COMPOSITIONS OF INFLUENZA VIRAL PROTEINS AND METHODS OF USE THEREOF

RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application Nos. 60/638,254, filed on December 21, 2004; 60/638,350, filed on December 21, 2004; 60/645,067, filed on January 19, 2005; 60/653,207, filed on February 15, 2005; 60/666,878, filed on March 31, 2005, 60/682,077, filed on May 18, 2005; and 60/741,202, filed November 30, 2005. The entire teachings of all of the above applications are incorporated herein by reference.

BACKGROUND OF THE INVENTION

Influenza is a contagious disease that usually results from an RNA virus. Three types of influenza viruses are known – influenza type A, B and C. The natural host for influenza type A is the aquatic bird. Influenza type A viruses can infect humans, birds, farm animals (e.g., pigs, horses) and aquatic animals (e.g., seals). Influenza type B viruses are usually found only in humans. Infection with influenza is generally characterized by fever, myalgia, headache, cough and muscle aches. In the elderly and infirm, influenza type B infection can result in disability and death. Influenza type B viruses can cause epidemics in humans. Influenza type C viruses can cause mild illness in humans and do not cause epidemics. Strategies to prevent and manage influenza infection include vaccines with inactivated viruses, nasal sprays and drugs, such as amantadine (1-aminoadamantine hydrochloride), rimantadine, zanamivir and oseltamivir. However, such strategies can be costly to maintain supply with demand and, thus, be limited in supply; may result in variable protection and less than satisfactory alleviation of symptoms, thereby ineffectively preventing or treating illness and, in some instances death, consequent to influenza infection. Thus, there is a need to develop new, improved and effective methods of treatment for preventing and managing influenza infection.

SUMMARY OF THE INVENTION

The present invention relates to compositions, fusion proteins and polypeptides comprising pathogen-associated molecular patterns (PAMPs) and influenza viral proteins. The compositions, fusion proteins and polypeptides of the invention can be employed in methods to stimulate an immune response in a subject.

In one embodiment, the invention is a composition comprising at least one Pam3Cys and at least a portion of at least one integral membrane protein of an influenza viral protein.

In another embodiment, the invention is a fusion protein comprising at least one pathogen-associated molecular pattern (PAMP) and at least one influenza M2 protein, wherein the pathogen-associated molecular pattern is not a Pam2Cys.

In a further embodiment, the invention is a composition comprising a pathogen-associated molecular pattern and an M2 protein, wherein the pathogen-associated molecular pattern is not a Pam2Cys.

In still another embodiment, the invention is a composition comprising at least a portion of at least one pathogen-associated molecular pattern and at least a portion of at least one influenza M2 protein, wherein, if the pathogen-associated molecular pattern includes a Pam2Cys, at least a portion of the Pam2Cys is not fused to the influenza M2 protein and at least a portion of the influenza M2 protein is not fused to the Pam2Cys.

In yet another embodiment, the invention is a fusion protein comprising at least a portion of at least one pathogen-associated molecular pattern and at least a portion of at least one influenza M2 protein, wherein, if the pathogen-associated molecular pattern includes a Pam2Cys, at least a portion of the Pam2Cys is not fused to the influenza M2 protein and at least a portion of the influenza M2 protein is not fused to the Pam2Cys.

In yet another embodiment, the invention is a method of stimulating an immune response in a subject, comprising the step of administering to the subject a composition that includes at least one Pam3Cys and at least a portion of at least one integral membrane protein of an influenza viral protein.

In still another embodiment, the invention is a method of stimulating an immune response in a subject, comprising the step of administering to the subject a

composition that includes a fusion protein comprising at least one pathogen-associated molecular pattern and at least one influenza M2 protein, wherein the pathogen-associated molecular pattern is not a Pam2Cys.

In an additional embodiment, the invention is a method of stimulating an immune response in a subject, comprising the step of administering to the subject a composition that includes at least one pathogen-associated molecular pattern and at least one influenza M2 protein, wherein the pathogen-associated molecular pattern is not a Pam2Cys and the M2 protein is not an M2e protein.

In still another embodiment, the invention is a method of stimulating an immune response in a subject, comprising the step of administering to the subject a composition that includes a composition comprising at least a portion of at least one pathogen-associated molecular pattern and at least a portion of at least one influenza M2 protein, wherein, if the pathogen-associated molecular pattern includes a Pam2Cys, at least a portion of the Pam2Cys is not fused to the influenza M2 protein and at least a portion of the influenza M2 protein is not fused to the Pam2Cys.

In a further embodiment, the invention is a method of stimulating an immune response in a subject, comprising the step of administering to the subject a composition that includes a fusion protein comprising at least a portion of at least one pathogen-associated molecular pattern and at least a portion of at least one influenza M2 protein, wherein, if the pathogen-associated molecular pattern includes a Pam2Cys, at least a portion of the Pam2Cys is not fused to the influenza M2 protein and at least a portion of the influenza M2 protein is not fused to the Pam2Cys.

The compositions, fusion proteins and polypeptides of the invention can be employed to stimulate an immune response in a subject. Advantages of the claimed invention include, for example, cost effective compositions, fusion proteins and polypeptides that can be produced in relatively large quantities for use in the prevention and treatment of influenza infection. The claimed compositions, fusion proteins, polypeptides and methods can be employed to prevent or treat influenza infection and, therefore, avoid serious illness and death consequent to influenza infection.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 depicts the amino acid sequence of *Salmonella typhimurium* flagellin type 2 (fljB/STF2) with the hinge region underlined (SEQ ID NO: 1).

Figure 2 depicts the nucleic acid sequence (SEQ ID NO: 2) encoding SEQ ID NO: 1. The nucleic acid sequence encoding the hinge region is underlined.

Figure 3 depicts the amino acid sequence of fljB/STF2 without the hinge region (also referred to herein as "fljB/STF2Δ" or "STF2Δ") (SEQ ID NO: 3).

Figure 4 depicts the nucleic acid sequence (SEQ ID NO: 4) encoding SEQ ID NO: 3.

Figure 5 depicts the amino acid sequence of *E.coli* flagellin fliC (also referred to herein as "*E.coli* fliC") with the hinge region underlined (SEQ ID NO: 5).

Figure 6 depicts the nucleic acid sequence (SEQ ID NO: 6) encoding SEQ ID NO: 5. The nucleic acid sequence encoding the hinge region is underlined.

Figure 7 depicts the amino acid sequence of *S. muenchen* flagellin fliC (also referred to herein as "*S. muenchen* fliC") with the hinge region underlined (SEQ ID NO: 7).

Figure 8 depicts the nucleic acid sequence (SEQ ID NO: 8) encoding SEQ ID NO: 7. The nucleic acid sequence encoding the hinge region is underlined.

Figure 9 depicts the amino acid sequence of pMT/STF2. The linker is underlined and the sequence of the BiP secretion signal is bolded (SEQ ID NO: 9).

Figure 10 depicts the nucleic acid sequence (SEQ ID NO: 10) of SEQ ID NO: 9. The nucleic acid sequence encoding the linker is underlined and the nucleic acid sequence encoding the BiP sequence is bolded.

Figure 11 depicts the nucleic acid sequence (SEQ ID NO: 17) encoding a multimer (4 units) of the amino-terminus of an M2 protein (also referred to herein as "4xM2e").

Figure 12 depicts an amino acid sequence (SEQ ID NO: 18) encoded by SEQ ID NO: 17.

Figure 13 depicts the amino acid sequence (SEQ ID NO: 31) of a fusion protein (referred to herein as "fljB/STF2-4xM2e" or "fljB/STF2.4xM2e")

comprising fljB/STF2 and four, 24-amino acid sequences of an amino-terminus of an M2 protein.

Figure 14 depicts the nucleic acid sequence (SEQ ID NO: 32) encoding SEQ ID NO: 31.

Figure 15 depicts a Pam3Cys.M2e fusion protein. The amino acid sequence (SEQ ID NO: 13) of M2e is shown in bold type.

Figure 16 depicts the activation of an antigen-presenting cell (APC) by Toll-like receptor (TLR) signaling.

Figures 17A and 17B depict plasmid constructs to express an amino-terminus of an M2 (e.g., SEQ ID NOS: 13, 47) of H1 and H5 (SEQ ID NO: 39) influenza A viral isolates. pMT: metallothionein promoter-based expression vector. BiP: secretion signal sequence of immunoglobulin-binding protein. STF2: full-length flagellin of *S. typhimurium*. STF2Δ: hinge region-deleted STF2. MCS: multiple cloning site.

Figure 18 depicts plasmid constructs designed to express HA of H1 and H5 influenza A virus isolates. AOX1: AOX1 promoter of pPICZα expression vector (Invitrogen Corporation, Carlsbad, CA). αf: secretion signal sequence of yeast. STF2: full-length flagellin of *S. typhimurium*. STF2Δ: hinge region-deleted STF2. MCS: multiple cloning site.

Figure 19 depicts the amino acid sequence (SEQ ID NO: 60) of the STF2Δ.HA fusion protein with the linker between STF2Δ (STF2 minus its hinge region) and HA underlined.

Figure 20 depicts the nucleic acid sequence (SEQ ID NO: 61) encoding SEQ ID NO: 60. The linker is underlined.

Figure 21 depicts the amino acid sequence (SEQ ID NO: 62) of the STF2Δ.HA (Puerto Rico 8 (PR8) strain of influenza A virus) fusion protein with the linker between STF2Δ and HA underlined.

Figure 22 depicts the nucleic acid sequence (SEQ ID NO: 63) encoding SEQ ID NO: 62. The linker is underlined.

Figure 23 depicts the amino acid sequence (SEQ ID NO: 64) of HA (PR8).

Figure 24 depicts the nucleic acid sequence (SEQ ID NO: 65) encoding SEQ ID NO: 64.

Figure 25 depicts the amino acid sequence (SEQ ID NO: 66) of *E. coli* fliC without the hinge region.

Figure 26 depicts the amino acid sequence of influenza A H5N1 HA (SEQ ID NO: 67).

Figure 27 depicts the nucleic acid sequence (SEQ ID NO: 68) encoding SEQ ID NO: 67.

Figure 28 depicts the amino acid sequence of pMT/STF2.4xM2e (H1) (SEQ ID NO: 83). The linker sequence between STF2 and 4xM2e is underlined and the *Drosophila* BiP secretion signal is bolded.

Figure 29 depicts the nucleic acid sequence (SEQ ID NO: 84) encoding SEQ ID NO: 83. The nucleic acid sequence encoding the linker is underlined and the nucleic acid sequence encoding the BiP secretion signal is bolded.

Figure 30 depicts the amino acid sequence pMT/STF2.4xM2e (H5) (SEQ ID NO: 85). The linker sequence between STF2 and 4xM2e is underlined and the BiP secretion signal is bolded.

Figure 31 depicts the nucleic acid sequence (SEQ ID NO: 86) encoding SEQ ID NO: 85. The nucleic acid sequence encoding the linker is underlined and the nucleic acid sequence encoding the BiP secretion signal is bolded.

Figure 32 depicts the amino acid sequence of pMT/STF2.4xM2e (H1H5) (SEQ ID NO: 87). The linker sequence between the STF2 and 4xM2e sequence is underlined and the BiP secretion signal is bolded.

Figure 33 depicts the nucleic acid sequence (SEQ ID NO: 88) encoding SEQ ID NO: 87. The nucleic acid sequence encoding the linker is underlined and the nucleic acid sequence encoding the BiP secretion signal is bolded.

Figure 34 depicts the amino acid sequence of pMT/STF2Δ (SEQ ID NO: 89). The linker sequence is underlined and the BiP secretion signal is bolded.

Figure 35 depicts the nucleic acid sequence (SEQ ID NO: 90) encoding SEQ ID NO: 89. The nucleic acid sequence encoding the linker is underlined and the nucleic acid sequence encoding the BiP secretion signal is bolded.

Figure 36 depicts the amino acid sequence of pMT/STF2Δ.4xM2e (H1) (SEQ ID NO: 91). The linker sequence is underlined and the BiP secretion signal sequence is bolded.

Figure 37 depicts the nucleic acid sequence (SEQ ID NO: 92) encoding SEQ ID NO: 91. The nucleic acid sequence encoding the linker is underlined and the nucleic acid sequence encoding the BiP secretion signal is bolded.

Figure 38 depicts the amino acid sequence of pMT/STF2Δ.4xM2e (H5) (SEQ ID NO: 93). The linker sequence is underlined and the BiP secretion signal is bolded.

Figure 39 depicts the nucleic acid sequence (SEQ ID NO: 94) encoding SEQ ID NO: 93. The nucleic acid sequence encoding the linker is underlined and the nucleic acid sequence encoding the BiP secretion signal is bolded.

Figure 40 depicts the amino acid sequence pMT/STF2Δ.4xM2e (H1H5) (SEQ ID NO: 95). The linker sequence is underlined and the BiP secretion signal is bolded.

Figure 41 depicts the nucleic acid sequence (SEQ ID NO: 96) encoding SEQ ID NO: 95. The nucleic acid sequence encoding the linker is underlined and the nucleic acid sequence encoding the BiP secretion signal is bolded.

Figure 42 depicts the amino acid sequence (SEQ ID NO: 99) of the *Salmonella muenchen* fliC without the hinge region, which is also referred to herein as "*S. muenchen* fliCΔ."

Figure 43 depicts the nucleic acid sequence of *Salmonella muenchen* fliC (SEQ ID NO: 100) encoding SEQ ID NO: 99.

Figure 44 depicts IL-8 secretion following stimulation of TLR5+ cells.

Figure 45 depicts TNF secretion following stimulation of TLR2+ cells.

Figure 46 depicts M2e-specific IgG.

Figure 47 depicts the OVA-specific IgG.

Figure 48 depicts the M2e-specific IgG serum titers.

Figure 49 depicts the M2e-specific serum IgG titer post-boost.

Figure 50 depicts the Pam3Cys.M2e dose response.

Figure 51 depicts the M2e-specific serum IgG titer.

Figure 52 depicts the rabbit IgG response to M2e.

Figure 53 depicts the immunogenicity of STF2.4xM2e in a rabbit 14 days post-prime.

Figure 54 depicts the survival following viral challenge.

DETAILED DESCRIPTION OF THE INVENTION

The features and other details of the invention, either as steps of the invention or as combinations of 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 of the invention. The principle features of this invention can be employed in various embodiments without departing from the scope of the invention.

In one embodiment, the invention is a composition comprising at least one Pam3Cys ([Palmitoyl]-Cys((RS)-2,3-di(palmitoyloxy)-propyl cysteine) and at least a portion of at least one integral membrane protein of an influenza viral protein. Pam3Cys (also referred to herein as "P2") is a Toll-like receptor 2 (TLR2) agonist.

The compositions can include, for example, two, three, four, five, six or more pathogen-associated molecular patterns (e.g., Pam2Cys, Pam3Cys) and two, three, four (e.g., SEQ ID NOS: 17 and 18), five, six or more integral membrane proteins of an influenza viral protein. When two or more PAMPs and/or two or more influenza viral proteins comprise the compositions, fusion proteins and polypeptides of the invention, they are also referred to as "multimers." For example, a multimer of the amino-terminus of an M2 protein can be four, 24-amino acid sequences (total of 96 amino acids), which is referred to herein as 4xM2 or 4xM2e ("M2e" refers to the 24 amino acid amino-terminus of the M2 protein or its ectodomain).

Pathogen-associated molecular pattern (PAMP) refers to a class of molecules (e.g., proteins, peptide, carbohydrates, lipids) found in microorganisms that when bound to a pattern recognition receptor (PRR) can trigger an innate immune response. The PRR can be a Toll-like receptor (TLR). Toll-like receptors refer to a family of receptor proteins that are homologous to the *Drosophila melangogaster* Toll protein. Toll-like receptors are type I transmembrane signaling receptor proteins characterized by an extracellular leucine-rich repeat domain and an intracellular domain homologous of that of the interleukin 1 receptor. Toll-like receptors include TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR 8, TLR9, TLR10, TLR11 and TLR12.

The pathogen-associated molecular pattern can be an agonist of a toll-like receptor, for example, a TLR2 agonist, such as Pam3Cys. "Agonist," as used herein in referring to a TLR, means a molecule that activates a TLR signaling pathway. A TLR signaling pathway is an intracellular signal transduction pathway employed by a particular TLR that can be activated by a TLR ligand or a TLR agonist. Common intracellular pathways are employed by TLRs and include, for example, NF-κB, Jun N-terminal kinase and mitogen-activated protein kinase. The pathogen-associated molecular pattern can include at least one member selected from the group consisting of a TLR1 agonist, a TLR2 agonist, a TLR 3 agonist, a TLR 4 agonist, a TLR 5 agonist, a TLR 6 agonist, a TLR 7 agonist, a TLR 8 agonist, a TLR 9 agonist, TLR10 agonist, a TLR11 agonist and a TLR12 agonist.

Influenza viruses are divided into three types (i.e., A, B, C) determined by the antigenic differences in ribonucleoprotein (RNP) and matrix (M) antigens of the viruses. Influenza A virus can cause epidemics and pandemics and has an avian intermediate host. Influenza B virus appears to naturally infect only humans and can cause epidemics in humans. It naturally infects humans and several other mammalian species, including swine and horses, and a wide variety of avian species. Influenza C virus has been isolated from humans and swine, but generally does not occur in epidemics and usually results in mild disease in humans.

Influenza A virus, influenza B virus and influenza C virus belong to the viral family *Orthomyxoviridae*. Virions of the genera influenza A virus, influenza B virus and influenza C virus contain a single stranded, negative sense, segmented RNA genome and are enveloped with a pleomorphic structure ranging in diameter from 80 – 120 nm. The single-stranded RNA genome is closely associated with a helical nucleoprotein and is present in seven (influenza C) or eight (influenza A and B) separate segments of ribonucleoprotein (RNP), each of which has to be present for successful replication of the virus. The segmented genome is enclosed within an outer lipoprotein envelope. Matrix protein 1 (MP1 or also referred to herein as "M1") lines the inside of the outer lipoprotein envelope and is bound to the RNP.

The outer lipoprotein envelope of the influenza virus has two types of protruding spikes. One of the protruding spikes is the integral membrane protein neuraminidase (NA), which has enzymatic properties. The other envelope spike is

the trimeric integral membrane protein haemagglutinin (HA), which participates in attachment of the virus particle to a cell membrane and can combine with specific receptors on a variety of cells, including red blood cells. The outer lipoprotein envelope makes the virion labile and susceptible to heat, drying, detergents and solvents.

Matrix protein 2 (M2 or M2 protein) is a proton-selective integral membrane ion channel protein of the influenza A virus. M2 is abundantly expressed at the plasma membrane of virus-infected cells, but is generally underexpressed by virions. For example, a portion of an M2 sequence of influenza A is MSLLTEVETPIRNEWGCRCNDSSDPLVVAASIIGILHLILWILDRLFFKCIYRL FKHGLKRGPSTEGVPESMREEYRKEQQNAVDADDSHFVSIELE (SEQ ID NO: 11), which is encoded by ATGAGCCTTCTAACCGAGGTCGAAACACCTATCAGAAACGAATGGGGGT GCAGATGCAACGATTCAAGTGACCCGCTTGTTGTTGCCGCGAGTATCATT GGGATCTTGCACTTGATATTGTGGATTCTTGATCGTCTTTTTTTCAAATGC ATCTATCGACTCTTCAAACACGGCCTTAAAAGAGGGCCTTCTACGGAAG GAGTACCTGAGTCTATGAGGGAAGAATATCGAAAGGAACAGCAGAATG CTGTGGATGCTGACGACAGTCATTTTGTCAGCATAGAGTTGGAGTAA (SEQ ID NO: 12). The native form of the M2 protein is a homotetramer (i.e., four identical disulfide-linked M2 protein molecules). Each of the units are helices stabilized by two disulfide bonds. M2 is activated by low pH. Each of the M2 protein molecules in the homotetramer consists of three domains: a 24 amino acid outer or N (amino)-terminal domain (e.g., SLLTEVETPIRNEWGCRCNDSSDP (SEQ ID NO: 13; also referred to herein as a "human consensus sequence"), which is encoded by ATGAGCCTGCTGACCGAGGTCGAAACACCGATCCGCAACGAATGGGGGT GCCGCTGCAACGATTCAAGTGACCCG (SEQ ID NO: 14); a 19 hydrophobic amino acid transmembrane region, and a 54 amino acid inner or C (carboxy)-terminal domain. The M2 protein can vary depending upon the influenza viral subtype (e.g., H1 and H5 subtypes of influenza A) and influenza viral source (e.g., Puerto Rico, Thailand, New York, Hong Kong), as shown, for example, in exemplary amino-terminal sequences of M2 proteins in Table 1 (*infra*).

The M2 protein has an important role in the life cycle of the influenza A virus. It is important in the uncoating stage where it permits the entry of protons into the viral particle, which lowers the pH inside the virus, resulting in dissociation of the viral matrix protein M1 from the ribonucleoprotein RNP. As a consequence, the virus coat is removed and the contents of the virus are released from the endosome into the cytoplasm of the host cell for infection.

The function of the M2 channel can be inhibited by antiviral drugs, such as amantadine and rimantadine, which prevent the virus from infecting the host cell. Such antiviral drugs usually bind the transmembrane region of the M2 protein and sterically block the ion channel created by the M2 protein, which prevents protons from entering and uncoating the virion.

As discussed above, M2, HA and NA are integral membrane proteins (e.g., proteins that extend from the outer surface of the virus to the inner surface of the virus) of influenza viruses (influenza A, B, C). "At least a portion," as used herein in reference to an integral membrane protein of an influenza virus, means any part of an entire integral membrane protein. For example, the 24 amino acid N-terminus of the M2 protein (e.g., SEQ ID NO: 13), EVETPIRNEWG (SEQ ID NO: 15), EVETPIRNE (SEQ ID NO: 19), EVETPIRNEW (SEQ ID NO: 34) or EVETPIRN (SEQ ID NO: 20) is at least a portion of an M2 protein; and PAKLLKERGRRGAIAGFLE (SEQ ID NO: 33) is at least a portion of an HA protein. SEQ ID NO: 15 encoded by GAGGTTGAGACCCCGATTCGCAACGAATGGGGT (SEQ ID NO: 97). The protein encoded by GAGGTCGAAACACCTATCAGAAACGAATGG (SEQ ID NO: 16) is also at least a portion of M2.

The compositions, fusion proteins and polypeptides of the invention can include at least one member selected from the group consisting of an influenza A viral protein, influenza B viral protein and an influenza C viral protein. The influenza viral protein can include an integral membrane protein that includes at least one member selected from the group consisting of a haemagglutinin integral membrane protein, a neuraminidase integral membrane protein and an M2 integral membrane protein.

The integral membrane protein can include an M2 protein that includes at least a portion of SLLTEVETPIRNEWGCRCNDSSDP (SEQ ID NO: 13) encoded by SEQ ID NO: 14 or at least a portion of SEQ ID NO: 47, encoded by AGCTTGCTGACTGAGGTTGAGACCCCGATTCGCAACGAATGGGGTTCCC GTTCCAACGATTCTTCCGACCCG (SEQ ID NO: 107). The M2 protein can further include at least one member selected from the group consisting of EVETPIRNEWG (SEQ ID NO: 15), EVETPIRNE (SEQ ID NO: 19), EVETPIRNEW (SEQ ID NO: 34); SLLTEVETPTRNEWESRSSDSSDP (SEQ ID NO: 39) (Flu A H5N1 M2e, 2004 Viet Nam Isolate with serine replacing cysteine); SLLTEVETPTRNEWECRCSDSSDP (SEQ ID NO: 40) (Flu A H5N1 M2e, 2004 Viet Nam Isolate); SLLTEVETLTRNGWGSRSSDSSDP (SEQ ID NO: 41) (Flu A H5N1 M2e, Hong Kong 97 Isolate with serine replacing cysteine); SLLTEVETLTRNGWGCRCSDSSDP (SEQ ID NO: 42) (Flu A H5N1 M2e, Hong Kong 97 Isolate); SLLTEVETPTRNGWESKSSDSSDP (SEQ ID NO: 43) (Flu A H7N2 M2e Chicken/New York 95 Isolate with serine replacing cysteine); SLLTEVETPTRNGWECKCSDSSDP (SEQ ID NO: 44) (Flu A H7N2 M2e, Chicken/ New York 95 Isolate); SLLTEVETLTRNGWESKSRDSSDP (SEQ ID NO: 45) (Flu A H9N2 M2e, Hong Kong 99 Isolate with serine replacing cysteine); and SLLTEVETLTRNGWECKCRDSSDP (SEQ ID NO: 46) (Flu A, Hong Kong 99 Isolate). Certain cysteine residues, for example, amino acids 16 and 18 of SEQ ID NO: 40; amino acids 17 and 19 of SEQ ID NOS: 42, 44 and 46 in the naturally occurring sequence of at least a portion of M2 protein are replaced with a serine (see, SEQ ID NOS: 41, 43, 45 and 47, respectively).

The integral membrane protein can include a haemagglutinin protein that includes, for example, at least a portion of SEQ ID NOS: 64 and 67, encoded by SEQ ID NOS: 65 and 68, respectively. The haemagglutinin protein can include at least a portion of at least one member selected from the group consisting of PAKLLKERGRRGAIAGFLE (SEQ ID NO: 33) (Influenza B); SLWSEEPAKLLKERGFFGAIAGFLEE (SEQ ID NO: 35) (Flu B); SLWSEENIPSIQSRGLFGAIAGFIEE (SEQ ID NO: 36) (FluA H1/H0); SLWSEENVPEKQTRGIFGAIAGFIEE (SEQ ID NO: 37) (Flu A H3/H0); SLWSEEEWEERERRRKKRGLFGAIAGFIEE (SEQ ID NO: 38) (Flu A H5/H0);

PAKLLKERGFFGAIAGFLEE (SEQ ID NO: 103) (Flu B);
NIPSIQSRGLFGAIAGFIEE (SEQ ID NO: 104) (Flu A H1/H0);
NVPEKQTRGIFGAIAGFIEE (SEQ ID NO: 105) (Flu A H3/H0); and
RERRRKKRGLFGAIAGFIEE (SEQ ID NO: 106) (Flu A H5/H0).

The composition comprising at least one Pam3Cys and at least a portion of at least one integral membrane protein of an influenza viral protein can further include at least one Pam2Cys (S-[2,3-bis(palmitoyloxy) propyl] cysteine). The composition of at least one Pam3Cys, at least one Pam2Cys and at least a portion of at least one integral membrane protein can be components of a fusion protein. The composition comprising at least one Pam3Cys and at least a portion of at least one integral membrane protein of an influenza viral protein can also be components of a fusion protein.

"Fusion protein," as used herein, refers to a protein generated from at least two similar or distinct components (e.g., Pam2Cys, Pam3Cys, PAMP, at least a portion of an integral membrane protein of an influenza viral protein) that are linked covalently or noncovalently. The components of the fusion protein can be made, for example, synthetically (e.g., Pam3Cys, Pam2Cys) or by recombinant nucleic acid techniques (e.g., transfection of a host cell with a nucleic acid sequence encoding a component of the fusion protein, such as at least a portion of a PAMP, or at least a portion of an integral membrane protein of an influenza viral protein). One component of the fusion protein (e.g., Pam2Cys, Pam3Cys, PAMP, at least a portion of an integral membrane protein of an influenza viral protein) can be linked to another component of the fusion protein (e.g., Pam2Cys, Pam3Cys, PAMP, at least a portion of an integral membrane protein of an influenza viral protein) using chemical conjugation techniques, including peptide conjugation, or using molecular biological techniques, including recombinant technology, such as the generation of a fusion protein construct. Exemplary fusion proteins of the invention include SEQ ID NO: 31 (Figure 13), encoded by SEQ ID NO: 32 (Figure 14); SEQ ID NO: 62 (Figure 21), encoded by SEQ ID NO: 63 (Figure 22); SEQ ID NO: 60 (Figure 19), encoded by SEQ ID NO: 61 (Figure 20); SEQ ID NO: 83 ((Figure 28), encoded by SEQ ID NO: 84 (Figure 29); SEQ ID NO: 85 (Figure 30), encoded by SEQ ID NO: 86 (Figure 31); SEQ ID NO: 87 (Figure 32), encoded by SEQ ID NO: 88 (Figure

33); SEQ ID NO: 91 (Figure 36), encoded by SEQ ID NO: 92 (Figure 37); SEQ ID NO: 93 (Figure 38), encoded by SEQ ID NO: 94 (Figure 39); SEQ ID NO: 95 (Figure 40), encoded by SEQ ID NO: 96 (Figure 41); and Pam3Cys, such as depicted in Figure 15.

Fusion proteins of the invention can be designated by components of the fusion proteins separated by a "." or "-." For example, "STF2.M2e" refers to a fusion protein comprising one fljB/STF2 protein and one M2e protein; and "STF2Δ.4xM2e" refers to a fusion protein comprising one fljB/STF2 protein without the hinge region and (4) 24-amino acid sequences of the N-terminus of the M2 protein (SEQ ID NO: 47).

A component of the fusion protein can include MKATKLVLGAVILGSTLLAGCSSN (SEQ ID NO: 21) encoded by ATGAAAGCTACTAAACTGGTACTGGGCGCGGTAATCCTGGGTTCTACTCT GCTGCTGGCAGGTTGCTCCAGCAAC (SEQ ID NO: 22).

The fusion proteins of the invention can further include a linker between at least one component of the fusion protein (e.g., Pam3Cys, Pam2Cys, PAMP) and at least one other component of the fusion protein (e.g., at least a portion of an integral membrane protein of an influenza viral protein) of the composition, a linker between at least two of similar components of the fusion protein (e.g., Pam3Cys, Pam2Cys, PAMP, at least a portion of an integral membrane protein of an influenza viral protein) or any combination thereof. "Linker," as used herein in reference to a fusion protein of the invention, refers to a connector between components of the fusion protein in a manner that the components of the fusion protein are not directly joined. For example, one component of the fusion protein (e.g., Pam3Cys, Pam2Cys, PAMP) can be linked to a distinct component (e.g., at least a portion of an integral membrane protein of an influenza viral protein) of the fusion protein. Likewise, at least two or more similar or like components of the fusion protein can be linked (e.g., two PAMPs can further include a linker between each PAMP, or two integral membrane proteins can further include a linker between each integral membrane protein).

Additionally or alternatively, the fusion proteins of the invention can include a combination of a linker between distinct components of the fusion protein and

similar or like components of the fusion protein. For example, a fusion protein can comprise at least two PAMPs, Pam3Cys and/or Pam2Cys components that further includes a linker between, for example, two or more PAMPs; at least two integral membrane proteins of an influenza viral antigen that further include a linker between them; a linker between one component of the fusion protein (e.g., PAMP) and another distinct component of the fusion protein (e.g., at least a portion of at least one integral membrane protein of an influenza viral protein), or any combination thereof.

The linker can be an amino acid linker. The amino acid linker can include synthetic or naturally occurring amino acid residues. The amino acid linker employed in the fusion proteins of the invention can include at least one member selected from the group consisting of a lysine residue, a glutamic acid residue, a serine residue and an arginine residue. The amino acid linker can include, for example, SEQ ID NOS: 24 (KGNSKLEGQLEFPRTS), 26 (EFCRYPAQWRPL), 27 (EFSRYPAQWRPL) and 29 (KGNSKLEGQLEFPRTSPVWWNSADIQHSGGRQCDGYLQNSPLRPL), encoded by the nucleic acid sequences of SEQ ID NOS: 23 (AAGGGCAATTCGAAGCTTGAAGGTCAATTGGAATTCCCTAGGACTAGT), 25 (GAATTCTGCAGATATCCAGCACAGTGGCGGCCGCTC), 28 (GAATTCTCTAGATATCCAGCACAGTGGCGGCCGCTC) and 30 (AAGGGCAATTCGAAGCTTGAAGGTCAATTGGAATTCCCTAGGACTAGTC CAGTGTGGTGGAATTCTGCAGATATCCAGCACAGTGGCGGCCGCCAGTG TGATGGATATCTGCAGAATTCGCCCTTGCGGCCGCTC), respectively.

The compositions of the invention can further include a linker between at least two integral membrane proteins of the composition.

The compositions, fusion proteins and polypeptides of the invention can further include a PAMP that is a TLR5 agonist. The TLR5 agonist can be a flagellin. The flagellin can be at least one member selected from the group consisting of fljB/STF2 (*S. typhimurium* flagellin B, Genbank Accession Number AF045151), at least a portion of fljB/STF2, *E. coli* flagellin fliC (also referred to herein as "*E. coli* fliC") (Genbank Accession Number AB028476), at least a portion

of *E. coli* flagellin fliC, *S. muenchen* flagellin fliC (also referred to herein as "*S. muenchen* fliC") and at least a portion of *S. muenchen* flagellin fliC.

In one embodiment, the flagellin includes the polypeptides of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, and SEQ ID NO: 7; at least a portion of SEQ ID NO: 1, at least a portion of SEQ ID NO: 3, at least a portion of SEQ ID NO: 5, at least a portion of SEQ ID NO: 7; and a polypeptide encoded by SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6 and SEQ ID NO: 8; or at least a portion of a polypeptide encoded by SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6 and SEQ ID NO: 8. "At least a portion," as used herein in reference to a flagellin (e.g., fljB/STF2, *E. coli* fliC, *S. muenchen* fliC), refers to any part of the flagellin that can initiate an intracellular signal transduction pathway for a TLR. "At least a portion," is also referred to herein as a "fragment."

The pathogen-associated molecular pattern can be a TLR2 agonist. The TLR2 agonist can include at least a portion of a bacterial lipoprotein (BLP), such as SEQ ID NO: 21 or a polypeptide encoded by SEQ ID NO: 22.

In another embodiment, the invention is a fusion protein comprising at least one pathogen-associated molecular pattern and at least one influenza M2 protein, wherein the pathogen-associated molecular pattern is not Pam2Cys. The fusion proteins of the invention can further include at least a portion of at least one member selected from the group consisting of an M2 protein, an HA protein and an NA protein. The M2 protein can include at least a portion of SEQ ID NO: 13, EVETPIRNEWG (SEQ ID NO: 15), EVETPTRNE (SEQ ID NO: 19) or EVETPIRNEW (SEQ ID NO: 34). The HA protein can include at least a portion of PAKLLKERGRRGAIAGFLE (SEQ ID NO: 33).

The fusion proteins of the invention can further include a linker between at least one pathogen-associated molecular pattern and at least one M2 protein; a linker between at least two M2 proteins; a linker between at least two PAMPs or any combination thereof.

In still another embodiment, the invention is a fusion protein comprising at least two Pam2Cys and at least one influenza M2 protein.

The pathogen-associated molecular pattern of the compositions, fusion proteins and polypeptides of the invention can include a TLR5 agonist, such as a

flagellin. The flagellin can include at least one member selected from the group consisting of fljB/STF2, *E.coli* fliC, and *S. muenchen* fliC.

In one embodiment, the compositions, fusion proteins and polypeptides of the invention can include a flagellin that includes fljB/STF2 that includes at least a portion of SEQ ID NO: 1, such as the fljB/STF2 that includes SEQ ID NO: 3 or a nucleic acid sequence that encodes at least of portion of SEQ ID NO: 2, such as SEQ ID NO: 4.

In another embodiment, the compositions, fusion proteins and polypeptides of the invention can include a flagellin that includes includes *E. coli* fliC that includes at least a portion of SEQ ID NOS: 5, 9, such as *E. coli* fliC that includes SEQ ID NO: 66 or a nucleic acid sequence that encodes at least of portion of SEQ ID NOS: 6, 10.

In yet another embodiment, the compositions, fusion proteins and polypeptides of the invention can include a flagellin that includes *S. muenchen* fliC that includes at least a portion of SEQ ID NO: 7, such as *S. muenchen* fliC that includes SEQ ID NO: 99 or a nucleic acid sequence that encodes at least of portion of SEQ ID NO: 8, such as SEQ ID NO: 100.

The flagellin employed in the compositions, fusion proteins and polypeptides of the invention can lack a hinge region or at least a portion of a hinge region. Hinge regions are the hypervariable regions of a flagellin that link the amino-terminus and carboxy-terminus of the flagellin. Example of hinge regions include amino acids 177-416 of SEQ ID NO: 1 that are encoded by nucleic acids 531-1248 of SEQ ID NO: 2; amino acids 174-422 of SEQ ID NO: 5 that are encoded by nucleic acids 522-1266 of SEQ ID NO: 6; or amino acids 173-464 of SEQ ID NO: 60 that are encoded by nucleic acids 519-1392 of SEQ ID NO: 61.

"At least a portion of a hinge region," as used herein, refers to any part of the hinge region of the PAMP that is less than the entire hinge region. "At least a portion of a hinge region" is also referred to herein as a "fragment of a hinge region." For example, the hinge region of *S. typhimurium* flagellin B (fljB, also referred to herein as fljB/STF2 or STF2) is amino acids 175-415 of SEQ ID NO: 1, which are encoded by nucleic acids at position 541-1246 of SEQ ID NO: 2. A

fragment of the hinge region of fljB/STF2 can be, for example, amino acids 200-300 of SEQ ID NO: 1.

The compositions, fusion proteins and polypeptides of the invention can also include at least a portion of an influenza viral protein placed in or fused to a portion of the pathogen-associated molecular pattern, such as a region of the pathogen-associated molecular pattern that contains or contained a hinge region. For example, the hinge region of the pathogen-associated molecular pattern or at least a portion of the hinge region of the pathogen-associated molecular pattern can be removed from the pathogen-associated molecular pattern and replaced with at least a portion of an influenza viral antigen (e.g., M2, such as SEQ ID NOS: 13, 19 and 39-59). A linker can further be included between the influenza viral antigen and the pathogen-associated molecular pattern in such a replacement.

The pathogen-associated molecular pattern of the fusion proteins of the invention can be fused to a carboxy-terminus, the amino-terminus or both the carboxy- and amino-terminus of an influenza protein, such as an integral membrane protein of an influenza viral protein (e.g., M2, HA, NA). The fusion proteins of the invention can include at least one pathogen-associated molecular pattern between at least two influenza M2 proteins, which can, optionally, include a linker between the pathogen-associate molecular pattern and the M2 protein.

The pathogen-associated molecular pattern of the fusion proteins of the invention can include a TLR2 agonist, such as at least one Pam2Cys, at least one Pam3Cys or any combination thereof. Thus, the fusion proteins of the invention can include at least one member selected from the group consisting of Pam2Cys and a Pam3Cys.

The fusion proteins comprising at least one pathogen-associated molecular pattern and at least a portion of at least one M2 protein can further include at least a portion of a haemagglutinin membrane protein; at least a portion of a neuraminidase membrane protein; at least one member selected from the group consisting of an influenza B viral protein and an influenza C viral protein; or any combination thereof. The influenza B viral protein and/or influenza C viral protein can be an integral membrane protein.

In yet another embodiment, the invention is a composition comprising a pathogen-associated molecular pattern and an M2 protein.

In an additional embodiment, the invention is a composition comprising at least a portion of at least one pathogen-associated molecular pattern and at least a portion of at least one influenza M2 protein, wherein, if the pathogen-associated molecular pattern includes a Pam2Cys, at least a portion of the Pam2Cys is not fused to the influenza M2 protein and at least a portion of the influenza M2 protein is not fused to the Pam2Cys.

"Fused to," as used herein means covalently or noncovalently linked or recombinantly produced together.

In another embodiment, the invention is a fusion protein comprising at least a portion of at least one pathogen-associated molecular pattern and at least a portion of at least one influenza M2 protein, wherein, if the pathogen-associated molecular pattern includes a Pam2Cys, at least a portion of the Pam2Cys is not fused to the influenza M2 protein and at least a portion of the influenza M2 protein is not fused to the Pam2Cys.

In still another embodiment, the invention includes a polypeptide that includes SEQ ID NOS: 9, 31, 64, 60, 83, 85, 87, 89, 91, 93 and 95 and a polypeptide encoded by SEQ ID NOS: 10, 32, 63, 61, 84, 86, 88, 90, 91, 94 and 96.

In an additional embodiment, the invention includes a polypeptide having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98% and at least about 99% sequence identity to the polypeptides of SEQ ID NOS: 9, 31, 64, 60, 83, 85, 87, 89, 91, 93 and 95 and the nucleic acids of SEQ ID NOS: 10, 32, 63, 61, 84, 86, 88, 90, 91, 94 and 96.

The percent identity of two amino acid sequences (or two nucleic acid sequences) can be determined by aligning the sequences for optimal comparison purposes (e.g., gaps can be introduced in the sequence of a first sequence). The amino acid sequence or nucleic acid sequences at corresponding positions are then compared, and the percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % identity = # of identical positions/total # of positions x 100). The length of the protein or nucleic

acid encoding a PAMP, at least a portion of an influenza viral protein, a fusion protein of the invention or a polypeptide of the invention aligned for comparison purposes is at least 30%, preferably, at least 40%, more preferably, at least 60%, and even more preferably, at least 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or 100%, of the length of the reference sequence, for example, the nucleic acid sequence of a PAMP, at least a portion of an integral membrane protein of an influenza viral protein, or a polypeptide or fusion protein, for example, as depicted in SEQ ID NOS: 9, 31, 64, 60, 83, 85, 87, 89, 91, 93 and 95 and SEQ ID NOS: 10, 32, 63, 61, 84, 86, 88, 90, 91, 94 and 96.

The actual comparison of the two sequences can be accomplished by well-known methods, for example, using a mathematical algorithm. A preferred, non-limiting example of such a mathematical algorithm is described in Karlin *et al.* (*Proc. Natl. Acad. Sci. USA, 90*:5873-5877 (1993), the teachings of which are hereby incorporated by reference in its entirety). Such an algorithm is incorporated into the BLASTN and BLASTX programs (version 2.2) as described in Schaffer *et al.* (*Nucleic Acids Res., 29*:2994-3005 (2001), the teachings of which are hereby incorporated by reference in its entirety). When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., BLASTN; available at the Internet site for the National Center for Biotechnology Information) can be used. In one embodiment, the database searched is a non-redundant (NR) database, and parameters for sequence comparison can be set at: no filters; Expect value of 10; Word Size of 3; the Matrix is BLOSUM62; and Gap Costs have an Existence of 11 and an Extension of 1.

Another mathematical algorithm employed for the comparison of sequences is the algorithm of Myers and Miller, CABIOS (1989), the teachings of which are hereby incorporated by reference in its entirety. Such an algorithm is incorporated into the ALIGN program (version 2.0), which is part of the GCG (Accelrys, San Diego, California) sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 is used. Additional algorithms for sequence analysis are known in the art and include ADVANCE and ADAM as described in Torellis and Robotti (Comput. Appl. Biosci., 10: 3-5 (1994),

the teachings of which are hereby incorporated by reference in its entirety); and FASTA described in Pearson and Lipman (*Proc. Natl. Acad. Sci USA, 85*: 2444-2448 (1988), the teachings of which are hereby incorporated by reference in its entirety).

In a further embodiment, the invention is host cells and vectors that include the nucleic acid sequences of the invention. The host cells can be prokaryotic (e.g., *E. coli*) or eukaryotic (e.g., insect cells, such as *Drosophila* Dmel2 cells; Baculovirus; CHO cells; yeast cells, such as Pichia) host cells.

The percent identity between two amino acid sequences can also be accomplished using the GAP program in the GCG software package (Accelrys, San Diego, California) using either a Blossom 63 matrix or a PAM250 matrix, and a gap weight of 12, 10, 8, 6, or 4 and a length weight of 2, 3, or 4. In yet another embodiment, the percent identity between two nucleic acid sequences can be accomplished using the GAP program in the GCG software package (Accelrys, San Diego, California), using a gap weight of 50 and a length weight of 3.

The nucleic acid sequence encoding a PAMP, at least a portion of an integral membrane protein of an influenza viral protein, fusion proteins of the invention and polypeptides of the invention can include nucleic acid sequences that hybridize to, for example, a fljB/STF2 (e.g., SEQ ID NOS: 2, 4), a fliC (e.g., SEQ ID NOs: 6, 8, 100), at least a portion of an integral membrane protein of an influenza viral protein (e.g., SEQ ID NOS: 11, 13, 15, 18, 19, 21, 33, 35-59, 64 and 67) and fusion proteins of the invention (e.g., SEQ ID NOS: 31, 64 and 60) under selective hybridization conditions (e.g., highly stringent hybridization conditions). As used herein, the terms "hybridizes under low stringency," "hybridizes under medium stringency," "hybridizes under high stringency," or "hybridizes under very high stringency conditions," describe conditions for hybridization and washing of the nucleic acid sequences. Guidance for performing hybridization reactions, which can include aqueous and nonaqueous methods, can be found in Aubusel, F.M., *et al.*, Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (2001), the teachings of which are hereby incorporated herein in its entirety.

For applications that require high selectivity, relatively high stringency conditions to form hybrids can be employed. In solutions used for some membrane

based hybridizations, addition of an organic solvent, such as formamide, allows the reaction to occur at a lower temperature. High stringency conditions are, for example, relatively low salt and/or high temperature conditions. High stringency are provided by about 0.02 M to about 0.10 M NaCl at temperatures of about 50°C to about 70°C. High stringency conditions allow for limited numbers of mismatches between the two sequences. In order to achieve less stringent conditions, the salt concentration may be increased and/or the temperature may be decreased. Medium stringency conditions are achieved at a salt concentration of about 0.1 to 0.25 M NaCl and a temperature of about 37°C to about 55°C, while low stringency conditions are achieved at a salt concentration of about 0.15 M to about 0.9 M NaCl, and a temperature ranging from about 20°C to about 55°C. Selection of components and conditions for hybridization are well known to those skilled in the art and are reviewed in Ausubel *et al.* (1997, Short Protocols in Molecular Biology, John Wiley & Sons, New York N.Y., Units 2.8-2.11, 3.18-3.19 and 4-64.9).

In a further embodiment, the compositions, fusion proteins and polypeptides of the invention can be employed in methods of stimulating an immune response in a subject. In one embodiment, the method of the invention can include a method of stimulating an immune response in a subject, comprising the step of administering to the subject a composition that includes at least one Pam3Cys and at least a portion of at least one integral membrane protein of an influenza viral protein. In another embodiment, the invention can include a method of stimulating an immune response in a subject, comprising the step of administering to the subject a composition that includes a fusion protein comprising at least one pathogen-associated molecular pattern and at least one influenza M2 protein. In a further embodiment, the invention can include a method of stimulating an immune response in a subject, comprising the step of administering to the subject a composition that includes at least one pathogen-associated molecular pattern and at least one influenza M2 protein, wherein the pathogen-associated molecular pattern is not a Pam2Cys and the M2 protein is not an M2e.

In yet another embodiment, the invention is a method of stimulating an immune response in a subject, comprising the step of administering to the subject a composition that includes a composition comprising at least a portion of at least one

pathogen-associated molecular pattern and at least a portion of at least one influenza M2 protein, wherein, if the pathogen-associated molecular pattern includes a Pam2Cys, at least a portion of the Pam2Cys is not fused to the influenza M2 protein and at least a portion of the influenza M2 protein is not fused to the Pam2Cys.

In a further embodiment, the invention is a method of stimulating an immune response in a subject, comprising the step of administering to the subject a composition that includes a fusion protein comprising at least a portion of at least one pathogen-associated molecular pattern and at least a portion of at least one influenza M2 protein, wherein, if the pathogen-associated molecular pattern includes a Pam2Cys, at least a portion of the Pam2Cys is not fused to the influenza M2 protein and at least a portion of the influenza M2 protein is not fused to the Pam2Cys.

A subject treated by the methods of the invention can be a mammal, such as a primate or a rodent (e.g., mouse, rat). In a particular embodiment, the subject is a human. A subject is also referred to herein as "an individual."

"Stimulating an immune response," as used herein, refers to the generation of antibodies to at least a portion of an influenza viral protein (e.g., an integral membrane, such as M2, HA, NA of influenza A, B and/or C). Stimulating an immune response in a subject can include the production of humoral and/or cellular immune responses that are reactive against the influenza viral protein. In stimulating an immune response in the subject, the subject may be protected from infection by the influenza virus or conditions associated with infection by the influenza virus that may diminish or be halted as a consequence of stimulating an immune response in the subject.

The compositions, fusion proteins and polypeptides of the invention can be administered to a subject with or without an adjuvant to coordinate the innate and adaptive immune mechanisms and induce a potent antibody response accompanied by minimal non-specific inflammation. The induced immune response may provide protection against homologous and heterologous strains of influenza viruses and thereby may provide protection against circulating influenza viruses and against potential pandemic influenza caused by introduction of the H5 avian strain into the human population.

Strategies to manage infection and illness consequent to influenza viral infection have not changed significantly in the past four decades. Due to the seasonal nature of the disease, the distinct types of influenza virus (A and B) that threaten the human population, and the genetic instability of each type, it is necessary to reformulate a multivalent compositions (e.g., compositions containing more than one type of influenza viral protein) for immunizing and vaccinating subjects each year, based on epidemiological prediction of strains likely to be circulating in a population in the an upcoming flu season. Certain compositions, such as vaccines are produced from stocks of selected prototype viral strains grown in embryonated chicken eggs. Limitations of the currently available techniques include, for example, uncertain prediction of circulating strains; the ability to grow the appropriate strains in chicken eggs; the egg-based production system carries risks of product contamination; the product produced in eggs cannot be used in subjects with egg allergies; and risk that the multivalent composition will not confer protection against a pandemic strain of virus to which the a subject has no pre-existing immunity.

Generally, the dominant protective component of an influenza composition, such as a vaccine, is the viral haemagglutinin, the major virulence factor associated with the influenza A virus. Neutralizing antibodies to HA arise in response to natural infection or administration with influenza A virus and provide sterilizing immunity to subsequent exposure to a virus expressing that particular HA.

There are several antigenically distinct phenotypes of HA. Most human influenza isolates express the H1 or H3 phenotype, while avian viral strains may express H5, H7, or H9. Even within a particular phenotype such as H1, the virus may change by "antigenic drift" (point mutation) and "antigenic shift" (genetic re-assortment) of the HA antigen that may render the virus resistant to immune responses directed against earlier virus strains, whether that immunity arose in response to infection or to vaccination. Thus, the efficacy of traditional compositions employed to prevent influenza infection is limited against a pandemic strain such as one of the avian strains to which the human population has not developed immunity. The long manufacturing process prevents the efficient production of traditional compositions to prevent influenza infection against an

emerging pandemic strain. The compositions, fusion proteins and polypeptides of the invention may prevent influenza infection in a manner that is cost-effective to produce and that can be stockpiled in preparation for an influenza pandemic.

Subtypes of the influenza A virus are generally named according to the particular antigenic determinants of hemagglutinin (H, about 13 major types) and neuraminidase (N, about 9 major types). For example, subtypes include influenza A (H2N1), A(H3N2), A(H5N1), A(H7N2), A(H9N2), A(H1/H0), A(H3/H0) and A(H5/H0). In the last century, three subtypes of influenza A resulted in pandemics: H1 in 1918 and 1977; H2 in 1957 and H3 in 1968. In 1997, an H5 avian virus and in 1999, an H9 virus resulted in outbreaks of respiratory disease in Hong Kong.

New strains of the influenza virus emerge due to antigenic drift, a process whereby mutations within the virus antibody-binding sites accumulate over time. As a consequence of antigenic drift, the influenza virus can circumvent the infected subject's immune system, which may not be able to recognize and confirm immunity to a new influenza strain despite the immunity to different strains of the virus. Influenza A and B undergo antigenic drift.

Influenza A can also undergo antigenic shift resulting in a new virus subtype. Antigenic shift is a sudden change in viral antigenicity usually associated with recombination of the influenza genome that can occur when a cell is simultaneously infected by two different strains of influenza A virus.

In the 20th century, three influenza pandemics occurred in 1918, 1957, and 1968. The 1918 "Spanish flu" pandemic was clearly the most lethal, causing more than 500,000 deaths in the U.S. and as many as 50,000,000 deaths worldwide. Recent sequence and phylogenetic analysis suggest that the causative agent of the 1918 pandemic was an avian strain that adapted to humans (Taubenberger, J.K., *et al.*, *Nature 437*:889). A similar threat may be occurring today.

Since 1996, there have been nearly 200 confirmed cases of avian influenza infection in humans with an apparent increase in incidence in southeast Asia in 2004 (Zeitlin, G.A., *et al., Curr Infect Dis Rep 7:*193). More recently, migratory wild birds have carried the disease as far as the Middle East and Eastern Europe (Fereidouni, S.R. *et al., Vet Rec 157:*526; Al-Natour, M.Q., *et al., Prev Vet Med 70:*45; Liu, J., *et al. Science 309:*1206; Chen, H., *et al. Nature 436:*191). With the

growing incidence of human cases, close proximity of humans and domesticated bird flocks that are potential carriers of the disease, spread through migratory fowl, and the ease of human-to-human spread on a global scale (as experienced with severe acute respiratory syndrome (Poutanen, S.M., *et al. N Engl J Med 348:* 1995; *MMWR Morb Mortal Wkly Rep 52:* 1157)), there is a need to develop new, improved compositions, fusion proteins and polypeptides to protect subjects, in particular humans, from the potentially disastrous effects of another influenza pandemic.

The compositions, fusion proteins and polypeptides of the invention may be refractory to the genetic instability of the prototypical influenza targets, HA and neuraminidase (NA), which requires annual selection of multiple strains for use in preventing influenza infection. A composition, fusion protein and polypeptide based on a genetically stable antigen may provide long-lasting immunity to influenza infection, be useful year after year, and be particularly valuable in case of an influenza A pandemic.

M2 has genetic stability. The amino terminal 24 amino acid sequence (SEQ ID NO: 13, also referred to herein as "M2e") has changed little in human pathogenic influenza virus strains isolated since 1933 (Neirynck, S., *et al. Nature Medicine 5:*1157). In mammals, M2 is poorly immunogenic in its native form; however, when administered with adjuvants or conjugated to an appropriate carrier backbone, M2e induces the production of specific antibodies that correlate with protection from subsequent live virus challenge (Neirynck, S., *et al. Nature Medicine 5:*1157; Frace, A.M., *et al. Vaccine 17:*2237; Mozdzanowska, K. *et al. Vaccine 21:* 2616; Fran, J., *et al. Vaccine 22:*2993). Antibodies to M2e also confer passive protection in animal models of influenza A infection (Treanor, J.J., *et al. J. Virol 64:*1375; Liu, W., *et al. Immunol Lett 93:*131), not by neutralizing the virus and preventing infectivity, but rather by killing infected cells and disrupting the viral life cycle (Zebedee, S.L., *et al. J. Virol 62:*I2762; Jegerlehner, A., *et al. J. Immunol 172:*5598). It has been proposed that one mechanism of protection is antibody-dependent NK cell activity (Jegerlehner, A., *et al. J. Immunol 172:*5598).

Immunization of pigs with an M2-nucleoprotein fusion protein exacerbated disease rather than protecting (Heinen, P.P., *et al. J. Gen Virol 83:*1851). However,

these data were confounded by the multiple variables examined (fusion protein linking M2 to hepatitis B core antigen versus DNA immunization linking M2 to nucleoprotein), the dose of viral challenge, and the virus strain. More recently, immunization of ferrets with M2e peptide in the context of a complex carrier resulted in reduced lung viral titers upon subsequent challenge without exacerbation of clinical symptoms (Fran, J., *et al. Vaccine 22:*2993). Compositions, fusion proteins and polypeptides of the invention that include M2, in particular M2e, may limit the severity of influenza illness while allowing the host immune response to develop adaptive immunity to the dominant neutralizing influenza antigen, HA.

The compositions, fusion proteins and polypeptides of the invention can be employed in methods of stimulating an immune response in a subject. The compositions, fusion proteins and polypeptides of the invention can be administered alone or with currently available influenza vaccines and drugs. However, because the sequence of M2e is highly conserved across strains, HA/NA subtypes, and geographically and temporally-distinct isolates, the compositions, fusion proteins and polypeptides of the invention that include M2e may stimulate an immune response in a subject to M2e that may provide protection against a possible pandemic arising from the introduction of a totally new HA/NA subtype into a population nature to that subtype. The same genetic conservation lends itself to providing broad protection against a potential bioterrorism use of any influenza strain, such as influenza A.

The M2e sequence of certain avian influenza A isolates differs slightly from that of human isolates, but is highly-conserved among the avian isolates, as shown in Table 1 (*infra*). The compositions, fusion proteins and polypeptides of the invention that include M2e may target circulating human pathogenic strains of influenza A (H1 and H3 subtypes) as well as avian strains that present a pandemic threat (H5 subtypes).

Exemplary M2e amino acid sequences of the compositions, fusion proteins and polypeptides of the invention are shown in Table 1. The M2e amino acid sequences were based on Fan, *et al. Vaccine 22:*2993 (2004) or the NCBI Protein Database (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html). Variants in reference to A/New Caledonia/20/99 sequence are denoted by bolded and underlined

letters. A cysteine (C) residue in the naturally occurring M2 sequence (e.g., SEQ ID NOS: 40, 42, 44 and 46, *supra*; and SEQ ID NOS: 48, 49 and 50, in Table 1, *infra*) can be substituted with serine (S) residue (e.g., SEQ ID NOS: 39, 41, 43 and 45, *supra*; and SEQ ID NOS: 54, 73 and 74 in Table 1, *infra*). Such substitution may improve solubility and structural integrity of the compositions, fusion proteins and polypeptides of the invention.

Table 1

Representative source	Subtype	Host	Amino acid sequences
Human with serine replacing cysteine			SLLTEVETPIRNEWGSR SNDSSDP (SEQ ID NO: 47)
A/Puerto Rico/8/34	H1N1	Human	SLLTEVETPIRNEWGCR CNGSSDP (SEQ ID NO: 48) SLLTEVETPIRNEWGSR SNGSSDP (SEQ ID NO: 54)
A/Wisconsin/3523/88	H1N1	Human	SLLTEVETPIRNEWGCK CNDSSDP (SEQ ID NO: 49) SLLTEVETPIRNEWGSK SNDSSDP (SEQ ID NO: 73)
A/New Caledonia/20/99	H1N1	Human	SLLTEVETPIRNEWGCR CNDSSDP (SEQ ID NO: 50) SLLTEVETPIRNEWGSR SNDSSDP (SEQ ID NO: 74)
A/Aichi/470/68	H3N1	human	SLLTEVETPIRNEWGCR CNDSSDP (SEQ ID NO: 51)
A/Hebei/19/95	H3N2	human	SLLTEVETPIRNEWECR CNGSSDP (SEQ ID NO: 52) SLLTEVETPIRNEWESR SNGSSDP (SEQ ID NO: 75)
A/Chicken/Nakorn-Patom/Thailand	H5N1	avian	SLLTEVETPTRNEWECR CSDSSDP (SEQ ID NO: 53)
A/Thailand/1(KAN-1)/04	H5N1	avian	SLLTEVETPTRNEWECR CSDSSDP (SEQ ID NO: 53) SLLTEVETPTRNEWESR SSDSSDP (SEQ ID NO: 76)
A/Hong Kong/156/97	H5N1	human	SLLTEVETLTRNGWGCR CSDSSDP (SEQ ID NO: 55) SLLTEVETLTRNGWGSR SSDSSDP (SEQ ID NO: 77)
A/Viet Nam/1203/2004	H5N1	human	SLLTEVETPTRNEWECR

			CSDSSDP (SEQ ID NO: 56) SLLTEVETPTRNEWESR SSDSSDP (SEQ ID NO: 78)
A/Chicken/New York/95	H7N2	avian	SLLTEVETPTRNGWECK CSDSSDP (SEQ ID NO: 57) SLLTEVETPTRNGWESK SSDSSDP (SEQ ID NO: 79)
A/Chicken/Hong Kong/G9/97	H9N2	avian	SLLTEVETPTRNGWGCR CSGSSDP (SEQ ID NO: 58) SLLTEVETPTRNGWGSR SSGSSDP (SEQ ID NO: 80)
A/Hong Kong/1073/99	H9N2	human	SLLTEVETLTRNGWECK CRDSSDP (SEQ ID NO: 59) SLLTEVETLTRNGWESK SRDSSDP (SEQ ID NO: 81)

In a particular embodiment, the compositions, fusion proteins and polypeptides of the invention include a pathogen-associated molecular pattern. Certain PAMPs (e.g., TLR ligands, TLR agonists) bind TLR, which act as initiators of the innate immune response and gatekeepers of the adaptive immune response (Medzhitov, R., *et al. Nature:388*:394; Medzhitov, R., *et al., Cold Spring Harb Symp Quant Biol 64:*429; Pasare, C., *et al. Semin Immunol 16*:23; Barton, G.M., *et al. Curr Opin Immunol 14*:380; Bendelac, A., *et al. J Exp Med 195*:F19). TLRs are the best characterized type of Pattern Recognition Receptor (PRR) expressed on antigen-presenting cells (APC). APC utilize TLRs to survey the microenvironment and detect signals of pathogenic infection by engaging the cognate ligands of TLRs, Pathogen-Associated Molecular Patterns (PAMPs). PAMP and TLR interaction triggers the innate immune response, the first line of defense against pathogenic insult, manifested as release of cytokines, chemokines and other inflammatory mediators; recruitment of phagocytic cells; and important cellular mechanisms which lead to the expression of costimulatory molecules and efficient processing and

presentation of antigens to T-cells. TLRs control both innate and the adaptive immune responses.

TLRs recognize PAMPs including bacterial cell wall components such as lipoproteins (TLR2) and lipopolysaccharides (TLR4), bacterial DNA sequences that contain unmethylated CpG residues (TLR9), and bacterial flagellin (TLR5). The binding of PAMPs to TLRs activates well-characterized immune pathways that can be mobilized for the development of more potent compositions, fusion proteins and polypeptides of the invention. The compositions, fusion proteins and polypeptides can be generated in a manner that ensure that those cells that are exposed to protective antigen(s) of the pathogenic agent also receive an innate immune signal (TLR activation) and vice versa. This can be effectively achieved by designing the compositions, fusion proteins and polypeptides to include at least a portion of at least one PAMP and at least a portion of at least one influenza viral protein (e.g., an integral membrane protein). The compositions, fusion proteins and polypeptides of the invention can trigger signal transduction pathways in their target cells that result in the display of co-stimulatory molecules on the cell surface, as well as antigenic peptide in the context of major histocompatibility complex molecules (see Figure 16).

Figure 16 depicts the activation of an APC by TLR signaling. The composition, fusion protein or polypeptide of the invention includes a PAMP that binds to a TLR, promoting differentiation and maturation of the APC, including production and display of co-stimulatory signals. The composition, fusion protein or polypeptide can be internalized by its interaction with the TLR and processed through the lysosomal pathway to generate antigenic peptides, which are displayed on the surface in the context of the major histocompatibility complex.

An "effective amount," when referring to the amount of a composition, fusion protein or a polypeptide of the invention, refers to that amount or dose of the composition, fusion protein, or a polypeptide, that, when administered to the subject is an amount sufficient for therapeutic efficacy (e.g., an amount sufficient to stimulate an immune response in the subject). The compositions, fusion proteins, or polypeptides of the invention can be administered in a single dose or in multiple doses.

The methods of the present invention can be accomplished by the administration of the compositions, fusion proteins or polypeptides of the invention by enteral or parenteral means. Specifically, the route of administration is by oral ingestion (e.g., drink, tablet, capsule form) or intramuscular injection of the composition, fusion protein or polypeptide. Other routes of administration as also encompassed by the present invention including intravenous, intradermal, intraarterial, intraperitoneal, or subcutaneous routes, and nasal administration. Suppositories or transdermal patches can also be employed.

The compositions, fusion proteins or polypeptides of the invention can be administered *ex vivo* to a subject's autologous dendritic cells. Following exposure of the dendritic cells to the composition, fusion protein or polypeptide of the invention, the dendritic cells can be administered to the subject.

The compositions, fusion proteins or polypeptides of the invention can be administered alone or can be coadministered to the patient. Coadminstration is meant to include simultaneous or sequential administration of the composition, fusion protein or polypeptide of the invention individually or in combination. Where the composition, fusion protein or polypeptide are administered individually, the mode of administration can be conducted sufficiently close in time to each other (for example, administration of the composition close in time to administration of the fusion protein) so that the effects on stimulating an immune response in a subject are maximal. It is also envisioned that multiple routes of administration (e.g., intramuscular, oral, transdermal) can be used to administer the compositions and fusion proteins of the invention.

The compositions, fusion proteins or polypeptide of the invention 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 extract. Suitable pharmaceutically acceptable carriers include water, salt solutions (such as Ringer's solution), alcohols, oils, gelatins and carbohydrates such as lactose, amylose or starch, fatty acid esters, hydroxymethycellulose, 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, fusion proteins or polypeptides of the invention. The preparations can also be combined, when desired, with other active substances to reduce metabolic degradation. The compositions, fusion proteins or polypeptides of the invention can be administered by is oral administration, such as a drink, intramuscular or intraperitoneal injection. The compositions, fusion proteins , or polypeptides alone, or when combined with an admixture, can be administered in a single or in more than one dose over a period of time to confer the desired effect (e.g., alleviate prevent viral infection, to alleviate symptoms of viral infection).

When parenteral application is needed or desired, particularly suitable admixtures for the compositions, fusion proteins or polypeptides are injectable, sterile solutions, preferably oily or aqueous solutions, as well as suspensions, emulsions, or implants, including suppositories. In particular, carriers for parenteral administration include aqueous solutions of dextrose, saline, pure water, ethanol, glycerol, propylene glycol, peanut oil, sesame oil, polyoxyethylene-block polymers, and the like. Ampules are convenient unit dosages. The compositions, fusion proteins or polypeptides can also be incorporated into liposomes or administered via transdermal pumps or patches. Pharmaceutical admixtures suitable for use in the present invention are well-known to those of skill in the art and are described, for example, in Pharmaceutical Sciences (17th Ed., Mack Pub. Co., Easton, PA) and WO 96/05309 the teachings of which are hereby incorporated by reference.

The compositions, fusion proteins and polypeptides of the invention can be administered to a subject on a carrier. "Carrier," as used herein, means any composition that presents the compositions, fusion proteins and polypeptides of the invention to the immune system of the subject to generate an immune response in the subject. The presentation of the compositions, fusion proteins and polypeptides of the invention would preferably include exposure of antigenic portions of the influenza viral protein to generate antibodies. The components (PAMP and an integral membrane protein of an influenza virus) of the compositions, fusion proteins and polypeptides of the invention are in close physical proximity to one another on the carrier. The compositions, fusion proteins and polypeptides of the

invention can be attached to the carrier by covalent or noncovalent attachment. Preferably, the carrier is biocompatible. "Biocompatible," as used herein, means that the carrier does not generate an immune response in the subject (e.g., the production of antibodies). The carrier can be a biodegradable substrate carrier, such as a polymer bead or a liposome. The carrier can further include alum or other suitable adjuvants.

The dosage and frequency (single or multiple doses) administered to a subject can vary depending upon a variety of factors, including prior exposure to a viral antigen, the duration of viral infection, prior treatment of the viral infection, the route of administration of the composition, fusion protein or polypeptide; size, age, sex, health, body weight, body mass index, and diet of the subject; nature and extent of symptoms of influenza exposure, influenza infection and the particular influenza virus responsible for the infection (e.g., influenza A, B, C), the source of the influenza virus (e.g., Hong Kong, Puerto Rico, Wisconsin, Thailand) kind of concurrent treatment (e.g., nasal sprays and drugs, such as amantadine, rimantadine, zanamivir and oseltamivir), complications from the influenza exposure, influenza infection or other health-related problems. Other therapeutic regimens or agents can be used in conjunction with the methods and compositions, fusion proteins or polypeptides of the present invention. For example, the administration of the compositions, fusion proteins or polypeptides can be accompanied by other viral therapeutics or use of agents to treat the symptoms of the influenza infection (e.g., nasal sprays and drugs, such as amantadine, rimantadine, zanamivir and oseltamivir). Adjustment and manipulation of established dosages (e.g., frequency and duration) are well within the ability of those skilled in the art.

The present invention is further illustrated by the following examples, which are not intended to be limiting in any way.

EXEMPLIFICATION

EXAMPLE 1: FLAGELLIN-M2e FUSION PROTEINS

M2e is conserved across multiple influenza A subtypes (also referred to herein as "strain"). M2e is at least a portion of the M2 protein, in particular, a 24

amino-terminus (also referred to herein as an "ectodomain") of the M2 protein. The M2 ectodomain is relatively small amino acid sequence (24 amino acids) compared to HA (about 566 amino acids) and NA (about 469 amino acids). The M2e sequence of exemplary avian influenza A isolates differs from that of human isolates, but is highly-conserved among the avian isolates (see Table 1, *supra*). Four tandem copies of M2e fused to the carboxy terminus of a flagellin STF2 (full-length or STF2 hinge region-deleted) were generated. The STF2 without the hinge region is also referred to herein as "STF2Δ."

Construction of Fusion Protein

The carboxy-terminal fusion of the synthetic 4xM2e sequence (4 consecutive 24 amino acid sequences) with STF2 was constructed as follows. The pET24A vector was purchased from Novagen, San Diego, CA. The strategy employed the Seamless Cloning Kit (Catalog number 214400) from Stratagene (La Jolla, CA www.stratagene.com) performed by DNA 2.0 Inc. (Menlo Park, CA). The gene encoding the fusion protein was in pDrive 4xM2E G00448 and was used as a PCR template for insert preparation for construction of the C-terminal fusion expression construct with STF2. The synthetic 4xM2E construct pDrive 4xM2E G00448 was used as a template for PCR as outlined in the Seamless Cloning Kit (Catalog number 214400) from Stratagene (La Jolla, CA). The expected product from this amplification includes the 318 bp and the restriction enzyme sites incorporated into the oligonucleotides used to amplify this insert. The procedure was as follows:

PCR conditions

1 μL -20 ng of pDrive 4xM2E G00448
5 μL of 10x cloned Pfu polymerase buffer
1 μL of 40 mM dNTP mix
1 μL -10 pmol of forward primer 4xM2Eforbs1
1 μL -10 pmol of reverse primer 4xM2Erevwsto
40 μL ddH_2O

Immediately before starting the thermal cycling 1 μL of *PfuTurbo* DNA Polymerase the following were added.

4xM2Eforbs1 primer sequence:

5'–CGCTCTTCAMTGAGCTTGCTGACTGAGGTTGAGACCCCGATTC (SEQ ID NO: 69)

4xM2Erevwsto primer sequence:

5'-

CGCTCTTCACGCTTATTATCTAGACGGGTCTGAGCTATCGTTAGAGCGAG (SEQ ID NO: 70)

This reaction was cycled as follows on a Thermo Hybaid PxE thermal cycler (Waltham, MA).

Initial cycle

Temperature	Duration
95°	3 minutes
65°	1 minute
72°	1 minute

Subsequent nine cycles

Temperature	Duration
95°	45 seconds
65°	35 seconds
72°	1 minute

At this point the following was added to each reaction.

5 µL of 10x cloned Pfu polymerase buffer

1 µL of 5-methyl dNTP mix

44 µL ddH_2O

Subsequently the following thermal cycling was repeated five times.

Temperature	Duration
95°	45 seconds
65°	35 seconds

72°	1 minute

The 100 µL product was brought to a volume of 300 µL by the addition of TE buffer. The resulting product was phenol chloroform (Invitrogen Carlsbad, CA-Catalog number 15593-031) extracted once and chloroform extracted once. The amplification product was then ethanol precipitated by addition of 30 µL of Sodium acetate buffer pH 5.2 and 750 µL of 100% Ethanol. The DNA pellet was washed twice with 300 µL 70% Ethanol allowed to air dry for ten minutes and then resuspended in 50 µL TE buffer.

Amplification of Vector STF2 in pET24.

The previously constructed pET24a/STF2.M2e construct was used as a template for PCR as outlined in the Seamless Cloning Kit (Catalog number 214400) from Stratagene (La Jolla, CA). The expected product from this amplification includes the whole of the pET24 plasmid plus the STF2 sequences but does not include the single copy of M2E that exists in this construct. The procedure was as follow:

PCR conditions

1 µL -40 ng of STF2.M2E pET22-2
5 µL of 10x cloned Pfu polymerase buffer
1 µL of 40 mM dNTP mix
1 µL -10 pmol of primer 4xMECpET24
1 µL -10 pmol of primer 4xM2EC-STF2
40 µL ddH_2O

Immediately before starting the thermal cycling the following were added:
1 µL of *PfuTurbo* DNA Polymerase
4xMECpET24 primer sequence:
5'-GCTCTTCAGCGGCTGAGCAATAACTAGCATAACCCCTTGGG (SEQ ID NO: 71)
4xM2EC-STF2 primer sequence:

5'-CGCTCTTCACAGACGTAACAGAGACAGCACGTTCTGCGG (SEQ ID NO: 72)

This reaction was cycled as follows on a Thermo Hybaid PxE thermal cycler (Waltham, MA).

Initial cycle

Temperature	Duration
95°	3 minutes
65°	1 minute
72°	18 minutes

Subsequent nine cycles

Temperature	Duration
95°	45 seconds
65°	35 seconds
72°	18 minutes

At this point the following was added to each reaction.

5 μL of 10x cloned Pfu polymerase buffer

1 μL of 5-methyl dNTP mix

44 μL ddH_2O

Subsequently the following thermal cycling was repeated five times.

Temperature	Duration
95°	45 seconds
65°	35 seconds
72°	18 minutes

The 100 μL product was brought to a volume of 300 μL by the addition of TE buffer. The resulting product was phenol chloroform (Invitrogen Carlsbad, CA-Catalog number 15593-031) extracted once and chloroform extracted once. The amplification product was then ethanol precipitated by addition of 30 μL of Sodium

acetate buffer pH 5.2 and 750 µL of 100% Ethanol. The DNA pellet was washed twice with 300 µL 70% Ethanol allowed to air dry for ten minutes and then resuspended in 50 µL TE buffer.

Digestion and ligation of Vector and Insert amplifications

Eam 1104 I digests were set up separately for vector and insert as follows:

30 µL of amplified product after ethanol precipitation

5 µL of 10x Universal buffer (Supplied with Seamless Cloning Kit)

4 µL *Eam* 1104 I restriction enzyme (Supplied with Seamless Cloning Kit)

11 µL ddH_2O

Digests were mixed gently and incubated at 37°C for one hour and ligation reactions of vector and insert products were prepared as above performed as follows (Reagents supplied with Seamless Cloning Kit):

Ingredients added in order listed:

9 µL ddH_2O

5 µL of *Eam* 1104 I digested 4xM2E amplified insert

5 µL of *Eam* 1104 I digested STF2.M2E pET22-2 amplified vector

2 µL 10x Ligase buffer

2 µL 10 mM rATP

1 µL T4 DNA Ligase (diluted from stock 1:16)

1 µL *Eam* 1104 I restriction enzyme

The ligation reactions were mixed gently and incubated for 30 minutes at 37°C. The ligations were then stored on ice until transformed into XL-10 competent cells (Stratagene Catalog number 200314) later than same day.

Transformation of Ligation into XL-10 Competent Cells

Eppendorf tubes were chilled for ten minutes while the XL-10 (Stratagene Catalog number 200314) competent cells thawed on ice.

50 µL of competent cells were aliquoted from the stock tube per ligation.

2 µL of β-mercaptoethanol stock which is provided with the XL-10 cells.

This mixture was incubated for ten minutes on ice gently mixing every 2 minutes.

Seamless cloning ligation reaction (4µl) was added, swirled gently and then

incubated on ice for 30 minutes. The tubes were heat shocked for 35 seconds at 42°C in a water bath. The tubes were incubated on ice for at least two minutes. SOC medium (400 µL) were added to the cells and incubated for one hour at 37°C with agitation.

Two LB agar kanamycin (50µg/mL) plates are used to plate 200 µL and 10 µL of the transformed cells and allowed to grow overnight.

Screening of Kanamycin Resistant Clones

Recombinant candidates were grown up for minipreps in Luria Broth containing Kanamycin (25 ug/mL) and extracted using the QIAprep Spin Miniprep Kit (Qiagen Valencia, CA Catalog Number 27106). Candidate clones were screened by restriction enzymes (New England Biolabs Beverly, MA) and positive clones were grown up in 100 mL of Luria Broth containing kanamycin (25 ug/mL) and extracted using the Qiagen HiSpeed Plasmid Midi Kit (Catalog number 12643). These clones were submitted to GENEWIZ (North Brunswick, NJ) for sequencing.

Production and Purification of STF2.4xM2E Fusion Protein

STF2.4xM2e in *E. coli* BLR(DE3)pLysS host (Novagen, San Diego, CA, Catalog #69053) was retrieved from glycerol stock and scaled up to 5 L. Cells were grown in LB medium containing 15 µg/ml Kanamycin and 12.5 µg/ml Teteracycline to OD_{600} = 0.4 and induced with 1 mM IPTG for 3 h at 37°C. The cells were harvested by centrifugation (7000 rpm x 7 minutes in a Sorvall RC5C centrifuge) and resuspended in 2x PBS, 1% glycerol, DNAse, 1 mM PMSF, protease inhibitor cocktail and 1 mg/ml lysozyme. The suspension was passed through a microfluidizer to lyse the cells. The lysate was centrifuged (45,000 g for one hour in a Beckman Optima L ultracentrifuge) to separate the soluble fraction from inclusion bodies. Protein was detected by SDS-PAGE in the soluble and insoluble fractions.

The soluble fraction was applied to Sepharose Q resin in the presence of high salt via batch method to reduce DNA, endotoxin, and other contaminants. The flow through containing the protein of interest was loaded onto 30 ml Q Sepharose column (Amersham Biosciences). Bound protein was eluted using a linear gradient from Buffer A to B. (Buffer A: 100 mM Tris-Cl, pH 8.0. Buffer B: 100 mM Tris-Cl,

1 M NaCl, pH 8.0). Eluted protein was further purified using a 45 ml Source Q column that provided greater resolution needed to resolve contaminating proteins. Bound protein was eluted with a linear gradient from Buffer A to B (Buffer A: 100 mM Tris-Cl, pH 8.0 Buffer B: 100 mM Tris-Cl, 1 M NaCl, pH 8.0).

Final purification of protein was completed using Superdex-200 gel filtration chromatography. The column was developed with 100 mM Tris, 150 mM NaCl and 1% glycerol plus 1% Na-deoxycholate to remove the LPS. Buffer exchange was carried out using overnight dialysis against buffer containing 50 mM Tris, 100 mM NaCl and 1% glycerol was done to remove Na-deoxycholate. Protein concentration was determined by the MicroBCA Protein Assay Reagent Kit (Pierce Biotechnology). Purified preparations of STF2.4xM2e yielded a single band visible with Coomassie stain that migrated with an apparent molecular weight of about 64 kDa on 12% SDS polyacrylamide gels.

EXAMPLE 2: EXPRESSION AND PURIFICATION OF FLAGELLIN (STF2 AND STF2Δ) FUSION PROTEIN CONSTRUCTS ENCODING INFLUENZA A M2 ECTODOMAIN SEQUENCES

The consensus M2e sequences from several influenza A strains of human and avian origin are depicted in Table 1. To facilitate the cloning of the M2e sequence, two vector cassettes, pMT/STF2 and pMT/STF2Δ, each containing a multiple cloning site (MCS) were generated (See Figures 17A and 17B). To generate pMT/STF2, the 1.5 kb gene encoding full length flagellin of *Salmonella typhimurium* fljb type 2, or STF2, was fused to the Ig binding protein (BIP) secretion signal of pMTBIP/V5-His vector (Invitrogen Corporation, Carlsbad, CA) for expression in *Drosophila*. The BiP sequence is included at the 5' end of the construct as a secretion signal for expression in *Drosophila*. A chemically-synthesized 4xM2e gene representing the H1, H2 and H3 consensus sequence, SLLTEVETPIRNEWGSRSNDSSDP (SEQ ID NO: 47, Table 1), was cloned into the MCS of pMT/STF2 to create *pMT/STF2.4xM2e(H1)*.

A similar strategy prophetically is employed to clone two H5-associated M2e sequences, SLLTEVETPTRNEWECRCSDSSDP (SEQ ID NO: 56) (A/Viet Nam/1203/2004) and SLLTEVETLTRNGWGCRCSDSSDP (SEQ ID NO: 55)

(A/Hong Kong/156/97). Codon-optimized chemically synthesized genes containing four tandemly repeated copies of the indicated H5-associated M2e sequence prophetically are cloned into pMT/STF2 to generate *STF2.4xM2e(H5VN)* and *STF2.4xM2e(H5HK)*, respectively. To generate a construct that contains multiple M2e forms, the heterologous 4xM2e sequence(s) prophetically are inserted into either of the primary constructs.

"Heterologous sequences," as used herein, means sequences from different species. For example, the H1 sequence is a human sequence and the H5 sequence is an avian sequence. Thus, the H1 and H5 sequences are heterologous sequences (e.g., SLLTEVETPTRNEWESRSSDSSDPLESLLTEVETPTRNEWESRSSDSSDPESSLLTEVETPTRNEWESRSSDSSDPGSSLLTEVETPTRNEWESRSSDSSDP (SEQ ID NO: 101), encoded by tctctgctgactgaagtagaaactccaacgcgtaatgaatgggaatcccgttctagcgactcctctgatcctctcgagtccctgctgacggaggttgaaaccccgacccgcaacgagtgggaaagccgttcctccgattcctctgatccggagagcagcctgctgaccgaggtagaaaccccgacccgtaatgagtgggaatctcgctcctctgattcttctgaccgggatcctctctgctgaccgaagtggagactccgactcgcaacgaatgggagagccgttcttctgactcctctgacccg (SEQ ID NO: 102).

Primary constructs comprise at least one pathogen-associated molecular pattern (e.g., STF2, STF2Δ) and at least a portion of at least one integral membrane protein (e.g., M2e, such as SEQ ID NOS: 13 and 47). If there is more than one integral membrane in a primary construct, the integral membrane proteins are from the same species.

A heterologous construct includes at least two integral membrane proteins such as H1 (human) and H5 (avian), for example, in SEQ ID NOS: 87 and 88.

To generate pMT/STF2Δ, the hyper-variable region that spans amino acids 170 to 415 of the full-length flagellin gene of SEQ ID NO: 2 was deleted and replaced with a short (10 amino acid) flexible linker (GAPVDPASPW, SEQ ID NO: 98) designed to facilitate interactions of the amino and carboxy terminal sequences necessary for TLR5 signaling. The protein expressed from this construct retains potent TLR5 activity whether expressed alone or in fusion with test antigen. Thus, a second series of M2e constructs prophetically is generated based on pMT/STF2Δ.

Drosophila Dmel-2 cells (Invitrogen Corporation, Carlsbad, CA) grown at room temperature in Schneider's medium supplemented with 10% FBS and antibiotics prophetically is transfected with the constructs described above using Cellfectin reagent (Invitrogen) according to the manufacturer's instructions. Twenty-four hours post transfection, cells prophetically is induced with 0.5 mM $CuSO_4$ in medium lacking FBS and incubated for an additional 48 hours. Conditioned media (CM) prophetically is harvested from induced cultures and screened for protein expression by SDS-PAGE and Western blot analyses using anti-flagellin and anti-M2e specific antibodies. The identity, TLR bioactivity of the fusion protein, antigenicity assessed by ELISA and *in vivo* mouse studies for immunogenicity prophetically is performed.

EXAMPLE 3: CONSTRUCTION AND EXPRESSION OF FLAGELLIN-HEMAGLUTININ (HA) CONSTRUCTS

The gene encoding HA from genomic DNA from the in-house laboratory strain PR8, an attenuated derivative of A/Puerto Rico/8/34 was isolated (SEQ ID NO: 68, encoding SEQ ID NO: 67). The gene was fused to the STF2Δ cassette that has been previously constructed in pPICZΔ generating STF2Δ.HAPR8 (SEQ ID NO: 63, encoding SEQ ID NO: 62) (See Figure 18). Purified recombinant protein was tested for immunogenicity and efficacy in BALB/c mice. The gene encoding H5N1 of the A/Vietnam/1203/04 strain was custom synthesized and fused to STF2Δ cassette generating STF2Δ.HAH5 (SEQ ID NO: 61, encoding SEQ ID NO: 60). Both human and avian HA constructs were transformed into *Pichia pastoris* strains GS115 and X-33 (Invitrogen Corporation, Carlsbad, CA). Selected clones were screened for expression by fractionation on SDS-PAGE gel and staining by Coommassie Blue and Western blot analysis using anti-HA and anti-flagellin antibodies.

EXAMPLE 4: GENERATION OF A PAM3CYS FUSION PROTEIN

M2e (SEQ ID NO: 47) was chemically coupled to a tri-palmitoylcysteine (Pam3Cys) moiety through the amino terminal serine residue of the peptide. The structure of the fusion protein (Pam3Cys.M2e) is shown in Figure 15. The

chemical name for Pam3Cys.M2e is [Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ser-Leu-Leu-Thr-Glu-Val-Glu-Thr-Pro-Ile-Arg-Asn-Glu-Trp-Gly-Ser-Arg-Ser-Asn-Asp-Ser-Ser-Asp-Pro-OH acetate salt]. The molecular mass of Pam3Cys.M2e is 3582.3 daltons.

Pam3Cys.M2e was synthesized using a solid phase peptide synthesis methodology based on a well established Fmoc-strategy (Houben-Weyl, 2004. Synthesis of peptides and peptidomimetics, Vol. 22, Georg Thieme Verlag Stuttgart, NY). The synthetic scheme and manufacturing process for Pam3Cys.M2e is diagrammed in the flow chart below. The Pam3Cys.M2e is a fusion protein (chemically linked) and is also referred to herein as a "lipidated peptide."

The first step in the synthesis included solid phase peptide synthesis. The amino acid sequence of Pam3Cys.M2e was assembled on an H-Pro-2-chlorotrityl chloride resin by solid phase peptide synthesis. This resin is highly suitable for the formation of peptides with the Fmoc-strategy. The peptide chain was elongated by successive coupling of the amino acid derivatives. Each coupling step was preceded by an Fmoc-deprotection step and both steps were accompanied by repeated washing of the resin. After coupling of the last amino acid derivative, the final Fmoc-deprotection step was performed. Finally, the peptide resin was washed and dried under reduced pressure. During solid phase peptide synthesis color indicator tests were performed for each step to monitor the completion of the Fmoc-cleavage and the subsequent coupling of the amino acid derivatives.

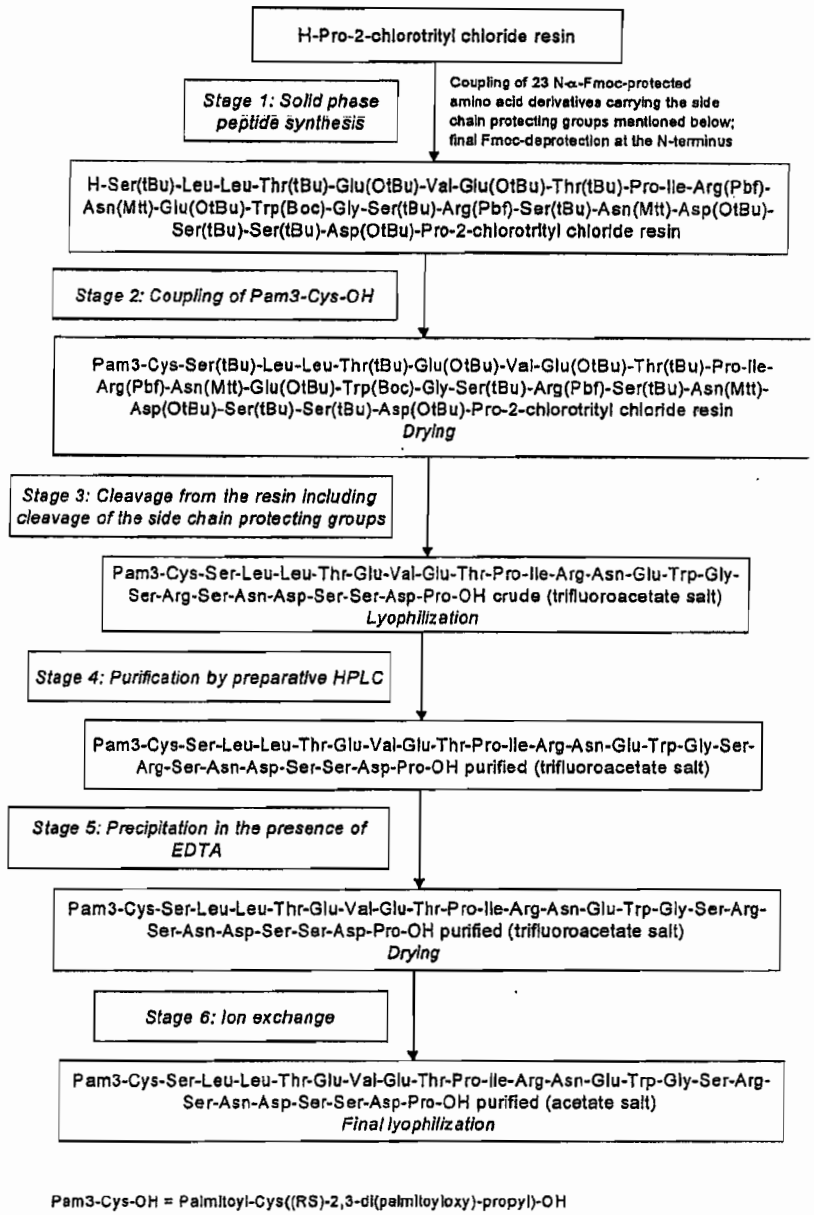
Stage 2 of the synthesis included coupling of Pam3Cys-OH. Pam3Cys-OH was pre-activated with N,N'-dicyclohexyl-carbodiimide (DCCI) in the presence of 1-hydroxybenzotriazole (HOBt). The resulting solution was filtered and added to the peptide resin. At the end of the reaction time the peptide resin was washed and dried under reduced pressure. Color indicator tests were performed to control the coupling of Pam3Cys-OH.

Stage 3 of the synthesis included cleavage from the resin including cleavage of the side chain protecting groups. The peptide resin was treated with trifluoroacetic acid (TFA). The product was precipitated from the reaction mixture and lyophilized.

Stage 4 of the synthesis included purification by preparative reverse phase HPLC. The crude material obtained from Stage 3 was purified by preparative HPLC on a reverse phase column using a TFA system. The fractions were collected, checked by analytical HPLC and pooled accordingly. Pooled fractions from the TFA runs were lyophilized.

Stage 5 of the synthesis included precipitation in the presence of EDTA. The purified material from Stage 4 was precipitated from an aqueous solution of EDTA. The product was filtered off and dried under reduced pressure.

Stage 6 of the synthesis included ion exchange chromatography. The last stage of manufacturing Pam3Cys.M2e was the exchange from the trifluoroacetate salt into the acetate salt by ion exchange. The material from Stage 5 was loaded onto an ion exchange column and eluted with acetic acid. Fractions were checked by thin layer chromatography and the combined product-containing fractions were filtered and lyophilized to yield the final product.



Pam3-Cys-OH = Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH

The purity specification for the Pam3Cys.M2e drug substance was ≥ 80% by RP-HPLC. The specification was based on the purity achieved with three non-GMP lots of Pam3Cys.M2e made from the same GMP batch of M2e-peptide intermediate resin. The purity of the three non-GMP lots of Pam3Cys.M2e was 80.2%, 80.3% and 80.8%, for lots D.001.Pam3Cys.M2e, D.002.Pam3Cys.M2e and D.003.Pam3Cys.M2e, respectively.

EXAMPLE 5: IMMUNOGENICITY

MATERIALS and METHODS

SYNTHESIS AND PURIFICATION OF PAM3CYS.M2E

Pam3Cys.M2e was prepared by Genemed Synthesis and Bachem using solid phase synthesis methodologies and FMOC chemistry as described above. Mass spectroscopy analysis was used to verify the molecular weight of the final product.

ENDOTOXIN ASSAY

Endotoxin levels of the STF2.4xM2e and the Pam3Cys.M2e were measured using the QCL-1000 Quantitative Chromogenic LAL test kit (BioWhittaker #50-648U), following the manufacturer's instructions for the microplate method.

TLR5 BIOACTIVITY ASSAY

HEK293 cells constitutively express TLR5 and secrete several soluble factors, including IL-8, in response to TLR5 signaling. HEK293 cells were seeded in 96-well microplates (50,000 cells/well) and test proteins were added and incubated overnight. The next day, the conditioned medium was harvested, transferred to a clean 96-well microplate and frozen at -20°C. After thawing, the conditioned medium was assayed for the presence of IL-8 in a sandwich ELISA using an anti-human IL-8 matched antibody pair (Pierce, #M801E and #M802B) following the manufacturer's instructions. Optical density was measured using a microplate spectrophotometer (FARCyte, Amersham). Results are reported as pg of IL8 per ml as determined by inclusion of a standard curve for IL8 in the assay.

TLR2 BIOACTIVITY ASSAY

RAW264.7 cells (ATCC) express TLR2 and secrete several soluble factors, including TNFα, in response to TLR2 signaling. RAW264.7 cells were seeded in 96-well microplates (50,000 cells/well), test compounds were added and incubated overnight. The next day, the conditioned medium was harvested, transferred to a clean 96-well microplate and frozen at -20°C. After thawing, the conditioned medium was assayed for the presence of TNFα in a sandwich ELISA using an anti-mouse TNFα matched antibody pair (Pierce) following the manufacturer's

instructions. Optical density was measured using a microplate spectrophotometer (FARCyte, Amersham). Results are reported as ng of TNF per ml as determined by reference to a standard curve for TNF included in the assay.

MOUSE IMMUNOGENICITY

Female BALB/c mice (National Cancer Institute) were used at the age of about 6-8 weeks. Mice were divided into groups of 5 to 10 mice per group, and immunized subcutaneously on each side of the base of the tail on days 0 and 21 with the indicated concentrations of STF2.4xM2e or Pam3Cys.M2e fusion protein. On days 10 (primary) and 28 (boost), individual mice were bled by retro-orbital puncture. Sera were harvested by clotting and centrifugation of the heparin-free blood samples.

MOUSE SERUM ANTIBODY DETERMINATION

M2e-specific IgG levels were determined by ELISA. 96-well ELISA plates were coated overnight at 4°C with 100 µl /well of a 5 µg/ml solution of the M2e peptide in PBS. Plates were blocked with 200 µl/well of Assay Diluent Buffer (ADB; BD Pharmingen) for one hour at room temperature. The plates were washed three times in PBS containing 0.05% Tween-20 (PBS-T). Dilutions of the sera in ADB were added (100 µl/well) and the plates were incubated overnight at 4°C. The plates were washed three times with PBS-T. Horse radish peroxidase, or HRP-labeled goat anti-mouse IgG antibodies (Jackson Immunochemical) diluted in ADB were added (100 µl/well) and the plates were incubated at room temperature for 1 hour. The plates were washed three times with PBS-T. After adding TMB Ultra substrate (3,3',5,5'-tetramentylbenzidine; Pierce) and monitoring color development, the O.D. 450 was measured on a Tecan Farcyte microspectrophotometer.

RABBIT IMMUNOGENICITY

Female and male NZW rabbits (Covance Research Products) were used at the age of about 13-17 weeks. Rabbits were divided into groups of 3 male and 3 female per group, and immunized *i.m.* on alternating thighs on days 0 and 21 and 42

with the indicated concentrations of Pam3Cys.M2e peptide or STF2.4xM2e fusion protein. Animals were bled on day -1 (prebleed), 14 (primary) and 28 and 42 (boost). Sera were prepared by clotting and centrifugation of samples.

RABBIT SERUM ANTIBODY DETERMINATION

M2e-specific IgG levels were determined by ELISA. 96-well ELISA plates were coated overnight at about 4°C with 100 µl/well M2e peptide in PBS (5 µg/ml). Plates were blocked with 200 µl/well of Assay Diluent Buffer (ADB; BD Pharmingen) for one hour at room temperature. The plates were washed three times in PBS-T. Dilutions of the sera in ADB were added (100 µl/well) and the plates were incubated overnight at about 4°C. The plates were washed 3x with PBS-T. Bound IgG was detected using HRP-conjugated goat anti-rabbit IgG (Jackson Immunochemical). The plates were washed three times with PBS-T. After adding TMB Ultra substrate (Pierce) and monitoring color development, O.D. 450 was measured on a Molecular Devices Spectramax microspectrophotometer. Results are reported as the Delta O.D. which is determined by subtracting the O.D. 450 reading for the prebleed of each animal from the O.D. 450 for each animal post-immunization.

BALB/C MOUSE EFFICACY MODEL

In a typical experiment, about 5-6 week old female BALB/c mice (10-20 per group) were obtained and allowed to acclimate for one week. Fusion proteins formulated in PBS or other suitable formulation were administered by s.c. injection. Mice were immunized on days 0 and 14. On day 21, sera was harvested by retro-orbital puncture and evaluated for M2e specific IgG by ELISA. Mice were challenged by intranasal administration of 1xLD90 of the well characterized mouse adapted Influenza A strain, A/Puerto Rico/8/34 (H1N1). Mice were monitored daily for 14 days for survival and weight loss. Mice that lost about 30% of their initial body weight were humanely sacrificed, and the day of sacrifice recorded as the day of death. Efficacy data were reported as survival times.

RESULTS

IN VITRO BIOACTIVITY

These assays were based on cell lines expressing the relevant TLR and screened for the ability to produce either IL8 or TNF-α in response to TLR triggering. In Figure 44, the ability of STF2.4xM2e (■) or STF2.OVA(○) to stimulate TLR5 dependent IL8 production was evaluated following the stimulation of TLR5 positive, HEK293 cells. The results indicate that both fusion proteins stimulated IL8 production in a dose dependent manner and that the activity of the PAMP was retained in the context of the fusion.

TLR2 activity was similarly evaluated for Pam3Cys.M2e following stimulation of TLR2 positive RAW264.7cells. In Figure 45, the experimental groups are: the known endotoxin, LPS, as a positive control (♦), LPS plus the inhibitor of endotoxin polymixin B (PMB) as a negative control (○), free Pam3Cys as a positive control for TLR2 signalling (■), free Pam3Cys plus PMB (□), Pam3Cys.M2e (♦) and Pam3Cys.M2e plus PMB (◇). The results showed similar activity profiles for Pam3Cys.M2e and the free TLR2 ligand Pam3Cys. The addition of polymyxin B (PMB) did not reduce its activity, indicating that there is no or low endotoxin contamination.

PHYSICAL LINKAGE OF PAMP AND ANTIGEN ENHANCES IMMUNOGENICITY

Using mouse models of immunogenicity, chemical coupling of Pam3Cys to M2e enhances the immunogenicity of the M2e antigen as compared to either the M2e peptide delivered alone or the M2e peptide co-delivered with free Pam3Cys. In the experiment shown in Figure 46, groups of mice were immunized on days 0 and 21 with PBS as a negative control (*), the free TLR2 ligand, Pam3CSK-4 (◊), M2e peptide alone (○), free Pam3CSK-4 mixed with M2e peptide (□), or the fusion of Pam3Cys and M2e referred to as Pam3.M2e (♦). The relevant the molar ratio of M2e peptide delivered was held constant. On day 28, sera were harvested and analyzed for M2e-specific antibody titers by ELISA. The results show that chemical coupling of Pam3Cys to the M2e (Pam3Cys.M2e) generates a detectable serum antibody response to the M2e antigen.

Physical linkage between the TLR5 ligand STF2 and antigen was demonstrated using the model antigen ovalbumin (OVA). Mice received a single s.c. immunization with STF2, OVA, STF2.OVA fusion protein, STF2 + OVA mixture or PBS alone. Dosages were calculated to deliver 12 μg equivalents of STF2 and OVA per group. Seven days later, sera were harvested and OVA-specific antibodies were examined by ELISA. Data shown in Figure 47 depict IgG1 titers at a 1:100 dilution of the sera. These results demonstrate that physical linkage of the TLR5 ligand and antigen results in optimal immunogenicity in vivo.

PAMP LINKED ANTIGENS ARE MORE IMMUNOGENIC THAN CONVENTIONAL ADJUVANT

Groups of 5 BALB/c mice were immunized on day 0 and 14 with 30 μg of Pam3Cys.M2e (♦), 22.5 μg of M2e which is the molar equivalent of M2e in 30 μg of Pam3Cys.M2e (◇), 22.5 mg of M2e adsorbed to the conventional adjuvant Alum (□), or 25 mg of the recombinant protein STF2.4xM2e (■). A group receiving PBS was included as a negative control (○). Sera were harvested 7 days post the second dose and M2e specific IgG were evaluated by ELISA. The results shown in Figure 48 indicate that M2e alone is poorly immunogenic in that it failed to elicit antibody titers above background. The conventional adjuvant Alum provided a modest enhancement in the immune response to M2e. The PAMP linked M2e constructs; however, provided the greatest enhancement in immunogenicity. These results indicate direct linkage of PAMPs with portions of an integral membrane protein of an influenza viral protein can elicit immune responses that are more potent than those elicited by the conventional adjuvant Alum.

DOSE AND IMMUNOGENICITY

Dose ranging studies were carried out to further assess the potency of Pam3Cys.M2e and STF2.4xM2e. For STF2.4xM2e, BALB/c mice were immunized on day 0 and 14 with dilutions of STF2.4xM2e that ranged from 0.25 to 25 μg of STF2.4xM2e per immunization. The prefix D002 refers to the specific batch of STF2.4xM2e used in this experiment, while R-028 refers to a historical reference batch of STF2.4xM2e used in this experiment. Seven days following the last

immunization (Day 21) mice were bled and M2e-specific IgG responses were evaluated by ELISA. The results shown in Figure 49 demonstrate that immunization with doses as low as 0.25 μg per immunization of STF2.4xM2e induced detectable levels of M2e-specific IgG, with the optimal dose in mice falling in the range of about 2.5 to about 25 μg.

For Pam3Cys.M2e, BALB/c mice were immunized on day 0 and 14 with 0.05 to 30 μg of Pam3Cys.M2e per immunization. Seven days following the last immunization (Day 21) mice were bled and M2e-specific IgG responses were evaluated by ELISA. The results shown in Figure 50 demonstrate that immunization with concentrations as low as 0.05 μg of Pam3Cys.M2e induced detectable levels of M2e-specific IgG, with the optimal dose for mice in this study of about 30 μg.

IMMUNOGENICITY IN MULTIPLE MOUSE STRAINS

The immunogenicity of Pam3Cys.M2e was evaluated in multiple mouse strains including BALB/c (●), C57BL/6 (■), CB6/F1 (◆), DBA/2 (▲), Cr:NIH (Swiss) (X) and C3H/HeN (*). Groups of five for each strain were immunized on day 0 and 14 with 30 μg of Pam3Cys.M2e per immunization. Sera were harvested on day 21 and levels of M2e-specific IgG evaluated by ELISA. All strains exhibited significant levels of M2e-specific IgG indicating that the immunogenicity of Pam3Cys.M2e is not dependent on a particular MHC (Figure 51).

IMMUNOGENICITY IN RABBITS

Studies aimed at evaluating the immunogenicity of Pam3Cys.M2e and STF2.4xM2e in a second species, rabbit, were carried out. In the first study, rabbits (3 females and 3 males/group) were immunized with 500, 150, 50, 15 or 5 μg (i.m.) of Pam3Cys.M2e on day 0, 21 and 42. As a control, an additional group received the formulation buffer F111 (10 mM Tris, 10 mM histidine, 75 mM NaCl, 5% sucrose, 0.02% Polysorbate-80, 0.1 mM EDTA, 0.5% ethanol, 20 mg/mL hydroxypropyl-beta-cyclodextrin, pH 7.2). On day 7 post-boost 2, peripheral blood was obtained and the anti-M2e antibody titers were evaluated by ELISA. The results shown in Figure 52 depict the individual rabbit antibody titers at a 1:125

dilution of the sera. The data suggest a dose-response relationship between the amount of Pam3Cys.M2e used for prime/boost vaccinations and the level of the antibody titer achieved.

In the second study, rabbits (3 females and 3 males/group) were immunized with 500, 150, 50, 15 or 5 μg (i.m.) of STF2.4xM2e. As a control, an additional group received saline alone. On day 14 post-immunization, peripheral blood was obtained and the anti-M2e antibody titers were evaluated by ELISA. Notably, significant M2e-specific IgG responses were detectable by day 14 post-prime in all animals immunized (Figure 53). The results indicate that STF2.4xM2e elicits a rapid and consistent immune response in rabbits.

EFFICACY IN THE MOUSE CHALLENGE MODEL

The efficacy of the Pam3Cys.M2e and STF2.4xM2e was evaluated in BALB/c mice using the well characterized mouse adapted strain, Influenza A/Puerto Rico/8/34 (PR/8) as the challenge virus. Groups of ten mice were immunized s.c. on day 0 and 14 with 30 μg of Pam3Cys.M2e in the formulation buffer F111 (■), 30 μg of Pam3Cys.M2e in the proprietary buffer F120 (10 mM Tris, 10 mM histidine, 10% sucrose, 0.02% Polysorbate-80, 0.1 mM EDTA, 0.5% ethanol, 0.075% docusate sodium, pH 7.2) (▲), 30 μg of Pam3Cys.M2e in the buffer F119 (10 mM Tris, 10 mM histidine, 75 mM NaCl, 5% sucrose, 0.02% Polysorbate-80, 0.1 mM EDTA, 0.5% ethanol, 0.1% docusate sodium, pH 7.2), 30 μg of STF2.4xM2e in the buffer F105 (10 mM Tris, 10 mM histidine, 75 mM NaCl, 5% sucrose, 0.02% Polysorbate-80, 0.1 mM EDTA, 0.5% ethanol, pH 7.2), 3 μg of STF2.4xM2e in buffer F105 (10 mM Tris, 10 mM histidine, 75 mM NaCl, 5% sucrose, 0.02% Polysorbate-80, 0.1 mM EDTA, 0.5% ethanol, pH 7.2) (●) or 0.3 μg of STF2.4xM2e in buffer F105 (□). A group receiving PBS alone was included as a negative control (○), and a convalescent group with immunity to PR/8 following a sublethal challenge with the virus was included as a positive control (◇). On day 28, animals were challenge with an LD90 of the PR/8 challenge stock. Weight loss and survival was followed for 14 days post challenge (Figure 54).

Animals in the convalescent group which had successfully cleared an earlier non-lethal infection with PR/8 demonstrated 100% protection to a subsequent viral

challenge. Animals receiving the PBS buffer alone exhibited morbidity beginning on days 7 and 8, with 80% lethality occurring by day 10, while animals immunized with 30 μg of Pam3Cys.M2e in F111 demonstrated enhanced survival, with 50% of mice surviving the challenge. Animals receiving Pam3Cys.M2e in F119 exhibited morbidity beginning on days 8 and 9 with 80% of the mice surviving. Animals receiving Pam3Cys.M2e in buffer F120 (10 mM Tris, 10 mM histidine, 10% sucrose, 0.02% Polysorbate-80, 0.1 mM EDTA, 0.5% ethanol, 0.075% docusate sodium, pH 7.2) or the STF2.4xM2e protein exhibited the mildest disease course with 90 to 100% of the mice in these groups surviving the lethal challenge. These results demonstrate that both Pam3Cys.M2e and STF2.4xM2e can confer protective immunity to a challenge with influenza A in vivo.

DISCUSSION

Salmonella typhimurium flagellin (fljB) is a ligand for TLR5. A recombinant protein consisting of full-length flijB (STF2) fused to four tandem repeats of M2e was expressed in E. coli and purified to > 95% purity with low endotoxin levels. In reporter cell lines, this protein (STF2.4xM2e) triggered IL8 production in a TLR5-dependent fashion. Mice immunized with dilutions of STF2.4xM2e that ranged from 0.25 μg to 25 μg, formulated in the buffer F105 which is without a conventional adjuvant or carrier, mounted a vigorous antibody response. The potency of the recombinant protein was further demonstrated in rabbit immunogenicity studies where animals receiving as little as 5 μg of protein seroconverted after a single dose. The efficacy of the PAMP fusion protein was demonstrated in the mouse challenge model using Influenza A/Puerto Rico/8/34 as the challenge virus. Mice immunized with as little as about 0.3 μg of the protein per dose exhibited mild morbidity with 100% of the mice surviving the challenge.

Synthetic tripalmitoylated peptides mimic the acylated amino terminus of lipidated bacterial proteins and are potent activators of TLR2. In these studies, a tripalmitoylated peptide consisting of three fatty acid chains linked to a cysteine residue and the amino terminus of the Influenza A M2 ectodomain (M2e) was synthesized using standard solid-phase peptide chemistries. This peptide (Pam3Cys.M2e) triggered TNFα production in a TLR2-dependent fashion in

reporter cell lines. When used to immunize mice without adjuvant, Pam3Cys.M2e generated an antibody response that was more potent than M2e when mixed with free Pam3CSK-4. Pam3Cys.M2e was also found to be immunogenic in rabbits where a dose response relationship was observed between the amount of Pam3Cys.M2e used for immunization and the antibody titer achieved. The efficacy of the Pam3Cys.M2e peptide in a number of different formulations was evaluated in the mouse challenge model using Influenza A/Puerto Rico/8/34 as the challenge virus. Pam3Cys.M2e formulated in F119 and F120 exhibited the mildest morbidity with about 80 to about 100% of the mice surviving the challenge.

EQUIVALENTS

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that 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 fusion protein comprising at least one flagellin or a portion thereof and at least one influenza M2 protein or a portion thereof.

2. The fusion protein of Claim 1, wherein the M2 protein includes at least a portion of SEQ ID NO: 11.

3. The fusion protein of Claim 2, further including a linker between at least one flagellin and at least one M2 protein.

4. The fusion protein of Claim 2, further including a linker between at least two M2 proteins.

5. The fusion protein of Claim 2, wherein the M2 protein includes SEQ ID NO: 15.

6. The fusion protein of Claim 1, wherein the flagellin is a TLR5 agonist.

7. The fusion protein of Claim 1, wherein the flagellin is at least one member selected from the group consisting of a fljB/STF2, a *E.coli* fliC, and a *S. muenchen* fliC.

8. The fusion protein of Claim 7, wherein the flagellin includes the fljB/STF2, and wherein the fljB/STF2 includes SEQ ID No: 1 or at least a portion of SEQ ID NO: 1.

9. The fusion protein of Claim 8, wherein the fljB/STF2 includes SEQ ID NO: 3 or at least a portion of SEQ ID NO: 3.

10. The fusion protein of Claim 7, wherein the flagellin includes the *E. coli* fliC, and wherein the *E.coli* fliC that includes SEQ ID NO: 5 or at least a portion of SEQ ID NO: 5.

11. The fusion protein of Claim 10, wherein the *E. coli* fliC includes SEQ ID NO: 66 or at least a portion of SEQ ID NO: 66.

12. The fusion protein of Claim 7, wherein the flagellin includes the *S. muenchen* fliC and wherein the *S. muenchen* fliC includes SEQ ID NO: 7 or at least a portion of SEQ ID NO: 7.

13. The fusion protein of Claim 12, wherein the *S. muenchen* fliC includes SEQ ID NO: 99 or at least a portion of SEQ ID NO: 99.

14. The fusion protein of Claim 1, wherein the flagellin is fused to a carboxy-terminus of the influenza M2 protein.

15. The fusion protein of Claim 1, wherein the flagellin is fused to an amino-terminus of the influenza M2 protein.

16. The fusion protein of Claim 1, wherein the flagellin is between at least two influenza M2 proteins.

17. The fusion protein of Claim 1, further including at least a portion of a haemagglutinin membrane protein.

18. The fusion protein of Claim 1, further including at least a portion of a neuraminidase membrane protein.

19. The fusion protein of Claim 1, further including at least one member selected from the group consisting of an influenza B viral protein and an influenza C viral protein.

20. The fusion protein of Claim 19, wherein the influenza B viral protein is an integral membrane protein.

21. The fusion protein of Claim 19, wherein the influenza C viral protein is an integral membrane protein.

22. A composition comprising a at least one flagellin or at least a portion thereof and at least one M2 protein or a portion thereof.

23. The fusion protein or the composition of any of the preceding claims, wherein the portion of the M2 protein includes an ectodomain of the M2 protein or at least a portion of the ectodomain of the M2 protein.

24. The fusion protein or the composition of Claim 23, further including at least one haemagglutinin membrane protein or at least a portion of the haemagglutinin membrane protein.

25. The fusion protein or the composition of Claim 24, wherein the haemagglutinin protein is at least one member selected from the group consisting of SEQ ID NO: 64, SEQ ID NO: 67, SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 105 and SEQ ID NO: 106.

26. The fusion protein or the composition of Claim 23, wherein the ectodomain includes SEQ ID NO: 13.

27. The fusion protein or the composition of any of the preceding claims, wherein the M2 protein includes SEQ ID NO: 18.

28. The fusion protein or the composition of any one of the preceding claims, wherein the flagellin lacks a hinge region or at least a portion of the hinge region.

29. The fusion protein of any one of the preceding claims, wherein the fusion protein is a recombinant fusion protein.

30. A composition that includes SEQ ID NO: 31 or at least a portion of SEQ ID NO: 31.

31. The use of the composition or the fusion protein of any one of the preceding claims in therapy.

32. The fusion protein or the composition of any one of the preceding claims for use in a method of stimulating an immune response in a subject.