Title: Methods and Compositions for Providing Protective Immunity in the Elderly

Methods and Compositions for Providing Protective Immunity in the Elderly

Cross Reference to Related Applications

This application claims priority to the filing date of US Provisional Application No. 61/292,593 filed January 6, 2010; the disclosure of which is incorporated by reference.

Background of the Invention

Many elderly humans (greater than 65 years of age) have been immunized or exposed to many diseases, such as influenza, yet many may still require immunization in order to remain healthy. The immune response to active immunization tends to decrease with age.

Improving the response to influenza vaccines has been a major initiative since the 1970s (Mostow SR, et al. (1973) Inactivate vaccines in Volunteer studies with very high doses of influenza vaccine purified by zonal centrifugation Postgrad Med. J. 49: 152-158). Numerous investigators have explored the effect of doses of hemagglutinin (HA) greater than the standard 15 µg dose. The most thorough and well documented effort to provide an efficacious flu vaccine for the elderly has involved FluZone®. Three successive trials (Keitel et al. (2006) Safety of high doses of influenza vaccine and effect on antibody responses in elderly persons. Arch. Intern. Med. 166: 1121-1127 (Table 1); Couch et al. (2007) Safety and immunogenicity of a high dose trivalent influenza vaccine among elderly subjects Vaccine 25: 7656-7663 (Table 2) and Falsey et al., (2009) Randomized, double-blind controlled phase 3 trial comparing the immunogenicity of high-dose and standard-dose influenza vaccine in adults 65 years and older. J. Infect Dis. 200: 172-180 (Table 3)) have shown that increasing the dose of HA from 15 µg to 60 µg, focusing on the HA component, yields an improvement in HA1 titers of about 1.6 to 1.7 fold.

Table 1. Keitel, Arch. Int. Med. 2006

		HAI Titers	
H1	Dose (µg)	Pre	Post
Std Vx	15	20	37
	30	20	50
	60	22	61

Table 2. Elderly, Couch, Vaccine 2007

	HAI Titers			HAI Titers	
H1	All points pre	All points post	H1	No prior vx pre	No prior vx post
Std 15 μg	11	21	Std 15 μg	7.4	24
Hi 60 μg	9	36	Hi 60 μg	6.1	54

Table 3. Falsey, Treanor, JID, 2009, Standard dose vs. 60μg in 3876 subjects

		HAI Titers				
H1	Dose	Day 0	Day 28	% SC	%SP	Fold x
High	60μg	28.5	115.6	48.6	89.9	4.1
Std	15μg	29.4	67.3	23.1	76.8	2.3
VAX125	2μg	24.6	69.6	35	70	2.8
	3μg	51	149.3	40	95	2.9

More aggressive studies with doses as high as 405 μg (Keitel, et al. (1994) High doses of purified influenza A virus hemagglutinin significantly augment serum and nasal secretion antibody responses in healthy young adults. J Clin Microbiol 32: 2468-2473; (Table 4) Keitel et al. (1996) Increased doses of purified influenza hemagglutinin and subvirion vaccines enhance antibody responses in the elderly Clin Diagn Lab immunol 3: 507-510 (Table 5)) resulted in an approximately 2.1 fold improvement in HA1 titers.

Table 4. Keitel, Couch, J. Clin. Microbiol. 1994, Bromelain Cleaved, Pure HA vs. Std. VX

		HAI Titers			HAI Titers		
H1	Dose (μg)	Lo Pre	Lo Post	% SC	Hi Pre	Hi Post	%SC
Mono	15	6	97	100	42	128	53
Purified	45	7	100	100	39	294	87
	135	6	100	100	37	274	87
	405	6	100	34	34	239	100
Std VX	15	20	55				
Tri-valent	15	17	63				
Purified	45	14	86				
	135	16	73				

Table 9. Elderly, Treanor, JID 2006

H1Bv	Pre	Post	%SC
Std VX	15	38	21
15μg	14	25	12
45μg	18	42	26
135μg	17	38	20

In general, the elderly are a difficult population for influenza vaccination. While increasing the amount of vaccine administered provides some benefit, it is not a proportional increase. Adjuvants may also help increase immunogenicity but they also increase the possibility of adverse reactions. Thus, there has been limited improvement in boosting the immune response in elderly to influenza by increasing the amount of HA administered. There is still a need for improved vaccine formulations for the elderly which provide increased immunity for the elderly.

Summary of the Invention

The invention is generally directed to methods of stimulating immune responses in older and elderly humans. The present invention includes compositions and methods of stimulating an immune response in a human, comprising the step of administering to the human a composition comprising at least a portion of at least one flagellin and at least a portion of at least one antigen, wherein the composition is administered to the human in a dose sufficient to provoke an immune response where the human is an elderly human, that is a human who is older than 49 years of age. In certain embodiments the compositions are administered to humans who are older than 55 years of age, or older than 60 years of age or older than 65 years of age or older than 70 years of age or older than 75 years of age or older than 80 years of age.

The compositions of the present invention comprise at least a portion of at least one flagellin and at least a portion of at least one antigen. The flagellin and the antigen may be associated in different ways. For example, the flagellin and the antigen may be part of a fusion protein in which the flagellin portion is associated with the antigen by constructing a DNA or RNA sequence that when expressed produces a protein containing both flagellin and antigen portions as part of a protein. The flagellin portion and antigen portion can be associated in other ways, for example each of these portions may be associated with a particle or some type of carrier where both the flagellin portion and the antigen portion may be attached to the carrier or particle.

The present invention also relates to compositions and methods of stimulating an immune response in an elderly human population, comprising the step of administering to the human population a composition that includes a fusion protein comprising at least a portion of at least one flagellin and at least a portion of at least one antigen, wherein seroconversion rate is at least 50% or at least 60% or at least 70% or at least 80% or at least 90% or at least 95% or at least 99%

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The present invention also relates to compositions and methods of stimulating an immune response in an elderly human population, comprising the step of administering to the human population a composition that includes a fusion protein comprising at least a portion of at least one flagellin and at least a portion of at least one antigen, wherein the seroprotection rate is at least 50% or at least 60% or at least 70% or at least 80% or at least 90% or at least 95% or at least 99%.

Figures

Figure 1 - Amino Acid Sequence of STF2.HA1 (VAX125)

Figure 2A and B – Graphic representation of peak GM HAI response measured 14 days after vaccination with VAX125 among subjects ≥ 65 years old (Figure 2A). GM HAI titers measured from day 0 to 28 after a single dose of VAX125 ranging from 0.5 to 8 μg among subjects ≥ 65 years old (Figure 2B)

Figure 3 – Plasmid map for Vax 128A, Vax 128B and Vax 128C

Figure 4 – Nucleotide sequence for Vax 128A (HL184 STF2.HA1-2 CA7 (CA07 H1N1))

Figure 5 – Amino acid sequence for Vax 128A (HL184 STF2.HA1-2 CA7 (CA07 H1N1))

Figure 6 – Nucleotide sequence for Vax 128B (HL185 STF2.HA1-2 CA7 (CA07 H1N1))

Figure 7 – Amino acid sequence for Vax 128B (HL185 STF2.HA1-2 CA7 (CA07 H1N1))

Figure 8 – Nucleotide sequence for Vax 128C (HL186 STF2.HA1-2 CA7 (CA07 H1N1))

Figure 9 – Amino acid sequence for Vax 128C (HL186 STF2.HA1-2 CA7 (CA07 H1N1))

Figure 10 - Comparison of immune response by dose among 112 subjects 18-49 years old and 100 subjects ≥ 65 years old who received a single IM dose of VAX128 (CA07). Note: responses to VAX128A-C have been pooled

Detailed Description of the Invention

The features and other details of the invention, either as steps of the invention or as combinations of the parts of the invention will now be more particularly described, and pointed out in the claims. It will be understood that the particular embodiments of the invention are shown by way of illustration and not as limitations to the invention. The principle features of this invention can be employed in various embodiments without departing from the scope of the invention.

In an embodiment, the invention is a method of stimulating an immune response in a human, comprising the step of administering to the human a composition comprising at least one portion of at least one antigen and at least one portion of at least one flagellin, wherein the composition is administered to a human that is at least 49 years old. Administration of the composition to the human can provide protective immunity against an infection consequent to exposure of the human to a source of the antigen.

The compositions of the present invention comprise at least a portion of at least one flagellin and at least a portion of at least one antigen. The flagellin and the antigen may be associated in different ways. For example, the flagellin and the antigen may be part of a fusion protein in which the flagellin portion is associated with the antigen by constructing a DNA or RNA sequence that when expressed produces a protein containing both flagellin and antigen portions as part of a protein. The flagellin portion and antigen portion can be associated in other ways, for example each of these portions may be associated with a particle or a carrier where both the flagellin portion and the antigen portion, including but not limited to proteins, lipoproteins, carbohydrates, polysaccharides and/or lipids, may be attached to the carrier or particle. The particle or carrier can be any vehicle by which the flagellin portion and the antigen portion can be associated such that when introduced to a human an immune response is generated. Particle formulations may be made of a variety of materials, including but not limited to lipids, proteins, waxes or amino acids, polysaccharides, polyacrylic substances or organic acids. In one embodiment, the flagellin portion and antigen portion may be associated with virosomes as, for example, described in US Patent Publication No. 20100136053 which is herein incorporated by

Table 5. Elderly, Keitel, Clin. Diag. Lab. 1996, Purified HA vs. Normal Subvirion

		HAI Titers	
H1	Dose (μg)	pre	Post
Subvirion	15	34	42
	45	42	56
	135	32	84
Purified	15	36	42
	45	36	84
	135	22	120

Other studies demonstrating the effects of influenza vaccine in the elderly are shown in the tables below.

Table 6 Increased dose or booster in Elderly, Cools et al. J. Med. Virol. 2009, 15μg, 15 + 15μg, 30μg, 30 + 15μg

	HAI Titers				HAI Titers			
H3 only	Day 0	Day 25	SC%	SP%	Day 84	Day 109	SC%	SP%
15 + pbo	22.7	57.8	33.3	66.3		49.3	21.7	63.9
15 + 15	22.2				48.6	53.9	34.5	61.9
30 + pbo	18.4	70.6	45.3	73.3			35.4	64.9
30 + 15	20.6				53.5	66.4	43.6	74.2

Table 7 AVP-MSD Vaxigrip vs. Fluad in Elderly, Squarcione, Sgricia, Biasio, Pernetti Vaccine 2003.

H1	Vaxigrip	Fluad
Pre-GMT	10.4	11.3
Post d21 GMT	87.1	154.4
Seroprotection %		
Under 75 years of age	74.9	86.4
Over 75 years of age	66.1	78.9
Whole population	71.6	83.4
Seroconversion		
Under 75 years of age	66.9	77.9
Over 75 years of age	59.5	73.1
Whole population	63.7	76.0

Table 8. Fluad vs Fluzone, Frey, Poland, Vaccine 2003, Adults 18-64

H1	GMT day 28-1	GMT day 28-2	Med req'd Yr 1	Med req'd Yr 2
Fluad	951	216	39%	35%
Fluzone	850	263	26%	24%

reference. Other potential particles to which the antigen(s) and flagellin(s) may be attached may include but are not limited to virus-like particles, biodegradable microspheres, liposomes, peptide nanoparticles, nanoparticles and self-assembling virus like particles as described in WO2010/002818 herein incorporated by reference. In one embodiment flagellin and antigen are associated with self-assembling peptide nanoparticles (SAPN). SAPN are described in PCT patent application WO 2009/109428 herein incorporated by reference. These nanoparticles are comprised of aggregates of a continuous peptidic chain comprising two oligomerization domains connected by a linker segment wherein one or both oligomerization domains incorporate T-cell epitopes and/or B-cell epitopes within their peptide sequence.

Antigens that can be used in combination with flagellin in the compositions and methods of the present invention are any antigen that will provoke an immune response in a human. Antigens used in the compositions of the present invention include viral antigens such as influenza viral antigens (e.g. hemagglutinin (HA) protein, matrix 2 (M2) protein, neuraminidase), respiratory synctial virus (RSV) antigens (e.g. fusion protein, attachment glycoprotein), papillomaviral (e.g. human papilloma virus (HPV), such as an E6 protein, E7 protein, L1 protein and L2 protein), Herpes Simplex, rabies virus and flavivirus viral antigens (e.g. Dengue viral antigens, West Nile viral antigens), hepatitis viral antigens including antigens from HBV and HC. Antigens used in the compositions of the present invention include bacterial antigens including those from *Streptococcus pneumonia, Haemophilus influenza, Staphylococcus aureus, Clostridium difficile* and enteric gram-negative pathogens including *Escherichia, Salmonella, Shigella, Yersinia, Klebsiella, Pseudomonas,Enterobacter, Serratia, Proteus.* Antigens used in the compositions of the present invention include fungal antigens including those from *Candida spp., Aspergillus spp., Crytococcus neoformans, Coccidiodes spp., Histoplasma capsulatum, Pneumocystis carinii, Paracoccidiodes brasiliensis, Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, and Plasmodium malariae.*

The ratio of flagellin to antigen in the compositions of the present invention may be from about 100:1 to about 1:100. In other embodiments the ratio of flagellin to antigen may be from about 1:20 to about 20:1. In a preferred embodiment of flagellin and influenza antigen the ratio of flagellin to influenza antigen is from about 1:20 to about 1:5. In some embodiments, especially those where flagellin and influenza antigen are linked in a fusion protein the ratio of flagellin to influenza antigen may be from 5:1 to 1:5.

The compositions of the present invention may include one or more types of flagellin associated with one or more type of antigens. With respect to particle carriers several different flagellin may be associated with one or more antigens which may be on the same or different particles.

In preferred embodiments the antigen contained within the compositions of the present invention is an antigen from influenza virus. A preferred antigen is hemagglutinin (HA). In preferred embodiments the HA sequences are conjugated to flagellin or to engineered flagellins as described in WO 2009/128950 herein incorporated by reference. Preferred embodiments of the present invention that utilize HA as an antigen are VAX 125, VAX 128A, VAX 128B and VAX 128C. VAX125, also known as STF2.HA1(SI), is a recombinant fusion protein that consists of Salmonella typhimurium flagellin type 2 (STF2), a TLR5 ligand, fused at its C-terminus to the globular head (amino acids 62-284) of the HA1 domain of the HA of influenza A/Solomon Islands/3/2006 (H1N1) and has a molecular mass of 77,539 kDa. By molecular weight approximately two-thirds of the vaccine composition is flagellin and one-third is influenza HA globular head. Thus, a 3 ug dose consists of 2 ug of flagellin and 1 ug of influenza HA. The amino acid sequence of VAX 125 (SEQ ID 1) is shown in Figure 1. VAX128A, also known as HL184 STF2.HA1-2 CA7, is a recombinant fusion protein that consists of Salmonella typhimurium flagellin type 2 (STF2), a TLR5 ligand, fused at its C-terminus to the globular head of the HA1 domain of the HA of Influenza A/California/07/2009 (H1N1) a pandemic strain of influenza. The nucleotide (SEQ ID 2) and amino acid (SEQ ID 3) sequences of VAX 128A are shown in Figures 4 and 5, respectively. VAX128B, also known as HL185 STF2.HA1-2 CA7, is an R3 fusion where the D3 domain of the flagellin has been removed and replaced with the globular head of the HA1 domain of the HA of Influenza A/California/07/2009 (H1N1). The nucleotide (SEQ ID 4) and amino acid (SEQ ID 5) sequences of VAX 128B are shown in Figures 6 and 7, respectively. VAX128C, also known as HL186 STF.2.HA1-2 CA 7, is a R32x fusion wherein the D3 domain of the flagellin has been removed and replaced with the globular head of the HA1 domain of the HA of Influenza A/California/07/2009 (H1N1) and the C-terminus of this flagellin is fused to the globular head of the HA1 domain of the HA of Influenza A/California/07/2009 (H1N1). Thus, the fusion protein contains two copies of the HA antigen per flagellin unit. The nucleotide (SEQ ID 6) and amino acid (SEQ ID 7) sequences of VAX 128B are shown in Figures 8 and 9, respectively. In VAX128C there are two copies of the HA globular head and the proportion of HA in the vaccine composition increases to 54% from 33% in the VAX128A construct.

about 4μg to about 50μg, 4μg to about 30μg, from about 4μg to about 20μg, from about 4μg to about 10μg, from about 4μg to about 8μg, from about 5μg to about 50μg, 5μg to about 30μg, from about 5μg to about 20μg, from about 5μg to about 10μg, from about 5μg to about 9μg, and from about 5μg to about 8μg. With respect to fusion protein compositions comprising flagellin and influenza antigen the dosage refers to the amount of protein present in the vaccine given to the human. Some of the protein quantity relates to the antigen and some of the protein quantity relates to the flagellin. For example, in VAX 125 and VAX 128A and VAX128B the HA1 antigen comprises about 33% of the molecular weight of the construct. In VAX128C there are two copies of the HA globular head and the proportion of HA in the vaccine increases to 54% from 33% in the VAX128A construct. In embodiments that use antigens other than HA it may be more useful to characterize the amount of the dosage by the amount of antigen, especially of the antigen is of a non-proteinaceous origin. In any event, the flagellin/antigen compositions of the present invention provide superior properties to the prior art compositions in treatment of the elderly.

With respect to compositions in which the flagellin and antigen are delivered via particles the dosage will depend on the type of particle used and the ratio of flagellin to antigen present in the composition. With respect to compositions in which flagellin and the antigen are present in ratios other than 1:1 the dosage will depend on the ratio of components. With respect to antigens other than influenza antigens the dosage will depend on the antigen used and the ratio of flagellin to the antigen. One of skill in the art may readily be able to determine the optimal dosing of the flagellin compositions based on the carrier, antigen and ratio of flagellin to antigen.

The antigen component of the fusion protein compositions of the present invention are preferably influenza antigens (influenza A, influenza B, influenza C antigen). In preferred embodiments of the compositions at least one member selected from the group consisting of a hemagglutinin influenza antigen (e.g., the fusion protein includes SEQ ID NO. 1 (Figure 1)), a matrix 2 (M2, such as an extodomain of M2, also referred to as "M2e") or a neuraminidase influenza antigen. The influenza antigen can include an H1N1 influenza antigen. In preferred embodiments, the antigen is fused to a flagellin molecule sequence such as described in WO 2009/128950 which is herein incorporated by reference. The compositions of the present invention may comprise one or more fusion proteins with one or more antigen sequences included in each fusion protein.

Advantages of Applicant's claimed methods include the ability to generate a protective immune response in an elderly population employing relatively low doses of antigens to infectious agents (e.g. influenza). The elderly are typically at high risk for disease consequent to exposure to an infectious agent, such as an influenza antigen, due, in part, to their inability to mount a suitable and sustainable immune response to an infectious antigen. A proposed approach to increase an elderly human's response to an antigen is to increase the dose of antigen and the frequency of dosing. However, such alternative treatment protocols have resulted in less than optimal immune responses to antigens and diminished the ability to provide protective immunity to disease consequent to exposure to antigens in elderly populations (See the tables herein provided concerning prior art products and studies). The methods employed herein improve immune responses in elderly populations so that an excess of 40% of the elderly population mount a suitable and sustainable immune response to an antigen administered in a relatively low dose, which also has the advantage of minimal side effects. The compositions of the present invention are more immunogenic and less reactogenic than prior art vaccines.

In one embodiment the human to be treated with the compositions and methods of the present invention is between 49 years old and about 64 years old. In another embodiment, the human may be older than 64 years old. In certain embodiments the compositions are administered to humans who are older than 55 years of age, or older than 60 years of age or older than 65 years of age or older than 70 years of age or older than 75 years of age or older than 80 years of age. The dose of compositions of the present invention may be selected to optimize the effects in the elderly while seeking to reduce any untoward side-effect. For example in the fusion protein compositions comprising flagellin and HA (as described in the case of VAX 125, VAX 128A, VAX 128B and VAX 128C) the does may be selected to optimize the immunogenic response while attempting to keep reactogenicity low. At least one dose selected from the group consisting of a 0.1μg, 0.5μg, 1μg dose, 2μg dose, 3μg dose, 4μg dose, 5μg dose, 6μg dose, 7μg dose, 8μg dose, 9μg dose, 10μg dose, 15μg dose, 20μg dose, 25μg dose and a 30μg dose may be sufficient to induce an immune response in elderly humans. The dose of the fusion protein may be administered to the human within a range of doses including from about 0.1μg to about 500μg, 1μg to about 100μg, 1μg to about 50μg, from about 1μg to about 30μg, from about 1μg to about 25μg, from about 1μg to about 20μg, from about 1μg to about 15μg, from about 1μg to about 10μg, from about 2μg to about 50μg, 2μg to about 30μg, from about 2μg to about 20μg, from about 2μg to about 10μg, from about 2μg to about 8μg, from about 3μg to about 50μg, 3μg to about 30μg, from about 3μg to about 20μg, from about 3μg to about 10μg, from about 3μg to about 8μg, from about 3μg to about 5μg, from

The composition can be administered intramuscularly to the human in a single or in multiple doses. The method can further include the step of administering at least one subsequent dose of the flagellin/antigen composition to the human.

The immunogenic compositions for use according to the present invention may be delivered as a standard 0.5 ml injectable dose and contain from about 0.1μg to about 50μg of antigen. In a preferred embodiment of the immunogenic compositions for use according to the present invention is a standard 0.5 ml injectable dose and contains from about 3μg to about 20μg of antigen. The vaccine volume may be between 0.25 and 1.0 ml, suitably between 0.5 ml and 1.0 ml, in particular a standard 0.5ml. A vaccine dose according to the present invention may be provided in a smaller volume than conventional dosing. Low volume doses according to the present invention are suitably below 0.5ml, typically below 0.3ml and usually not less than 0.1 ml.

Accordingly, in one aspect of the invention it is provided for a composition, method or use as claimed herein wherein the immune response produced by administration of the composition in a human population where the humans are about 49 years old to about 64 years old or where the humans are older than 55 years of age, or older than 60 years of age or older than 65 years of age or older than 70 years of age or older than 75 years of age or older than 80 years of age and has a seroconversion rate of greater than 50%, greater than 60%, greater than 65% greater than 70%, greater than 75%, greater than 80%, greater than 85%, greater than 90%, greater than 95% or greater than 99%.

Accordingly, in one aspect of the invention it is provided for a composition, method or use as claimed herein wherein the immune response produced by administration of the composition in a human population where the humans are about 49 years old to about 64 years old or where the humans are older than 55 years of age, or older than 60 years of age or older than 65 years of age or older than 70 years of age or older than 75 years of age or older than 80 years of age and has a seroprotection rate of greater than 50%, greater than 60%, greater than 65%, greater than 70%, greater than 75%, greater than 80%, greater than 85%, greater than 90%, greater than 95% or greater than 99%.

Seroresponsive means an increase in HAI antibody titer of at least fourfold with a minimum post vaccination titer of 40.

Seroprotection means achievement of minimum post vaccination HAI titer of 40 among subjects with prevaccination titers of <40.

Seroconversion rate for anti-HA antibody response is defined as the proportion of subjects in each group having protective post-vaccination titer ≥ 1:40. The seroprotection rate is the percentage of subjects who have an HAI titer before vaccination of < 1:10 and ≥ 1:40 after vaccination. However, if the initial titer is ≥ 1:10 then there needs to be at least a fourfold increase in the amount of antibody after vaccination.

In another aspect of the invention it is provided for a composition, method or use as claimed herein wherein the immune response produced by administration of the compositions of the present invention induces functional (HAI) antibodies in the majority of elderly recipients in a dose dependent fashion. In certain embodiments the composition will induce a neutralizing antibody response of greater than a titer about 50 after 7 days or after 14 days or after 28 days. In other embodiments the composition will induce a neutralizing antibody response of greater than about a titer of 100 after 7 days or after 14 days or after 28 days. In other embodiments the composition will induce a neutralizing antibody response of greater than a titer of about 150 after 7 days or after 14 days or after 28 days. In other embodiments the composition will induce a neutralizing antibody response of greater than about 200 after 7 days or after 14 days or after 28 days.

Accordingly, in one aspect of the invention it is provided for a composition, method or use as claimed herein wherein the immune response produced by administration of the composition in an elderly population meets or exceeds one of the following criteria:

- a seroconversion rate of greater than 50%, greater than 60%, greater than 70%, greater than 80%, greater than 90%, greater than 95% or greater than 99%.
- a seroprotection rate of greater than 50%, greater than 60%, greater than 70%, greater than 80%, greater than 90%, greater than 95% or greater than 99%.
- an average of four-fold or greater increase in neutralizing antibody titer at post vaccination.

An "effective amount" when referring to the amount of a composition and a fusion protein administered to the human, refers to that amount or dose of the composition that, when administered to the subject is an amount sufficient for therapeutic efficacy (e.g., an amount sufficient to stimulate an immune response in a subject, an amount sufficient to provide protective immunity in the subject).

The methods of the present invention can be accomplished by the administration of the compositions and fusion proteins of the invention by enteral or parenteral means. Specifically, the route of administration is by intramuscular injection of the composition and fusion protein. Other routes of administration are also encompassed by the present invention including intravenous, intradermal, interaarterial, interperitoneal, intranasal, transdermal, suppositories or subcutaneous routes.

The compositions that include the fusion proteins can be administered alone or as admixtures with conventional excipients, for example, pharmaceutically, or physiologically, acceptable organic, or inorganic carrier substances suitable for enteral or parenteral application which do not deleteriously react with the composition. Suitable pharmaceutically acceptable carriers inclue water, salt solutions (such as Ringer's solution), alcohols, oils, gelatins and carbohydrates such as lactose, amylose or starch, fatty acid esters, hydroxymethylcellulose, and polyvinyl pyrolidine. Such preparations can be sterilized and, if desired, mixed with auxillary agents such as lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, coloring and/or aromatic substances and the like which do not deleteriously react with the compositions administered to the human. Preferred diluents for diluting the vaccines of the present invention include but are not limited to 150mM NaCl with histidine and trehalose.

The compositions, fusion proteins and proteins of the invention can be administered to a subject on a support that presents the compositions, proteins and fusion proteins of the invention to the immune system of the subject to generate an immune response in the subject. The presentation of the compositions, proteins and fusion proteins of the invention would preferably include exposure of antigenic portions of the fusion protein to generate antibodies. The support is biocompatible. "Biocompatible" as used herein, means that the support does not generate an immune response in the subject (e.g., the production of antibodies).

The dosage and frequency (single or multiple doses) administered to a subject can vary depending upon a variety of factors, including, for example, prior exposure to an infection consequent to exposure to the antigen: health, body weight, body mass index, and diet of the subject or health-related problems. Other therapeutic regimens or agents can be used in conjunction with the methods and compositions, proteins or polypeptides of the present invention.

The composition can be administered to the human in a single dose or in multiple doses, such as at least two doses. When multiple doses are administered to the subject, a second or third dose can be administered days (e.g., 1, 2, 3, 4, 5, 6, 7), weeks (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10), months (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10) or years (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10) after the initial dose. For example, a second dose of the composition can be administered about 7 days, about 14 days or about 28 days following administration of a first dose of the composition that includes the fusion protein.

Ranges may be expressed herein as from about one particular value, and/or to about another particular value. When such a range is expressed, another aspect includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by use of the antecedent about, it will be understood that the particular value forms another aspect. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint.

The dose of fusion protein is made in reference to microgram doses. The invention is generally directed to methods of treating elderly populations of human to stimulate immune responses to antigens, in particular a protective immune response, employing relatively low doses of the antigenic composition that include the antigen. A description of example embodiments of the invention follows.

Examples

Example 1

Phase II, Open-Label, Escalating Dose-Ranging Study

STF2.HA1(SI) (VAX125) is a recombinant fusion protein that consists of *Salmonella typhimurium* flagellin type 2 (STF2), a TLR5 ligand, fused at its C-terminus to the globular head (amino acids 62-284) of the HA1 domain of the HA of influenza A/Solomon Islands/3/2006 (H1N1) and has a molecular mass of 77,539 kDa (SEQ ID 1). Vaccine was supplied in glass vials at either 20 μg/mL or 2 μg/mL, and diluted in dilution buffer (150mM NaCl with histidine and trehalose) to the final concentration on the day of administration. Study materials were prepared by non-blinded pharmacists and provided to blinded clinical staff. Vaccine was administered at a final volume of 0.5 mL by deep intramuscular injection in the deltoid muscle.

A total of 120 community-living adults who were ≥ 65 years of age (the mean age was 72 with a range of 64-84) enrolled across the six dose groups. Subjects were healthy as ascertained by medical history, screening physical examination and screening laboratory analysis. Subjects with active medical conditions, abnormal screening laboratory tests (CBC and differential, and AST and ALT), known allergies to vaccine components, influenza vaccination within the previous 6 months, or receipt of other vaccines within 30 days were excluded from participation.

The study was conducted in an open-label, dose escalation design. Twenty subjects were enrolled in each dose group at up to 3 study sites. Subjects received a single dose at dose levels of 0.5 μg, 1.0 μg, 2.0 μg, 3.0 μg, 5 μg, and 8 μg (Table 1). Subjects were evaluated in the clinic following vaccination at 30 minutes, 4 hours, Day 1, and by phone interview at Day 3. Subjects enrolled in the 5 and 8 μg groups remained under observation at the study site for four hours after vaccination. Subjects were further evaluated during clinic visits on study days 7, 14, and 28 following vaccination. Safety follow-up by telephone contact also occurred at 6 and 12 months after vaccination.

Table 1. Demographic and Baseline characteristics

	VAX125 dose (μg)						
	0.5	1	2	3	5	8	Total
	n=20	n=20	n=20	n=20	n=20	n=20	n=120
Male (%)	10 (50)	7 (35)	10 (50)	5 (25)	12 (60)	9 (45)	54 (44)
Age (mean)	72.8	71.2	71.7	71.9	70.7	70.6	71.5
Age (range)	65-84	65-81	65-79	67-81	65-77	65-80	65-84
Race – White	20 (100)	20 (100)	18 (90)	20 (100)	19 (95)	20 (100)	117 (98)
-- Black	0 (0)	0 (0)	1 (5)	0 (0)	1 (5)	0 (0)	2 (2)
-- Asian	1 (0)	1 (0)	1 (5)	0 (0)	0 (0)	0 (0)	1 (1)

Immunogenicity

The ability of antibody generated in response to *E. coli*-derived hemagglutinin antigen to inhibit hemagglutination by egg-grown influenza virus is shown in Tables 2 and 3. The geometric mean titer

(GMT) of HAI antibody on day 28 after a single dose increased in a dose dependent manner, with the highest post-vaccination GMT seen in subjects who received 5 μg (Table 2). Four-fold or greater antibody responses were seen in the majority of subjects who received doses of 5 or 8 μg. Among subjects who received doses of 5 or 8 μg, a total of 30 (75%) of 40 subjects had four-fold or greater serum HAI antibody responses. In the 5 and 8 μg dose groups 18 (45%) subjects had titers of 1:40 or greater at the beginning of the study before vaccination. Among the 22 subjects with pre-vaccination serum HAI antibody of 1:40 or less, 21 (98%) achieved a titer of at least 1:40 by day 28 after vaccination (Table 3). The kinetics of the antibody titers by group in this study are shown in Figure 2.

The anti-flagellin response as assessed by IgG ELISA appears in Table 4. Levels of anti-flagellin IgG were low in these subjects prior to vaccination. VAX125 induced substantial increases in anti-flagellin antibodies. There was no apparent relationship between the level of anti-flagellin antibody detected prior to vaccination and either the frequency or severity of side effects, or the serum antibody response to the influenza hemagglutinin.

The immune response generated by VAX125 was compared to the H1N1 component of the standard (15 μg) and high dose (60 μg) Fluzone as reported by Falsey et al. (Table 5). The GMT increase in subjects 65 years and older after standard Fluzone increases by about 2-fold and after the high dose by about 4-fold. In contrast the GMT increase in subjects 65 years and older after VAX125 the 5 μg dose increases by more than 10-fold. This translates to higher seroconversion rates and seroprotection rates in the VAX125 groups.

Table 2. Geometric mean (GMT) HAI antibody titers, mean fold response (MFR) among 120 subjects ≥ 65 years old who received a single dose of VAX125

	Dose (µg)											
	0.5		1		2		3		5		8	
Day	n=20	95% CI	n=20	95% CI	n=20	95% CI	n=20	95% CI	n=20	95% CI	n=20	95% CI
0	27	14-52	27	15-50	25	12-51	51	29-89	18	10-32	30	16-58
7	30	16-58	39	21-70	51	25-105	106	50-225	61	34-107	155	86-279
14	43	22-83	77	43-138	70	34-143	160	82-311	226	121-420	234	127-431
28	48	24-95	75	44-126	70	34-143	149	79-283	219	115-413	211	120-370
MFR												
14	1.6	1-2.5	2.8	1.6-5.1	2.8	1.6-4.9	3.1	2.1-4.8	12.6	6-25	7.7	4-16
28	1.7	1-2.9	2.7	1.6-4.7	2.8	1.6-4.9	2.9	1.9-4.6	11.7	6-25	7	4-14

Table 3. Seroconversion (SC) and seroprotection (SP) rates among 120 subjects ≥ 65 years old who received a single dose of VAX125

	Dose (ug) of VAX125 (STF2.HA1 (SI))					
	0.5 n=20	1 n=20	2 n=20	3 n=20	5 n=20	8 n=20
Seroconversion						
day 14 (%)	3 (15)	8 (40)	8 (40)	8 (40)	16 (80)	13 (65)
day 28 (%)	3 (15)	7 (35)	6 (30)	7 (35)	16 (80)	14 (70)
Seroprotection						
day 0 (%)	10 (50)	11 (55)	7 (35)	15 (75)	7 (35)	11 (55)
day 14 (%)	13 (65)	17 (85)	14 (70)	19 (95)	19 (95)	20 (100)
day 28 (%)	14 (70)	18 (90)	13 (65)	19 (95)	19 (95)	20 (100)

Seroconversion (increase in HI titer of at least 4 fold with a min titer of 40
Seroprotection (min post-vac. titer of 1:40),

Table 4. Geometric mean anti-flagellin serum IgG titers (ug/ml) after single dose of VAX125 in adults ≥ 65 years old

	Dose (ug/ml)					
Day post vaccination	0.5 n=20	1 n=20	2 n=20	3 n=20	5 N=20	8 n=20
0	5	2	4	2	3	2
7	8	8	39	17	41	34
14	19	26	103	49	173	93
28	16	21	72	35	125	58
Mean Fold response						
day 14	4	14	24	25	55	49
day 28	3	11	16	18	39	31

Table 5. Comparative seroresponse against H1N1 antigens in adults over 65 for VAX125 and Fluzone standard dose and high dose

	VAX125 dose (ug)			Fluzone® dose (ug)	
	3	5	8	15	60
Number of subjects	20	20	20	1252	2543
GMT (A/H1N1)					
Pre-vaccination	51	18	30	29	29
Post-vaccination	160	226	234	67	116
Fold increase	3.1	12.6	7.7	2.3	4.1
Seroconversion (%)	40	80	65	23	49
Seroprotection (%)	95	95	100	77	90

A/H1N1 Solomon Islands for VAX125 and H1N1 New Caledonia for Fluzone.
Fluzone (Sanofi) standard dose 15 ug, HD high dose 60ug each antigen.
From Falsey et al. JID 2009;200:172-80

Reactogenicity.

Vaccine was well tolerated in all dose groups (Table 6). No side effect was graded as severe (3) or life-threatening (4) and there were serious no adverse events. Moderate arm pain was observed in

about 20% of subjects of subjects who received a dose of 2 μg or greater. Systemic adverse events were usually mild and did not appear to be dose-depended.

C-reactive protein (CRP) levels were measured at baseline and on day 1 (Table 7). The mean fold increase in CRP showed a dose-dependence, with a mean increase of less than 5-fold in subjects receiving the two highest doses. At doses of 3 μg or greater 57% of 60 subjects experienced at least a 2-fold increase in CRP. However, the majority (or none) of CRP increases were not associated with local or systemic symptoms of greater than mild severity or for subjects in stage 1 associated with increases in IL-6, IL-8 or IL-10 (data not shown).

Table 6. *Number of subjects over 65 years with symptoms after vaccination with VAX125*

	Dose (μg) of VAX125 (STF2.HA1 (SI))					
	0.5	1	2	3	5	8
Local symptoms	n=20	n=20	n=20	n=20	n=20	n=20
Grade 0	14 (70)	9 (45)	12 (60)	6 (30)	4 (20)	6 (30)
Grade 1	5	10 (50)	3 (15)	10 (50)	12 (60)	10 (50)
Grade 2	1	1 (5)	5 (25)	4 (20)	4 (20)	4 (20)
Grade 3	0	0	0	0	0	0
Grade 4	0	0	0	0	0	0
Systemic symptoms						
Grade 0	16 (80)	18 (90)	10 (50)	12 (60)	11 (55)	12 (60)
Grade 1	4 (20)	0	6 (30)	4 (20)	6 (30)	7 (35)
Grade 2	0	2 (10)	4 (20)	4 (20)	3 (15)	1 (5)
Grade 3	0	0	0	0	0	0
Grade 4	0	0	0	0	0	0

Table 7. CRP response among adults ≥ 65 years old after one dose of VAX125

	Dose (μg)					
	0.5	1	2	3	5	8
No. of subjects	n=20	n=20	n=20	n=20	n=20	n=20
GM CRP day 0	1.0	1.3	1.2	1.7	1.6	1.4
GM CRP day 1	1.0	2.0	2.2	3.4	4.7	4.0
Max. CRP day 1	4.9	11.9	13.7	13.5	22.8	33.7
GM CRP fold inc.	1.1	1.6	1.9	2.0	2.9	2.8
No. (%) ≥ 2-fold inc.	1 (5)	4 (20)	7 (35)	10 (50)	13 (65)	11 (55)

Discussion

VAX125 was both safe and immunogenic at the dose range tested, but the elderly appear much less sensitive to the VAX125 in terms of immune and cytokine response. The peak GMT HAI titers in the 3μg elderly group was 160 which was similar to the 0.1μg dose in young adults. The peak titers in the 0.3 and 0.5μg dose in the young adults were higher than the elderly (Table 8). There is a four-fold or greater serum HAI responses in 61 of 96 (64%) subjects who received doses of 0.5 μg or greater, including in 46 of 72 subjects who received doses from 0.5 μg to 2 μg. The GM HAI titer after a single 0.5 μg dose of VAX125 was similar to the titer observed in the 5 μg dose in subjects over 65 years, suggesting that it requires nearly a 10-fold increase in dose to obtain the same titer in the elderly.

Table 8. Geometric mean HAI antibody titers, seroprotection and seroconversion rates for H1N1 Solomon Island among young adult subjects who received one IM dose of VAX125.

HAI GMT						MFR		SR	SP
Dose	No. of Subjects	Day 0	Day 14	Day 28	Day 180	Day 14	Day 28	NO. (%)	B < 40
0.1	8	87	123	174	293	1.4	2	1(13)	1/1
0.3	8	113	293	381	226	2.6	3.4	4 (50)	3/3
1	8	160	698	905	587	4.4	5.7	4 (50)	2/2
2	8	95	587	587	226	6.2	6.2	5 (63)	1/1
3	8	174	987	640	351	5.7	3.7	3 (38)	1/1
5	8	62	1522	1174	359	24.7	19	7 (88)	2/2
8	8	40	320	349	147	8	8.7	5 (63)	2/3
Control	16	91	87	84	-	1	1	0	0/2
0.5	24	37	-	285	-	-	7.8	16 (67)	9/10
1	16	108	494	473	-	4.6	4.4	8 (50)	3/4
2	16	62	795	761	-	12.9	12.3	13 (81)	5/5

Table 9 is a comparison of the mean and maximum CRP values for healthy subjects older than about 65 years old and subjects 18-49 years old. The geometric means do not vary until you reach the 3μg group where the mean is nearly double in the young adults. The upper 95% confidence limit and the maximum value are both less in the elderly.

Table 9. Comparison of CRP in elderly and young adults after VAX125.

Dose (μg)	1		2		3	
Age group	>65	18-49	>65	18-49	>65	18-49
No. subjects	20	24	20	24	20	8
Geometric mean	2	2.2	2.2	2.4	3.4	5.9
Upper 95% CI	3.2	4.7	3.4	4.6	5	19.9
Maximum	11.9	43.7	13.7	33.2	13.5	83

While these data may suggest that the optimal dose in the elderly is greater than 3μg, doses as low as this still produce an HAI response to the H1N1 antigen that is better than the standard Fluzone TIV and is similar to the response observed with the high dose Fluzone formulation (Table 10).

Table 10. Immunological response of VAX125 compared to standard dose and high dose Fluzone in elderly subjects 65 years or older.

	VAX125		Fluzone TIV	
Dose of H1N1	2 μg	3 μg	15 μg	60 μg
No. of subjects	20	20	1252	2543
SP (%)	70	95	77	90
SC (%)	35	40	23	49
GMT HAI Titer	70	149	67	116

Table 11 Comparative Seroresponse against H1N1 antigens in adults over 65 for VAX 125 and Fluzone standard dose and high dose

	VAX 125 dose (μg)			Fluzone dose (μg)	
	3	5	8	15	60
Number of Subjects	20	20	20	1252	2543
GMT (H1N1)					
Prevaccination	51	19	30	29	29
Post vaccination	160	234	234	67	116
Fold Increase	3.1	12.6	7.7	2.3	4.1
Seroconversion (%)	40	80	65	23	49
Seroprotection (%)	95	95	100	77	90

Table 12. Clinical Evaluation of VAX 125 Phase I

			VAX 125 dose range (μg)		
	Timing	Control N=16	0.1-0.3 N=16	1-3 N=56	5-8 N=16
GMT	Day 0	91	99	102	50
	Day 14	87	190	672	698
	Day 28	84	258	640	640
GMT fold increase	At day 14	1.0	1.9	6.6	14.1
	At day 28	0.9	2.6	6.2	12.9
	Seroresponse*	0	5 (31%)	33 (59%)	12 (75%)
	Seroprotection	0 of 2	4 of 4	12 of 13 (92%)	5 of 6 (83%)

*Seroresponse: increase in HAI antibody titer of at least fourfold with a minimum post vaccination titer of 40

**Seroprotection: achievement of minimum post vaccination HAI titer of 40 among subjects with prevaccination titers of <40.

VAX125 induces functional (HAI) antibodies in the majority of elderly recipients in a dose dependent fashion. The 5.0 and 8.0 μg doses elicited high HAI GMT (226 and 234, respectively) and high GMT fold response (12.6 and 7.7, respectively) values. The 5.0 μg dose was the most immunogenic. The 5.0 μg and 8.0 μg doses elicited high seroconversion rates (80% and 65%, respectively) and high seroprotection rates (95% and 100%, respectively). The peak GMT values in healthy adults ≥ 65 were similar to those observed with 0.5 μg dose in young adults. This suggests that a high dose formulation is required for the elderly. The ability to produce a high dose influenza vaccine in *E. coli* for the elderly is unique to this product.

Example 2

Construction and Expression of Vax 128A, Vax 128B and Vax 128C

Cloning and Expression

E. coli clones producing each of the three vaccine candidates Vax 128A, Vax 128B and Vax 128C were produced in a similar manner, but with differences in plasmid preparation specific to each construct. Plasmids encoding for STF2.HA1 (CA07) and STF2R3.HA1 (CA07) were mutated from plasmids encoding for STF2.HA1 (CA04) and STF2R3.HA1 (CA04). The plasmid encoding for STF2R3.2xHA1 (CA07) was produced from the STF2.HA1 (CA07) and STF2R3.HA1 (CA07) plasmids. Details of the plasmid construction and clone selection follow. A plasmid map for the vaccine candidates is shown in Figure 3.

Plasmid Construction for Vax 128A (STF2. HA1 (CA07))

A multi step PCR procedure was performed to produce the plasmid encoding for STF2.HA1 (CA07). A plasmid encoding for STF2.HA1 (CA04) was initially produced, and then mutated to code for STF2.HA1 (CA07). To make STF2.HA1 (CA04), DNA encoding STF2 was fused with DNA encoding the HA1 globular head domain of the (CA04) HA protein. DNA encoding STF2 was amplified from the pET24a-STF2.HA1 FL plasmid. DNA encoding the HA globular head protein was synthesized by an outside vendor (DNA 2.0, Menlo Park, CA) and amplified by PCR. After gel purification of both the SFT2 and HA1 (CA07) DNA, the fragments were fused together. The final PCR product (DNA encoding the STF2.HA1 (CA04) fusion protein) was digested with restriction enzymes NdeI and EcoRI and ligated to the pET24a plasmid. Since there is only one residue difference between the HA portion of STF2.HA1-2 (CA04) and STF2.HA1 (CA07), STF2.HA1 (CA07) was made by site-directed mutagenesis of STF2.HA1 (CA04). The recombinant DNA sequence was confirmed by an outside vendor (Genewiz Inc.).

Plasmid Construction for Vax 128B (STF2R3. HA1 (CA07))

As with STF2.HA1 (CA07), a plasmid encoding for STF2R3.HA1 CA4 was initially produced and then mutated to code for STF2R3.HA1 (CA07). To make STF2R3.HA1 (CA04), DNA encoding portions of the N and C terminal sections of STF2 was fused with DNA encoding the HA1 globular head domain of the CA4 HA protein. This has the effect of replacing the D3 domain of STF2 with HA1-2 (CA04) in the fusion protein. DNA encoding for the portions of the N and C terminal sections of STF2 was PCR amplified from the pET24a-STF2.HA1 (CA04) plasmid. DNA encoding for HA1 (CA04) was PCR amplified from the same

plasmid. Gel purified STF2 and HA1 (CA04) fragments were fused, and the final PCR product was digested with restriction enzymes NdeI and EcoRI and ligated to the pET24a plasmid. Since there is only one residue difference between the HA portion of STF2R3.HA1 (CA04) and STF2R3.HA1 (CA07), STF2R3.HA1 (CA07) was made by site-directed mutagenesis of STF2R3.HA1 (CA04). The recombinant DNA sequence was confirmed by an outside vendor (Genewiz Inc.).

Plasmid Construction for Vax 128C (STF2R3. 2×HA1 (CA07))

The plasmid encoding for STF2R3.2×HA1 (CA07) was made from a fusion of the DNA from the STF2R3.HA1 (CA07) plasmid and the STF2.HA1 (CA07) plasmid. Both plasmids were digested separately with NdeI and MfeI enzymes. Specific fragments of the STF2R3.HA1 (CA07) (as insert) and STF2.HA1 (CA07) (as vector) were gel purified and ligated to form a complete DNA sequence encoding for STF2R3.2xHA1 (CA07). The common restriction site is in the C terminal section of STF2. The recombinant DNA sequence was confirmed by an outside vendor (Genewiz Inc.).

Cloning

The DNA was used to transform commercially available *E. coli* BLR(DE3) cells (Novagen, Cat. No. 69053). *E. coli* BLR(DE3) cells from Novagen are a recA derivative of a BL21 strain of *E. coli* that improves plasmid monomer yield and may help to stabilize included plasmids.. The cells are *lon* and *ompT* protease deficient, and are lysogenic for lambda prophage that contains an IPTG inducible T7 RNA polymerase. The resulting BLR(DE3) clones, were used to produce a research cell bank for each vaccine candidate. To generate the banks, a selected clone was grown in LB media, supplemented with 0.5% Glucose, and 25 µg/mL kanamycin. Cells were frozen in 1 mL cryovials at -60 to -80 °C following the addition of glycerol to 7% final concentration. In protein expression studies, proteins corresponding to the expected molecular weights of the vaccine candidates were easily visualized by Coomassie Blue staining and by Western blot analyses using an anti-flagellin monoclonal antibody.

Protein Production

The constructs described below are all comprised of combinations of *Salmonella typhimurium fljB* flagellin (STF2) and a portion of the globular head domain of the protective hemagglutinin antigen from the novel H1N1 emerging in 2009 (A/California 07/2009).

Manufacture of VAX128A

A pET24a plasmid encoding production of STF2.HA1-2 CA07 is used to transform commercially available *E. coli* BLR(DE3) cells. These cells are expanded to manufacture both research and cGMP master cell banks. To produce the target protein, a vial of banked cells was expanded in shake flasks using synthetic media and grown to a specific optical density. A specific amount of cells were then transferred to a bioreactor containing a different synthetic media. The bioreactor was run under automatic control (T=30C, pH = 7, DO >= 30%) in batch mode until the glucose was exhausted. At that point a synthetic feed media is added to the bioreactor at a specific flow rate. After three hours, target protein production was initiated in the culture by addition of IPTG to a concentration of 2 mM. The cells were harvested four hours after induction and the cell paste separated from the conditioned media by centrifugation. After freezing and thawing, the cells were re-suspended in a Tris acetate buffer and lysed by high pressure homogenization. The lysate supernatant was collected after centrifugation. Proteins were precipitated using 14% PEG, and then re-suspended in 6M urea at pH 4. After adjusting the solution pH to 8, the buffer was exchanged into a Tris/acetate buffer by TFF. Triton X-114 and PEG were added and the resulting solution separated into detergent and aqueous phases by centrifugation. The aqueous phase was retained and filtered. Proteins were denatured by addition of urea and diluted to a concentration of 2 grams per liter protein in a 6 molar urea solution at pH 8. Target protein was refolded in batch mode by a ten-fold dilution into a refolding buffer. Initial purification of active, monomeric vaccine was performed by bind and elute anion exchange chromatography, with target protein and impurity elution effected by increasing salt concentration in a step-wise fashion. After filtration and pH adjustment of the AEX eluate to 6.8, active, monomeric vaccine was further purified by utilizing a hydroxyapatite (CHT) chromatography media in binding mode. Elution of impurities and target protein was effected by increasing phosphate concentration in steps. After filtration, the material was buffer-exchanged into a formulation buffer by TFF and dilution, filtered, aliquoted, and stored at -70C. The resulting bulk drug substance was diluted to the target drug product concentration in the same formulation buffer and stored at -70C prior to administration.

Manufacture of VAX128B

A pET24a plasmid encoding production of STF2R3.HA1-2 CA07 was used to transform commercially available *E. coli* BLR(DE3) cells. These cells were expanded to manufacture both research and cGMP master cell banks. To produce the target protein, a vial of banked cells was expanded in

shaker flasks using synthetic media and grown to a specific optical density. A specific amount of cells were then transferred to a bioreactor containing a different synthetic media. The bioreactor was run under automatic control (T=30C, pH = 7, DO >= 30%) in batch mode until the glucose was exhausted. At that point a synthetic feed media was added to the bioreactor at a specific flow rate. After three hours, target protein production was initiated in the culture by addition of IPTG to a concentration of 2 mM. The cells were harvested four hours after induction and the cell paste separated from the conditioned media by centrifugation. After freezing and thawing, the cells were re-suspended in a Tris acetate buffer and lysed by high pressure homogenization. The lysate pellet was collected after centrifugation, re-suspended in a phosphate buffer, and collected again by subsequent centrifugation. The pellet resuspension and collection process was repeated and the washed pellet was solubilized in a 6M urea buffer at pH 8. After mixing, solubilized proteins were recovered by centrifugation and diluted to 2 grams protein per liter of solution. The target protein was refolded into its proper conformation by rapidly diluting the denatured proteins into a nine fold excess of refolding buffer under flow. The denatured protein solution and refolding buffer were mixed in line at a 1 to 9 flow rate ratio, held for an average of 30 minutes before loading on an anion exchange chromatography column. Active, monomeric vaccine was bound to the AEX media with target protein and impurity elution effected by increasing salt concentration in steps. After filtration, the anion exchange elution was diluted 10% with water and loaded onto a different anion exchange chromatography column for further purification. Elution of impurities and target protein was effected by increasing Tris/acetate concentration in steps. After filtration, the material was buffer-exchanged into a formulation buffer by TFF and dilution, filtered, aliquoted, and stored at -70C. The resulting bulk drug substance was diluted to the target drug product concentration in the same formulation buffer and stored at -70C prior to administration.

Manufacture of VAX128C

A pET24a plasmid encoding production of STF2R3.2xHA1-2 CA07 was used to transform commercially available *E. coli* BLR(DE3) cells. These cells were expanded to manufacture both research and cGMP master cell banks. To produce the target protein, a vial of banked cells was expanded in shaker flasks using synthetic media and grown to a specific optical density. A specific amount of cells were then transferred to a bioreactor containing a synthetic media. The bioreactor was run under automatic control (T=30C, pH = 7, DO >= 30%) in batch mode until the glucose was exhausted. At that point a synthetic feed media was added to the bioreactor at a specific flow rate. After three hours,

target protein production was initiated in the culture by addition of IPTG to a concentration of 2 mM. The cells were harvested four hours after induction and the cell paste separated from the conditioned media by centrifugation. After freezing and thawing, the cells were re-suspended in a Tris/acetate buffer and lysed by high pressure homogenization. The lysate pellet was collected after centrifugation, re-suspended in a phosphate buffer, and collected again by subsequent centrifugation. The pellet re-suspension and collection process was repeated and the washed pellet was solubilized in a 6M urea buffer at pH 8. After mixing, solubilized proteins were recovered by centrifugation and diluted to 2 grams protein per liter of solution. Target protein was refolded in batch mode by a ten-fold dilution into a refolding buffer. Initial purification of active, monomeric vaccine was performed by bind and elute anion exchange chromatography, with impurity and target protein elution effected by increasing salt concentration in steps. After filtration and pH adjustment of the AEX eluate to 6.8, active, monomeric vaccine was further purified by utilizing a hydroxyapatite (CHT) chromatography media in binding mode. Elution of impurities and target protein was effected by increasing phosphate concentration in a step-wise fashion. After filtration, the material was buffer exchanged into a formulation buffer by TFF and dilution, filtered, aliquoted, and stored at -70C. The resulting bulk drug substance was diluted to the target drug product concentration in the same formulation buffer and stored at -70C prior to administration.

Synthetic shake flask media

Component	Amount
Water	940 mL
KH_2PO_4	7.8 g/L
Citric Acid	1.0 g/L
$(NH_4)_2SO_4$	2.33 g/L
Trace Metals Solution	1.0 mL/L
Thiamine HCl	0.01 g/L
Glucose	10 g/L
$MgSO_4$	1 g/L
$CaCl_2$	0.04 g/L
Kanamycin	0.0075 g/L
10N NaOH	As needed to adjust final pH to 7.0-7.2

Synthetic bioreactor media

Component	Amount
Water	As needed to obtained calculated final volume
KH_2PO_4	2.2 g/L
$(NH_4)_2SO_4$	4.5 g/L
Citric Acid	1.0 g/L
Trace Metals Solution	1.0 mL/L
Thiamine HCl	0.01 g/L
Antifoam	100 µL per L
10N NaOH	As needed to adjust base MRBR to pH to 6.0-6.5
Glucose	20 g/L
$MgSO_4$	1 g/L
$CaCl_2$	0.04 g/L
Kanamycin	0.0075 g/L
10N NaOH Solution	As needed to adjust final MRBR to pH to 6.0-6.5

Synthetic feed media

Component	Amount
Water	As needed to obtained calculated final volume
Dextrose	180.0 g/L
L-Alanine	40.0 g/L
KH_2PO_4	1.5 g/L
$(NH_4)_2HPO_4$	10.0 g/L
NaH_2PO_4	4.0 g/L
$(NH_4)_2SO_4$	5.0 g/L

Component	Amount
Citric Acid	4.0 g/L
Sulfuric Acid	As needed to adjust pH to 5.0-5.4
$MgSO_4$	2.3 g/L
Trace Metals Solution	6 mL/L
$CaCl_2$	2 g/L
Thiamine HCl	0.01 g/L
$FeSO_4$	0.25 g/L
10N NaOH Solution	As needed to adjust pH to 5.95-6.05

Trace Metals Solution

Component	Amount
Water	As needed to obtained calculated final volume
EDTA	5.0 g/L
$FeSO_4 \cdot 7H_2O$	10.0 g/L
$ZnSO_4 \cdot 7H_2O$	2.0 g/L
$MnSO_4 \cdot H_2O$	2.0 g/L
$CoCl_2 \cdot 6H_2O$	0.2 g/L
$CuSO_4 \cdot 5H_2O$	0.1 g/L
$Na_2MoO_4 \cdot 2H_2O$	0.2 g/L
H_3BO_3	0.1 g/L
Sulfuric Acid	As needed to remove precipitate

Formulation buffer (pH7)

Component	Amount
Tris	10 mM
Histidine	10 mM

Component	Amount
Trehalose	5% (w/v)
NaCl	150 mM
Polysorbate-80	0.02% (w/v)
EDTA	0.1 mM
Ethanol	0.5% (v/v)
WFI	q.s.

Example 3

Phase I Escalating Dose Ranging Study of Vax 128 A, B, C

A Phase I escalating dose ranging study to evaluate the safety and immunogenicity of VAX128 A, B, and C H1N1 influenza vaccine constructs in healthy adults 18-49 years of age and in adults ≥65 years of age was conducted. These 3 novel influenza vaccine constructs are comprised of the globular head of the HA1 domain of the A/California/07, Novel H1N1 (VAX128) genetically fused to the TLR5 ligand, flagellin, and produced in *E. coli*. In Vax128A the HA1 was fused to the C-terminus of flagellin, while in VAX128B HA1 replaced the D3 domain of flagellin and in VAX128C HA1 was replaced the D3 domain and was fused to the C-terminus. Vaccine was supplied in glass vials at either 20 µg/mL or 2 µg/mL, and diluted in dilution buffer (150mM NaCl with histidine and trehalose) to the final concentration on the day of administration. Study materials were prepared by non-blinded pharmacists and provided to blinded clinical staff. Vaccine was administered at a final volume of 0.5 mL by deep intramuscular injection in the deltoid muscle.

116 healthy adult subjects 18-49 and 100 healthy adults ≥ 65 years old were enrolled in a double blind, placebo controlled clinical trial conducted at two centers. Subjects received one of the 3 VAX128 vaccine constructs or placebo in a dose escalation study in a ratio of 3:3:3:1. Vaccines were administered IM at doses ranging from 0.5-20 µg. Subjects were followed for safety and sera were tested by hemagglutination-inhibition (HAI) against egg-grown virus on days 0, 7, 14, and 28. Serum C-reactive protein (CRP), cytokine levels, and anti-flagellin antibody were also assessed.

Table 13. Number (%) of young adults subjects with local and systemic symptoms in VAX128-01, n=112

Dose ranges	VAX128A				VAX128B		VAX128C			Placebo
	<2.5	2.5	4	8	< 16	16	< 16	16	20	0
	N=6	n=12	n=12	n=6	n=18	n=12	n=18	n=12	N=6	n=10
Temp. > 99° F	0	0	1 (8)	1 (17)	1 (6)	0	4 (22)	0	2 (33)	0
Local										
None	0	3 (25)	3 (25)	0	3 (17)	2 (17)	4 (22)	1 (8)	1 (17)	8 (80)
Mild	3 (50)	2 (17)	4 (33)	3 (50)	7 (39)	7 (58)	9	3 (25)	1 (17)	2 (20)
Moderate	2 (33)	7 (58)	1 (8)	2 (33)	8 (44)	3 (25)	4 (22)	6 (50)	4 (67)	0
Severe	1 (17)	0	1 (8)	1 (17)	0	0	1 (6)	2 (17)	0	0
Systemic										
None	3 (50)	9 (75)	11 (92)	5 (83)	12 (67)	9 (75)	12 (67)	7 (58)	5 (83)	6 (60)
Mild	3 (50)	1 (8)	0	1 (17)	5 (28)	1 (8)	3 (17)	2 (17)	1 (17)	3 (30)
Moderate	0	2 (17)	0	0	1 (6)	1 (8)	3 (17)	1 (8)	0	1 (10)
Severe	0	0	1 (8)	0	0	1 (8)	0	2 (17)	0	0
CRP day 1 > 40	0	0	0	1 (17)	1 (6)	4 (33)	0	0	0	0
CRP fold > 40	0	0	0	0	3 (17)	1 (8)	1 (6)	0	0	0
WBC increase	0	1 (8)	0	0	0	2 (17)	0	2 (17)	0	0
LFT increase	0	2 (17)	0	0	1 (6)	2 (17)	0	0	0	0
IL-6 increase	0	0	0	0	3 (17)	3 (25)	1 (6)	0	0	0

Table14. Number (%) of subjects ≥ 65 with local and systemic symptoms or lab abnormities in VAX128-01, n=100

Dose ranges (μg)	VAX128A		VAX128B		VAX128C		Placebo
	12.5-4	8	1.25-12	16	1.25-12	16-20	0
	n=9	n=21	n=18	n=12	n=15	n=15	n=10
Temp. > 99° F	0	3 (14)	0	0	0	1 (7)	0
Local							
None	4 (44)	10 (48)	8 (44)	2 (17)	3 (20)	5 (33)	10 (100)
Mild	2 (22)	9 (43)	8 (44)	4 (33)	10 (67)	7 (47)	0
Moderate	3 (33)	2 (10)	2 (11)	6 (50)	2 (13)	3 (20)	0
Severe	0	0	0	0	0	0	0
Systemic							
None	9 (100)	17 (81)	13 (72)	7 (58)	13 (87)	13 (87)	10 (100)
Mild	0	4 (19)	4 (22)	4 (33)	2 (13)	1 (7)	0
Moderate	0	0	0	1 (8)	0	1 (7)	0
Severe	0	0	1 (6)	0	0	0	0
CRP day 1 > 40	0	1 (5)	1 (6)	1 (8)	1 (7)	1 (7)	0
CRP fold > 40	0	0	0	2 (17)	1 (7)	1 (7)	0
WBC increase	0	3 (14)	1 (6)	1 (8)	1 (7)	2 (13)	0
LFT increase	1 (11)	0	1 (6)	1 (8)	0	0	0
IL-6 increase	0	0	1 (6)	1 (8)	0	0	0

Table 15a GMT HAI titers, seroconversion (SC) and seroprotection (SP) rates among 112 subjects 18-49 years old who received a single IM dose of VAX128 (CA07) construct A, B or C (construct groups combined).

		GMT HAI on day				MFR on day					
Dose (µg)	No. of Subjects	0	7	14	28	14	28	SC	SP	SC%	SP%
placebo	10	9	10	10	10	1	1	0	2	0	20
0.5	9	14	29	86	63	6	5	7	7	78	78
1.25	9	7	25	218	148	32	22	7	7	78	78
2.5	18	12	83	231	196	20	20	16	17	89	94
4	18	16	148	453	359	29	23	17	17	94	94
8	12	13	150	320	190	25	15	9	10	75	83
12	6	16	1280	1810	905	114	57	6	6	100	100
16	24	10	301	567	433	53	40	19	19	79	79
20	6	20	359	640	453	32	23	5	6	83	100

Table 15b. GMT HAI titers, seroconversion (SC) and seroprotection (SP) rates among 100 subjects ≥ 65 years old who received a single IM dose of VAX128 (CA07) construct A, B or C (construct groups combined).

		GMT HAI on day				MFR on day					
Dose (µg)	No. of Subjects	0	7	14	28	D14 fold	D28 fold	SC	SP	SC%	SP%
placebo	10	20	20	21	21	1	1	0	3	0	30
1.25	9	10	13	13	16	2	2	2	3	22	33
2.5	9	22	50	93	69	4	3	4	8	44	89
4	9	27	74	109	93	4	3	5	8	56	89
8	30	12	36	88	84	7	7	19	23	63	77
12	6	16	80	226	202	14	13	3	5	50	83
16	15	14	101	221	175	16	13	9	10	60	67
20	12	7	34	127	113	18	16	9	9	75	75

Table 16 GMT HAI titers, seroconversion (SC) and seroprotection (SP) rates among 100 subjects ≥ 65 years old who received a single IM dose of VAX128 (CA07) construct A, B or C.

			GMT HAI on day				MFR on day					
Vaccine	dose	Subj.	0	7	14	28	14	28	SC	SP	SC%	SP%
VAX128A	1.25	3	16	25	40	40	2.5	2.5	1	2	33	67
	2.5-4	6	11	40	71	63	6.3	5.7	4	6	67	100
	8	21	13	26	56	56	4.4	4.4	11	15	52	71
VAX128B	1.25	3	5	5	5	5	1.0	1.0	0	0	0	0
	2.5-4	6	22	45	80	80	3.6	3.6	3	5	50	83
	8-12	9	17	93	373	296	21.8	17.3	8	9	89	100
	16	12	8	53	143	107	17.0	12.7	7	7	58	58
VAX128C	1.25	3	13	16	20	20	1.6	1.6	1	1	33	33
	2.5-4	6	57	127	180	101	3.2	1.8	2	5	33	83
	8-12	6	7	57	127	127	18.0	18.0	3	4	50	67
	16-20	15	12	70	202	184	16.8	15.3	11	12	73	80

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Table 17. Immune response by age cohort

		GMT HAI on day				MFR on day					
Age	Group	0	7	14	28	14	28	SC	SC%	SP	SP%
65-69	N=30	12.9	56.6	113.1	94.0	8.8	7.3	18	60	24	80
70-79	N=46	10.0	26.6	66.8	61.0	6.7	6.1	24	52	30	65
80-90	N=14	32.8	144.9	275.8	249.8	8.4	7.6	9	64	12	86

Table 18. HAI Titers for elderly adults 65 and over by VAX128 construct and dose

				HAI on day				Fold on day			
Subject	Age	Vaccine	Dose	0	7	14	28	14	28	SC	SP
1	69	B	1.25	5	5	5	5	1	1	N	N
2	76	B	1.25	5	5	5	5	1	1	N	N
3	78	P	0	10	10	10	10	1	1	N	N
4	71	C	2.5	10	40	80	20	8	2	Y	Y
5	79	C	1.25	10	10	10	10	1	1	N	N
6	65	C	1.25	20	40	80	80	4	4	Y	Y
7	66	C	2.5	40	80	40	40	1	1	N	Y
8	70	A	1.25	80	160	160	160	2	2	N	Y
9	72	C	1.25	10	10	10	10	1	1	N	N

10	79	B	2.5	5	5	10	5	2	1	N	N
11	74	A	2.5	10	20	160	160	16	16	Y	Y
12	67	A	2.5	40	80	80	20	2	0.5	N	Y
13	86	A	4	20	80	80	80	4	4	Y	Y
14	65	A	2.5	10	20	20	40	2	4	N	Y
15	68	A	4	5	40	160	160	32	32	Y	Y
16	74	B	4	5	40	80	40	16	8	Y	Y
17	84	C	2.5	20	640	2560	640	128	32	Y	Y
18	69	A	1.25	10	20	80	80	8	8	Y	Y
19	90	B	2.5	160	160	640	2560	4	16	Y	Y
20	68	P	0	20	10	10	10	0.5	0.5	N	N
21	72	P	0	40	40	40	40	1	1	N	Y
22	71	B	1.25	5	5	5	5	1	1	N	N
23	68	B	2.5	40	40	40	40	1	1	N	Y
24	77	A	1.25	5	5	5	5	1	1	N	N
25	65	B	8	80	2560	2560	2560	32	32	Y	Y
26	74	C	4	2560	2560	2560	2560	1	1	N	Y
27	65	C	4	5	5	5	5	1	1	N	N
28	83	C	12	5	640	2560	2560	512	512	Y	Y
29	85	C	4	320	160	320	160	1	0.5	N	Y
30	89	B	4	160	320	160	160	1	1	N	Y
31	67	B	8	5	80	320	160	64	32	Y	Y
32	84	P	0	40	80	80	80	2	2	N	Y
33	66	B	4	5	20	80	80	16	16	Y	Y
34	69	A	8	10	5	5	5	0.5	0.5	N	N
35	70	B	8	5	5	20	40	4	8	Y	Y
36	68	A	4	5	40	40	40	8	8	Y	Y
37	71	C	8	5	5	20	20	4	4	N	N
38	71	B	12	20	80	640	320	32	16	Y	Y
39	69	A	8	20	160	320	320	16	16	Y	Y
40	65	B	8	5	80	80	40	16	8	Y	Y
41	66	A	8	40	80	80	80	2	2	N	Y
42	70	A	8	10	40	160	160	16	16	Y	Y
43	67	C	16	40	2560	2560	2560	64	64	Y	Y
44	73	A	8	20	20	20	40	1	2	N	Y
45	69	C	8	5	80	160	320	32	64	Y	Y
46	80	C	12	40	40	40	40	1	1	N	Y
47	77	C	8	5	160	640	320	128	64	Y	Y
48	84	A	8	80	80	320	160	4	2	Y	Y

49	74	B	8	20	160	640	320	32	16	Y	Y
50	66	A	8	5	40	40	20	8	4	Y	Y
51	69	B	8	40	80	2560	2560	64	64	Y	Y
52	77	A	8	20	40	40	40	2	2	N	Y
53	75	C	16	160	320	320	320	2	2	N	Y
54	75	P	0	80	80	80	80	1	1	N	Y
55	74	B	12	40	40	40	40	1	1	N	Y
56	76	B	12	20	160	2560	2560	128	128	Y	Y
57	77	C	12	5	20	20	20	4	4	N	N
58	72	P	0	20	20	20	20	1	1	N	N
59	81	B	16	5	5	5	5	1	1	N	N
60	83	C	16	160	2560	2560	2560	16	16	Y	Y
61	77	C	20	5	5	10	10	2	2	N	N
62	84	B	16	40	640	2560	2560	64	64	Y	Y
63	66	A	8	160	320	320	160	2	1	N	Y
64	72	B	16	5	5	20	10	4	2	N	N
65	77	C	20	5	20	160	160	32	32	Y	Y
66	72	C	20	5	80	160	80	32	16	Y	Y
67	65	A	8	5	40	160	80	32	16	Y	Y
68	70	A	8	5	40	640	320	128	64	Y	Y
69	73	A	8	5	5	40	40	8	8	Y	Y
70	79	C	20	10	80	320	160	32	16	Y	Y
71	77	B	16	5	10	20	10	4	2	N	N
72	71	A	8	5	5	20	10	4	2	N	N
73	70	C	20	5	40	160	80	32	16	Y	Y
74	72	A	8	40	640	640	640	16	16	Y	Y
75	65	B	16	5	2560	2560	640	512	128	Y	Y
76	65	P	0	20	20	20	20	1	1	N	N
77	69	B	16	5	20	20	20	4	4	N	N
78	81	A	8	10	10	20	20	2	2	N	N
79	69	P	0	20	20	20	20	1	1	N	N
80	74	C	20	5	5	5	5	1	1	N	N
81	74	C	20	5	40	320	640	64	128	Y	Y
82	69	C	20	5	5	10	5	2	1	N	N
83	68	B	16	20	80	2560	2560	128	128	Y	Y
84	68	B	16	10	160	2560	2560	256	256	Y	Y
85	85	B	16	20	640	2560	2560	128	128	Y	Y
86	73	A	8	5	20	40	80	8	16	Y	Y
87	71	P	0	5	5	5	5	1	1	N	N

88	72	C	20	20	20	80	80	4	4	Y	Y
89	68	A	8	80	80	320	320	4	4	Y	Y
90	71	C	20	40	160	160	320	4	8	Y	Y
91	70	C	20	5	80	2560	2560	512	512	Y	Y
92	79	B	16	10	40	80	40	8	4	Y	Y
93	79	B	16	5	5	5	5	1	1	N	N
94	67	A	8	5	5	10	10	2	2	N	N
95	74	C	20	5	320	2560	2560	512	512	Y	Y
96	81	B	16	5	40	40	40	8	8	Y	Y
97	74	A	8	5	5	5	80	1	16	Y	Y
98	75	A	8	5	5	5	5	1	1	N	N
99	72	P	0	10	10	20	20	2	2	N	N
100	79	A	8	5	5	20	20	4	4	N	N

Table 19. Antibody response after one or two doses of the egg-based novel H1N1 (CA09) as measured by the HAI assay

Product, Author	CSL vaccine, Greenberg				Novartis vaccine, Clark	
Age group	18-49	50-64	18-49	50-64	18-49	18-49
Dose (ug)	15	15	30	30	7.5	7.5
Adjuvant	none	none	none	none	MF59	MF59
No. doses and Regimen	1, Day 0	1, Day 0	1, Day 0	1, Day 0	1, Day 0	2, Day 0 & 7
No. subjects	n=58	n=62	n=62	n=58	n=25	n=26
HAI Day 0	21	19	20	13	6	7
HAI Day 21	307	157	514	174	172	282
Mean fold resp.	14	8	25	13	28	59
Seroconversion	76	66	82	74	76	92
Seroprotection	100	94	98	88	80	96

In young adults, the maximum tolerated dose (MTD) for VAX128A was 8 μg and VAX128B 16μg. VAX128C was safe at 20μg, the highest dose tested (Table 13). Serum antibody responses were seen by HAI after doses as low as 0.5 μg. Doses of 1.25 μg to 2.5 μg induced a GMT of 1:250 with over 90% seroconversion and seroprotection (Table 15a and 15b). In adults ≥ 65 years, all three vaccines were safe at the highest doses tested of 8 μg, 12 μg and 20 μg for VAX128A, B and C, respectively (Table 14). Doses of 2.5 to 4 μg were associated reciprocal HAI titers of 70-180 and doses of 8-16 μg were associated HAI titers of 60 to 370 (Table 16). Table 17 shows the immune response by age cohort and table 18 shows the response for each individual tested that was 65 year of age and older.

Altering the configuration of the HA1 and flagellin was used to produce influenza vaccines that are safe with a large therapeutic window. VAX128C with two copies of the HA1 globular head had the highest MTD and was the most immunogenic. The globular head of the influenza HA expressed in a prokaryotic system was able to induce a functional antibody response. Vigorous responses were seen at relatively low doses of HA antigen indicating that the addition of flagellin provided a substantial adjuvanting effect.

Within this disclosure, any indication that a feature is optional is intended provide adequate support (e.g., under 35 U.S.C. 112 or Art. 83 and 84 of EPC) for claims that include closed or exclusive or negative language with reference to the optional feature. Exclusive language specifically excludes the particular recited feature from including any additional subject matter. For example, if it is indicated that A can be drug X, such language is intended to provide support for a claim that explicitly specifies that A consists of X alone, or that A does not include any other drugs besides X. "Negative" language explicitly excludes the optional feature itself from the scope of the claims. For example, if it is indicated that element A can include X, such language is intended to provide support for a claim that explicitly specifies that A does not include X. Non-limiting examples of exclusive or negative terms include "only," "solely," "consisting of," "consisting essentially of," "alone," "without", "in the absence of (e.g., other items of the same type, structure and/or function)" "excluding," "not including", "not", "cannot," or any combination and/or variation of such language.

Similarly, referents such as "a," "an," "said," or "the," are intended to support both single and/or plural occurrences unless the context indicates otherwise. For example "a dog" is intended to include support for one dog, no more than one dog, at least one dog, a plurality of dogs, etc. Non-limiting

examples of qualifying terms that indicate singularity include "a single", "one," "alone", "only one," "not more than one", etc. Non-limiting examples of qualifying terms that indicate (potential or actual) plurality include "at least one," "one or more," "more than one," "two or more," "a multiplicity," "a plurality," "any combination of," "any permutation of," "any one or more of," etc. Claims or descriptions that include "or" between one or more members of a group are considered satisfied if one, more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process unless indicated to the contrary or otherwise evident from the context.

Where ranges are given herein, the endpoints are included. Furthermore, it is to be understood that unless otherwise indicated or otherwise evident from the context and understanding of one of ordinary skill in the art, values that are expressed as ranges can assume any specific value or subrange within the stated ranges in different embodiments of the invention, to the tenth of the unit of the lower limit of the range, unless the context clearly dictates otherwise.

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. 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 invention is not entitled to antedate such publication by virtue of prior invention.

While this invention has been particularly shown and described with references to example embodiments thereof, it will be understood by those skilled in the art that the various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

Claims

What is claimed is:

1. A method of stimulating an immune response in a human, comprising the step of administering to the human a composition that includes a fusion protein comprising at least a portion of at least one flagellin and at least a portion of at least one antigen, wherein the fusion protein is administered to the human in a dose greater than about a 0.5μg dose and the human is at least 49 years old.

2. The method of claim 1, wherein administration of the composition to the human provides protective immunity against an infection consequent to exposure of the human to a source of antigen.

3. The method of claim 1, wherein the human is between 49 years old and about 64 years old.

4. The method of claim 1, wherein the human is at least 65 years old.

5. The method of claim 1, wherein the dose is at least one dose selected from the group consisting of a 0.5 μg dose, 1 μg dose, 2 μg dose, 3 μg dose, 4 μg dose, 5 μg dose, 6 μg dose, 7 μg dose, 8μg dose, 10μg dose, 15μg dose, 20μg dose, 25μg dose and a 30μg dose.

6. The method of claim 5, wherein the dose is at least one dose selected from the group consisting of a 0.5 μg dose, 1 μg dose, 2 μg dose, 3 μg dose, 4 μg dose, 5 μg dose, 6 μg dose, 7 μg dose, and 8μg dose.

7. The method of claim 5, wherein the dose is at least one dose selected from the group consisting of a 5 μg dose, 6 μg dose, 7 μg dose, 8μg dose, 10μg dose, 15μg dose, 20μg dose, 25μg dose and a 30μg dose.

8. The method of claim 1, wherein the dose is at least one dose selected from the group consisting of an 8μg dose, 10μg dose, 15μg dose, 20μg dose, 25μg dose and a 30μg dose.

9. The method of claim 1, wherein the antigen is an influenza antigen.

10. The method of claim 9, wherein the influenza antigen is a hemagglutinin influenza antigen

11. The method of claim 9, wherein the influenza antigen is an influenza A antigen.

12. The method of claim 9, wherein the influenza antigen is an influenza B antigen.

13. The method of claim 9, wherein the influenza antigen is an influenza C antigen.

14. The method of claim 1, wherein the fusion protein is administered intramuscularly to the human.

15. The method of claim 1, further including the step of administering at least one subsequent dose of the fusion protein to the human.

16. The method of claim 8, wherein the fusion protein includes SEQ ID No. 1.

17. A method of stimulating an immune response in an elderly human population, comprising the step of administering to the human population a composition that includes a fusion protein comprising at least a portion of at least one flagellin and at least a portion of at least one antigen, wherein the seroresponse rate is at least 75%.

18. A method of stimulating an immune response in an elderly human population, comprising the step of administering to the human population a composition that includes a fusion protein comprising at least a portion of at least one flagellin and at least a portion of at least one antigen, wherein the seroprotection rate is at least 95%.

19. A method of stimulating an immune response in an elderly human population, comprising the step of administering to the human population a composition that includes a fusion protein comprising at least a portion of at least one flagellin and at least a portion of at least one antigen, wherein the seroconversion rate is at least 50%.

20. A method of stimulating an immune response in an elderly human population, comprising the step of administering to the human population a composition that includes a fusion protein comprising at least a portion of at least one flagellin and at least a portion of at least one influenza antigen.

21. A method of stimulating an immune response in a human, comprising the step of administering to the human a composition that includes comprising at least a portion of at least one flagellin and at least a portion of at least one antigen associated with a particle, wherein the human is at least 49 years old.

22. The method of claim 18, wherein the particle is a virosome, liposome, biodegradable microsphere, self-assembling peptide nanoparticle, virus-like particle or combinations thereof.

23. The method of claim 22, wherein the particle is a self-assembling peptide nanoparticle.

24. The method of claim 22, wherein the particle is a virosome.

25. The method of claim 19, wherein administration of the composition to the human provide protective immunity against an infection consequent to exposure of the human to a source of antigen.

26. The method of claim 19, wherein the human is between 49 years old and about 64 years old.

27. The method of claim 19, wherein the human is at least 65 years old.

28. The method of claim 19, wherein the dose is at least one dose selected from the group consisting of a 8μg dose, 10μg dose, 15μg dose, 20μg dose, 25μg dose and a 30μg dose.

29. The method of claim 19, wherein the antigen is an influenza antigen.

30. The method of claim 24, wherein the influenza antigen is a hemagglutinin influenza antigen

31. The method of claim 24, wherein the influenza antigen is an influenza A antigen.

32. The method of claim 24, wherein the influenza antigen is an influenza B antigen.

33. The method of claim 24, wherein the influenza antigen is an influenza C antigen.

34. A method of stimulating an immune response in an elderly human population, comprising the step of administering to the human population a composition comprising at least a portion of at least one flagellin and at least a portion of at least one antigen, wherein the seroresponse rate is at least 75%.

35. A method of stimulating an immune response in an elderly human population, comprising the step of administering to the human population a composition comprising at least a portion of at least one flagellin and at least a portion of at least one antigen, wherein the seroprotection rate is at least 95%.

36. A method of stimulating an immune response in an elderly human population, comprising the step of administering to the human population a composition comprising at least a portion of at least one flagellin and at least a portion of at least one antigen, wherein the seroconversion rate is at least 50%.

37. A method of stimulating an immune response in an elderly human population, comprising the step of administering to the human population a composition comprising at least a portion of at least one flagellin and at least a portion of at least one influenza antigen.

38. The nucleic acid sequence as set forth in Seq ID 2.

39. The nucleic acid sequence as set forth in Seq ID 4.

40. The nucleic acid sequence as set forth in Seq ID 6.

41. The polypeptide sequence as set forth in Seq ID 3.

42. The polypeptide sequence as set forth in Seq ID 5.

43. The polypeptide sequence as set forth in Seq ID 7.

44. A method of stimulating an immune response in an elderly human population, comprising the step of administering to the human population a composition comprising at least 3 μg of SEQ ID No. 1, wherein the immune response produced by administration of the composition in an elderly population meets or exceeds one of the following criteria:

- a seroconversion rate of greater than 50%, greater than 60%, greater than 70%, greater than 80%, greater than 90%, greater than 95% or greater than 99%.
- a seroprotection rate of greater than 50%, greater than 60%, greater than 70%, greater than 80%, greater than 90%, greater than 95% or greater than 99%.
- an average four-fold or greater increase in neutralizing antibody titer at post vaccination.