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(54) Titre : UTILISATION DE LA FLAGELLINE DANS L'IMMUNOTHERAPIE DE YERSINIA PESTIS

(54) Title: USE OF FLAGELLIN IN THE IMMUNOTHERAPY OF YERSINIA PESTIS

(57) Abrégé/Abstract:

The invention provides a fusion protein comprising a flagellin adjuvant and a Yersinia pestis antigen. Also provided are compositions comprising a flagellin adjuvant and a Yersinia pestis antigen. The invention also discloses methods of making a fusion protein comprising a flagellin adjuvant and a Yersinia pestis antigen. The invention further provides pharmaceutical formulations and methods for inducing an immune response against Yersinia pestis.

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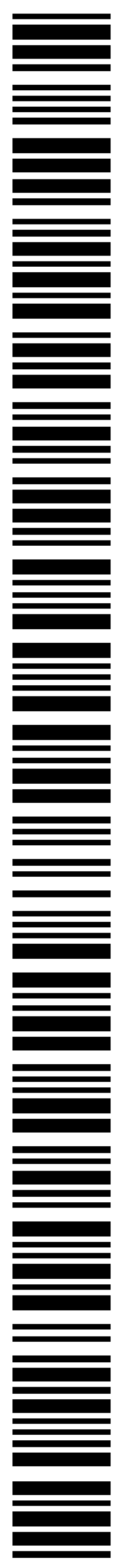
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(54) Title: USE OF FLAGELLIN IN THE IMMUNOTHERAPY OF YERSINIA PESTIS

(57) Abstract: The invention provides a fusion protein comprising a flagellin adjuvant and a *Yersinia pestis* antigen. Also provided are compositions comprising a flagellin adjuvant and a *Yersinia pestis* antigen. The invention also discloses methods of making a fusion protein comprising a flagellin adjuvant and a *Yersinia pestis* antigen. The invention further provides pharmaceutical formulations and methods for inducing an immune response against *Yersinia pestis*.



WO 2006/066214 A3

USE OF FLAGELLIN IN THE IMMUNOTHERAPY OF *YERSINIA PESTIS*

Statement of Federal Support

This invention was made with government support under grant number P01-AI060642 from the National Institutes of Health. The United States government has certain rights to this invention.

Field of the Invention

The present invention concerns the use of a flagellin adjuvant, antigens from *Yersinia pestis* and fusion proteins thereof to produce an immune response against *Y. pestis* (e.g., in the prophylactic treatment of *Y. pestis* infection).

Background of the Invention

Although the shift in vaccine development from complete pathogens to individual antigens has led to safer vaccines, efficacy has been markedly reduced. Vaccine adjuvants promote strong adaptive responses to soluble recombinant protein antigens. The proinflammatory effects of toll-like receptor (TLR) agonists such as gram-negative LPS and bacterial CpG DNA has led to evaluation of their adjuvant properties and effects on dendritic cells (Jäger et al. (2002) *Curr. Opin. Immunol.* 14:178–182; Ko et al. (2003) *Clin. Cancer res.* 9:3222–3234; Medzhitov (2001) *Nat. Immunol.* 1:135–145). Most TLR agonists function as adjuvants by stimulating the production of cytokines and the maturation of dendritic cells, thereby linking innate and adaptive immunity.

Yersinia pestis, the causative agent of plague, is a gram-negative organism responsible for approximately 200 millions deaths during three major pandemics. In humans,

plague has three forms designated by the nature of the infection: bubonic, pneumonic and septicemic. Whereas bubonic plague is spread via bites from infected fleas, the pneumonic form may be transmitted person-to-person. Without medical treatment, pneumonic plague is a rapidly progressing disease with a mortality rate approaching 100% (McSorley et al. (2002) *J. Immunol.* **169**:3914–3919; Means et al. (2003) *J. Immunol.* **170**:5165–5175).

The use of whole-cell vaccines for plague has raised safety concerns. Immunization with the F1 antigen of *Y. pestis* and an appropriate adjuvant elicits a protective response that correlates with the titer of anti-F1 IgG antibodies (Davila and Celis (2000) *J. Immunol.* **165**:539–547; Brunner et al. (2000) *J. Immunol.* **165**:6278–6286). A synergistic protective effect is obtained when animals are immunized with both F1 and V antigens or a recombinant F1/V fusion protein (Ciacci-Woolwine et al. (1998) *Infect. Immun.* **66**:1127–1134; Moors et al. (2001) *Infect. Immun.* **69**:4424–4429; Gewirtz et al. (2001) *J. Clin. Invest.* **107**:99–109; Steiner et al. (2000) *J. Clin. Invest.* **105**:1769–1777). Although highly variable responses were observed, a phase 1 clinical trial demonstrated that intramuscular immunization with a vaccine containing F1 and V is immunogenic in humans (Eaves-Pyles et al. (2001) *J. Immunol.* **166**:1248–1260).

It would be desirable to provide improved reagents, pharmaceutical formulations and methods for producing immune responses against pathogens such as *Y. pestis*.

Summary of the Invention

A first aspect of the invention is a fusion protein comprising, consisting of, or consisting essentially of: (a) a flagellin adjuvant comprising, consisting of, or consisting essentially of (i) a flagellin N-terminal constant region; (ii) a flagellin C-terminal constant region; and (b) a *Yersinia pestis* antigen between the N-terminal constant region and the C-terminal constant region (e.g., inserted into or in place of part or all of the flagellin hypervariable region, which is optionally partially or entirely deleted).

A further aspect of the invention is a nucleic acid encoding a fusion protein as described above. In some embodiments, the nucleic acid is operably associated with a promoter.

A further aspect of the invention is a vector comprising a nucleic acid as described above.

A further aspect of the invention is a host cell comprising a nucleic acid or vector as described above. In some embodiments, the host cell expresses the encoded fusion protein.

A further aspect of the invention is a method of making a fusion protein as described above, the method comprising culturing a host cell comprising a nucleic acid encoding the fusion protein described above in a suitable culture medium under conditions sufficient for the fusion protein to be production. Optionally, the fusion protein is collected from the host cell or from the culture medium.

A further aspect of the invention is a composition (*e.g.*, for mucosal delivery) comprising, consisting of, or consisting essentially of (a) a flagellin adjuvant, and (b) a *Yersinia pestis* antigen (with the *Y. pestis* antigen and the flagellin adjuvant being separate or coupled to one another, *i.e.*, in the form of a fusion protein, for example, a fusion protein as described herein).

A further aspect of the present invention is a pharmaceutical formulation comprising a fusion protein or a composition as described above in a pharmaceutically acceptable carrier.

A further aspect of the invention is a method of inducing an immune response (*e.g.*, producing antibodies and/or inducing a cell-mediated immune response) to a *Y. pestis* antigen in a subject, comprising administering a fusion protein, a composition, or a pharmaceutical formulation as described above to the subject in an amount effective to induce an immune response to the *Y. pestis* antigen in the subject.

A still further aspect of the invention is a method of treating a subject for a *Y. pestis* infection (*e.g.*, vaccinating a patient against a *Y. pestis* infection) comprising administering a fusion protein, a composition, or a pharmaceutical formulation as described above to the subject in an amount effective to treat *Y. pestis* infection (*e.g.*, to produce a prophylactic protective immune response and/or to produce a protective immune response against a *Y. pestis* infection in the subject).

In particular embodiments of the methods of the invention, the subject is a mammalian subject, a primate subject, or a human subject.

A further aspect of the invention is the use of a fusion protein or composition as described herein for the preparation of a medicament for carrying out a method of treatment as described herein.

According to another aspect, there is provided an isolated fusion protein comprising:

(a) an isolated flagellin adjuvant comprising:

(i) a flagellin N-terminal constant region; and

(ii) a flagellin C-terminal constant region; and

(b) a *Yersinia pestis* antigen between the flagellin N-terminal constant region and the flagellin C-terminal constant region, wherein the *Y. pestis* antigen comprises *Y. pestis* F1 antigen, *Y. pestis* V antigen, or a fusion peptide of *Y. pestis* F1 antigen and *Y. pestis* V. antigen; and

wherein the flagellin N-terminal constant region and/or the flagellin C-terminal constant region of the isolated flagellin adjuvant comprises a TLR5 recognition site and the isolated fusion protein is able to activate the TLR5 pathway.

According to a further aspect, there is provided an immunogenic composition for mucosal administration, comprising in a pharmaceutically acceptable carrier:

(a) an isolated flagellin adjuvant comprising:

(i) a flagellin N-terminal constant region; and

(ii) a flagellin C-terminal constant region; and

(b) a *Yersinia pestis* antigen between the flagellin N-terminal constant region and the flagellin C-terminal constant region, wherein the *Y. pestis* antigen comprises *Y. pestis* F1 antigen, *Y. pestis* V antigen, or a fusion peptide of *Y. pestis* F1 antigen and *Y. pestis* V. antigen; and

wherein the flagellin N-terminal constant region and/or the flagellin C-terminal constant region of the isolated flagellin adjuvant comprises a TLR5 recognition site and the isolated flagellin adjuvant is able to activate the TLR5 pathway.

These and other aspects of the invention are set forth in the description of the invention that follows.

Brief Description of the Drawings

Figure 1 depicts TNF α expression in the lungs of BALB/c mice given flagellin or mutant flagellin 229 by i.t. instillation.

Figure 2 depicts the effect of flagellin on tumor growth. The open circles represent the mice given the Fra-1 antigen and the inactive form of flagellin. The closed circles represent the mice given Fra-1 antigen and the active form of flagellin.

Figure 3 shows that immunization with flagellin and the F1 antigen of *Yersinia pestis* results in substantial anti-F1 antibody production. **(Panel a)** Female BALB/c mice were immunized intratracheally (i.t.) or intranasally (i.n.) with 10 μ g F1 + 1 μ g flagellin (FliC). Control animals were immunized i.t. with 10 μ g F1 alone or with 1 μ g of the 229 mutant flagellin. Mice were boosted in an identical manner at 4 weeks and plasma was collected 2 weeks later for analysis by ELISA. Numbers within the bars indicate ratio of IgG1/IgG2a isotypes. * indicates statistical significance over controls and ** indicates i.n. titers are statistically greater than i.t. ($p < 0.007$). **(Panel b)** Anti-F1 antibody titers from mice immunized i.n. with 10 μ g F1 + 1 μ g flagellin. Each line represents one mouse and arrows indicate booster immunizations. **(Panel c)** Female BALB/c mice were immunized i.t. with 10 μ g F1 and increasing amounts of FliC or 5 μ g of 229 and boosted at 4 weeks. Plasma anti-F1 IgG titers were determined 2 weeks post-boost. **(Panel d)** A group of female BALB/c mice was immunized i.n. with 5 μ g of flagellin alone and boosted in an identical manner at 4 weeks. Anti-FliC antibody titers were determined 2 weeks later (mean anti-FliC titer = 8.5×10^5) and flagellin-immune mice were then immunized and boosted with 10 μ g F1 + 1 μ g FliC i.n. Two weeks post-boost, anti-F1 titers were determined and compared to titers of flagellin-naïve animals immunized with 10 μ g F1 + 1 μ g FliC or 229. Bars represent mean antibody titers $\pm$ s.e.m. Seven female BALB/c mice were used per immunization group.

Figure 4 shows that flagellin stimulates antigen-specific responses and requires T cells. **(Panel a)** Groups of 7 female BALB/c mice were immunized i.n. with 10 μ g F1 antigen + 1 μ g flagellin (FliC) and boosted at 4 weeks with PBS, 1 μ g FliC alone, 10 μ g F1 alone or

10 μ g F1 + 1 μ g FliC. Plasma was collected 3 weeks after boosting for analysis by ELISA. * indicates statistical significance over animals boosted with PBS or FliC alone and ** indicates that boosting with F1 + FliC results in antibody titers statistically greater than F1 antigen alone ($p < 0.01$). Bars represent mean antibody titers $\pm$ s.e.m. **(Panel b)** A group of 7 athymic nude mice (BALB/cAnNCr-*nu/nu*) was immunized and boosted i.n. with 10 μ g F1 + 1 μ g FliC. Plasma was collected 2 weeks post-boost for analysis of anti-F1 IgG titers by ELISA. * indicates statistical significance compared to normal BALB/c mice immunized in an identical manner ($p < 0.001$).

Figure 5 depicts the requirements for the adjuvant effects of flagellin. TNFR^{-/-} (**panel a**) or IL6^{-/-} (**panel b**) and wild-type B6;129 control mice were immunized i.t. with 10 μ g F1 antigen + 1 μ g flagellin (FliC) or mutant flagellin (229). * indicates TNFR^{-/-} titers are statistically less than B6;129 control ($p < 0.001$). C3H/HeJ (*Tlr4* P712H mutant) and wild-type C3H/HeN mice were immunized with 10 μ g F1 + 1 μ g FliC or 229 (**panel c**). IFN α/β R^{-/-} (**panel d**) and IFN γ ^{-/-} (**panel e**) mice and corresponding wild-type controls were immunized i.n. with 10 μ g F1 + 1 μ g FliC or 229. Seven female mice were used in each immunization group. Mice were boosted in the same manner at 4 weeks and plasma was collected 2 weeks later for analysis of anti-F1 IgG titers by ELISA. Bars represent mean antibody titers $\pm$ s.e.m.

Figure 6 shows that flagellin promotes a protective response for intranasal infection with *Yersinia pestis* CO92. Groups of 15 female BALB/c mice (**panel a**) were immunized i.n. with 10 μ g F1 antigen + 1 μ g flagellin (FliC) or PBS alone and boosted in an identical manner at 4 weeks. Plasma was collected 2 weeks post-boost for analysis of antibody titers by ELISA (mean anti-F1 titer = 9.4×10^5). One week later mice were challenged i.n. with a dose of *Y. pestis* CO92 equivalent to 100 $\times$ LD₅₀. Mice were monitored for 30d post-challenge. Groups of 10 antibody-deficient IgH^{-/-} mice (**panel b**) were immunized i.n. with 10 μ g F1 + 1 μ g FliC or PBS alone and boosted in an identical manner at 4 weeks. Mice were challenged two weeks later i.n. with a dose of *Y. pestis* equivalent to 155 $\times$ LD₅₀. Groups of 10 wild-type C57BL/6 mice (**panel c**) and female IFN γ ^{-/-} mice (**panel d**) were immunized and boosted i.n. with 10 μ g F1 + 1 μ g FliC. Plasma was collected 2 weeks post-boost for analysis of antibody titers by

ELISA (anti-F1 titer $\geq 1 \times 10^6$). One week later mice were challenged i.n. with a dose of *Y. pestis* equivalent to $150 \times \text{LD}_{50}$ and monitored for 16d post-challenge

Figure 7 shows that flagellin is an effective adjuvant in nonhuman primates. Female cynomolgus monkeys (*Macaca fascicularis*) were immunized intranasally (n=6) or intramuscularly (n=6) with 150 μ g F1/V fusion protein + 50 μ g flagellin. Control animals (n=3) were immunized i.n. and i.m. with PBS alone. No significant change in body temperature occurred over 12h and TNF- α was not detected in plasma collected at 4h, 12h and 24h post-immunization. Animals were boosted in an identical manner at 4 weeks and plasma was collected 2 weeks later for analysis by ELISA. Bars indicate mean anti-F1/V antibody titers $\pm$ s.e.m. and * indicates statistical significance over intranasal immunization ($p < 0.006$).

Figure 8 shows that a fusion protein containing flagellin and the *Yersinia pestis* F1 and V proteins retains flagellin biological activity.

Detailed Description of the Preferred Embodiments

The present invention is based, in part, on the discovery that flagellins, and fragments thereof, can function as adjuvants, including as mucosal adjuvants, to enhance the immune response mounted in a subject against *Yersinia pestis*.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. The terminology used in the description of the invention herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention.

As used in the description of the invention and the appended claims, the singular forms "a," "an" and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise.

By "consisting essentially of" as used herein, it is meant that the indicated peptide, protein, fusion protein, nucleic, compound, composition, and the like does not include any

other material elements (*i.e.*, elements that materially impact the structure and/or function of the peptide, protein, fusion protein, nucleic acid, compound or composition).

In representative embodiments of the invention, the peptides, proteins, fusion proteins, nucleic acids and/or cells of the invention are "isolated." By "isolated" it is meant that the peptide, protein, fusion protein, nucleic acid and/or cell is at least partially purified away from other components.

1. *Yersinia pestis* Antigens.

The terms "immunogen" and "antigen" are used interchangeably herein and mean any compound (including peptides and proteins) to which a cellular and/or humoral immune response can be directed.

The present invention can be practiced with any suitable *Y. pestis* antigen. Antigens obtained from *Y. pestis* that can be used to carry out the present invention are known and described in, for example, U.S. Patent Nos. 6,706,522; 6,638,510; and 5,985,285. In particular embodiments, the antigen is a *Y. pestis* V antigen and/or a *Y. pestis* F1 antigen (these terms including the entire protein and fragments thereof, which may be at least about 10, 15, 20, 30, or 50 contiguous amino acids in length), such as described in U.S. Patent No. 5,985,285; or fusions of the *Y. pestis* V antigen and F1 antigens (again, these terms including the entire protein and fragments thereof, which may be at least about 10, 15, 20, 30 or 50 contiguous amino acids in length). In some embodiments, the antigen comprises all or a fragment of a mature *Y. pestis* V protein and/or F1 protein or, alternatively, can comprise all or a fragment of a *Y. pestis* V and/or F1 precursor. Suitable fragments comprise one or more epitopes that induce an immune response and, optionally, confer protection. In representative embodiments, the fragment comprises all or part of the extracellular portion of the protein. Further, as used herein, a "*Y. pestis* antigen" or "antigen from *Y. pestis*" or like terms include, without limitation, naturally occurring *Y. pestis* antigens and modified forms thereof that induce an immune response in a subject, optionally a protective immune response. For example, the native antigen can be modified to increase safety and/or immunogenicity.

Y. pestis antigens may be in the form of a fusion peptide, such as an F1/V fusion peptide or a V/F1 fusion peptide, including but not limited to those described in U.S. Patent No. 5,985,285 to Titball et al., and S. Leary et al., (1997) *Microbial Pathogenesis* 23: 167-179. Where two antigens are joined as a fusion peptide, they may be joined directly to one

another or joined by a peptide linking or "hinge" segment (*e.g.*, a segment of 2, 3, 4, 6, 8, 10, 15, 20, 30, 50 or more amino acids).

2. Flagellins.

The inventor has determined that flagellin can function as an adjuvant, including acting as a mucosal adjuvant, to enhance the active immune response mounted by a host to a *Y. pestis* antigen. As used herein, the term "adjuvant" has its ordinary meaning as understood by those skilled in the art. For example, an adjuvant can be defined as a substance that increases the ability of an antigen to stimulate an immune response against the antigen in the subject. In particular embodiments, the adjuvant increases the immune response against the antigen by at least about 2, 3, 4, 5, 10, 15, 20, 30, 40, 50, 60, 75, 100, 150, 500, 1000-fold or more. In other embodiments, the adjuvant reduces the amount of antigen required to achieve a particular level of immune response (cellular and/or humoral and/or mucosal), *e.g.*, a reduction of at least about 15%, 25%, 35%, 50%, 65%, 75%, 80%, 85%, 90%, 95%, 98% or more. An adjuvant can further be a substance that prolongs the time over which an immune response, optionally protective immune response, is sustained (*e.g.*, by at least about a 2-fold, 3-fold, 5-fold, 10-fold, 20-fold longer time period or more). In some instances there may be no significant immune response elicited in the host in the absence of an adjuvant.

Flagellin proteins are known and described, for example, in U.S. Patent Nos. 6,585,980, 6,130,082; 5,888,810; 5,618,533; 4,886,748 and U.S. Patent Publication No. US 2003/0044429 A1; and Donnelly et al., (2002) *J. Biol. Chem.* 43: 40456. Most gram-negative bacteria express flagella, which are surface structures that provide motility. The flagella are formed from a basal body, a filament, and a hook that connects the two. The filament is formed of a long polymer of a single protein, flagellin, with a small cap protein at the end. Polymerization of flagellin is mediated by conserved regions at the N- and C- termini, whereas the intervening regions of the flagellin protein are very diverse among species.

In illustrative embodiments of the invention, a fusion protein is provided comprising a flagellin adjuvant and one or more *Y. pestis* antigens. In general, fusion proteins of the invention comprise, consist essentially of, or consist of: (a) a flagellin adjuvant comprising (i) a flagellin N-terminal constant region; and (ii) a flagellin C-terminal constant region; and (b) a *Y. pestis* antigen, wherein the *Y. pestis* antigen is between the N-terminal constant region and the C-terminal constant region. In some embodiments, the flagellin hypervariable region

between the constant regions is deleted (in whole or in part); in other embodiments the hypervariable region is present. When the hypervariable region is present (in whole or in part) the antigen can be inserted (i) within the hypervariable region, (ii) between the flagellin N-terminal constant region and the hypervariable region, or (iii) between the flagellin C-terminal constant region and the hypervariable region.

Further, the N-terminal constant and C-terminal constant regions can be linked by a hinge region. The hypervariable region or a *Y. pestis* antigen can function as a hinge region. Additionally, or alternatively, a segment of about 2, 3, 4, 6, 8, 10, 15, 20, 30, 50 or more amino acids can function as a hinge region.

Conserved regions of flagellin are well known in the art and have been described, for example, in Mimori-Kiyosue et al., (1997) *J. Mol. Virol.* 270:222-237; Iino et al., (1977) *Ann. Rev. Genet.* 11:161-182; and Schoenhals et al., (1993) *J. Bacteriol.* 175:5395-5402. As is understood by those skilled in the art, the size of the constant region will vary somewhat depending on the source of the flagellin protein. In general, the N-terminal constant domain includes the approximately 170 or 180 N-terminal amino acids of the protein, whereas the C-terminal constant domain typically spans the approximately 85 to 100 C-terminal amino acids. The central hypervariable region varies considerably by size and sequence among bacteria, and accounts for most of the difference in molecular mass. The N- and C-terminal constant regions of flagellin proteins from a variety of bacteria are known, and others can be readily identified by those skilled in the art using known alignment techniques, which are facilitated by the elucidation of the crystal structure of the flagellin monomer (Samatey et al., (2001) *Nature* 41:331).

The terms "flagellin N-terminal constant region" and "flagellin C-terminal constant region" as used herein includes active fragments (e.g., fragments of at least about 50, 100 or 120 amino acids in length) and modifications of any of the foregoing that enhance the immune response to the *Y. pestis* antigen (e.g., by activating the TLR5 pathway). For example, the native flagellin regions can be modified to increase safety and/or immune response. In some embodiments, the flagellin N-terminal and/or C-terminal constant region comprises the full-length region or, alternatively, can comprise only a fragment of one or both regions.

In particular embodiments, the N-terminal and/or C-terminal constant region comprises a TLR5 recognition site(s) and is able to activate the TLR5 pathway.

In representative embodiments, the N-terminal constant region comprises the N-terminal RINSA domain (amino acids 31–52 of the *S. dublin* flagellin) as described by Eaves-Pyles et al. (2001) *J. Immunology* 167: 7009-7016, or a homolog or modified form thereof that enhances the immunogenicity of the *Y. pestis* antigen. In other embodiments, the N-terminal constant region comprises the D1 and D2 domains, and the C-terminal constant region comprises the D1 and D2 domains (Eaves-Pyles et al. (2001) *J. Immunology* 167: 7009-7016) or a modified form thereof that enhances the immunogenicity of the *Y. pestis* antigen.

In other embodiments, the flagellin N-terminal and/or C-terminal constant region comprises, consists of, or consists essentially of the peptide GAVQNRFN~~S~~AIT (**SEQ ID NO:4**) as described by U.S. Patent Publication No. US 2003/0044429 A1 to Alderem et al., or a homolog or modification thereof that enhances the immunogenicity of the *Y. pestis* antigen.

In still other embodiments, the N-terminal constant domain comprises the "motif N" (e.g., amino acids 98-108 of the *S. muenchen* flagellin) and/or the C-terminal constant domain comprises the "motif C" (e.g., amino acids 441-449 of *S. muenchen* flagellin) identified by Kanneganti et al., (2004) *J. Biol. Chem.* 279:5667-5676, or a homolog or modified form thereof that enhances an immune response to the *Y. pestis* antigen.

In other illustrative embodiments, the N-terminal constant domain comprises amino acids 88 to 97 of the *P. aeruginosa* flagellin (see, e.g., Verma et al., (2005) *Infect. Immun.* 73:8237-8246) or a homolog or modified form thereof that enhances an immune response to the *Y. pestis* antigen.

Regions of the flagellin protein involved in TLR5 signaling have been identified by Smith et al. (2003) *Nat. Immunol.* 4:1247-1253 (e.g., amino acids 78-129, 135-173 and 394-444 of *S. typhimurium* flagellin or homologs or modified forms thereof).

The flagellin N-terminal constant, C-terminal constant and hypervariable regions can be derived from flagellins from any suitable source, with some or all of these regions being derived from the same organism or from different organisms. A number of flagellin genes have been cloned and sequenced (see, e.g., Kuwajima et al., (1986) *J. Bact.* 168:1479; Wei et al., (1985) *J. Mol. Biol.* 186:791-803; and Gill et al., (1983) *J. Biol. Chem.* 258:7395-7401). Non-limiting sources of flagellins include but are not limited to *S. enteritidis*, *S. typhimurium*, *S. dublin*, *H. pylori*, *V. cholera*, *S. marcesens*, *S. flexneri*, *S. enterica*, *T. pallidum*, *L.*

pneumophila, *B. burgdorferi*, *C. difficile*, *A. tumefaciens*, *R. meliloti*, *B. clarridgeiae*, *R. lupine*, *P. mirabilis*, *B. subtilis*, *P. aeruginosa*, and *E. coli*.

Non-limiting examples of fusion proteins of the invention are provided in the working Examples herein.

Optionally, the fusion protein can comprise any other peptide or protein. For example, the fusion protein can further comprise one or more antigens from other organisms. In representative embodiments, the fusion protein further comprises an immunomodulatory compound. For example, it is known in the art that immune responses can be enhanced by an immunomodulatory cytokine or chemokine (*e.g.*, α -interferon, β -interferon, γ -interferon, ω -interferon, τ -interferon, interleukin-1 α , interleukin-1 β , interleukin-2, interleukin-3, interleukin-4, interleukin 5, interleukin-6, interleukin-7, interleukin-8, interleukin-9, interleukin-10, interleukin-11, interleukin 12, interleukin-13, interleukin-14, interleukin-18, B cell growth factor, CD40 Ligand, tumor necrosis factor- α , tumor necrosis factor- β , monocyte chemoattractant protein-1, granulocyte-macrophage colony stimulating factor, lymphotoxin, CCL25 [MECK], and CCL28 [TECH]).

The invention also provides a composition comprising a flagellin adjuvant and a *Y. pestis* antigen. According to this embodiment, the flagellin adjuvant can be a full-length flagellin or can be a flagellin peptide comprising the N-terminal constant and/or C-terminal constant regions as described in more detail above. Further, also as described above, the flagellin adjuvant can be coupled (*i.e.*, fused) to a *Y. pestis* antigen to form a fusion protein. In exemplary embodiments, the *Y. pestis* antigen is a *Y. pestis* F1 protein, a *Y. pestis* V protein, or a fusion thereof. The composition can comprise one or more *Y. pestis* antigens fused to the flagellin adjuvant and, optionally, one or more *Y. pestis* antigens present in the composition are not fused to the flagellin adjuvant. In other embodiments, the flagellin adjuvant is not coupled to a *Y. pestis* antigen.

Unless indicated otherwise, the fusion protein is administered *per se* as a protein (or a nucleic acid encoding the protein) and not as part of live, killed, or recombinant bacterium- or virus-vectored vaccine.

4. Recombinant Nucleic Acids and Production of Fusion Proteins.

Except as otherwise indicated, standard methods known to those skilled in the art may be used for cloning genes, amplifying and detecting nucleic acids, generating fusion

constructs, expressing peptides in host cells or organisms, and the like. Such techniques are known to those skilled in the art. *See, e.g.,* Sambrook *et al.*, "Molecular Cloning" A Laboratory Manual 2nd Ed. (Cold Spring Harbor, NY, 1989); F. M. Ausubel *et al.* Current Protocols in Molecular Biology (Green Publishing Associates, Inc. and John Wiley & Sons, Inc., New York).

As used herein, "nucleic acid" encompasses both RNA and DNA, including cDNA, genomic DNA, synthetic (*e.g.*, chemically synthesized) DNA and chimeras of RNA and DNA. The nucleic acid may be double-stranded or single-stranded. The nucleic acid may be synthesized using oligonucleotide analogs or derivatives (*e.g.*, inosine or phosphorothioate nucleotides). Such oligonucleotides can be used, for example, to prepare nucleic acids that have altered base-pairing abilities or increased resistance to nucleases.

The fusion protein of the invention can be produced in, and optionally purified from, cultured cells or organisms expressing a heterologous nucleic acid encoding the fusion protein for a variety of purposes (*e.g.*, to produce immunogenic compositions, as a diagnostic or research reagent, and the like).

In some embodiments, the fusion protein can be collected and, optionally, purified from the host cell. For example, the fusion protein can be collected from the conditioned medium. According to this embodiment, it may be advantageous to express the fusion protein operably associated with a secretory signal sequence. Alternatively, the fusion protein can be isolated from the host cell (*e.g.*, the host cell can be lysed and the fusion protein isolated therefrom).

In other embodiments, the host cells are collected and the fusion protein is not isolated therefrom.

Generally, the heterologous nucleic acid is incorporated into an expression vector (viral or non-viral). Suitable expression vectors include but are not limited to plasmids, bacteriophage, bacterial artificial chromosomes (bacs), yeast artificial chromosomes (yacs), cosmids, virus vectors, and the like. Expression vectors compatible with various host cells are well known in the art and contain suitable elements for transcription and translation of nucleic acids. Typically, an expression vector contains an "expression cassette," which includes, in the 5' to 3' direction, a promoter, a coding sequence encoding the fusion protein operatively associated with the promoter, and, optionally, a termination sequence including a stop signal for RNA polymerase and a polyadenylation signal for polyadenylase.

Expression vectors can be designed for expression of polypeptides in prokaryotic or eukaryotic cells. For example, polypeptides can be expressed in bacterial cells such as *E. coli*, insect cells (*e.g.*, in the baculovirus expression system), yeast cells, mammalian cells, or plant cells. Examples of vectors for expression in yeast *S. cerevisiae* include pYepSec1 (Baldari et al., (1987) *EMBO J.* 6:229-234), pMFa (Kurjan and Herskowitz, (1982) *Cell* 30:933-943), pJRY88 (Schultz et al., (1987) *Gene* 54:113-123), and pYES2 (Invitrogen Corporation, San Diego, Calif.). Baculovirus vectors available for expression of nucleic acids to produce proteins in cultured insect cells (*e.g.*, Sf9 cells) include the pAc series (Smith et al., (1983) *Mol. Cell. Biol.* 3:2156-2165) and the pVL series (Lucklow, V.A., and Summers, M.d. (1989) *Virology* 170:31-39).

Additionally, the expression vector will generally include expression control sequences (*e.g.*, transcription/translation control signals and polyadenylation signals), which are operably associated with the nucleic acid sequence encoding the fusion protein of the invention. It will be appreciated that a variety of promoter/enhancer elements can be used depending on the level and tissue-specific expression desired. The promoter can be constitutive or inducible (*e.g.*, the metallothionein promoter or a hormone inducible promoter), depending on the pattern of expression desired. The promoter can be native or foreign and can be a natural or a synthetic sequence. By foreign, it is intended that the promoter is not found in the wild-type host into which the promoter is introduced. The promoter is chosen so that it will function in the target cell(s) of interest. Moreover, specific initiation signals are generally provided for efficient translation of inserted protein coding sequences. These translational control sequences, which can include the ATG initiation codon and adjacent sequences, can be of a variety of origins, both natural and synthetic. In embodiments of the invention wherein the expression vector comprises two open reading frames to be transcribed, the open reading frames can be operatively associated with separate promoters or with a single upstream promoter and one or more downstream internal ribosome entry site (IRES) sequences (*e.g.*, the picornavirus EMC IRES sequence).

Examples of mammalian expression vectors include pCDM8 (Seed, (1987) *Nature* 329:840) and pMT2PC (Kaufman et al. (1987), *EMBO J.* 6:187-195). When used in mammalian cells, the expression vector's control functions are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma, adenovirus 2, cytomegalovirus and Simian Virus 40.

The invention further provides a host cell comprising (transiently or stably) a nucleic acid encoding a fusion protein of the invention. Suitable host cells are well-known in the art and include prokaryotic and eukaryotic cells. See *e.g.*, Goeddel, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, Calif. (1990). It is well-known that proteins can be expressed in bacterial cells such as *E. coli*, insect cells (*e.g.*, the baculovirus expression system), yeast cells, plant cells or mammalian cells (*e.g.* human, rat, mouse, hamster, bovine, porcine, ovine, caprine, equine, feline, canine, lagomorph, simian and the like). The host cell can be a cultured cell such as a cell of a primary or immortalized cell line. The host cell can be a cell in a microorganism, animal or plant being used essentially as a bioreactor. In particular embodiments of the present invention, the host cell is an insect cell that allows for replication of expression vectors. For example, the host cell can be from *Spodoptera frugiperda*, such as the Sf9 or Sf21 cell lines, *drosophila* cell lines, or mosquito cell lines, *e.g.*, *Aedes albopictus* derived cell lines. Use of insect cells for expression of heterologous proteins is well documented, as are methods of introducing nucleic acids, such as vectors, *e.g.*, insect-cell compatible vectors (such as baculovirus vectors), into such cells and methods of maintaining such cells in culture. See, for example, *Methods in Molecular Biology*, ed. Richard, Humana Press, NJ (1995); O'Reilly et al., *Baculovirus Expression Vectors, A Laboratory Manual*, Oxford Univ. Press (1994); Samulski et al., *J. Vir.* 63:3822-8 (1989); Kajigaya et al., *Proc. Nat'l. Acad. Sci. USA* 88: 4646-50 (1991); Ruffing et al., *J. Vir.* 66:6922-30 (1992); Kimbauer et al., *Vir.* 219:37-44 (1996); Zhao et al., *Vir.* 272:382-93 (2000); and U.S. Patent No. 6,204,059 to Samulski et al. In particular embodiments of the present invention, the insect cell is an Sf9 cell.

Vectors can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" refer to a variety of art-recognized techniques for introducing foreign nucleic acids (*e.g.*, DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, electroporation, microinjection, DNA-loaded liposomes, lipofectamine-DNA complexes, cell sonication, gene bombardment using high velocity microprojectiles, and viral-mediated transfection. Suitable methods for transforming or transfecting host cells can be found in Sambrook et al. (*Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and other laboratory manuals.

In further embodiments of the present invention, the host cell can be stably transformed with the heterologous nucleic acid sequence encoding the fusion protein. "Stable transformation" as used herein generally refers to the integration of the heterologous nucleic acid sequences into the genome of the host cell in contrast to "transient transformation" wherein the heterologous nucleic acid sequence introduced into the host cell does not integrate into the genome of the host cell. The term "stable transformant" can further refer to stable maintenance of an episome (*e.g.* Epstein-Barr Virus (EBV)) in the cell.

When producing stably transformed cells, often only a small fraction of cells (in particular, mammalian cells) integrate a foreign nucleic acid into their genome. In order to identify and select these integrants, a nucleic acid that encodes a selectable marker (*e.g.*, resistance to antibiotics) can be introduced into the host cells along with the nucleic acid of interest. Preferred selectable markers include those that confer resistance to drugs, such as G418, hygromycin and methotrexate. Nucleic acids encoding a selectable marker can be introduced into a host cell on the same vector as that comprising the nucleic acid of interest or can be introduced on a separate vector. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (*e.g.*, cells that have incorporated the selectable marker gene will survive, while the other cells die).

The fusion protein can also be produced in a transgenic plant in which the isolated nucleic acid encoding the fusion protein is inserted into the nuclear or plastidic genome. Plant transformation is known as the art: *See, in general, Methods in Enzymology* Vol. 153 ("Recombinant DNA Part D") 1987, Wu and Grossman Eds., Academic Press and European Patent Application EP 693554.

Foreign nucleic acids can be introduced into plant cells or protoplasts by several methods. For example, nucleic acid can be mechanically transferred by microinjection directly into plant cells by use of micropipettes. Foreign nucleic acid can also be transferred into a plant cell by using polyethylene glycol which forms a precipitation complex with the genetic material that is taken up by the cell (Paszkowski et al. (1984) *EMBO J.* 3:2712-22). Foreign nucleic acid can be introduced into a plant cell by electroporation (Fromm et al. (1985) *Proc. Natl. Acad. Sci. USA* 82:5824). In this technique, plant protoplasts are electroporated in the presence of plasmids or nucleic acids containing the relevant genetic construct. Electrical impulses of high field strength reversibly permeabilize biomembranes allowing the introduction of the plasmids. Electroporated plant protoplasts reform the cell

wall, divide, and form a plant callus. Selection of the transformed plant cells comprising the foreign nucleic acid can be accomplished using phenotypic markers.

Cauliflower mosaic virus (CaMV) can be used as a vector for introducing foreign nucleic acids into plant cells (Hohn et al. (1982) "Molecular Biology of Plant Tumors," Academic Press, New York, pp. 549-560; Howell, U.S. Pat. No. 4,407,956). CaMV viral DNA genome is inserted into a parent bacterial plasmid creating a recombinant DNA molecule which can be propagated in bacteria. The recombinant plasmid can be further modified by introduction of the desired DNA sequence. The modified viral portion of the recombinant plasmid is then excised from the parent bacterial plasmid, and used to inoculate the plant cells or plants.

High velocity ballistic penetration by small particles can be used to introduce foreign nucleic acid into plant cells. Nucleic acid is disposed within the matrix of small beads or particles, or on the surface (Klein et al. (1987) *Nature* **327**:70-73). Although typically only a single introduction of a new nucleic acid segment is required, this method also provides for multiple introductions.

A nucleic acid can be introduced into a plant cell by infection of a plant cell, an explant, a meristem or a seed with *Agrobacterium tumefaciens* transformed with the nucleic acid. Under appropriate conditions, the transformed plant cells are grown to form shoots, roots, and develop further into plants. The nucleic acids can be introduced into plant cells, for example, by means of the Ti plasmid of *Agrobacterium tumefaciens*. The Ti plasmid is transmitted to plant cells upon infection by *Agrobacterium tumefaciens*, and is stably integrated into the plant genome (Horsch et al., (1987) *Science* **227**:1229-1231; Fraley et al. (1983) *Proc. Natl. Acad. Sci. USA* **80**:4803).

The fusion protein can be expressed from the nucleic acid operably associated with other peptides or proteins, for example, operably associated with purification signals (such as poly His) or as a chimera with other proteins (*e.g.*, cytokines).

5. Methods of Administering the Fusion Proteins, Flagellin Adjuvants, and Compositions of the Invention.

The present invention can be practiced for therapeutic and prophylactic purposes, in accordance with known techniques (*see, e.g.*, PCT Application WO 2004/101737 to Pizzo et al.).

Generally, the present invention is practiced prophylactically to prevent infection by *Y. pestis* and/or to reduce and/or ameliorate the effects of infection by *Y. pestis*. In other embodiments, however, the methods of the invention are practiced to treat a subject that has already been infected by *Y. pestis*. Immunogenic compositions for use in the inventive methods are described below. Boosting dosages can further be administered over a time course of weeks, months or years. In chronic infection, initial high doses followed by boosting doses may be advantageous.

By the term "treat," "treating" or "treatment of" (or grammatically equivalent terms) it is meant that the severity of the subject's condition is reduced or at least partially improved or ameliorated and/or that some alleviation, mitigation or decrease in at least one clinical symptom is achieved and/or there is a delay in the progression of the condition and/or prevention or delay of the onset of a disease or disorder. The term "treat," "treats," "treating," or "treatment of" and the like also include prophylactic treatment of the subject (*e.g.*, to prevent the onset of infection or cancer). As used herein, the term "prevent," "prevents," or "prevention" (and grammatical equivalents thereof) are not meant to imply complete abolition of disease and encompasses any type of prophylactic treatment that reduces the incidence of the condition, delays the onset and/or progression of the condition, and/or reduces the symptoms associated with the condition. Thus, the term "treat," "treating" or "treatment of" (or grammatically equivalent terms) refer to both prophylactic and therapeutic regimens.

The terms "vaccination" or "immunization" are well-understood in the art, and are used interchangeably herein unless otherwise indicated. For example, the terms vaccination or immunization can be understood to be a process that increases an organism's immune response to antigen and therefore to resist or overcome infection. In the case of the present invention, vaccination or immunization against *Y. pestis* increases the organism's immune response and resistance to *Y. pestis*.

As used herein, a "treatment effective amount" is an amount that is sufficient to treat (as defined herein) the subject.

An "active immune response" or "active immunity" is characterized by "participation of host tissues and cells after an encounter with the immunogen. It involves differentiation and proliferation of immunocompetent cells in lymphoreticular tissues, which lead to synthesis of antibody or the development of cell-mediated reactivity, or both." Herbert B. Herscovitz, *Immunophysiology: Cell Function and Cellular Interactions in Antibody*

Formation, in IMMUNOLOGY: BASIC PROCESSES 117 (Joseph A. Bellanti ed., 1985).

Alternatively stated, an active immune response is mounted by the host after exposure to immunogens by infection or by vaccination. Active immunity can be contrasted with passive immunity, which is acquired through the "transfer of preformed substances (antibody, transfer factor, thymic graft, interleukin-2) from an actively immunized host to a non-immune host." *Id.*

A "protective" immune response or "protective" immunity as used herein indicates that the immune response confers some benefit to the subject in that it prevents or reduces the incidence and/or severity of disease. Alternatively, a protective immune response or protective immunity may be useful in the therapeutic treatment of existing disease.

The present invention can be practiced for both medical and veterinary purposes. Subjects to be treated by the methods of the invention include both avian and mammalian subjects, mammalian subjects including but not limited to humans, non-human primates (*e.g.*, monkeys, apes, baboons, and chimpanzees), dogs, cats, rabbits, goats, horses, pigs, cattle, sheep, and the like (including both male and female subjects and subjects of all ages including infant, juvenile, adolescent and adult subjects). Subjects may be treated for any purpose, such as for eliciting a protective immune response; for eliciting the production of antibodies in that subject (typically an animal subject) which antibodies can be collected and used for other purposes such as diagnostic purposes or administering to other subjects to produce passive immunity therein, *etc.* In particular embodiments, the subject has or is considered at risk for *Y. pestis* infection.

In some embodiments the subjects are aged subjects, *e.g.*, human subjects 50 or 60 years old or more, where other adjuvants such as alum are generally less effective.

Accordingly, in particular embodiments, the invention provides a method of inducing an immune response against *Y. pestis* in a subject (such as a mammalian subject, *e.g.*, human or primate), the method comprising administering a fusion protein of the invention or a pharmaceutical composition thereof to the subject in an immunogenically effective amount. In representative embodiments, the method is practiced to protect a subject (such as a mammalian subject, *e.g.*, human or primate) from the effects of *Yersinia pestis* infection, the method comprising administering a fusion protein of the invention or a pharmaceutical composition thereof to the subject in an amount effective to protect the subject from the effects of *Yersinia pestis* infection. Optionally, these methods are practiced by delivering the

fusion protein or pharmaceutical composition to a mucosal surface (*e.g.*, by intranasal or inhalation administration).

The invention further provides a method of inducing an immune response to *Y. pestis* in a subject (such as a mammalian subject, *e.g.*, human or primate), the method comprising administering a flagellin adjuvant and a *Y. pestis* antigen, or a pharmaceutical composition(s) thereof, to the subject in an immunogenically effective amount. In representative embodiments, the method is practiced to protect a subject (such as a mammalian subject, *e.g.*, human or primate) from the effects of *Y. pestis* infection, the method comprising administering a flagellin adjuvant and a *Y. pestis* antigen, or a pharmaceutical composition(s) thereof, to the subject in an amount effective to protect the subject from the effects of *Y. pestis* infection. Optionally, this method is practiced by delivering the fusion protein or pharmaceutical composition to a mucosal surface (*e.g.*, by intranasal or inhalation administration). The flagellin adjuvant and *Y. pestis* antigen can be administered in the same or separate compositions. If administered as separate compositions, they can optionally be administered concurrently. As used herein, the term "concurrently" means sufficiently close in time to produce a combined effect (that is, concurrently can be simultaneously, or it can be two or more events occurring within a short time period [*e.g.*, minutes or hours] before or after each other).

Administration can be by any route known in the art. As non-limiting examples, the route of administration can be by inhalation (*e.g.*, oral and/or nasal inhalation), oral, buccal (*e.g.*, sublingual), rectal, vaginal, topical (including administration to the airways), intraocular, transdermal, by parenteral (*e.g.*, intramuscular [including administration to skeletal, cardiac and/or diaphragm muscle], intravenous, subcutaneous, intradermal, intrapleural, intracerebral and intra-arterial, and intrathecal) routes, as well as direct tissue or organ injection, or by administration to the central nervous system (*e.g.*, stereotactic administration to the brain).

In particular embodiments, administration is to a mucosal surface, *e.g.*, by intranasal, inhalation, intra-tracheal, oral, rectal or vaginal administration, and the like. In general, mucosal administration refers to delivery to a mucosal surface such as a surface of the respiratory tract, gastrointestinal tract, urinary tract, reproductive tract, etc.

Methods of administration to the respiratory tract include but are not limited to transmucosal, intranasal, inhalation or intratracheal administration or administration to the

lungs. Other methods of mucosal administration include oral, buccal (*e.g.*, sub-lingual), intra-tracheal, rectal, vaginal and intra-ocular administration.

The protein(s) of the invention can be delivered *per se* or by delivering a nucleic acid intermediate that encodes the protein(s) and is expressed in the subject to produce the protein(s), such as described in U.S. Patent No. 5,589,466 to Felgner et al.

Immunomodulatory compounds, such as immunomodulatory chemokines and cytokines (preferably, CTL inductive cytokines) can be co-administered to a subject. Cytokines may be administered by any method known in the art. Exogenous cytokines may be administered to the subject, or alternatively, a nucleotide sequence encoding a cytokine may be delivered to the subject using a suitable vector, and the cytokine produced *in vivo*. In particular embodiments, the cytokine is provided as fusion protein with the flagellin adjuvant and/or *Y. pestis* antigen. For example, a fusion protein comprising a flagellin adjuvant, a *Y. pestis* antigen, and an immunomodulatory cytokine (*e.g.*, interferon- γ) can be administered. Alternatively, a fusion protein comprising the cytokine and the *Y. pestis* antigen or the flagellin adjuvant can be administered.

In addition to their use for prophylactic or therapeutic purposes, the fusion proteins and compositions of the present invention can be administered to subjects for the purpose of producing antibodies to a *Y. pestis* antigen, which antibodies are in turn useful for diagnostic or therapeutic/prophylactic purposes in human and animal subjects.

6. Pharmaceutical Compositions.

The invention further provides pharmaceutical compositions (*e.g.*, immunogenic compositions) comprising a fusion protein of the invention in a pharmaceutically acceptable carrier. In particular embodiments, the pharmaceutical composition is formulated for mucosal delivery. By "pharmaceutically acceptable" it is meant a material that is not toxic or otherwise undesirable.

In representative embodiments, the fusion protein is present in the pharmaceutical composition in an "immunogenically effective" amount. An "immunogenically effective amount" is an amount that is sufficient to evoke an active immune response (*i.e.*, cellular and/or humoral) in the subject to which the pharmaceutical composition is administered. Optionally, the dosage is sufficient to produce a protective immune response (prophylactic or therapeutic after onset of infection). The degree of protection conferred need not be complete

or permanent, as long as the benefits of administering the pharmaceutical composition outweigh any disadvantages thereof. Immunogenically effective amounts depend on the protein, the manner of administration, the stage and severity of the disease being treated, the weight and general state of health of the subject, and the judgment of the prescribing physician.

Dosages of pharmaceutically active compounds can be determined by methods known in the art, *see, e.g., Remington's Pharmaceutical Sciences* (Maack Publishing Co., Easton, Pa). In particular embodiments, the dosage of the fusion proteins of the present invention ranges from about 0.1, 0.5, 1, 10, 25, 250, 100, 150 or 250 μg to about 300, 500, 1000, 2500, 5000 or 10,000 μg of fusion protein for a typical (*e.g.* 70 kg) subject. In particular embodiments, dosages are in the range of about 50 to 2000 μg , about 100 to 1500 μg , or about 250 to 1000 μg for a typical subject. The initial dose can be followed by boosting dosages over weeks, months or years of from about 1 μg to 250, 500 or 1000 μg depending on the subject's response to the initial dosage.

The invention also provides a pharmaceutical composition comprising: (a) a flagellin adjuvant; and (b) a *Yersinia pestis* antigen. In particular embodiments, the pharmaceutical composition is formulated for mucosal administration. As described in more detail herein, the *Y. pestis* antigen can be a *Y. pestis* F1 antigen, a *Y. pestis* V antigen, or a fusion peptide thereof. Optionally, the *Y. pestis* antigen is coupled to the flagellin adjuvant, *i.e.*, is in the form of a fusion protein with the flagellin antigen. According to this embodiment, the composition can further comprise one or more additional *Y. pestis* antigens that are not coupled to the flagellin adjuvant (*i.e.*, is not part of a fusion protein with the flagellin adjuvant).

Optionally, the *Y. pestis* antigen is present in an immunogenically effective amount, as defined herein. Further, in some embodiments, the flagellin adjuvant is present in an "adjuvant effective amount." An "adjuvant effective amount" is an amount of the flagellin adjuvant that is sufficient to enhance or stimulate the active immune response (cellular and/or humoral) mounted by the host against the *Y. pestis* antigen, optionally an active mucosal immune response. In particular embodiments, the active immune response (*e.g.*, a mucosal immune response) by the host is enhanced by at least about 2, 3, 4, 5, 10, 15, 20, 30, 40, 50, 60, 75, 100, 150, 500, 1000-fold or more. In other embodiments, an "adjuvant effective amount" is an amount of the flagellin adjuvant that reduces the amount of antigen required to

achieve a specified level of immunity (cellular and/or humoral), optionally mucosal immunity, for example, a reduction of at least about 15%, 25%, 35%, 50%, 65%, 75%, 80%, 85%, 90%, 95%, 98% or more in the amount of antigen. As a further option, an "adjuvant effective amount" can refer to an amount of the flagellin adjuvant that accelerates the induction of the immune response in the host and/or reduces the need for booster immunizations to achieve protection. As yet another alternative, an "adjuvant effective amount" can be an amount that prolongs the time period over which an immune response, optionally protective immune response, is sustained (*e.g.*, by at least about a 2-fold, 3-fold, 5-fold, 10-fold, 20-fold longer time period or more).

Dosages of the flagellin adjuvant and *Y. pestis* antigen (if not in the form of a fusion protein) can be determined by those skilled in the art. In particular embodiments, dosages of the flagellin adjuvant are in the range from about 0.1, 0.5, 1, 10, 25, 50, 100 or 150 µg to about 200, 250, 300, 500, 1000, or 2500 µg for a typical (*e.g.*, 70 kg) subject. In particular embodiments, dosages are from about 10 to 1000 µg, or from about 50 to 500 µg, or from about 150 to 300 µg for a typical subject. Suitable dosages of the *Y. pestis* antigen can range from about 0.1, 0.5, 1, 10, 25, 50, 100 or 150 µg to about 200, 300, 500, 1000, 1500, 2000, 2500 or 5000 µg for a typical (*e.g.* 70 kg) subject. In particular embodiments, the dosage of the *Y. pestis* antigen is from about 50 to 2000 µg, from about 150 to about 1500 µg, or from about 300 to about 1000 µg for a typical subject. The initial dose can be followed by boosting dosages over weeks, months or years of from about 1 µg to about 1000 µg depending on the subject's response to the initial dosage.

The pharmaceutical compositions of the invention can optionally comprise other medicinal agents, pharmaceutical agents, stabilizing agents, buffers, carriers, diluents, salts, tonicity adjusting agents, wetting agents, and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, *etc.*

For injection, the carrier will typically be a liquid. For other methods of administration, the carrier may be either solid or liquid. For inhalation administration, the carrier will be respirable, and is typically in a solid or liquid particulate form.

While adjuvants beyond flagellin are generally not required, the composition can optionally comprise an additional adjuvant, such as complete or incomplete Freund's

adjuvant, aluminum phosphate, aluminum hydroxide, alum, cytokines, TLR ligands, and the like.

The concentration of the proteins in the pharmaceutical compositions can vary widely, *e.g.*, from less than about 0.01% or 0.1% up to at least about 2% to as much as 20% to 50% or more by weight, and will be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected.

The proteins can be formulated for administration in a pharmaceutical carrier in accordance with known techniques. *See, e.g.*, Remington, *The Science And Practice of Pharmacy* (9th Ed. 1995). In the manufacture of a pharmaceutical composition according to the invention, the protein(s) (including physiologically acceptable salts thereof) is typically admixed with, *inter alia*, an acceptable carrier. The carrier can be a solid or a liquid, or both, and is optionally formulated with the compound as a unit-dose formulation, for example, a tablet. A variety of pharmaceutically acceptable aqueous carriers can be used, *e.g.*, water, buffered water, 0.9% saline, 0.3% glycine, hyaluronic acid, pyrogen-free water, pyrogen-free phosphate-buffered saline solution, bacteriostatic water, or Cremophor EL[R] (BASF, Parsippany, N.J.), and the like. These compositions can be sterilized by conventional techniques. One or more proteins can be incorporated in the formulations of the invention, which can be prepared by any of the well-known techniques of pharmacy.

The pharmaceutical compositions can be packaged for use as is, or lyophilized, the lyophilized preparation generally being combined with a sterile aqueous solution prior to administration. The compositions can further be packaged in unit/dose or multi-dose containers, for example, in sealed ampoules and vials.

The pharmaceutical compositions can be formulated for administration by any method known in the art according to conventional techniques of pharmacy. For example, the compositions can be formulated to be administered intranasally, by inhalation (*e.g.*, oral inhalation), orally, buccally (*e.g.*, sublingually), rectally, vaginally, topically, intrathecally, intraocularly, transdermally, by parenteral administration (*e.g.*, intramuscular [including administration to skeletal, cardiac and/or diaphragm muscle], intravenous, subcutaneous, intradermal, intrapleural, intracerebral and intra-arterial, intrathecal), topically (*e.g.*, to both skin and mucosal surfaces, including airway surfaces), as well as direct tissue or organ injection, and by administration to the central nervous system (*e.g.*, stereotactic administration to the brain).

In particular embodiments, the pharmaceutical composition is administered to a mucosal surface, *e.g.*, by intranasal, inhalation, intratracheal, oral, rectal or vaginal administration, and the like.

For intranasal or inhalation administration, the pharmaceutical composition can be formulated as an aerosol (this term including both liquid and dry powder aerosols). For example, the pharmaceutical composition can be provided in a finely divided form along with a surfactant and propellant. Typical percentages of the composition are 0.01-20% by weight, preferably 1-10%. The surfactant is generally nontoxic and soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride. Mixed esters, such as mixed or natural glycerides may be employed. The surfactant may constitute 0.1-20% by weight of the composition, preferably 0.25-5%. The balance of the composition is ordinarily propellant. A carrier can also be included, if desired, as with lecithin for intranasal delivery. Aerosols of liquid particles can be produced by any suitable means, such as with a pressure-driven aerosol nebulizer or an ultrasonic nebulizer, as is known to those of skill in the art. *See, e.g.*, U.S. Patent No. 4,501,729. Aerosols of solid particles can likewise be produced with any solid particulate medicament aerosol generator, by techniques known in the pharmaceutical art. Intranasal administration can also be by droplet administration to a nasal surface.

Injectable formulations can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. Alternatively, one can administer the pharmaceutical composition in a local rather than systemic manner, for example, in a depot or sustained-release formulation.

Extemporaneous injection solutions and suspensions can be prepared from sterile powders, granules and tablets of the kind previously described. For example, an injectable, stable, sterile composition of this invention in a unit dosage form in a sealed container can be provided. The composition can be provided in the form of a lyophilizate, which can be reconstituted with a suitable pharmaceutically acceptable carrier to form a liquid composition suitable for injection into a subject. The unit dosage form can be from about 1 μ g to about 10 grams of the composition of this invention. When the composition is substantially water-

insoluble, a sufficient amount of emulsifying agent, which is pharmaceutically acceptable, can be included in sufficient quantity to emulsify the composition in an aqueous carrier. One such useful emulsifying agent is phosphatidyl choline.

Pharmaceutical compositions suitable for oral administration can be presented in discrete units, such as capsules, cachets, lozenges, or tablets, as a powder or granules; as a solution or a suspension in an aqueous or non-aqueous liquid; or as an oil-in-water or water-in-oil emulsion. Oral delivery can be performed by complexing a compound(s) of the present invention to a carrier capable of withstanding degradation by digestive enzymes in the gut of an animal. Examples of such carriers include plastic capsules or tablets, as known in the art. Such formulations are prepared by any suitable method of pharmacy, which includes the step of bringing into association the compound(s) and a suitable carrier (which may contain one or more accessory ingredients as noted above). In general, the pharmaceutical compositions are prepared by uniformly and intimately admixing the compound(s) with a liquid or finely divided solid carrier, or both, and then, if necessary, shaping the resulting mixture. For example, a tablet can be prepared by compressing or molding a powder or granules containing the compound(s), optionally with one or more accessory ingredients. Compressed tablets are prepared by compressing, in a suitable machine, the composition in a free-flowing form, such as a powder or granules optionally mixed with a binder, lubricant, inert diluent, and/or surface active/dispersing agent(s). Molded tablets are made by molding, in a suitable machine, the powdered compound moistened with an inert liquid binder.

Pharmaceutical compositions suitable for buccal (sub-lingual) administration include lozenges comprising the compound(s) in a flavored base, usually sucrose and acacia or tragacanth; and pastilles comprising the compound(s) in an inert base such as gelatin and glycerin or sucrose and acacia.

Pharmaceutical compositions of this invention suitable for parenteral administration can comprise sterile aqueous and non-aqueous injection solutions of the compounds of this invention, which preparations are preferably isotonic with the blood of the intended recipient. These preparations can contain anti-oxidants, buffers, bacteriostats and solutes, which render the composition isotonic with the blood of the intended recipient. Aqueous and non-aqueous sterile suspensions, solutions and emulsions can include suspending agents and thickening agents. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous

carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like.

Pharmaceutical compositions suitable for rectal administration are preferably presented as unit dose suppositories. These can be prepared by admixing the compound(s) with one or more conventional solid carriers, such as for example, cocoa butter and then shaping the resulting mixture.

Pharmaceutical compositions of this invention suitable for topical application to the skin preferably take the form of an ointment, cream, lotion, paste, gel, spray, aerosol, or oil. Carriers that can be used include, but are not limited to, petroleum jelly, lanoline, polyethylene glycols, alcohols, transdermal enhancers, and combinations of two or more thereof. In some embodiments, for example, topical delivery can be performed by mixing a pharmaceutical composition of the present invention with a lipophilic reagent (*e.g.*, DMSO) that is capable of passing into the skin.

Pharmaceutical compositions suitable for transdermal administration can be in the form of discrete patches adapted to remain in intimate contact with the epidermis of the subject for a prolonged period of time. Compositions suitable for transdermal administration can also be delivered by iontophoresis (*see, for example, Pharmaceutical Research* 3:318 (1986)) and typically take the form of an optionally buffered aqueous solution of the compound(s). Suitable formulations can comprise citrate or bis/tris buffer (pH 6) or ethanol/water and can contain from 0.1 to 0.2M active ingredient.

Further, the compound(s) can be formulated as a liposomal formulations. The technology for forming liposomal suspensions is well known in the art. When the compound(s) or salt thereof is an aqueous-soluble salt, using conventional liposome technology, the same can be incorporated into lipid vesicles. In such an instance, due to the water solubility of the compound(s) or salt, the compound(s) or salt will be substantially entrained within the hydrophilic center or core of the liposomes. The lipid layer employed can be of any conventional composition and can either contain cholesterol or can be cholesterol-free. When the compound(s) or salt of interest is water-insoluble, again

employing conventional liposome formation technology, the salt can be substantially entrained within the hydrophobic lipid bilayer which forms the structure of the liposome. In either instance, the liposomes which are produced can be reduced in size, as through the use of standard sonication and homogenization techniques.

The liposomal formulations can be lyophilized to produce a lyophilizate which can be reconstituted with a pharmaceutically acceptable carrier, such as water, to regenerate a liposomal suspension.

Having described the present invention, the same will be explained in greater detail in the following examples, which are included herein for illustration purposes only, and which are not intended to be limiting to the invention. Abbreviations used herein are as follows: GM-CSF, granulocyte-macrophage colony stimulating factor; IL, interleukin; i.n., intranasal; i.t., intra-tracheal; NK, natural killer cell; NO, nitric oxide; s.c., subcutaneous; TLR, toll like receptor; TGF- β ; transforming growth factor beta.

EXAMPLE 1

Cancer Antigens and Vaccines Thereof

Effect of flagellin on innate immunity in the mouse lung. Non-surgical intratracheal (i.t.) instillation of 1 μ g of flagellin is sufficient to induce maximal production of TNF α after approximately 4 hr (**Fig. 1**). By 12–24 hr, cytokine levels in bronchoalveolar lavage fluid return to baseline levels. Note that a mutant flagellin that does not bind to TLR5 and thus lacks signaling activity, did not induce cytokine production. In addition to TNF α , several other cytokines including IL-6, G-CSF, and the chemokines, MIP-2 and KC were induced to relatively high levels. The increase in cytokine expression is followed by the transient infiltration of neutrophils (maximal at 12–24 hr). It is important to emphasize that the innate immune response initiated by flagellin does not result in severe tissue damaging inflammation. The induced inflammatory response is relatively moderate and acute in nature. These findings, in conjunction with those of other investigators, establish the in vivo potency of flagellin as an activator of innate immunity.

Adjuvant effect of flagellin in the mouse lung. In addition to analyzing the innate response induced by flagellin, the effects of flagellin on the antibody response were examined. BALB/c mice were immunized with 10 μ g F1 antigen with 1 μ g flagellin or an

inactive flagellin mutant protein by nonsurgical intra-tracheal (i.t.) or intra-nasal (i.n.) instillation. Four weeks later, the mice were boosted with the same regimens and then serum levels of anti-F1 IgG were determined two weeks later. Flagellin, but not the inactive mutant, exhibited extraordinarily potent adjuvant activity as measured by serum anti-F1 IgG levels. In animals immunized i.t., the antibody titers ranged from 20,000 to greater than 100,000, and from 90,000 to greater than 300,000 in the case of animals immunized i.n.. The results for i.n. immunization are presented in **Table 1**. Analysis of the cytokine requirements for this response using knockout mice indicate that neither IL-6, IL-12, nor TNF- α are required for the adjuvant effect of flagellin (**Figure 5**). Furthermore, the maximal adjuvant effect of flagellin is achieved with a dose of flagellin (1 μ g) that is 5-10x less than required for the maximal induction of the innate immune response in the respiratory tract of mice. Thus maximal adjuvant activity is achieved with a dose of flagellin that produces limited inflammation.

Flagellin	Total IgG Titer
Inactive mutant (7 mice)	<100
wild-type mouse 1	>300,000
mouse 2	>300,000
mouse 3	>300,000
mouse 4	90,000
mouse 5	>300,000
mouse 6	300,000

Table 1. I.n. immunization with *Y. pestis* F1 antigen and wild-type flagellin results in a strong anti-F1 IgG response. Mice were given two immunizations with 10 μ g F1 antigen and 1 μ g wild-type or mutant flagellin. Serum anti-F1 IgG titers were measured by ELISA.

EXAMPLE 2

Flagellin as an Adjuvant to Produce an Antigen-Specific Breast Cancer Response

Treatment of breast tumor *in vivo*. BALB/c mice were immunized with the Fra-1 antigen, an antigen that is over-expressed by many breast tumors, and either flagellin or an

inactive form of flagellin. The mice were boosted once prior to subcutaneous injection of D2F2 cells, a breast cancer tumor. The mice were monitored for the growth of tumors and the tumor volumes were determined. Data are shown in **Figure 2**. The open circles represent the mice given the Fra-1 antigen and the inactive form of flagellin. The closed circles represent the mice given Fra-1 antigen and the active form of flagellin. As can readily be seen, flagellin + Fra-1 antigen had a significant effect on the growth of the tumor.

Evaluation of whether flagellin can promote a protective immune response against D2F2 breast cancer cells in a mouse model. The ability of flagellin to promote a protective state of immunity against D2F2 breast cancer cells is evaluated using as an antigen, Fra-1, a protein that is over-expressed by these cancer cells. These experiments involve immunization and then challenge with D2F2 cells as well as challenge followed by immunization. In addition, the nature of the induced effectors—antibodies vs. cytolytic T cells and/or NK cells is determined.

A. Preparation of purified recombinant Fra-1 antigen. The pET22 expression vector encoding the cDNA encoding murine Fra-1 (kindly provided by Dr. Rong Xiang, The Scripps Research Institute) is used to express the Fra-1 protein in Rosetta-gami-pLysS bacteria. The protein is purified using the Talon affinity resin (via recognition of the His-tag on the Fra-1) and contaminating endotoxin is removed by passage of the purified protein over a Detoxi-gel column (McDermott et al. (2000) *Infect. Immun.* **68**:5525–5529). Using this procedure, mg amounts of several proteins have been obtained previously (*e.g.*, flagellin, F1 antigen, and IRAK-4 [a eukaryotic protein]).

B. Evaluation of whether flagellin promotes protective immunity against D2F2 breast cancer cells in BALB/c mice immunized with Fra-1 antigen and flagellin. Groups of 7 mice are immunized with 10 µg Fra-1 protein with or without 1 µg flagellin or mutant flagellin 229 intra-peritoneally (i.p.) or intramuscularly (i.m.). Two weeks later, the mice are boosted and then challenged with 1 x 10⁶ D2F2 cells (given subcutaneously (s.c.)). Tumor mass and the survival of the mice is followed over a period of approximately 3 months. In the absence of immunity, approximately 50% of the mice succumb within 4 weeks after challenge. The s.c. tumors are measured for width and length so that tumor volume can be

calculated. If a significant effect of flagellin is observed, the dose of flagellin that produces the maximal response is determined. Once the optimal dose of flagellin is established, it is determined if flagellin can promote the clearance of an existing tumor. Groups of 7 mice are challenged with D2F2 cells by s.c. injection and then immunized with Fra-1 with or without flagellin at the time of challenge or 1, 3, 7, and 21 days later. In each case, the mice are boosted after 2 weeks. Tumor mass and survival of the mice are determined.

C. Identification of the potential effectors in the flagellin-promoted response against Fra-1-expressing D2F2 cells. To identify the induced effectors in the flagellin-promoted response, the level of circulating anti-Fra-1 antibodies as well as the relative numbers of CD8⁺ cytolytic T cells (CTL) and NK cells are determined. BALB/c mice are immunized with Fra-1 and flagellin or mutant flagellin according to the protocol presented above.

1. Anti-Fra-1 antibodies. Two weeks after challenge with D2F2 cells, the mice are euthanized and serum samples are taken for analysis of circulating anti-Fra-1 antibodies by ELISA. Using appropriate anti-isotypic antibodies, the levels of circulating total IgG and IgM as well as IgG1 and IgG2a sub-isotypes are assessed.

2. Anti-Fra-1 specific CD8⁺ CTL. Anti-Fra-1 specific CD8⁺ CTL are determined by isolating splenocytes and assessing their cytolytic activity in a standard ⁵¹Cr-release assay. The lysis mediated by CD8⁺ CTL should be blocked by an anti-MHC class I antibody. Therefore, cell samples are analyzed in the presence and absence of this antibody. By varying the ratio of effectors-to-targets, a relative estimate of the increase in CD8⁺ CTL in mice immunized with Fra-1 and flagellin or mutant flagellin is obtained. The numbers of activated CD8⁺ CTL can also be determined using an ELISPOT assay for interferon- γ (IFN- γ) production. Using anti-CD8 MACS MicroBeads (Miltenyl Biotech), CD8⁺ splenocytes from immunized mice are isolated and the cells incubated for 24 h with irradiated D2F2 cells. The cells are analyzed by ELISPOT assay for IFN- γ production. If sufficient numbers of cells are present, intracellular cytokine staining (ICS) is used as a second method.

3. NK cells. The relative numbers of activated NK cells are assessed in a ^{51}Cr -release assay using YAC-1 cells as targets. Splenocytes are obtained from immunized and D2F2 challenged mice and assessed for cytolytic activity against YAC-1 cells. Since the expression of DX5 is associated with NK cells, the expression of this marker by flow cytometry is measured using a commercially available PE-labeled antibody. A stimulatory effect of flagellin on the expansion of NK cells should be associated with an increase in DX5 expression.

The efficacy of a recombinant flagellin protein containing Fra-1 epitopes in the protective response against D2F2 breast cancer cells. It is further determined if flagellin can serve as a vaccine vector and adjuvant. If this is the case, recombinant flagellin proteins encoding a number of epitopes from different tumor antigens are generated.

The available evidence indicates that human breast cancers exhibit substantial heterogeneity in the tumor-specific antigens that they express. For example, MAGE-3 is expressed in approximately 14% of breast cancers, whereas Her2/neu is expressed in 40% , NY-BR-62 in 60%, and NY-BR-85 in approximately 90% (Scanlan and Jäger (2001) *Breast Cancer Res.* 3:95–98). It is determined if a recombinant flagellin protein containing full-length Fra-1 antigen can function in the same manner as the two proteins. If this is the case, then the epitopes within Fra-1 that are involved in inducing a protective response are mapped. It would be a straightforward effort to do the same thing with other major breast cancer antigen targets. A flagellin protein expressing epitopes from a range of target antigens can be generated. Alternatively, a cocktail of flagellin proteins can be used – each flagellin expressing a subset of target epitopes. Furthermore, by introducing foreign epitopes are introduced into the hypervariable region of the protein – a region that is not involved in the interaction of flagellin with TLR5 (Donnelly and Steiner (2002) *J.Biol.Chem.* 277:40456–40461; Smith et al. (2003) *Nat. Immunol.* 4:1247–1253; Murthy et al. (2004) *J.Biol.Chem.* 279:5667–5675), it is unlikely that the biologic activities of flagellin are affected in any significant manner.

Generation of a flagellin/Fra-1 chimera. The full sequence for Fra-1 is inserted into the hypervariable region of flagellin and the resultant construct is cloned into the pET22a expression vector and the protein is expressed in Rosetta-gami-pLysS bacteria. Endotoxin is

removed using a Detoxi-gel column. The biologic activity of the chimeric protein is assessed using TNF α production by RAW264.7 cells. The chimera is titrated and its potency compared with wild-type flagellin.

Mapping of Fra-1 epitopes required for protection against D2F2 cells. The ability of the chimera to protect BALB/c mice against a challenge with D2F2 cells is evaluated. If the chimera induces protective immunity, overlapping truncations of the Fra-1 sequence are generated and tested for efficacy in the context of the flagellin. To reduce the number of constructs, three overlapping fragments covering the entire sequence of Fra-1 are prepared. If one of these truncations is active, additional truncations are generated to define the minimal sequence(s) that is required for protection. If none of the three truncations is functional by itself, these are tested in pairs to determine if the required sequences are in different parts of the protein. Using this approach, the minimal required sequences can be defined.

EXAMPLE 3

Flagellin is an Effective Mucosal Adjuvant for Immunization Against Lethal Respiratory Challenge with *Yersinia pestis*

METHODS:

Plasmids and cell culture. The coding sequence for the F1 antigen of *Yersinia pestis*, *cafI*, (plasmid containing the entire *caf* operon kindly provided by Dr. J.B. Bliska, State University of New York, Stony Brook) was subcloned into the *NdeI* and *XhoI* sites of the pET29a expression vector from Novagen (EMD Biosciences, Inc., Madison, WI). The recombinant F1/V fusion construct (Heat et al. (1998) *Vaccine* **16**:1131–1137) (provided by Drs. G. Andrews and P. Worsham, USAMRIID) was sequenced and subcloned into pET16b. Sequencing revealed the absence of 21 amino acids corresponding to the signal sequence of F1.

Reagents and antibodies. Purified, recombinant His-tagged flagellin from *Salmonella enteritidis* was prepared as described previously (Honko and Mizel (2004) *Infect. Immun.* **72**:6676–6679; McDermott et al. (2000) *Infect. Immun.* **68**:5525–5529). The 229 mutant flagellin, as well as the F1 and F1/V antigens were purified in an identical manner. Endotoxin levels were ≤ 1 pg/ μ g as detected by the QCL-1000® Chromogenic LAL Test Kit

from the Cambrex Corporation (East Rutherford, NJ). TNF- α was detected using the BD OptEIA ELISA kit (mono/mono) per the manufacturers instructions (BD Biosciences). An anti-F1 mouse monoclonal IgG1, clone YPF19 obtained from Research Diagnostics, Inc. (Flanders, NJ), was used as a control in the anti-F1 ELISA. Goat anti-mouse IgG-HRP was purchased from SouthernBiotech (Birmingham, AL). Goat anti-monkey IgG-HRP was purchased from Research Diagnostics, Inc. (Flanders, NJ).

Mice. Female BALB/cAnNCr mice were purchased from the Frederick Cancer Research and Development Center (Frederick, MD). Female IL-6^{-/-} mice (B6;129S2-*Il6* /J), TNFR1 mice (*tm1Kopf* ^{-/-} B6;129S-*Tnfrsf1a* *Tnfrsf1b* /J), IFN γ (*tm1Imx* *tm1Imx* ^{-/-} B6.129S7-*Ifngtm1Ts*/J) and control mice (C57BL/6J, B6;129SF2/J and 129/SvJ) were purchased from The Jackson Laboratory (Bar Harbor, ME). IFN α / β R^{-/-} mice were provided by Dr. C. Schindler, Columbia University, New York (Müller et al. (1994) *Science* 264:1918-1921). Mice were maintained in a specific-pathogen free facility and all research complied with federal and institutional guidelines set forth by the Wake Forest University Animal Care and Use Committee.

Nonsurgical intratracheal and intranasal immunization of mice. For intratracheal immunization, mice were anesthetized with Avertin (2,2,2-tribromoethanol, Sigma; *tert*-amyl alcohol, Fisher) by intraperitoneal injection and suspended from a length of wire by their front incisors. Using a sterile gel-loading tip inserted gently into the trachea, 10 μ g F1 antigen and the indicated amount of flagellin or the flagellin mutant 229 was administered in a total of 50 μ L pyrogen-free PBS. For intranasal immunization, small volumes (9–12 μ L total) containing antigen and adjuvant in PBS were administered to the nostrils of anesthetized mice. Mice were boosted at 4 weeks and plasma collected 2–3 weeks post-boost for analysis of antibody titers.

Immunization of monkeys. Fifteen healthy adult female cynomolgus monkeys (*Macaca fascicularis*) were maintained in accordance with federal and institutional guidelines set forth by the Wake Forest University Animal Care and Use Committee. Animals were anesthetized with 7-10mg/kg ketamine intramuscularly for immunizations and blood collection. For intranasal immunization, 150 μ g F1/V fusion protein and 50 μ g flagellin were

delivered dropwise (100 μ L/nostril) to animals in a recumbent position. Intramuscular immunizations were administered into the quadriceps in a volume of 1mL. Control animals received PBS intranasally and intramuscularly.

Analysis of plasma antibody titers by enzyme-linked immunosorbant assay (ELISA). Heparinized tubes (StatSpin; Fisher Scientific) or BD Vacutainer PST tubes were used for plasma collection. Plasma was then aliquoted and frozen at -70°C until analysis. ELISA plates were coated with 100 μ L of antigen at 10 μ g/mL in sterile PBS overnight at 4°C and blocked with 10% FCS in PBS. Duplicate or triplicate plasma dilutions were added and the plate was incubated overnight at 4°C, followed by secondary anti-Ig antibodies for 2h at room temperature. Peroxidase activity was detected with 3,3',5,5'-Tetramethylbenzidine (TMB) Liquid Substrate System (Sigma-Aldrich) and stopped with 2M H₂SO₄. End-point dilution titers were defined as the inverse of the highest dilution that resulted in an absorbance value (OD₄₅₀) >0.1 over that of naive plasma.

Respiratory challenge with *Yersinia pestis* CO92. The Centers for Disease Control (CDC) Division of Vector-Borne Infectious Diseases (Fort Collins, CO) provided a stock culture of *Yersinia pestis* CO92 biovar orientalis, a strain isolated from a fatal case of human primary pneumonic plague (Doll et al. (1994) *Am. J. Trop. Med. Hyg.* **51**:109–114). Heart infusion broth was inoculated with a single colony from a subculture plate and grown at 28°C to an approximate density of 1 $\times$ 10⁹ colony forming units (cfu)/mL. Mice were challenged intranasally with 10 μ L of culture diluted in PBS to $\sim$ 1.8 $\times$ 10⁷ cfu/mL, a dose equivalent to 150 $\times$ lethal dose 50% (LD50) of 1.2 $\times$ 10⁴ cfu (data not shown). Actual cfu/mL values were determined by plating serial dilutions onto tryptose blood agar plates. Survival was monitored for 14-30 days post challenge. All experiments were conducted according to CDC approved standard operating procedures for the BSL3 and ABSL3 facility at the Infectious Disease Unit at Virginia Tech (CDC approval #C20031120-0016).

Statistical analysis. Data are represented as individual values with means and standard error. The F test for equality of variance and Student's one-sided t-test were used to assign statistical significance at p<0.05 or p<0.01. SigmaStat3.1 (Systat Software, Inc., Richmond, CA) was used to determine LD₅₀ values by nonlinear regression.

RESULTS:

Immunization with flagellin promotes a potent adaptive response to the F1 antigen of *Yersinia pestis*. To determine the ability of flagellin to promote a humoral immune response against the F1 antigen of *Y. pestis*, BALB/c mice were immunized with 10µg F1 antigen and 1µg recombinant flagellin (FliC) intratracheally (i.t.) or intranasally (i.n.). Control animals were immunized with F1 antigen in PBS, or F1 and a mutant form of flagellin, designated 229. Four weeks later, mice were boosted in an identical manner and plasma was collected for analysis of circulating antibody titers at various times post-boost. No F1-specific IgG was detected in the groups of control mice. However, a vaccine containing F1 and flagellin stimulated a dramatic increase in total IgG titers (**Figure 3, panel a**), with high levels of F1-specific IgG1 and IgG2a. The mean ratio of IgG1 to IgG2a (indicated within the bars) ranged from 30-170 when flagellin and F1 were administered i.t. and was approximately 3 when given i.n. Although i.n. and i.t. immunization resulted in mixed Th responses, the bias towards an apparent Th2 response was more evident following intratracheal immunization. It is important to note that flagellin did not promote significant F1-specific IgE production. Mice immunized with F1 and flagellin exhibited sustained titers of anti-F1 IgG after two immunizations (**Figure 3, panel b**). A tertiary immunization at 16 weeks improved the antibody response in the two mice that had lower initial titers.

In a previous study, it was demonstrated that the innate response to flagellin in the lung was maximal with doses of flagellin in the range of 5–15µg (Honko and Mizel (2004) *Infect. Immun.* 72:6676–6679). To determine if there was a linear relationship between the magnitude of the cytokine response and the antibody response promoted by flagellin, BALB/c mice were immunized with F1 antigen and 1µg, 5µg and 15µg of flagellin (**Figure 3, panel c**). Anti-F1 IgG titers were not significantly different at these doses, indicating that the adjuvant effect is maximal at 1µg flagellin. However, we noticed a trend towards a greater IgG1/IgG2a ratio with increasing doses of flagellin. Thus it appears that a maximal adaptive response may not require a maximal innate response.

Pre-existing immunity to flagellin is an obvious concern when considering its use as an adjuvant. Therefore, we evaluated the effectiveness of immunization with flagellin and F1 in the presence of high titers of anti-flagellin antibodies. Female BALB/c mice were immunized and boosted with 5µg flagellin i.n. and anti-flagellin antibody titers were

determined. Flagellin-specific IgG titers were below the level of detection prior to immunization, and increased significantly with a mean anti-FliC IgG titer of 8.5×10^5 . These mice were then immunized and boosted with 10 μ g F1 and 1 μ g FliC i.n. Two weeks post-boost, anti-F1 IgG titers were similar between naive and FliC-immune mice (**Figure 3, panel d**), indicating that circulating anti-flagellin antibodies did not positively or negatively alter the response to flagellin. Our results, support the conclusion that flagellin is an effective adjuvant in the presence of prior immunity to flagellin.

The adjuvant effect of flagellin stimulates antigen-specific responses and is dependent on T lymphocytes. The development of immunological memory is an essential feature of an effective vaccine, preparing the immune system to respond rapidly following subsequent exposure to the antigen during infection. To evaluate the requirement for flagellin stimulation in the secondary immunization, four groups of BALB/c mice were immunized with F1 antigen and flagellin and subsequently boosted with PBS, flagellin alone, F1 antigen alone, or flagellin and F1 (**Figure 4, panel a**). F1-specific IgG titers in mice boosted with PBS or flagellin alone remained between 500-1100, values that are typical for post-primary responses. However, mice that received F1 antigen alone in the secondary immunization had dramatically increased anti-F1 IgG titers. Although flagellin was not required in the boost, there was a significant increase in anti-F1 antibody titers when flagellin was present. These findings are analogous to those reported in Pasare and Medzhitov (2004) *Immunity* **21**:733–741 using LPS as an adjuvant. The authors suggested that once CD4⁺ memory is established using LPS as an adjuvant, TLR stimulation is no longer required for activation of these lymphocytes. Although the memory response in our system remains to be fully characterized, the lack of an F1-specific IgG response in athymic nude mice (BALB/cAnNCr-*nu/nu*) immunized with flagellin and F1 (**Figure 4, panel b**) demonstrates a requirement for T cells in the humoral response to this vaccine.

TNF- α , IL-6 and interferons are not required for the adjuvant effects of flagellin. Previously, it was determined that flagellin induces high levels of TNF- α and IL-6 in the lung (**Example 1**; Honko and Mizel (2004) *Infect. Immun.* **72**, 6676–6679). Therefore, the role of these cytokines in the adjuvant activity of flagellin was evaluated. TNF- α is a pleiotropic cytokine that promotes dendritic cell maturation (Banchereau et al. (2000) *Annu.*

Rev. Immunol. **18**, 767–811). As shown in **Figure 5, panel a**, the anti-F1 antibody response remains extremely high in these mice, indicating that TNF- α is not required for the adjuvant effect of flagellin. However, flagellin-stimulated TNF- α production appears to enhance the antibody response against F1 antigen, as the titers in TNFR^{-/-} mice were reduced approximately two-fold relative to wild-type B6;129 mice. The role of IL-6, a cytokine that promotes B cell proliferation and differentiation (Kaminura et al. (2003) *Rev. Physiol Biochem. Pharmacol.* **149**:1–38), in the adjuvant activity of flagellin was evaluated using IL-6^{-/-} mice. There was no defect in anti-F1 IgG production in these mice following immunization with F1 and FliC (**Figure 5, panel b**), indicating that this cytokine is also not essential for the adjuvant effect of flagellin.

In vitro, flagellin stimulates nitric oxide and interferon- β (IFN- β) production via signaling through functional TLR5/4 heteromeric complexes (Mizel et al. (2003) *J. Immunol.* **170**:6217–6223). C3H/HeJ mice, which possess a nonfunctional mutant TLR4 (Poltorak et al. (1998) *Science* **282**:2085–2088), provide a model to separate the effects of TLR5/4 heteromeric and TLR5/5 homomeric signaling *in vivo*. In the lung, flagellin-stimulated production of TNF- α , IL-6, G-CSF (granulocyte colony stimulating factor), keratinocyte-derived chemokine (KC), macrophage inflammatory protein 2 (MIP-2) and MIP-1 α was not disrupted in C3H/HeJ mice (**Example 1**; Honko and Mizel (2004) *Infect. Immun.* **72**:6676–6679); however, interferon production was not evaluated. Type I IFNs are proposed to link innate and adaptive immune responses by stimulating the upregulation of MHC and costimulatory molecules on antigen-presenting cells (Le Bon and Tough (2002) *Curr. Opin. Immunol.* **14**:432–436). To determine the role of TLR5/4 signaling on the adjuvant effect of flagellin, anti-F1 antibody titers in C3H/HeJ mice were compared to their wild-type counterpart, C3H/HeN mice (**Figure 5, panel c**), following immunization with F1 antigen and flagellin. As there was no defect in antibody production, signaling via TLR5/4 complexes is not required for the adjuvant effects of flagellin. The role of interferons in the adjuvant activity of flagellin was evaluated directly by determining antibody responses in mice lacking either type I interferon signaling (IFN α/β R^{-/-}) or the ability to produce interferon- γ (IFN γ ^{-/-}) (**Figure 5, panels d and e**). As both strains of mice responded in a manner similar to wild-type mice immunized with F1 antigen and flagellin, interferons are also not required for the adjuvant effect of flagellin.

Flagellin promotes a protective response for intranasal challenge with *Yersinia pestis* CO92. The fundamental test of a vaccine is the ability to provide protection against challenge with a pathogen. As a model for respiratory infection, immunized and control mice were challenged intranasally with virulent *Y. pestis* CO92. To ensure that vaccination induced adequate protection, a challenge dose of ~150 times the lethal dose 50% (LD₅₀) for i.n. infection with *Y. pestis* was selected on the basis of recommendations from a recent Plague Vaccine Workshop sponsored by the NIAID and the FDA (October 13-14, 2004). BALB/c mice immunized and boosted i.n. with flagellin and F1 had a 93% survival rate, versus only 7% in the control group (**Figure 6, panel a**), following challenge with a dose equivalent to 100×LD₅₀. B cell-deficient IgH^{-/-} mice were used to evaluate the B cell/antibody dependence of the protective response (**Figure 6, panel b**). All control and immunized mice succumbed to *Y. pestis* infection at a dose of approximately 150×LD₅₀, indicating that protection at this challenge dose is B cell-mediated and thus presumably antibody-mediated. Previously, Elvin et al. examined the role of type 1 effector functions in protection from *Y. pestis* infection using Stat4^{-/-} animals that have defective IL-12 and IFN-γ-mediated cellular immune responses (Elvin and Williamson (2004) *Microb. Patho.* **37**:177–184). Whereas immunized Stat4^{-/-} mice produced similar levels of anti-F1 and anti-V IgG as their wild-type counterpart, these animals were less protected from high dose challenge. To address the role of IFN-γ-mediated protection following intranasal challenge in our system, groups of IFN-γ^{-/-} and wild-type C57BL/6 mice were immunized and boosted with F1 antigen and flagellin prior to challenge with 150×LD₅₀ *Y. pestis*. Wild-type C57BL/6 mice were completely protected by i.n. immunization with flagellin and F1, versus only 10% of controls (**Figure 6, panel c**). Immunized IFN-γ^{-/-} mice had 80% survival following challenge (**Figure 6, panel d**), indicating that IFN-γ-mediated responses are not required for protection. As these animals had high titers of anti-F1 IgG, these results confirm the importance of F1-specific antibodies in protection from *Y. pestis* infection and support the utility of circulating IgG titers as a correlate of protective efficacy. The observation that two IFNγ^{-/-} mice succumbed to infection suggests that IFN-γ may augment antibody-mediated protection from *Y. pestis*, possibly through the promotion of the respiratory burst in phagocytes.

Flagellin is an effective adjuvant in nonhuman primates. In view of the ability of flagellin to promote protective adaptive immune responses in murine models, we next evaluated the effectiveness of flagellin as an adjuvant in nonhuman primates. A recombinant fusion protein consisting of the F1 and V antigens of *Y. pestis* was used for immunization of female cynomolgus macaques. Groups of 6 monkeys were immunized with 150µg F1/V fusion and 50µg flagellin i.n. or intramuscularly (i.m.). Additional control animals (n=3) received PBS by both routes. Prior to immunization, the monkeys exhibited anti-flagellin antibody titers of approximately 9.8×10^4 . Monkeys immunized with flagellin exhibited no change in body temperature or plasma TNF- α levels during the first 24h following immunization, and no observable inflammation occurred at the site of injection. Animals were boosted in an identical manner at four weeks and plasma anti-F1/V IgG titers were determined two weeks later (**Figure 7**). Immunized monkeys exhibited a striking increase in F1/V-specific antibody titers. No antigen-specific IgE was detected. These results clearly establish that flagellin is an effective adjuvant for the development of an antibody response in nonhuman primates, even in the presence of circulating anti-flagellin antibodies.

EXAMPLE 4

Yersinia pestis Antigens and Vaccines

A fusion protein to induce an immune response against *Yersinia pestis* is produced in a like manner as described in **Example 2**, with a *Y. pestis* V antigen, a *Y. pestis* F1 antigen, or a fusion peptide thereof. Such fusion proteins can be used to induce an immune response, optionally a protective immune response, as described herein. Optionally, the response is a mucosal immune response. Specific non-limiting examples of suitable fusion proteins are:

Example A: FliC/F1/V amino acid sequence (SEQ ID NO:1)

```

1  MAQVINTNSL SLLTQNNLNK SQSSLSSAIE RLSSGLRINS AKDDAAGQAI
51  ANRFTSNIKG LTQASRNAND GISIAQTTEG ALNEINNNLQ RVRELSVQAT
101 NGTNSDSDLK SIQDEIQQRL EEIDRVSNQT QFNGVKVLSQ DNQMKIQVGA
151 NDGETITIDL QKIDVKSLGL DGFNVNGPKE ATVGDLSKF KTVTGRSMAD
201 LTASTTATAT LVEPARITLT YKEGAPITIM DNGNIDTELL VGTTLTGGYK
251 TGTTSTSVNF TDAAGDPMYL TFTSQDGNNH QFTTKVIGKD SRDFDISPKV

```

301 NGENLVGDDV VLATGSQDFF VRSIGSKGGK LAAGKYTDAV TVTVSNQGS I
 351 EGRNRAYEQN PQHFIEDLEK VRVEQLTGHG SSVLEELVQL VKDKNIDISI
 401 KYDPRKDSEV FANRVITDDI ELLKKILAYF LPEDAILKGG HYDNQLQNGI
 451 KRVKEFLESS PNTQWELRAF MAVMHFSLTA DRIDDDILKV IVDSMNHGHD
 501 ARSKLREELA ELTAEIKIYS VIQAEINKHL SSSGTINIHD KSINLMDKNL
 551 YGYTDEEIFK ASAEEKILEK MPQTTIQVDG SEKKIVSIKD FLGSENKRTG
 601 ALGNLKN SYS YNKDNNELSH FATTCS DKSR PLNDLVSQKT TQLSDITSRF
 651 NSAIEALNRF IQKYDSVMQR LLDDTSGKRS ATGDKITLAG KTMFIDKTAS
 701 GVSTLINEDA AAKKSTANP LASIDSALSK VDAVRSSLGA IQNRFDSA IT
 751 NLGNTVTNLN SARSRIEDAD YATEVSNMSK AQILQQAGTS VLAQANQVPQ
 801 NVLSLLRLEH HHHHH*

Example B: FliC/F1 amino acid sequence (SEQ ID NO:2)

1 MAQVINTNSL SLLTQNNLNK SQSSLSSAIE RLSSGLRINS AKDDAAGQAI
 51 ANRFTSNIKG LTQASRNAND GISIAQTTEG ALNEINNNLQ RVRELSVQAT
 101 NGTNSDSDLK SIQDEIQQRL EEIDRVSNQT QFNGVKVLSQ DNQMKIQVGA
 151 NDGETITIDL QKIDVKSLGL DGFNVNGPKE ATVGD LKSSF KNVTGRSMAD
 201 LTASTTATAT LVEPARITLT YKEGAPITIM DNGNIDTELL VGT LTLGGYK
 251 TGT TSTSVNF TDAAGDPMYL TFTSQDGNNH QFTTKVIGKD SRDFDISPKV
 301 NGENLVGDDV VLATGSQDFF VRSIGSKGGK LAAGKYTDAV TVTVSNQ RSA
 351 TGDKITLAGK TMFIDKTASG VSTLINEDAA AAKKSTANPL ASIDSALSKV
 401 DAVRSSLGAI QNRFDSA ITN LGNTVTNLNS ARSRIEDADY ATEVSNMSKA
 451 QILQQAGTSV LAQANQVPQN VLSLLRLEHH HHHH*

EXAMPLE C: FliC/V amino acid sequence (SEQ ID NO:3)

1 MAQVINTNSL SLLTQNNLNK SQSSLSSAIE RLSSGLRINS AKDDAAGQAI
 51 ANRFTSNIKG LTQASRNAND GISIAQTTEG ALNEINNNLQ RVRELSVQAT
 101 NGTNSDSDLK SIQDEIQQRL EEIDRVSNQT QFNGVKVLSQ DNQMKIQVGA
 151 NDGETITIDL QKIDVKSLGL DGFNVNGPKE ATVGD LKSSF KNVTGRSMIR
 201 AYEQNPQHFI EDLEKVRVEQ LTGHGSSVLE ELVQLVKDKN IDISIKYDPR

```

251  KDSEVFANRV ITDDIELLLKK ILAYFLPEDA ILKGGHYDNQ LQNGIKRVKE
301  FLESSPNTQW ELRAFMVAVMH FSLTADRIDD DILKVIVDSM NHHGDARSKL
351  REELAELTAE LKIYSVIQAE INKHLSSSGT INIHDKSINL MDKNLYGYTD
401  EEIFKASAEY KILEKMPQTT IQVDGSEKKI VSIKDFLGSE NKRTGALGNL
451  KNSYSYNKDN NELSHFATTC SDKSRPLNDL VSQKTTQLSD ITS RFNSAIE
501  ALNRFIQKYD SVMQRLLDDT SGKRSATGDK ITLAGKTMFI DKTASGVSTL
551  INEDAAAANK STANPLASID SALSKVDAVR SSLGAIQNRD DSAITNLGNT
601  VTNLNSARSR IEDADYATEV SNMSKAQILQ QAGTSVLAQA NQVPQNVLSL
651  LRLEHHHHHHH *

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Note: FliC obtained from *S. enteritidis*. In each of these fusion proteins, the N terminal constant region of FliC ends at amino acid residue 198, and the C terminal constant region (the first seven amino acids of which is denoted in bold and underlined) begins at amino acid residue 679 of Example A (**SEQ ID NO:1**), amino acid residue 348 of Example B (**SEQ ID NO:2**), and amino acid residue 524 of Example C (**SEQ ID NO:3**).

Example 5

Biological Activity of a Fusion Peptide Containing Flagellin and the F1 and V Antigens of *Yersinia pestis*

To prepare an expression plasmid encoding flagellin and *Yersinia pestis* F1 and V antigens as a single protein, the majority of the nucleotide sequence encoding the hypervariable region of the *S. enteritidis* flagellin was removed and replaced with the F1 and V sequences in tandem separated by an eighteen nucleotide bridge encoding 6 amino acids (*see* Example A, **SEQ ID NO:1**, above). The recombinant protein was produced in BL21 cells and purified by affinity chromatography on a metal affinity resin. Endotoxin and contaminating nucleic acid were removed using an Acrodisc chromatography filter. To determine if the resultant protein retained flagellin bioactivity, TLR5-negative and TLR5-positive RAW264.7 cells were incubated with the trifusion protein and the extent of tumor necrosis factor- α production was determined. The TLR5-negative RAW cells were used to control for any contaminating factors that might have an effect in this assay. As shown in **Figure 8**, a fusion protein containing flagellin and the *Yersinia pestis* F1 and V proteins

retains flagellin biological activity in TLR5-positive cells. The protein did not signal in TLR5-negative RAW264.7 cells.

Toll-like receptor 5 (TLR5)-negative RAW264.7 cells or TLR5-positive RAW264.7 cells (a cell line created by stably transfecting RAW264.7 cells with a construct encoding a TLR5-enhanced yellow fluorescent protein) were incubated with increasing concentrations of a fusion protein encoding flagellin and the Fl and V proteins of *Y. pestis* for 4 hours and then the culture medium was assayed by ELISA for the content of TNF- α .

To determine if flagellin + Fl + V or a single protein containing all three protects against a lethal challenge with *Y. pestis* CO92, C3H/HeJ mice were immunized and boosted with phosphate buffered saline only (PBS) or a vaccine containing three proteins — 1 mg flagellin + 5 mg each Fl and V or a vaccine containing a single protein containing flagellin, Fl, and V (flagellin/Fl/V; 10 mg). The mice were boosted after 4 weeks with the same regimens and then challenged with approximately 150 LD₅₀ of *Y. pestis* CO92. The mice were bled prior to the challenge and anti-Fl IgG titers were determined by ELISA. As shown in **Table 2**, flagellin + Fl and V antigens of *Yersinia pestis* or a fusion protein containing flagellin and the Fl and V antigens of *Yersinia pestis* provides complete protection against a lethal respiratory challenge with *Yersinia pestis*.

Immunization	Anti-Fl IgG titer	Anti-V IgG titer	<u>Survival</u>	
			Number	Percent
PBS	6.6×10^4	1.2×10^3	0/10	0
Flagellin + Fl +V	4.5×10^6	4.2×10^6	10/10	100
Flagellin/Fl/V	5×10^6	6.3×10^6	4/4	100

Table 2. Results of protection studies of mice immunized with flagellin + Fl and V antigens of *Y. pestis* or a fusion protein containing flagellin and the Fl and V antigens of *Y. pestis* against a lethal respiratory challenge with *Y. pestis*.

The foregoing is illustrative of the present invention. The claims should not be limited by the preferred embodiments set forth in the description, but should be given the broadest interpretation consistent with the description as a whole.

Claims:

1. An isolated fusion protein comprising:
 - (a) an isolated flagellin adjuvant comprising:
 - (i) a flagellin N-terminal constant region; and
 - (ii) a flagellin C-terminal constant region; and
 - (b) a *Yersinia pestis* antigen between the flagellin N-terminal constant region and the flagellin C-terminal constant region, wherein the *Y. pestis* antigen comprises *Y. pestis* F1 antigen, *Y. pestis* V antigen, or a fusion peptide of *Y. pestis* F1 antigen and *Y. pestis* V. antigen; and

wherein the flagellin N-terminal constant region and/or the flagellin C-terminal constant region of the isolated flagellin adjuvant comprises a TLR5 recognition site and the isolated fusion protein is able to activate the TLR5 pathway.
2. The fusion protein of claim 1, wherein the flagellin adjuvant comprises a deleted flagellin hypervariable region or the flagellin hypervariable region is absent from the flagellin adjuvant.
3. The fusion protein of claim 1 or 2, wherein the *Y. pestis* antigen is inserted (i) within the hypervariable region, (ii) between the flagellin N-terminal constant region and the hypervariable region, or (iii) between the flagellin C-terminal constant region and the hypervariable region.
4. The fusion protein of any one of claims 1 to 3, wherein the *Yersinia pestis* antigen is a fusion peptide comprising a *Y. pestis* F1 antigen and a *Y. pestis* V antigen.
5. A nucleic acid encoding the fusion protein of any one of claims 1 to 4.
6. A vector comprising the nucleic acid of claim 5.
7. A host cell comprising the nucleic acid of claim 5 or the vector of claim 6.
8. A method of making the fusion protein of any one of claims 1 to 4, the method comprising culturing the host cell of claim 7 in a culture medium under conditions sufficient for the fusion protein to be produced.
9. The method of claim 8, wherein the fusion protein is collected from the host cell or from the culture medium.

10. An immunogenic composition comprising the fusion protein of any one of claims 1 to 4 in a pharmaceutically acceptable carrier.
11. Use of an effective amount of the fusion protein of any one of claims 1 to 4 or the immunogenic composition of claim 10 for producing an immune response against *Yersinia pestis* in a mammalian subject.
12. Use an effective amount of the fusion protein of any one of claims 1 to 4 or the immunogenic composition of claim 10 for protecting a mammalian subject from the effects of *Yersinia pestis* infection.
13. The use of claim 11 or 12, wherein the fusion protein or immunogenic composition is for delivery to a mucosal surface.
14. The use of claim 13, wherein the use is by intranasal or inhalation administration.
15. The use of any one of claims 11 to 14, wherein the subject is a human subject at least 50 years old.
16. An immunogenic composition for mucosal administration, comprising in a pharmaceutically acceptable carrier:
 - (a) an isolated flagellin adjuvant comprising:
 - (i) a flagellin N-terminal constant region; and
 - (ii) a flagellin C-terminal constant region; and
 - (b) a *Yersinia pestis* antigen between the flagellin N-terminal constant region and the flagellin C-terminal constant region, wherein the *Y. pestis* antigen comprises *Y. pestis* F1 antigen, *Y. pestis* V antigen, or a fusion peptide of *Y. pestis* F1 antigen and *Y. pestis* V. antigen; and
 wherein the flagellin N-terminal constant region and/or the flagellin C-terminal constant region of the isolated flagellin adjuvant comprises a TLR5 recognition site and the isolated flagellin adjuvant is able to activate the TLR5 pathway.
17. The immunogenic composition of claim 16, wherein the *Yersinia pestis* antigen is a fusion peptide comprising a *Y. pestis* F1 antigen and a *Y. pestis* V antigen.
18. The immunogenic composition of claim 16 or 17, further comprising a second *Yersinia pestis* antigen not coupled to the flagellin adjuvant.

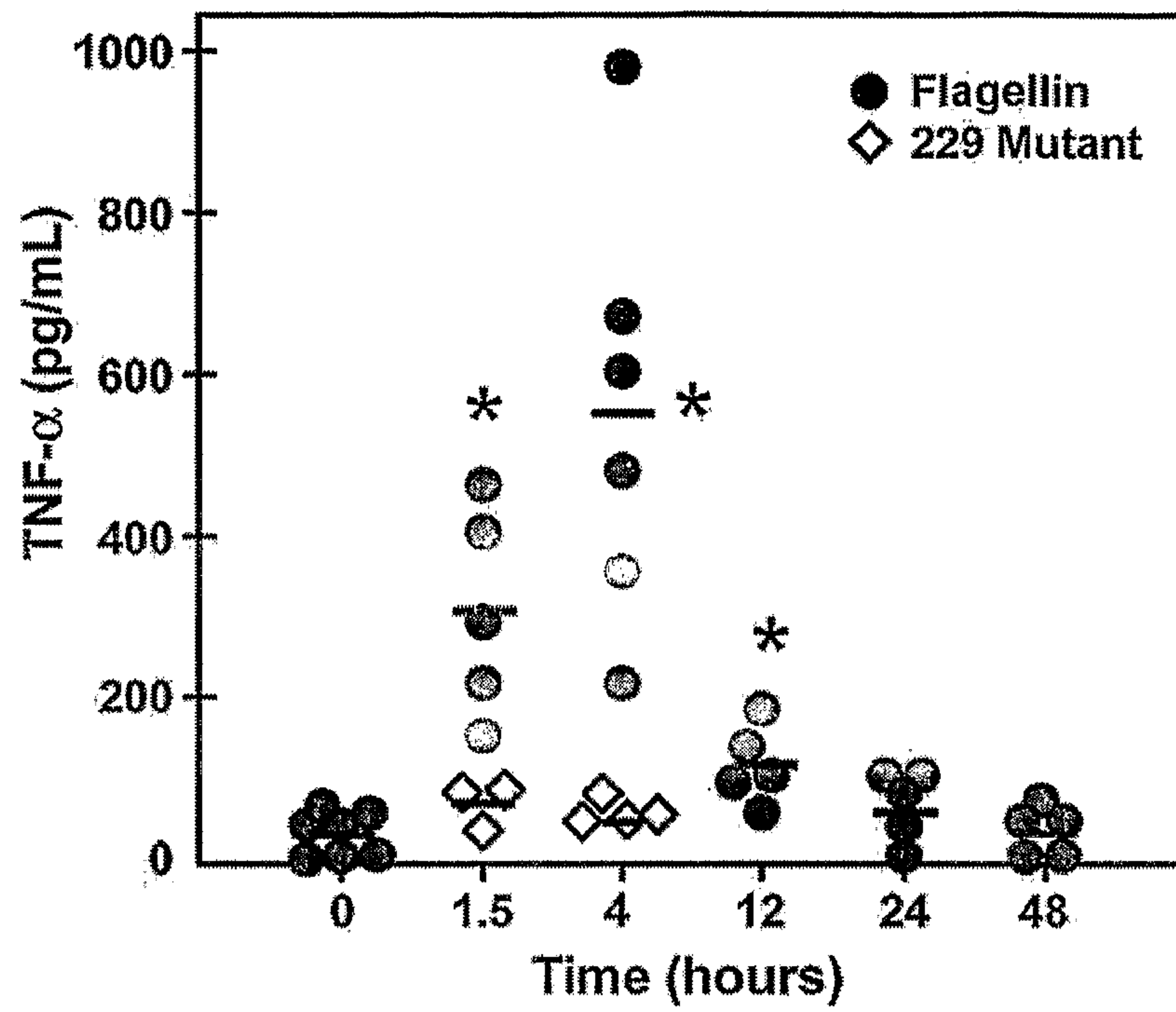
19. Use of an effective amount of the composition of any one of claims 16 to 18, mucosally, for producing an immune response against *Yersinia pestis* in a human subject.

20. Use of an effective amount of the composition of any one of claims 16 to 18, mucosally, for protecting a human subject from the effects of *Yersinia pestis* infection.

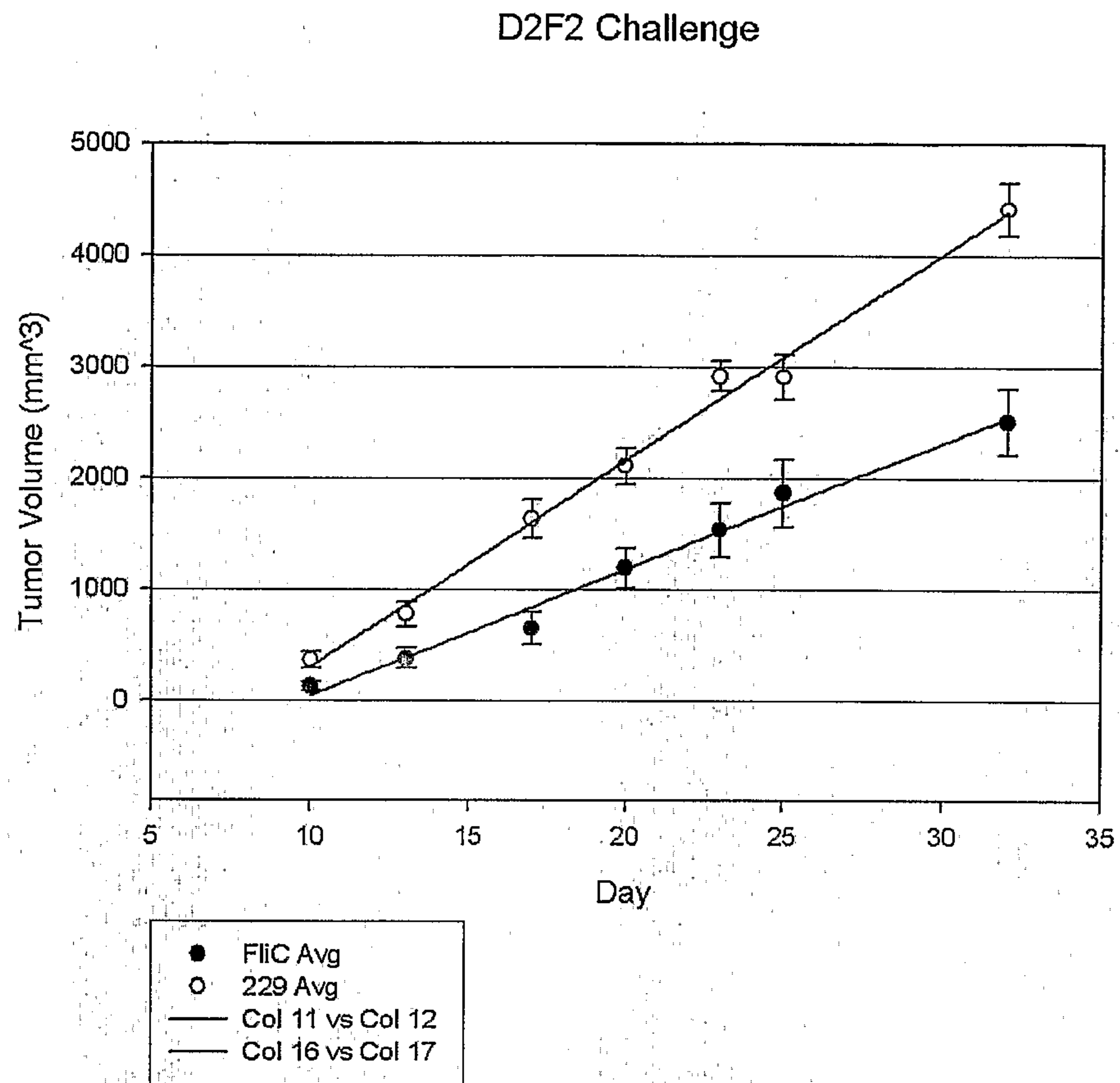
21. The use of claim 19 or 20, wherein the use is by intranasal or inhalation administration.

22. The use of claim 19 or 20, wherein said subject is a human subject at least 50 years old.

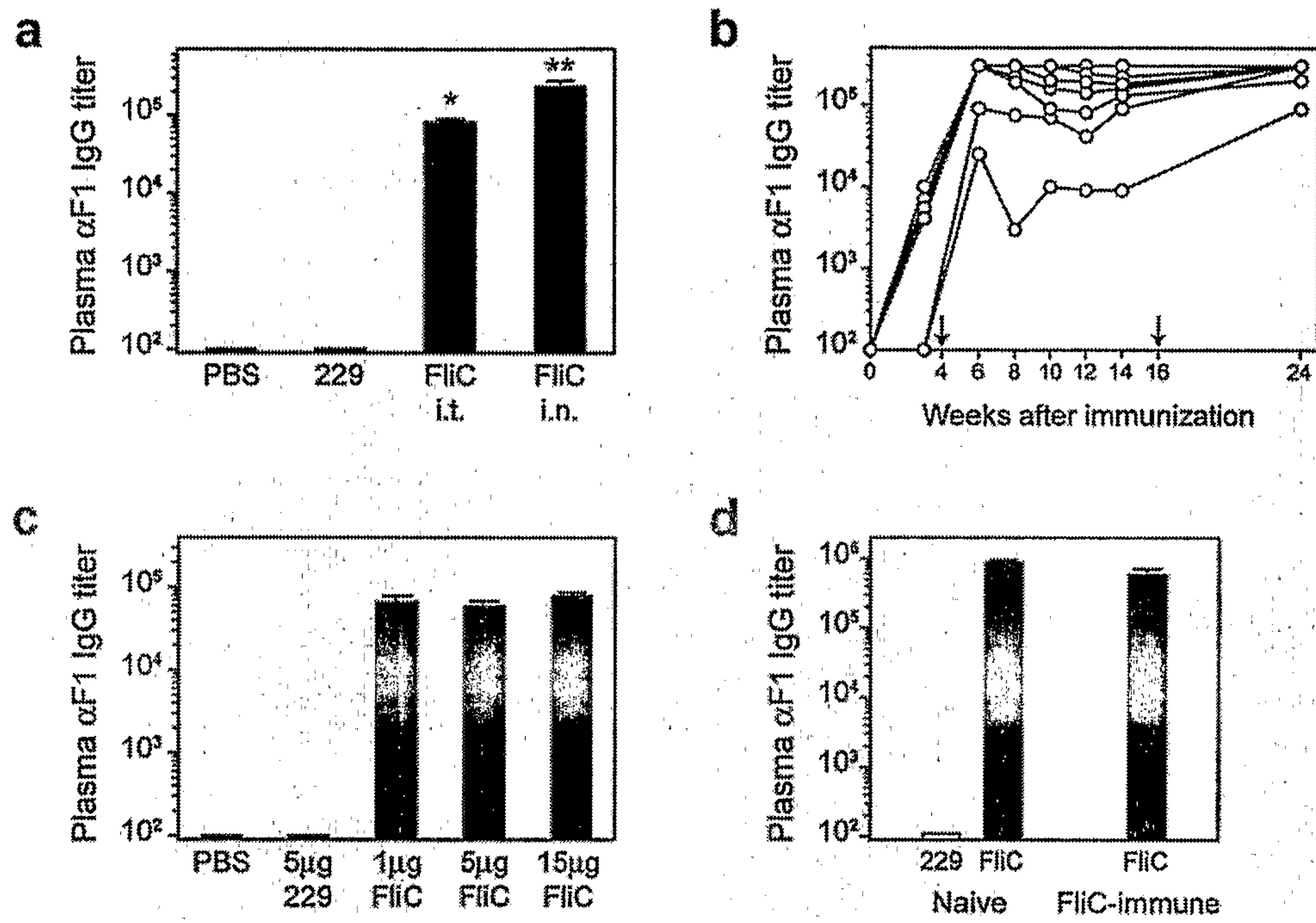
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*FIG. 1*

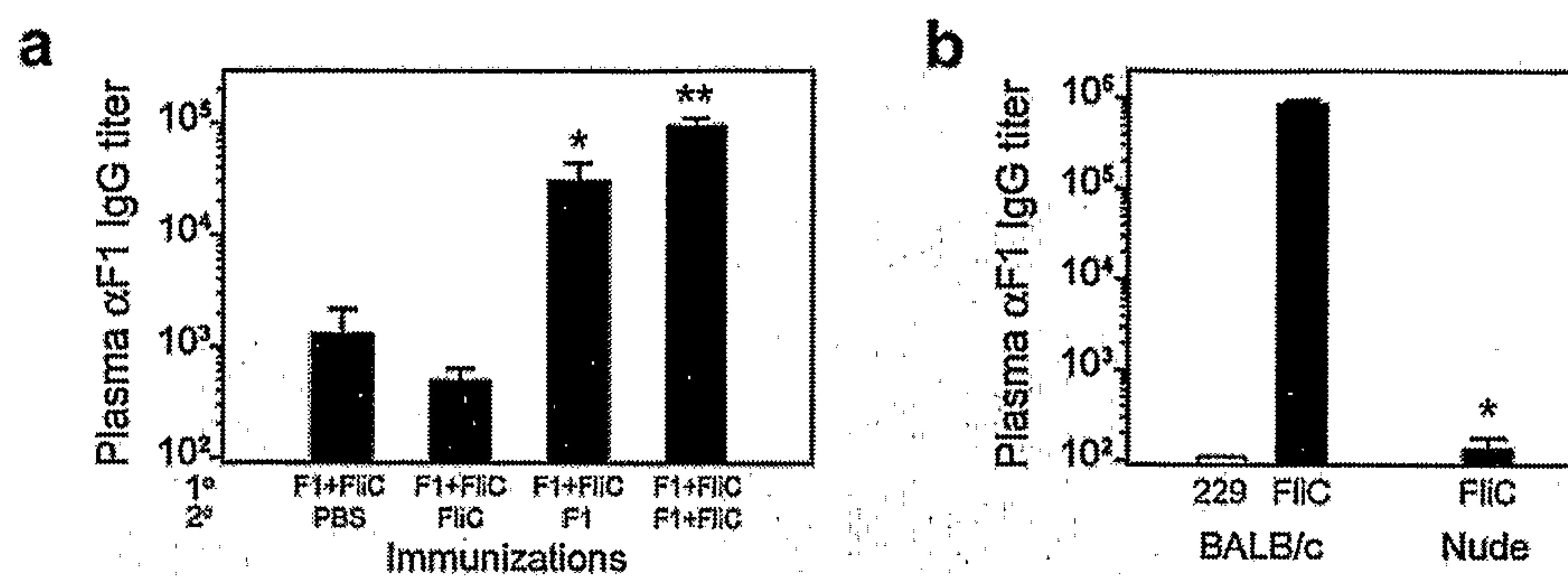
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**FIG. 2**

3/8

**FIG. 3**

4/8

**FIG. 4**

5/8

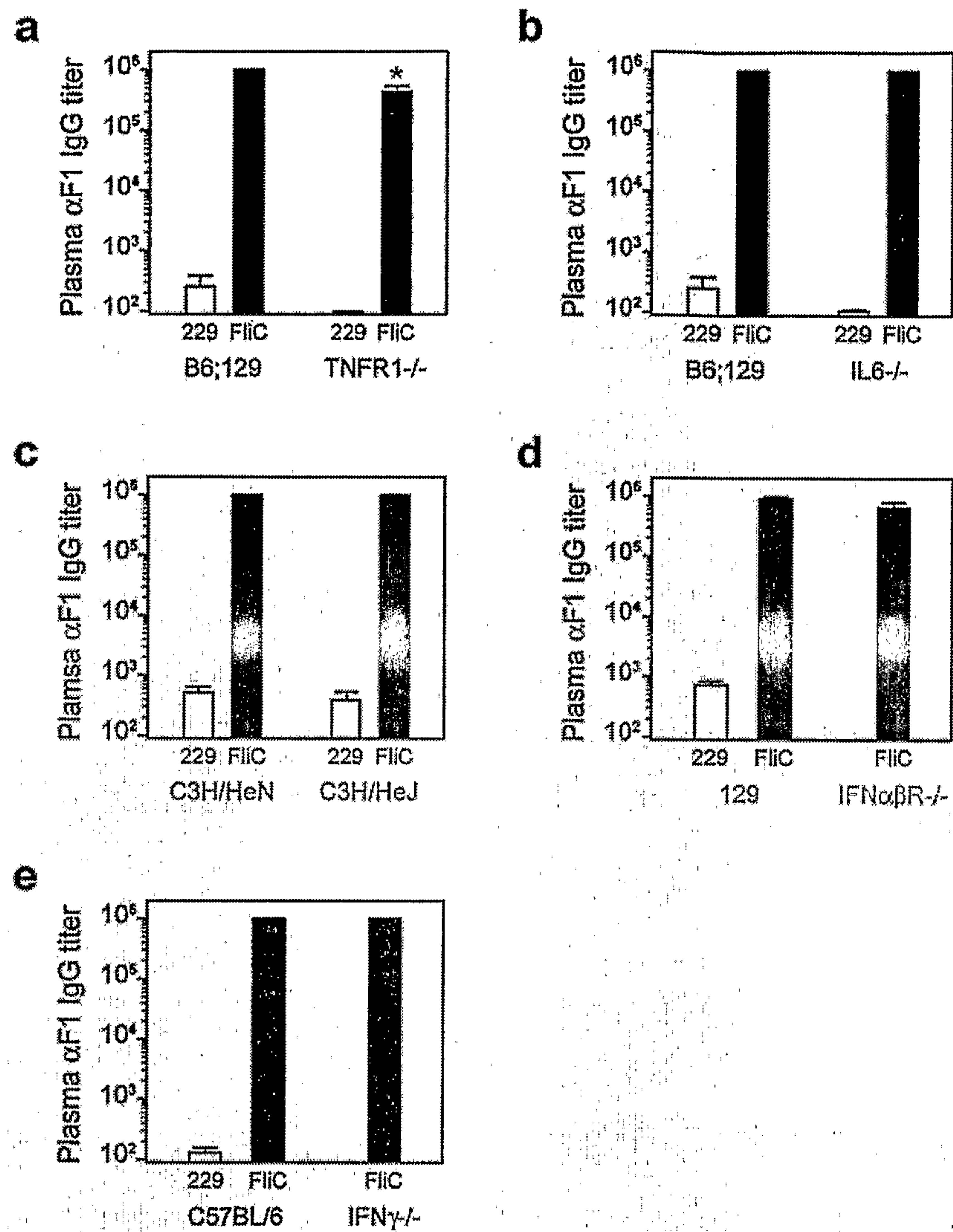
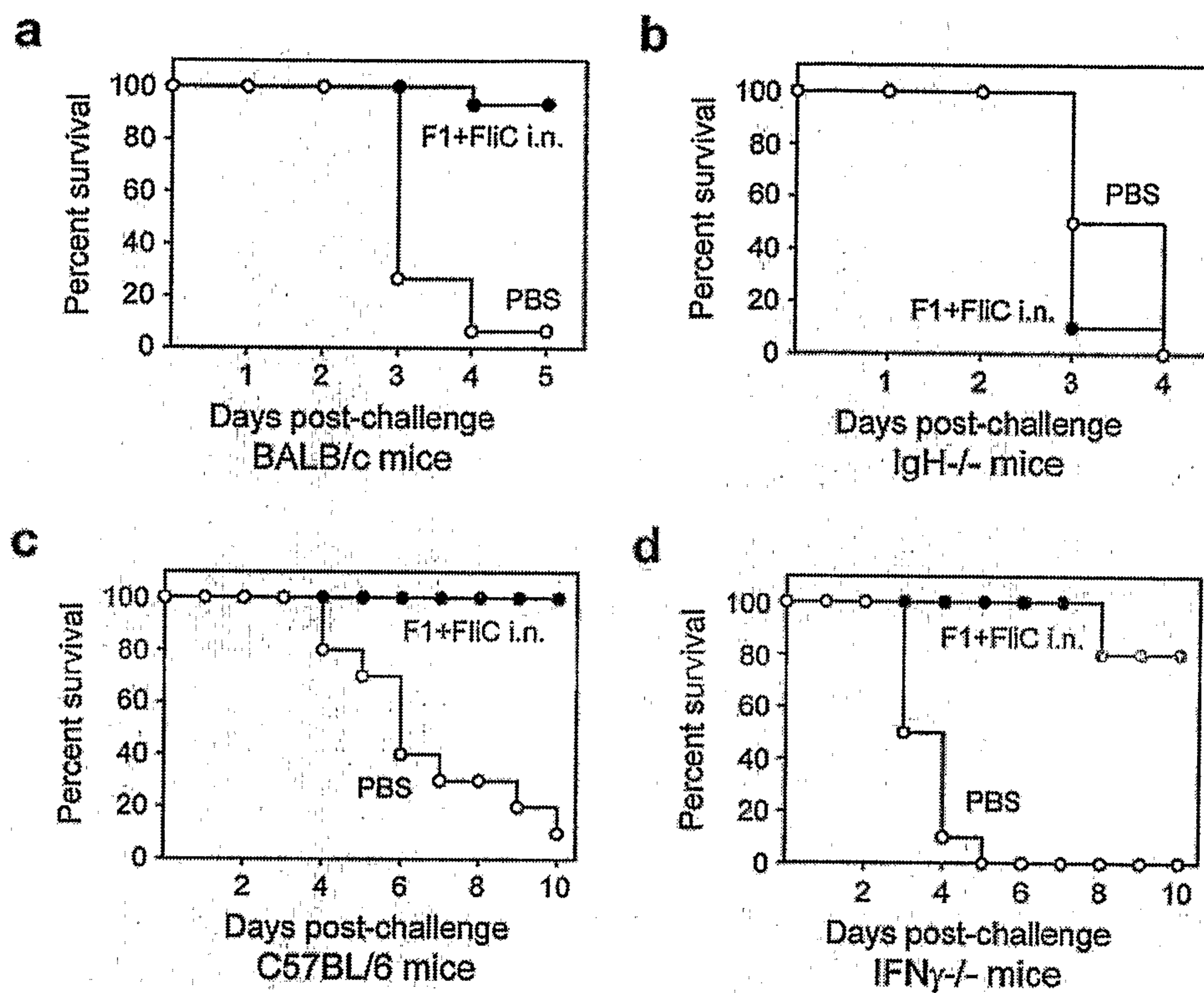
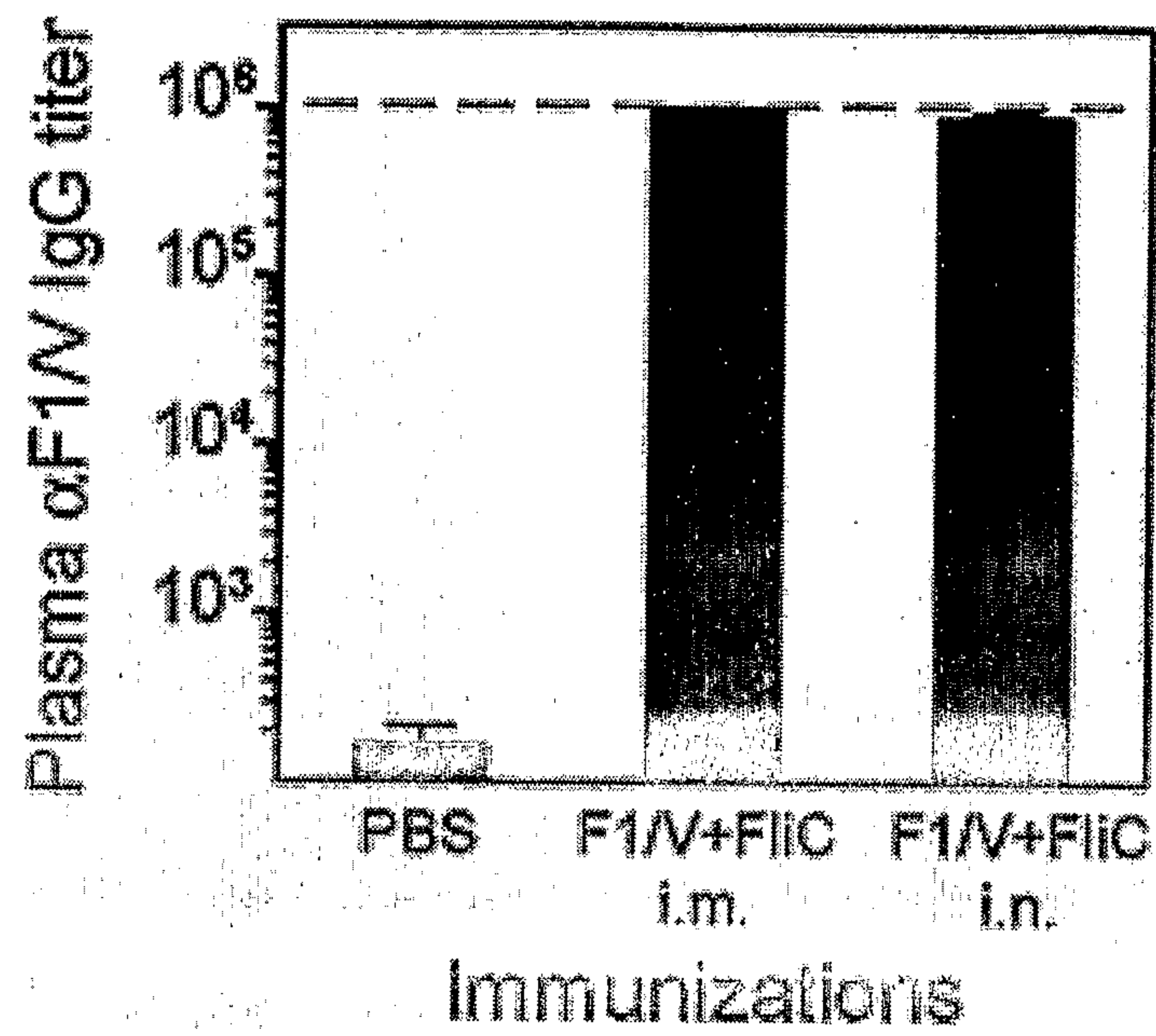


FIG. 5

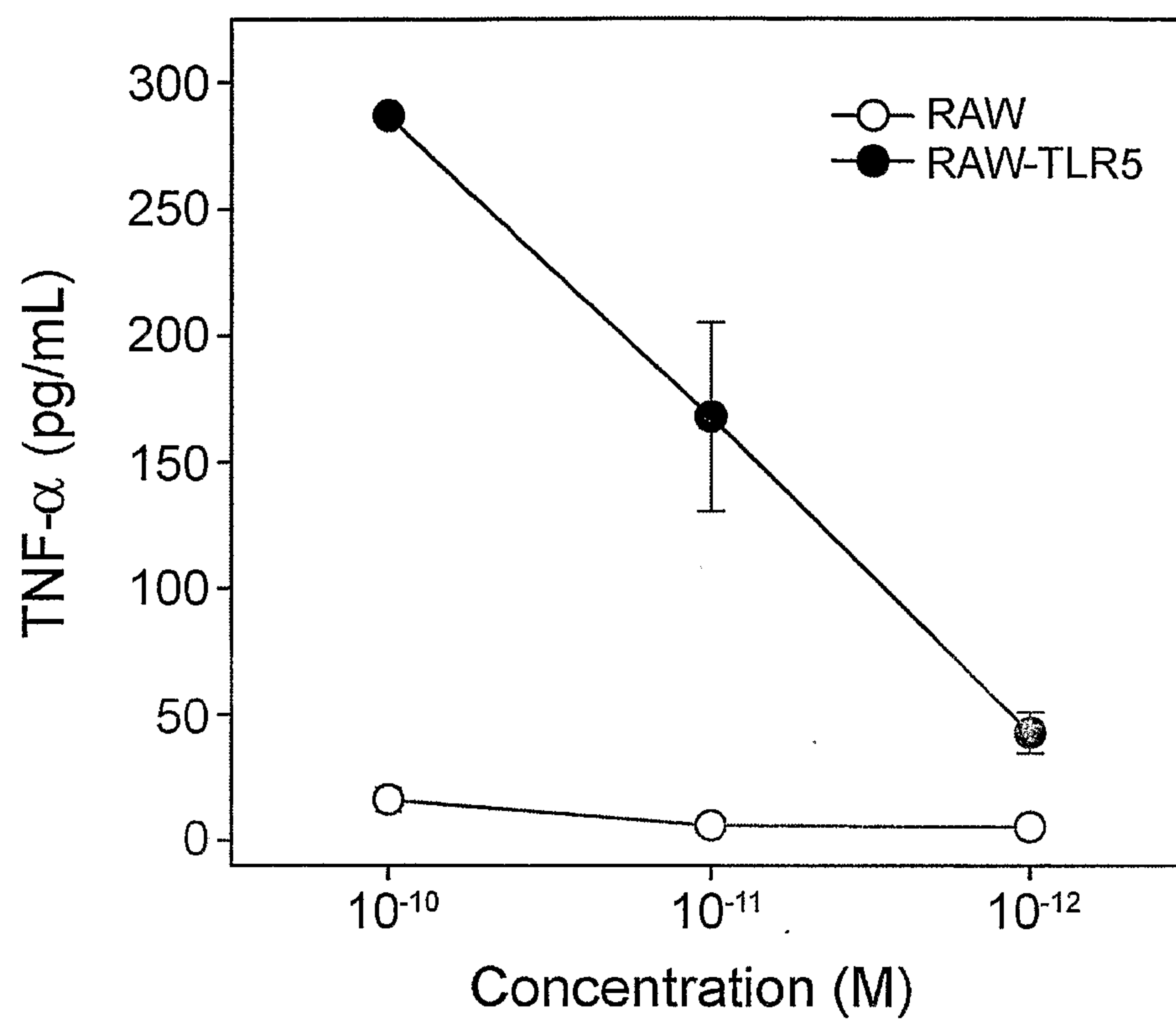
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**FIG. 6**

7/8

*FIG. 7*

8/8

*FIG. 8*