Tyr Ala Asn
565 570 575
Cys His Leu Ala Arg Ala Pro Asn His Ala Val Thr Arg Lys Asp
580 585 590
Lys Glu Ala Cys Val His Lys Ile Leu Arg Gin Gin Gin Gin Leu Phe
595 600 605
Gly Ser Asn Val Ala Asp Cys Ser Gly Asm Phe Cys Leu Phe Arg Ser
610 615 620
Glu Thr Lys Asp Leu Leu Phe Arg Asp Thr Val Cys Leu Ala Lys
625 630 635 640
Leu His Asp Arg Asn Thr Tyr Glu Lys Tyr Leu Gly Glu Glu Tyr Val
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<213> ORGANISM: Artificial
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<222> OTHER INFORMATION: Synthetic
<220> FEATURE:
<221> NAME/KEY: misc_feature
<223> OTHER INFORMATION: X is palmitoyl group anchored to a synthetic
diaminopropionic acid (Dap) residue
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (3)...(3)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
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Pro Gly Gly His His His His
20 25

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<213> ORGANISM: Artificial
<220> FEATURE:
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<210> SEQ ID NO 20
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20 25 30
Ser Ser Gly Leu Arg Ile Asn Ser Ala Lys Asp Asp Ala Ala Gly Gln
35 40 45
Ala Ile Ala Asn Arg Phe Thr Ala Asn Ile Lys Gly Leu Thr Gln Ala
50 55 60
Ser Arg Asn Ala Asn Asp Gly Ile Ser Ile Ala Gln Thr Thr Gly
65 70 75 90
Ala Leu Asn Gln Ile Asn Asn Leu Glu Arg Val Arg Glu Leu Ala
85 90 95
Val Gln Ser Ala Asn Ser Thr Asn Ser Gln Ser Asp Leu Asp Ser Ile
100 105 110
Gln Ala Glu Ile Thr Gln Arg Leu Asn Glu Ile Asp Arg Val Ser Gly
115 120 125
Gln Thr Gln Phe Asn Gly Val Lys Val Leu Ala Gln Asp Asn Thr Leu
130 135 140
Thr Ile Gln Val Gly Ala Asn Asp Gly Glu Thr Ile Asp Ile Asp Leu
145 150 155 160
Lys Gln Ile Asn Ser Glu Thr Leu Gly Leu Asp Thr Leu Asn Val Gln
165 170 175
Gln Lys Tyr Lys Val Ser Asp Thr Ala Ala Thr Val Thr Gly Tyr Ala
180 185 190
Asp Thr Thr Ile Ala Leu Asp Asn Ser Thr Phe Lys Ala Ser Ala Thr
195 200 205
Gly Leu Gly Gly Thr Asp Gln Ile Asp Gly Asp Leu Lys Phe Asp
210 215 220
Asp Thr Thr Gly Lys Tyr Tyr Ala Lys Val Thr Val Thr Gly Gly Thr
225 230 235 240
Gly Lys Asp Gly Tyr Tyr Glu Val Ser Val Asp Lys Thr Asn Gly Glu
245 250 255
Val Thr Leu Ala Gly Gly Ala Thr Ser Pro Leu Thr Gly Gly Leu Pro
260 265 270
Ala Thr Ala Thr Glu Asp Val Lys Val Val Ala Asn Ala Asp
275 280 285
Leu Thr Glu Ala Lys Ala Ala Leu Thr Ala Ala Gly Thr Val Gln Thr
290 295 300
 Ala Ser Val Val Lys Met Ser Tyr Thr Asp Asn Asn Gly Lys Thr Ile
305 310 315 320
Asp Gly Gly Leu Ala Val Lys Val Gly Asp Asp Tyr Ser Ala Thr
325 330 335
Gln Asn Lys Asp Gly Ser Ile Ser Ile Asn Thr Thr Lys Tyr Thr Ala
340 345 350
Asp Asp Thr Ser Lys Thr Ala Leu Asn Lys Leu Gly Gly Ala Asp
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<212> TYPE: PRT
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<400> SEQUENCE: 21

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Asn Asn Asn Leu Gln Arg Val Arg Glu Leu Ala Val Gln Ser Ala Asn 35 40 45
Ser Thr Asn Ser Gln Ser Asp Leu Ser Ile Gln Ala Glu Ile Thr 50 55 60
Gln Arg Leu Asn Gln Ile Glu Arg Val Ser Gly Thr Gln Phe Asn 65 70 75 80
Gly Val Lys Val Leu Ala Gln Asp Asn Thr Leu Thr Ile Gln Val Gly 85 90 95
Ala Asn Asp Gly Glu Thr Ile Asp Ile Asp Leu Lys Gln Ile Asn Ser 100 105 110
Gln Thr Leu Gly Leu Asp Thr Leu Asn Val Gln Gln Lys Tyr Lys Val 115 120 125
Ser Asp Thr Ala Ala Val Thr Gly Tyr Ala Asp Thr Thr Ile Ala 130 135 140
Leu Asp Asn Ser Thr Phe Lys Ala Ser Ala Thr Gly Leu Gly Gly Thr 145 150 155 160
Asp Gin Lys Ile Asp Gly Asp Leu Lys Phe Asp Asp Thr Thr Gly Lys 165 170 175
Tyr Tyr Ala Lys Val Thr Val Thr Gly Thr Gly Lys Asp Gly Tyr 180 185 190
Tyr Glu Val Ser Val Asp Lys Thr Asn Gly Glu Val Thr Leu Ala Gly 195 200 205
Gly Ala Thr Ser Pro Leu Thr Gly Leu Pro Ala Thr Ala Thr Glu 210 215 220
Asp Val Lys Asn Val Gln Val Ala Asn Ala Asp Leu Thr Glu Ala Lys 225 230 235 240
Ala Ala Leu Thr Ala Ala Gly Val Thr Gly Thr Ala Ser Val Val Lys 245 250 255
Met Ser Tyr Thr Asn Asn Gly Lys Thr Ile Asp Gly Gly Leu Ala 260 265 270
Val Lys Val Gly Asp Asp Tyr Tyr Ser Ala Thr Gln Asn Lys Asp Gly  
275 380 285  
Ser Ile Ser Ile Asn Thr Thr Lys Tyr Thr Ala Asp Asp Gly Thr Ser  
290 395 305  
Lys Thr Ala Leu Asn Leu Gly Gly Ala Asp Gly Lys Thr Glu Val  
310 315 320  
Val Ser Ile Gly Gly Lys Tyr Thr Ala Ala Ser Lys Ala Glu Gly His  
325 330 335  
Asn Phe Lys Ala Gln Pro Asp Leu Ala Glu Ala Ala Thr Thr Thr  
340 345 350  
Glu Asn Pro Leu Gln Lys Ile Asp Ala Ala Leu Ala Glu Val Asp Thr  
355 360 365  
Leu Arg Ser Asp Leu Ala Val Gln Asn Arg Phe Asn Ser Ala Ile  
370 375 380  
Thr Asn Leu Gly Asn Thr Val Asn Asn Leu Thr Ser Ala Arg  
385 390 395  
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"<223> OTHER INFORMATION: Synthetic  
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20 25 30  
Ser Thr Gly Ser Arg Ile Asn Ser Ala Lys Asp Asp Ala Ala Gly Leu  
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Gln Ile Ala Asn Arg Leu Thr Ser Gln Val Asn Gly Leu Asn Val Ala  
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Thr Lys Asn Ala Asn Gly Asp Gly Ile Ser Leu Ala Gln Thr Ala Glu Gly  
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85 90 95  
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100 105 110  
Asn Gly Glu Val Lys Gln Leu Gln Lys Glu Leu Asp Arg Ile Ser Asn  
115 120 125  
Thr Thr Thr Phe Gly Gly Arg Lys Leu Leu Asp Gly Ser Phe Gly Val  
130 135 140  
Ala Ser Phe Gln Val Gly Ser Ala Ala Asn Leu Ile Ser Val Gly  
145 150 155 160  
Ile Gly Gly Lys Leu Met Ile Lys Leu Lys Phe Gly Val Phe Phe  
165 170 175  
Thr Val Leu Ser Ser Ala Tyr Ala His Gly Thr Pro Gln Asn Ile  
180 185 190  
Thr Asp Leu Cys Ala Glu Tyr His Asn Thr Gln Ile His Thr Leu Asn  
195 200 205  
Asp Lys Ile Phe Ser Tyr Thr Glu Ser Leu Ala Gly Lys Arg Glu Met  
210 215 220  
Ala Ile Ile Thr Phe Lys Asn Gly Ala Thr Phe Gln Val Glu Val Pro  
225 230 235 240
-continued

Gly Ser Gln His Ile Asp Ser Gln Lys Lys Ala Ile Glu Arg Met Lys  
245  250  255

Asp Thr Leu Arg Ile Ala Tyr Leu Thr Glu Ala Lys Val Glu Lys Leu  
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Cys Val Trp Asn Asn Lys Thr Pro His Ala Ala Ala Ala Ile Ser Met  
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Ala Asn  
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<210> SEQ ID NO 23  
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<220> FEATURE:  
<223> OTHER INFORMATION: Synthetic  

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20  25  30

Ile Gln Ala Glu Ile Thr Gln  
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1  5  10  15

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20  25  30

Gln Ile Asn Ser Gln Thr Leu Gly Leu Asp Thr Leu  
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1  5  10  15

Leu Ala Val Gln Ser Ala Asn Ser Thr Ann Ser Gln Ser Asp Leu Asp  
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Ser Ile Gln Ala Glu Ile Thr Gln Arg Leu Asn Glu Ile Asp Arg Val  
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Asn Gly  
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Ser Ser Gly Leu Arg Ile Asn Ser Ala Lys Asp Asp Ala Ala Gly Gln 35 40 45
Ala Ile Ala Asn Phe Thr Ala Asn Ile Gly Leu Thr Gln Ala Ser 50 55 60
Arg Asn Ala Asn Asp Gly Ile Ser Ile Ala Gln Thr Thr Gly Ala 65 70 75 80
Leu Asn

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Asp Leu Gly Ala Val Gln Asn Arg Phe Asn Ser Ala Ile Thr Asn Leu 20 25 30

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Ile Thr Asn Leu Gly Asn Thr Val Asn Asn Leu Ser Ser 20 25

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Ala Leu Ala Gln Val Asp Thr Leu Arg Ser Asp Leu Gly Ala Val Gln 20 25 30
Asn Arg Phe Asn Ser Ala Ile Thr Asn Leu Gly Asn Thr Val Asn Asn 35 40 45
Leu Ser Ser 50

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Phe Leu Gly Ile Ala Glu Ala Ile Asp Ile Gly Asn Gly Trp Gly
The invention claimed is:

1. A method of treating an inflammatory disease comprising administering an effective amount of a pharmaceutical composition comprising particles comprising
   a) recombinant AvrA proteins; and
   b) linking groups comprising disulfide bonds, wherein the linking groups conjugate the recombinant AvrA proteins to form the particles, and wherein the size of the particles are between 50 nm and 200 nm in diameter, to a subject in need thereof.

2. The method of claim 1, wherein the inflammatory disease is inflammatory bowel disease, Crohn’s disease, ulcerative colitis, proctitis, fulminant colitis, proctosigmoiditis, left-sided colitis celiac disease, or enteric infection.

3. The method of claim 2, wherein the subject is in need of treatment as the subject is at risk of, exhibiting symptoms, or diagnosed with an inflammatory bowel disease.

4. The method of claim 2, wherein the subject is exhibiting symptoms of abdominal pain or bloody diarrhea or combination thereof.

5. The method of claim 3, wherein the composition is administered in combination with second anti-inflammatory agent.

6. The method of claim 1, wherein the recombinant AvrA is mAvrA(C186A).

7. The method of claim 1, wherein the recombinant AvrA proteins are fusion proteins comprising bovine serum albumin (BSA), green fluorescent protein (GFP), glutathione S-transferase (GST), polyhistidine, polylysine, or combinations thereof.

8. The method of claim 1, wherein the linking groups have the following formula,
wherein,

n is 1 to 100,

L forms amides to the recombinant AvrA proteins, and

X is at each occurrence individually and independently selected from O, NH, C==O, CH₂, OCH₂, CH₂O,

NHCH₂, CH₂NH, OCH₂CH₂, CH₃CH₂O, NHCH₂CH₂, or CH₃CH₂NH.

9. The method of claim 8, wherein X is (CH₂)n wherein n is 1 to 4.

* * * * *