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(54) **MUTANT BORDETELLA STRAINS AND METHODS FOR USE**

MUTIERTE BORDETELLA-STÄMME UND VERFAHREN ZUR VERWENDUNG

SOUCHES MUTANTES DE BORDETELLA ET PROCÉDÉS D'UTILISATION

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(73) Proprietors:

- **Institut Pasteur de Lille**
59019 Lille Cedex (FR)
- **Institut National de la Santé et de la**
Recherche Médicale (INSERM)
75654 Paris Cedex 13 (FR)

(72) Inventors:

- **SOLANS, Luis**
59110 Le Madeleine (FR)

• **LOCHT, Camille**

59019 Lille Cedex (FR)

• **TSICOPOULOS, Anne**

59019 Lille Cedex (FR)

• **AIT YAHIA SENDID, Saliha**

59019 Lille Cedex (FR)

(74) Representative: **Abel & Imray LLP**

Westpoint Building

James Street West

Bath BA1 2DA (GB)

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- RIGMOR THORSTENSSON ET AL: "A Phase I Clinical Study of a Live Attenuated Bordetella pertussis Vaccine - BPZE1; A Single Centre, Double-Blind, Placebo-Controlled, Dose-Escalating Study of BPZE1 Given Intranasally to Healthy Adult Male Volunteers", PLOS ONE, vol. 9, no. 1, 8 January 2014 (2014-01-08), pages e83449, XP055374316, DOI: 10.1371/journal.pone.0083449

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The complete document including Reference Table(s) and the Sequence Listing(s) can be downloaded from the EPO website

Description

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims the priority of U.S. provisional patent application serial number 62/314,843 filed on March 29, 2016.

SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing which has been filed electronically in ASCII format. Said ASCII copy, created on March 27, 2017, is named 7056-0073_SL.txt and is 2,134 bytes in size.

FIELD OF THE INVENTION

[0003] The invention relates generally to the fields of microbiology, immunology, allergy, and medicine. More particularly, the invention relates to live attenuated *Bordetella pertussis* strains deficient in pertactin, and their use as prophylactic and therapeutic agents in various disease settings.

BACKGROUND

[0004] Microbial organisms and their components have long been known to affect the immune systems of mammals. Infection with virulent bacteria and viruses can cause severe illness or death. Contributing toward this, purified components of bacteria and viruses can also cause pathology by inducing inflammatory responses or otherwise causing the immune system to behave in an undesirable manner. Despite this, vaccines including whole bacteria, viruses, or parts thereof have not only proven to be one of the most powerful tools that medicine has developed to prevent serious infections, but also can cause other beneficial effects. For example, in experimental models, a live attenuated pertussis vaccine candidate named BPZE1 (see WO2007104451A1) was found to not only protect against virulent *Bordetella pertussis*, but also to exert potent anti-allergic and anti-asthma effects by dampening hyperimmune responses to allergens (see WO2013066272A1).

[0005] Developing safe and effective vaccines nonetheless remains challenging for several reasons. Among these, despite modern molecular biology techniques and significant advances in our understanding of microbiology and immunology, it remains quite difficult to produce a vaccine product that is sufficiently attenuated to not cause significant pathology, while at the same time sufficiently immunogenic to induce an effective and long-lasting immune response against the target pathogen. In the case of live attenuated whole-cell bacterial vaccines, arriving at an optimal level of attenuation is particularly troublesome because overattenuation by reducing the amount or activity of virulence factors can result in a vaccine that is poorly immunogenic and/or unable to

survive or replicate in a subject for a sufficient time after administration to induce an immune response. Various *Bordetella* strains in which the expression of various toxins is eliminated, or reduced, are disclosed in US2842529, WO2010/012501, WO2103/066627, WO2007/104451, WO2013/066272, US8986709 and US9119804.

SUMMARY

[0006] The invention is set out in the appended set of claims.

[0007] Described herein is the development of pertactin-deficient *Bordetella* strains, and their use in inducing protective immune responses against pathologic *Bordetella* infection as well as in treating or preventing respiratory tract inflammation such as that observed in allergic asthma. Pertactin, an outer membrane protein of *Bordetella* bacteria, serves as a virulence factor by promoting adhesion to a variety of cells. In the experiments described below, it was discovered that a pertactin-deficient mutant of BPZE1, termed BPZE1P (deposited in accordance with the requirements of the Budapest Treaty with the Collection Nationale de Cultures de Microorganismes ("CNCM"), 25, Rue du Docteur Roux, Paris Cedex 15, 75724, France on December 12, 2016 under accession number CNCM-I-5150), was able to colonize the respiratory tract, induce antibody responses against *Bordetella*, and protect against and treat allergic lung disease. The discovery was surprising because as others have shown that pertactin was required for *Bordetella* to resist neutrophil-mediated clearance, *B. pertussis* deficient in this virulence factor would have been expected to be cleared too rapidly to allow the induction of a protective immune responses. See, Inatsuka et al. Infect. Immun. 2010; 78: 2901-2909.

[0008] In the absence of anti-pertactin antibodies, BPZE1P colonized lungs as efficiently as BPZE1 and induced protective immunity against *B. pertussis* challenge as efficiently as BPZE1. In the presence of anti-pertactin antibodies, BPZE1P colonized the mouse lungs significantly better than BPZE1. Therefore, pertactin-deficient *B. pertussis* strains such as BPZE1P may be advantageous in protecting against respiratory tract inflammation in subjects with high pre-existing titers of pertactin antibodies, including those previously vaccinated with pertactin-containing acellular vaccines.

[0009] Accordingly described herein are methods of reducing or preventing the development of airway inflammation in a subject by administering to the respiratory tract of the subject an amount of a composition including a pharmaceutically acceptable carrier and live pertactin-deficient *Bordetella* bacteria sufficient to colonize the respiratory tract of the subject and thereby reduce or prevent the development of airway inflammation in the subject. In these methods, the airway inflammation can be associated with one or more of airway resistance, eosinophil infiltration in the lungs of the subject, and/or

increased amounts of inflammatory cytokines in the lungs of the subject. Colonization of the respiratory tract of the subject can result in reduction or prevention of such airway resistance, eosinophil infiltration, and/or increased amounts of inflammatory cytokines.

[0010] Also described herein are compositions including a pharmaceutically acceptable carrier and live pertactin-deficient *Bordetella* bacteria capable of colonizing the respiratory tract of a subject and reducing or preventing the development of airway inflammation in the subject.

[0011] In the methods and compositions described herein, the live pertactin-deficient *Bordetella* bacteria can lack a functional gene encoding pertactin, also be deficient in tracheal cytotoxin (TCT), pertussis toxin (PTX), and/or dermonecrotic toxin (DNT). The live pertactin-deficient *Bordetella* bacteria can be BPZE1P. The airway inflammation can be caused by exposure to an allergen, and the subject can be one diagnosed with asthma, interstitial lung disease, or allergic rhinitis; one having greater than 10 ng per ml of anti-pertactin antibodies in its serum; or one that has previously been immunized with a vaccine containing pertactin or a pertactin-like antigen.

[0012] As used herein, "pertactin" is the outer surface membrane protein produced by *Bordetella pertussis* and its close relatives, such as *Bordetella parapertussis* that is involved in the binding of *Bordetella* bacteria to host cells as described in Leininger et al., Proc. Nat'l. Acad. Sci. USA, 1991, 88:345-9. Conserved regions in this protein, such as its passenger and autotransporter domains, contribute directly to the overall virulence and pathogenicity of these organisms.

[0013] As used herein, the abbreviation "PTX" refers to pertussis toxin, a major virulence factor of *B. pertussis*, which induces metabolic changes and alters immune responses in the host as described in Saukkonen et al., Proc. Natl. Acad. Sci. USA., 1992, 89:118-122.

[0014] As used herein the abbreviation "DNT" refers to pertussis dermonecrotic toxin (also called lethal toxin), a toxin found in *B. pertussis* which induces inflammation, vasoconstriction and dermonecrotic lesions in sites where *B. pertussis* colonize the respiratory tract. See Fukui-Miyazaki et al., BMC Microbiol. 2010, 10:247.

[0015] As used herein the abbreviation "TCT" refers to tracheal cytotoxin, a disaccharide tetrapeptide derivative of peptidoglycan synthesized by bordetellae, which induces the production of interleukin-1 and nitric oxide synthase, and causes stasis of cilia and lethal effects on respiratory epithelial cells. See Luker et al., Proc. Natl. Acad. Sci. USA., 1993, 90, 2365-2369

[0016] As used herein, a "pertactin-deficient" *Bordetella* strain is one that exhibits at least less than 50% (e.g., less than 50, 40, 30, 20, 10, 5, 4, 3, 2, or 1%) of the pertactin activity found in BPZE1 under the conditions described in the Examples section below, one that exhibits no detectable pertactin activity, or one that exhibits not detectable expression of pertactin as determined by

Western blotting.

[0017] The term "functional" when referring to a toxin or virulence factor in a bacterial strain means that (i) the toxin/virulence factor expressed by the strain has not been mutated to eliminate or at least reduce by greater than 50% its enzymatic activity compared to the non-mutated version of the toxin/factor, and/or (ii) that a bacterial strain expressing the toxin/factor has not been engineered or selected to eliminate or at least reduce by greater than 50% the number of molecules of that toxin/factor compared to the starting strain from which the engineered or selected strain was derived.

[0018] The term "mammal", "mammalian subject" or "subject" encompasses any of various warm-blooded vertebrate animals of the class Mammalia, including human beings.

[0019] Unless otherwise defined, all technical terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. In the case of conflict, the present specification, including definitions will control. In addition, the particular embodiments discussed below are illustrative only and not intended to be limiting.

DESCRIPTION OF THE DRAWINGS

[0020]

Figure 1A is a diagram showing the structure of a plasmid used in the construction of BPZE1P.

Figure 1B is another diagram showing the construction of BPZE1P, and photographs of gels showing the results of PCR amplification of the prn UPg and prn Log fragments.

Figure 1C is photographs of immunoblots showing the presence of pertactin in BPZE1, but not in BPZE1P lysates and supernatants.

Figure 2 is a graph showing the results of lung colonization in Balb/c mice using either BPZE1 or BPZE1P.

Figure 3 is a graph showing total IgG titers after BPZE1P or BPZE1 administration to mice.

Figure 4A is a graph showing BPZE1- and BPZE1P-mediated protection in Balb/c mice challenged intranasally with 10^6 viable *B. pertussis* bacteria of the BPSM strain.

Figure 4B is a graph showing BPZE1- and BPZE1P-mediated protection in Balb/c mice challenged intranasally with 10^6 viable *B. pertussis* bacteria of the BPSMP strain.

Figure 4C is a graph showing BPZE1- and BPZE1P-mediated protection in Balb/c mice challenged intranasally with 10^6 viable *B. pertussis* bacteria of the B1917 strain.

Figure 5 is a graph showing the fitness of BPZE1 and BPZE1P in mice preimmunized with an acellular *B. pertussis* vaccine (aPv).

Figure 6 is a diagram of the experimental protocol of

an assay for airway responsiveness in allergic mice vaccinated with BPZE1, BPZE1P or left unvaccinated, and a graph showing the results of the assay. Figure 7A is a graph showing the total airway cell population infiltration in the bronchoalveolar (BAL) fluid of the allergic mice of the experiments shown in Fig. 6.

Figure 7B is a graph showing the percentage of eosinophils in the cells in the BAL fluid of the allergic mice of the experiments shown in Fig. 6.

Figure 7C is a graph showing the percentage of neutrophils in the cells in the BAL fluid of the allergic mice of the experiments shown in Fig. 6.

Figure 7D is a graph showing the percentage of lymphocytes in the cells in the BAL fluid of the allergic mice of the experiments shown in Fig. 6.

Figure 7E is a graph showing the percentage of macrophages in the cells in the BAL fluid of the allergic mice of the experiments shown in Fig. 6.

Figure 8A is a graph showing the amount of IL-1 α normalized against total proteins measured in the lung lobe in the allergic mice of the experiments shown in Fig. 6.

Figure 8B is a graph showing the amount of IL-1 β normalized against total proteins measured in the lung lobe in the allergic mice of the experiments shown in Fig. 6.

Figure 8C is a graph showing the amount of IL-6 normalized against total proteins measured in the lung lobe in the allergic mice of the experiments shown in Fig. 6.

Figure 8D is a graph showing the amount of IL-13 normalized against total proteins measured in the lung lobe in the allergic mice of the experiments shown in Fig. 6.

Figure 8E is a graph showing the amount of CXCL1 normalized against total proteins measured in the lung lobe in the allergic mice of the experiments shown in Fig. 6.

Figure 8F is a graph showing the amount of CXCL9 normalized against total proteins measured in the lung lobe in the allergic mice of the experiments shown in Fig. 6.

Figure 8G is a graph showing the amount of CXCL10 normalized against total proteins measured in the lung lobe in the allergic mice of the experiments shown in Fig. 6.

Figure 8H is a graph showing the amount of GM-CSF normalized against total proteins measured in the lung lobe in the allergic mice of the experiments shown in Fig. 6.

Figure 9 is a diagram of the experimental protocol of an assay for airway responsiveness in a therapeutic model of allergic mice vaccinated with BPZE1, BPZE1P or left unvaccinated, and a graph showing the results of the assay.

DETAILED DESCRIPTION

[0021] Described herein are *Bordetella* strains deficient in pertactin and their use in stimulating anti-*Bordetella* immune responses as well as in preventing and treating respiratory tract inflammation.

General Methodology

[0022] Methods involving conventional microbiological, immunological, molecular biological, and medical techniques are described herein. Microbiological methods are described in *Methods for General and Molecular Microbiology* (3d Ed), Reddy et al., ed., ASM Press. Immunological methods are generally known in the art and described in methodology treatises such as *Current Protocols in Immunology*, Coligan et al., ed., John Wiley & Sons, New York. Techniques of molecular biology are described in detail in treatises such as *Molecular Cloning: A Laboratory Manual*, 2nd ed., vol. 1-3, Sambrook et al., ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2001; and *Current Protocols in Molecular Biology*, Ausubel et al., ed., Greene Publishing and Wiley-Interscience, New York. General methods of medical treatment are described in McPhee and Papadakis, *Current Medical Diagnosis and Treatment 2010*, 49th Edition, McGraw-Hill Medical, 2010; and Fauci et al., *Harrison's Principles of Internal Medicine*, 17th Edition, McGraw-Hill Professional, 2008.

Pertactin-Deficient *Bordetella* Strains

[0023] *Bordetella* species such as *Bordetella pertussis*, *Bordetella parapertussis*, and *Bordetella bronchiseptica* that are deficient in pertactin expression (e.g., those that express at least 50, 60, 70, 80, 90, 95, 96, 97, 98, or 99% less pertactin than do corresponding strains) can be used to generate immune responses against *Bordetella* species, as well as to treat and/or prevent respiratory tract inflammation such as that which occurs in allergic asthma. Live, attenuated, pertactin-deficient *Bordetella pertussis* and live, attenuated, pertactin-deficient *Bordetella parapertussis* are preferred for use for treating or preventing allergic respiratory tract inflammation in human subjects. The live attenuated pertactin-deficient *Bordetella* strains described herein can be made by adapting methods known in the art such as those described in the Examples section below. The starting strain can be any suitable *Bordetella* species. Examples of *Bordetella* species include *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*. *B. pertussis* is preferred for use as the starting strain for vaccines and methods for preventing pertussis infection. Several suitable *Bordetella* strains for use as starting strains are available from established culture collections (e.g., the American Type Culture Collection in Manassas, Virginia) or can be isolated from natural reservoirs (e.g., a patient having pertussis) by known techniques (e.g., as described in Aoya-

ma et al., Dev. Biol. Stand, 73:185-92, 1991).

[0024] *Bordetella* strains which express functional pertactin can be made deficient in this molecule or its activity by selection or, preferably for stability purposes, mutagenesis (e.g., deletion of the native *prn* gene as described below). Alternatively, *Bordetella* species deficient in pertactin can also be isolated from natural sources (e.g., human subjects or other mammals infected or colonized with such strains). Because insufficient attenuation of a pathogenic strain of *Bordetella* might cause a pathological infection in a subject, it is preferred that the pertactin-deficient *Bordetella* strain used also have lower levels of other functional virulence factors. On the other hand, to ensure that the pertactin-deficient *Bordetella* strain retains the ability to colonize a subject and exert a protective effect on respiratory tract inflammation, it must not be overly attenuated. Attenuation might be achieved by mutating the strain to reduce its expression of pertactin and one or more (e.g., 1, 2, 3, 4, 5 or more) of the following: pertussis toxin (PTX), dermonecrotic toxin (DNT), tracheal cytotoxin (TCT), adenylate cyclase (AC), lipopolysaccharide (LPS), filamentous hemagglutinin (FHA), or any of the *bvg*-regulated components. Attenuation might also be achieved by mutating the strain to reduce the biological activity of pertactin and one or more (e.g., 1, 2, 3, 4, 5 or more) of the following: pertussis toxin (PTX), dermonecrotic toxin (DNT), tracheal cytotoxin (TCT), adenylate cyclase (AC), lipopolysaccharide (LPS), filamentous hemagglutinin (FHA), or any of the *bvg*-regulated components. Examples of methods for making such mutants are described herein and in U.S. patent no. 9,119,804. In the experiments presented below, a *Bordetella* strain deficient in functional pertactin, functional PTX, functional DNT, and functional TCT was able to colonize the respiratory tract of subjects, induce immune responses against *Bordetella*, and reduce or prevent the development of allergic and inflammatory responses. Accordingly, *Bordetella* strains, such as BPZE1P, which are deficient in these four virulence factors and can colonize a subject and induce immune responses targeting *Bordetella* strains and/or reduce or prevent the development of allergic and inflammatory responses are preferred.

[0025] A variety of methods are known in the art for attenuating an infectious bacterial strain. These include passaging the strain *in vitro* until virulence is lost, non-specific chemical mutagenesis followed by screening and selection based on phenotype, and using targeted molecular biology techniques such as those described in the Examples section below (including allelic exchange) and in Methods for General and Molecular Microbiology (3d Ed), Reddy et al., ed., ASM Press. Using these methods, the genes encoding pertactin, PTX, and/or DNT can be deleted or mutated to an enzymatically inactive form (which is preferred where it is desired to retain the toxin's antigenicity). TCT production can be significantly (e.g., > than 99.99, 99.90, 99.8, 99.7, 99.6, 99.5, 99.0, 98, 97, 96, 95, or 90%) reduced by replacing

the native *ampG* gene (unlike other species, *B. pertussis* *ampG* does not actively recycle TCT-containing peptidoglycan) with a heterologous (e.g., from *E. coli* or another gram-negative species) *ampG* gene, or by mutating the native *ampG* gene such that it is active at recycling peptidoglycan.

[0026] Modification of a starting strain to reduce or remove toxin/virulence factor activity can be confirmed by sequencing the genomic DNA or genes encoding the toxins of the modified strains. Southern, Northern, and/or Western blotting might also be used to confirm that the target genes have been deleted or that expression of the target factors has been reduced or removed. Biological activity can also be evaluated to confirm reduction or removal of toxin/virulence factor activity. Once the modifications have been confirmed, the modified strains can be tested for the ability to colonize a subject and to induce protective immunity against *Bordetella* infection or to reduce or prevent the development of allergic and inflammatory responses by known methods such as those described in the Examples section below.

Compositions For Modulating Immune Responses

[0027] The live attenuated *Bordetella* strains described herein can be used in compositions that protect a mammalian subject from developing a *Bordetella* infection (e.g., pertussis), or to reduce the symptoms of such an infection. They can also be used to reduce or prevent the development of allergic and inflammatory responses in a subject such as asthma, allergic rhinitis, interstitial lung disease, food allergies, peanut allergy, venom allergies, atopic dermatitis, contact hypersensitivity, and anaphylaxis. For use in therapeutic or prophylactic compositions, the live attenuated *Bordetella* strains are typically formulated with a pharmaceutically acceptable excipient. Examples of pharmaceutically acceptable excipients include, e.g., buffered saline solutions, distilled water, emulsions such as an oil/water emulsion, various types of wetting agents, sterile solutions, and the like.

[0028] The vaccines can be packaged in unit dosage form for convenient administration to a subject. For example, a single dose of between 1×10^4 to 1×10^9 (e.g., 1×10^4 , 5×10^4 , 1×10^5 , 5×10^5 , 1×10^6 , 5×10^6 , 1×10^7 , 5×10^7 , 1×10^8 , 5×10^8 , or 1×10^9 +/- 10, 20, 30, 40, 50, 60, 70, 80, or 90%) live bacteria of the selected attenuated *Bordetella* strain and any excipient can be separately contained in packaging or in an administration device. The vaccine can be contained within an administration device such as a syringe, spraying device, or insufflator.

Formulations/Dosage/Administration

[0029] The present invention does not claim methods for treatment of the human or animal body by surgery or therapy.

[0030] The compositions described herein can be administered to a mammalian subject (e.g., a human being, a human child or neonate, a human adult, a human being at high risk from developing complications from pertussis, a human being with lung disease, a human being that is or will become immunosuppressed, and a human being having or at high risk for developing respiratory tract inflammation such as asthma, allergic rhinitis, or interstitial lung disease) by any suitable method that deposits the bacteria within the composition in the respiratory tract or other mucosal compartment. For example, the compositions may be administered by inhalation or intranasal introduction, e.g., using an inhaler, a syringe, an insufflator, a spraying device, etc.

[0031] The pertactin-deficient *Bordetella* strains described herein can be formulated as compositions for administration to a subject. A suitable number of live bacteria are mixed with a pharmaceutically suitable excipient or carrier such as phosphate buffered saline solutions, distilled water, emulsions such as an oil/water emulsions, various types of wetting agents, sterile solutions and the like. In some cases, the vaccine can be lyophilized and then reconstituted prior to administration. The use of pharmaceutically suitable excipients or carriers which are compatible with mucosal (particularly nasal, bronchial, or lung) administration are preferred for colonizing the respiratory tract. See Remington's Pharmaceutical Sciences, a standard text in this field, and USP/NF.

[0032] When formulated for mucosal administration, each dose of a composition can include a sufficient number of live pertactin-deficient *Bordetella* bacteria to result in colonization of the mucosal site, e.g., approximately (i.e., +/- 50%) 5×10^3 to 5×10^9 bacteria. For administration to human subjects, the dose can include approximately 1×10^6 , 5×10^6 , 1×10^7 , 5×10^7 , 1×10^8 , 5×10^8 , 1×10^9 , 5×10^9 , or 1×10^{10} live pertactin-deficient *Bordetella* bacteria. The dose may be given once or on multiple (2, 3, 4, 5, 6, 7, 8 or more) occasions at intervals of 1, 2, 3, 4, 5, or 6 days or 1, 2, 3, 4, 5, or 6 weeks, or 1, 2, 3, 4, 5, 6, or 12 months. Generally, sufficient amounts of a composition are administered to result in colonization and the protective and/or anti-inflammatory response. Additional amounts are administered after the induced protective and/or anti-inflammatory response wanes (e.g., after the subject resumes suffering from the symptoms of respiratory tract inflammation).

[0033] Subjects which can be administered a composition containing live pertactin-deficient *Bordetella* bacteria can include any capable of being colonized with a selected live pertactin-deficient *Bordetella* bacterial strain. For example, the subject can be a mammal such as a human being. Human subjects having, or at high risk of developing, respiratory tract inflammation such as those having or prone to developing allergic asthma or allergic rhinitis are preferred recipients of the composition. While the composition can be used in subjects regardless of their titers of anti-pertactin antibodies,

the composition may be used in those having measurable titers (e.g., greater than 10, 20, 50, 100, 200, or 500 ng per ml of serum) of anti-pertactin antibodies and those having previously been immunized with a vaccine containing pertactin or a pertactin-like antigen because pertactin-deficient *Bordetella* bacterial strains are not subject to pertactin-targeting immune responses.

[0034] The effectiveness of the compositions in dampening respiratory tract inflammation can be assessed by known methods, e.g., measuring the number of inflammatory cells, IgE titers, levels of pro-inflammatory cytokines/chemokines (such as eotaxin, GM-CSF, IFN γ , IL-4, IL-5, IL-8, IL-10, IL-12, IL-13, IL-17A, IL-17F, IL-18, and TNF α) in fluid taken from the respiratory tract (e.g., bronchoalveolar lavage fluid), or clinical parameters such as spirometry or the level of dyspnea, coughing, wheezing, or respiratory capacity. Improvement in any of one or more of these parameters (at least 10, 20, 30, 40, 50, 60, 70, 80, 90% or more improved compared to a subject not receiving the composition) indicates that the composition is effective. Animal models of allergic respiratory tract inflammation can also be used to assess the effectiveness of a composition, see e.g., U.S. patent no. 8,986,709.

Examples

Example 1 - construction and characterization of a pertactin-deficient strain of *B. pertussis*.

[0035] *Escherichia coli* DH5 α , SM10 and *B. pertussis* BPZE1, BPSM (Menozzi et al., Infect Immun 1994;62:769-778) and B1917 (Bart et al. Genome Announc 2014;2(6)) were used in this study. The *Bordetella* strains were cultured at 37°C on Bordet-Gengou agar (BG), supplemented with 1% glycerol and 10 % defibrinated sheep blood. After growth, the bacteria were harvested by scraping the plates and resuspended in phosphate-buffered saline (PBS) at the desired density. For liquid culture the *Bordetella* strains were grown at 37°C in modified Stainer-Scholte medium (Imaizumi et al. Infect Immun 1983;41:1138-1143) containing 1 g/l heptakis (2,6-di-o-methyl) β -cyclodextrin (Sigma). *E. coli* strains used for cloning procedures were grown in LB broth or LB agar plates. When required, streptomycin (Sm) was used at 100 μ g/ml, gentamycin (Gm) at 10 μ g/ml and ampicillin (Amp) at 100 μ g/ml.

[0036] To delete *prn*, the gene coding for pertactin, in BPZE1, a 739-bp fragment downstream (*prn* LO) of the *prn* gene and a 759-bp fragment upstream (*prn* UP) of the *prn* gene were cloned into pSS4940 to introduce the *prn* deletion in the BPZE1 and BPSM genomes by homologous recombination. Referring to Figs. 1A and 1B, the upstream and downstream *prn* flanking region were PCR amplified using *prn*_KO_fw (ATCCTCAAGCAA GACTGCGAGCTG) (SEQ ID NO:1)) and OL_*prn*_KO_rv (GGGGATAGACCCTCCTCGCTTG GATGCCAGGTGGAGAGCA) (SEQ ID NO:2)), and

OL_prn_KO_fw (TGCTCTCCACCTGGCATCCAAGC GAGGAGGGTCTATCCCC) (SEQ ID NO:3)) and prn_KO_rv (CCATCATCCTGTACGACCGCCT) (SEQ ID NO:4)), respectively, as primers. These fragments then served as template for a PCR elongation using prn_KO_fw and prn_KO_rv as primers. The resulting fragment (containing the *prn* deletion) was inserted into the TOPO blunt® vector (ThermoFisher Scientific) and then excised as a *KpnI*-*NotI* fragment. The excised *KpnI*-*NotI* fragment was inserted into *KpnI*- and *NotI*-digested pSS4940, a pSS4245 (Inatsuka et al., Infect Immun 2010;78 2901-2909) derivative. The resulting plasmid was transformed into *E. coli* SM10, which was then conjugated with BPZE1. Following two successive homologous recombination events, as described elsewhere (Mielcarek et al., PLoS Pathog 2006;2:e65), referring to Fig. 1B, PCR was used to confirm deletion of the entire *prn* gene by amplifying the flanking regions covering the construction using prnKO_UP (TTCTTGCGCG AACAGATCAAAC) (SEQ ID NO:5)) - prnKOin_UPrv (-CTGCTGGTCATCGGCGAAGT) (SEQ ID NO:6)) for the 5' region and prnKOin_LOfw (CGCCCATCTTCCCTG TTCC) (SEQ ID NO:7))-prnKO_LO (GAACAGGAAGTGAACAGGCG) (SEQ ID NO:8)) for the 3' region. A strain carrying the *prn* deletion was selected and named BPZE1P. The same strategy was used to construct a pertactin-deficient BPSM mutant, named BPSMP.

[0037] The presence of pertactin was tested by immunoblotting of BPZE1 and BPZE1P lysates and supernatants, using purified Prn (List Biological laboratories) as control for correct size of the band. For protein extraction, BPZE1 and BPZE1P strains were plated onto BG blood agar and incubated for 48h at 37° C. After growth, the bacteria were scraped off the plates, resuspended in 10 ml of Stainer-Scholte medium and grown for 4 days at 37°C. The bacteria were then harvested by centrifugation. The supernatants were recovered and treated with trichloroacetic acid (TCA) as described previously (Solans et al., PLoS Pathog 2014;10:e1004183) for protein concentration. The bacterial pellets were resuspended in PBS complemented with an EDTA-free protease inhibitor cocktail (Roche) and lysed using a French pressure cell. Bacterial debris were removed by centrifugation for 30 minutes at 15,000 x g, and the supernatants were recovered for immunoblotting. Proteins were separated by 12% SDS-PAGE and then transferred onto a Nitrocellulose membrane using the Criterion™ cell system (Bio-Rad). After blocking with 5% w/v skim milk powder in PBS 0.01% Tween 20 for 30 min, the membrane was incubated with an anti-pertactin monoclonal antibody at 1:1,000 dilution. Goat- anti mouse-HRP (Abcam) was then added at a 1:10,000 dilution, and the blot was developed using chemiluminescent substrates (GE Healthcare). As shown in Figure 1C, an anti-pertactin antibody reactive protein co-migrating with purified pertactin was detected in the supernatant of BPZE1, but not in the supernatant of BPZE1P. This protein was not detected in the bacterial cell lysate of either BPZE1 or

BPZE1P.

Example 2 - BPZE1P colonizes mice as well as BPZE1.

[0038] Groups of 18 six-week old mice were inoculated intranasally with 20 µl PBS containing 10⁶ viable bacteria as described previously (Mielcarek et al., *supra*). At the indicated time points (3 hours, 3 days, 7 days, 14 days, 21 days and 28 days), 3 mice per group were sacrificed, and lungs were harvested and homogenized for measuring total number of colony formation units (CFU). Statistical analysis was done by a 2-way ANOVA test, using post hoc comparison Bonferroni test with confidence intervals of 95%. Referring to Fig. 2, both BPZE1 and BPZE1P colonized the animals equally well. Both strains exhibited a peak of multiplication 3 days post vaccination and colonization persisted for 4 weeks. No statistically significant difference was observed between these strains in their ability to colonize the mouse lungs.

Example 3 - BPZE1P is as immunogenic and protective against challenge with virulent *B. pertussis* as BPZE1.

[0039] Immunity induced by BPZE1P in comparison with BPZE1 was measured by antibody titration of mouse immune serum after nasal vaccination. Groups of 8 mice were vaccinated intranasally with 10⁵ viable BPZE1 or BPZE1P. Four weeks later, the mice were bled, and total IgG titers were measured against total BPSM lysate. Blood was centrifuged for 5 min. at 5,000 x g to separate the serum from the cells. Antibody titers against *B. pertussis* were estimated using enzyme-linked immunosorbent assays (ELISA) as described previously (Mielcarek et al., *supra*), using total *B. pertussis* BPSM lysate at 1 µg of total protein per well. Statistical analysis was performed using GraphPad Prism software. As shown in Fig. 3, BPZE1- and BPZE1P-vaccinated mice exhibited much higher antibody titers than did naive control mice. No significant difference in antibody titers between BPZE1- and BPZE1P-vaccinated mice was detected.

[0040] The protective efficacy of BPZE1P compared with BPZE1 was tested in a suboptimal protection protocol by measuring the CFU counts in the lungs 7 days post challenge, and comparing a naïve group with the vaccinated groups. Groups of 8 six-week old mice were vaccinated intranasally with 20 µl PBS containing 10⁵ viable BPZE1 or BPZE1P, or were left unvaccinated. Four weeks later, all mice were challenged intranasally with 20 µl PBS containing 10⁶ viable BPSM, BPSMP or B1917. Three hours after the challenge 3 mice per group were sacrificed, and lungs were harvested and homogenized for CFU counting. The remaining 5 mice per group were sacrificed 7 days after challenge for CFU counting. Three hours post infection, 3 mice were euthanized, and their lungs were harvested to determine the CFU counts shortly after challenge. Seven days post-infection, the remaining 5 mice were euthanized, their lungs were harvested, and the CFU counts were measured. Statis-

tical analysis was done applying a parametric 2-way ANOVA test, using post hoc Bonferroni comparison test with a confidence interval of 95%. *, $p < 0.005$; **, $p < 0.001$; ***, $p < 0.0001$. As shown in Figs. 4A-C, vaccination with either strain protected against challenge with BPSM, BPSMP and B1917 equally well. These results show that the deletion of *prn* does not impact on the protective capacity of the live attenuated vaccine - either against the laboratory strain BPSM, its pertactin-deficient derivative BPSMP, or clinical isolate B1917.

Example 4 - BPZE1P increases pulmonary vaccine uptake in aPv-vaccinated mice.

[0041] The ability of BPZE1P to colonize the lungs of mice having pre-existing antibodies against pertactin was investigated. Groups of 8 six-week old mice were vaccinated subcutaneously with 1/5 of the human dose of the acellular pertussis vaccine (aPv; Infanrix, GSK; containing inactivated pertussis toxin, filamentous hemagglutinin and pertactin). Four weeks later, the mice were boosted with the same dose of aPv. Four weeks after boosting, the mice were infected intranasally with 10^6 BPZE1 or BPZE1P. Three hours post infection, 3 mice were euthanized, and the lungs were harvested to determine the CFU counts. Seven days post-infection, the remaining 5 mice were euthanized, the lungs harvested, and the CFU counts were measured. Statistical analysis was done applying a parametric 2-way ANOVA test, using post hoc Bonferroni comparison test with a confidence interval of 95%. ***, $p < 0.0001$. Referring to Fig. 5, at 3 hours post-administration, no significant difference in colonization was seen between the two strains. In contrast, seven days after inoculation, BPZE1P colonized the lungs significantly better than BPZE1. Mice infected with BPZE1P had almost 10^4 CFU in the lungs 7 days after administration, while the CFU counts in the lungs of the mice given BPZE1 reached 10^2 CFU. These data show that the deletion of the *prn* gene improves BPZE1 pulmonary take in mice pre-immunized with pertactin-containing aPy.

Example 5 - BPZE1P and BPZE1 protect equally well against allergic airway inflammation.

[0042] The effect of vaccination with BPZE1P on airway responsiveness was investigated in allergic mice as described in the protocol shown in Fig. 6. Groups of 4 weeks-old mice were vaccinated intranasally with 20 μ l PBS containing 10^6 viable BPZE1 or BPZE1P, or were left unvaccinated. Four weeks later, the mice were sensitized intranasally with 20 μ l of house dust mite (HDM; Stallergenes S.A.) of 5 index of reactivity (IR) of *Derma-tophagoides farinae* extract (Derm 5IR) or 20 μ l of PBS as control. Ten days later, the mice were challenged intranasally with 20 μ l of Derm 5IR or PBS daily for 5 days, and two days later, the mice were anesthetized and intubated intratracheally for mechanical ventilation using

the FlexiVent (SCIREQ®) device. The mice were then exposed to increasing concentrations of nebulized methacholine (0-50 mg/mL in PBS) (Sigma-Aldrich) to determine the resistance in their respiratory airways using plethysmography. Statistical analysis was done by applying a parametric 2-way ANOVA test, using the post hoc Bonferroni comparison test with a confidence interval of 95%. ***, $p < 0.0001$; **, $p < 0.001$; *, $p < 0.005$. In Fig. 6, comparisons between Derm 5IR and BPZE1 + Derm 5IR are represented the solid line, and comparisons between Derm 5IR and BPZE1P + Derm 5IR are represented the dashed line. Both the BPZE1- and the BPZE1P-vaccinated mice presented significantly less resistance in their airways after treatment with methacholine at 6, 12 and 25 mg/ml compared to the non-vaccinated mice. The resistance of the vaccinated mice was comparable to that of the PBS control group, which was not sensitized, nor challenged throughout the entire experiment.

Example 6 - Measurement of lung cell infiltration and cytokine profiles.

[0043] Airway cell population infiltration in the allergic mice of the experiment shown in Fig. 6 and discussed immediately above was assessed. After plethysmography measurements, Bronchoalveolar lavage (BAL) fluids were collected to measure the cell infiltration in the airway. Cells from the BAL fluids were harvested by centrifugation at 1,200 rpm for 5 min at 4°C, resuspended in PBS for cell counting using the Shandon cyto spin 4 (Thermo Fisher Scientific) and stained with May Grünwald Giemsa (DiffQuik®) for cell type counting. Total cell numbers were measured in the BAL fluid of mice (Fig. 7A), and percentages of eosinophils (Fig. 7B), neutrophils (Fig. 7C), lymphocytes (Fig. 7D) and macrophages (Fig. 7E) were calculated. Statistical analysis was done by applying a parametric one-way ANOVA test, using the post hoc Bonferroni comparison test with a confidence interval of 95%. ***, $p < 0.0001$; *, $p < 0.005$. BPZE1 or BPZE1P vaccination significantly reduced the recruitment of total cells in the airways after allergen exposure and challenge, compared to the non-vaccinated mice (Figure 7A). This reduction reflected essentially the reduction in eosinophil recruitment in the vaccinated mice (Figure 7B), whereas there was no significant change in the percentages of neutrophils or lymphocytes (Figure 7C and D) between the vaccinated and non-vaccinated mice. A small, but significant increase in the percentage of macrophages was observed in the mice that were vaccinated with BPZE1P (Figure 7E).

[0044] Following BAL, the right lung lobes were harvested and directly frozen in liquid Nitrogen for protein extraction. The lung lobes were resuspended in 1 mL of lysis buffer, PBS with 0.5% nonidet P40 and protease inhibitor cocktail (Roche®), and homogenized at 4°C using T-18 Ultra-Turrax (Ika®). The samples were centrifuged, and the supernatants were collected for total

protein quantification using the Pierce[™] BCA protein assay (Thermo Fisher Scientific), and for cytokine and chemokine measurements using the Cytokine 20-Plex Mouse Panel (Invitrogen[™], Thermo Fisher Scientific) per the manufacturer's specifications. Referring to Fig. 8, cytokine levels are represented as the normalization of the cytokine/chemokine quantification against total proteins measured in the lung lobe. Statistical analysis was done by applying a parametric one-way ANOVA test, using the post hoc Bonferroni comparison test with a confidence interval of 95%. **, $p < 0.001$. *, $p < 0.005$.

[0045] As shown in Figure 8, HDM (Derf 5IR)-treated mice produced significantly increased levels of IL1 α and IL1 β in the lungs, compared to non-treated mice. Vaccination with BPZE1 or BPZE1P prior to HDM sensitization significantly decreased these levels (Figure 8A and B). A similar trend was observed for IL6 and IL13 although the differences between the vaccinated and the non-vaccinated mice did not reach statistical significance (Figure 8C and D). Significantly lower levels of induced CXCL1 (KC), CXCL9 (MIG), CXCL10 (IP-10) and GM-CSF were observed in the vaccinated mice compared to the mice which were only treated with HDM (Figure 8F-H). Generally, there was no statistical difference between the BPZE1-vaccinated and the BPZE1P-vaccinated mice.

[0046] Example 7 - Vaccination of pre-sensitized subjects with either BPZE1 or BPZE1P present significantly lower levels of airway resistance compared to those that were not vaccinated. As shown in the diagram of Fig. 9, groups of 5-week-old mice were either sensitized intranasally with Derf 5IR or administrated PBS, and then either vaccinated with 10⁶ BPZE1 or BPZE1P, or left unvaccinated. Nine days later, the mice were challenged intranasally with Derf 5IR or PBS during 5 days. Two days after last challenge, the mice were anesthetized, and their resistance in the respiratory airway was measured by plethysmography as described above. Statistical analysis was done by applying a parametric 2-way ANOVA test, using the post hoc Bonferroni comparison test with a confidence interval of 95%. ***, $p < 0.0001$. **, $p < 0.001$. Comparisons between Derf 5IR and BPZE1 + Derf 5IR are represented by the solid line, and comparisons between Derf 5IR and BPZE1P + Derf 5IR are represented by the dashed line. The mice vaccinated with either BPZE1 or BPZE1P present significantly lower levels of airway resistance compared to those that were not vaccinated. Again, the airway resistance of the vaccinated mice was indistinguishable from that of the control group, which were not sensitized nor challenged with Derf 5IR.

Claims

1. Composition comprising a pharmaceutically acceptable carrier and a strain of live attenuated pertussis toxin (PTX)-deficient and pertactin-deficient *Bordetella* bacteria sufficient to colonize the respiratory tract of a subject for use in reducing or preventing

the development of airway inflammation in a subject diagnosed with asthma, interstitial lung disease, or allergic rhinitis wherein the strain of live attenuated pertussis toxin (PTX)-deficient and pertactin-deficient *Bordetella* bacteria is also deficient in tracheal cytotoxin (TCT) and dermonecrotic toxin (DNT).

2. Composition for use according to claim 1, wherein the airway inflammation is **characterized by** airway resistance, and the colonization of the respiratory tract of the subject results in reduction or prevention of the development of airway resistance in the subject.

3. Composition for use according to claim 1, wherein the airway inflammation is **characterized by** eosinophil infiltration in the lungs of the subject and the colonization of the respiratory tract of the subject results in reduction or prevention of the eosinophil infiltration.

4. Composition for use according to claim 1, wherein the airway inflammation is **characterized by** increased amounts of inflammatory cytokines in the lungs of the subject and the colonization of the respiratory tract of the subject results in reduction or prevention of the development of the increased amounts of inflammatory cytokines.

5. Composition for use according to claim 1, wherein the strain of live attenuated pertussis toxin (PTX)-deficient and pertactin-deficient *Bordetella* bacteria lacks a functional gene encoding pertactin.

6. Composition for use according to claim 1, wherein the strain of live attenuated pertussis toxin (PTX)-deficient and pertactin-deficient *Bordetella* bacteria is the BPZE1P strain deposited with the Collection Nationale de Cultures de Microorganismes under accession number CNCM-I-5150.

7. Composition for use according to claim 1, wherein the airway inflammation is caused by exposure to an allergen.

8. Composition for use according to claim 1, wherein the subject has greater than 10 ng per ml of anti-pertactin antibodies in its serum.

9. Composition for use according to claim 1, wherein the subject has previously been immunized with a vaccine containing pertactin or a pertactin-like antigen.

10. The BPZE1P strain deposited with the Collection Nationale de Cultures de Microorganismes under accession number CNCM-I-5150.

Patentansprüche

1. Verbindung enthaltend einen pharmazeutisch akzeptablen Trägerstoff und einen Bakterienstamm lebend abgeschwächter Pertussis-Toxin (PTX)-defizienter und Pertactin-defizienter *Bordetella* Bakterien, die ausreichen um den Atmungstrakt eines Subjekts zu bevölkern zur Verwendung in der Reduzierung oder Vorbeuge der Entwicklung einer Atemwegsentzündung in einem Subjekt, das mit Asthma, interstitieller Lungenkrankheit, oder allergischer Rhinitis diagnostiziert wurde, wobei der Bakterienstamm lebend abgeschwächter Pertussis-Toxin (PTX)-defizienter und Pertactin-defizienter *Bordetella* Bakterien auch in trachealem Zytotoxin (TCT) und dermonekrotischem Toxin (DNT) defizient ist. 5
2. Verbindung zur Verwendung nach Anspruch 1, wobei die Atemwegsentzündung durch Atemwegswiderstand charakterisiert ist und wobei die Bevölkering des Atmungstrakts des Subjekts in Reduktion oder Verhinderung der Entwicklung der Atemwegswiderstand in dem Subjekt resultiert. 20
3. Verbindung zur Verwendung nach Anspruch 1, wobei die Atemwegsentzündung durch die Infiltration von Eosinophilen in die Lunge des Subjekts charakterisiert ist und wobei die Bevölkering des Atmungstrakts des Subjekts in Reduktion oder Verhinderung der Infiltration der Eosinophile resultiert. 25 30
4. Verbindung zur Verwendung nach Anspruch 1, wobei die Atemwegsentzündung durch erhöhte Mengen inflammatorischer Zytokine in der Lunge des Subjekts charakterisiert ist und wobei die Bevölkering des Atmungstrakts des Subjekts in Reduktion oder Verhinderung der Entwicklung erhöhter Mengen inflammatorischer Zytokine resultiert. 35
5. Verbindung zur Verwendung nach Anspruch 1, wobei dem Bakterienstamm lebend abgeschwächter Pertussis-Toxin (PTX)-defizienter und Pertactin-defizienter *Bordetella* Bakterien ein funktionelles Gen, das Pertactin codiert, fehlt. 40 45
6. Verbindung zur Verwendung nach Anspruch 1, wobei der Bakterienstamm lebend abgeschwächter Pertussis-Toxin (PTX)-defizienter und Pertactin-defizienter *Bordetella* Bakterien der BPZE1P-Stamm ist, der bei der Collection Nationale de Microorganismes unter Zugangsnummer CNCM-I-5150 abgelegt ist. 50
7. Verbindung zur Verwendung nach Anspruch 1, wobei die Atemwegsentzündung durch das einem Allergen Aussetzen hervorgerufen ist. 55
8. Verbindung zur Verwendung nach Anspruch 1, wo-

bei das Subjekt mehr als 10 ng pro mL Antipertactinantikörper in seinem Serum hat.

9. Verbindung zur Verwendung nach Anspruch 1, wobei das Subjekt zuvor mit einem Impfstoff, der Pertactin oder ein Pertactin-ähnliches Antigen enthält, immunisiert wurde.
10. Der BPZE1P-Stamm, der bei der Collection Nationale de Microorganismes unter Zugangsnummer CNCM-I-5150 abgelegt ist.

Revendications

1. Composition comprenant un support pharmaceutiquement acceptable et une souche de bactérie *Bordetella* vivante atténuée déficiente pour la toxine coquelucheuse (PTX) et déficiente pour la pertactine, en une quantité suffisante pour coloniser les voies respiratoires d'un sujet pour une utilisation dans la réduction ou la prévention du développement d'une inflammation des voies respiratoires chez un sujet diagnostiqué avec de l'asthme, une maladie pulmonaire interstitielle ou une rhinite allergique, dans laquelle la souche de bactérie *Bordetella* vivante atténuée déficiente pour la toxine coquelucheuse (PTX) et déficiente pour la pertactine est également déficiente pour la cytotoxine trachéale (TCT) et pour la toxine dermonécrotique (DNT).
2. Composition pour utilisation selon la revendication 1, dans laquelle l'inflammation des voies respiratoires est **caractérisée par** une résistance des voies respiratoires, et la colonisation des voies respiratoires du sujet entraîne une réduction ou une prévention du développement d'une résistance des voies respiratoires chez le sujet.
3. Composition pour utilisation selon la revendication 1, dans laquelle l'inflammation des voies respiratoires est **caractérisée par** une infiltration d'éosinophiles dans les poumons du sujet et la colonisation des voies respiratoires du sujet entraîne une réduction ou une prévention de l'infiltration d'éosinophiles.
4. Composition pour utilisation selon la revendication 1, dans laquelle l'inflammation des voies respiratoires est **caractérisée par** des quantités accrues de cytokines inflammatoires dans les poumons du sujet, et la colonisation des voies respiratoires du sujet entraîne une réduction ou une prévention du développement des quantités accrues de cytokines inflammatoires.
5. Composition pour utilisation selon la revendication 1, dans laquelle la souche de bactérie *Bordetella* vivante atténuée déficiente pour la toxine coquelu-

cheuse (PTX) et déficient pour la pertactine est dépourvue d'un gène fonctionnel codant pour la pertactine.

6. Composition pour utilisation selon la revendication 1, dans laquelle la souche de bactérie *Bordetella* vivante atténuée déficient pour la toxine coquelucheuse (PTX) et déficient pour la pertactine est la souche BPZE1P déposée à la Collection Nationale de Cultures de Microorganismes sous le numéro d'accès CNCM-I-5150. 5 10
7. Composition pour utilisation selon la revendication 1, dans laquelle l'inflammation des voies respiratoires est causée par une exposition à un allergène. 15
8. Composition pour utilisation selon la revendication 1, dans laquelle le sujet présente plus de 10 ng par ml d'anticorps anti-pertactine dans son sérum. 20
9. Composition pour utilisation selon la revendication 1, dans laquelle le sujet a été préalablement immunisé avec un vaccin contenant de la pertactine ou un antigène analogue à la pertactine. 25
10. Souche BPZE1P déposée à la Collection Nationale de Cultures de Microorganismes sous le numéro d'accès CNCM-I-5150. 30

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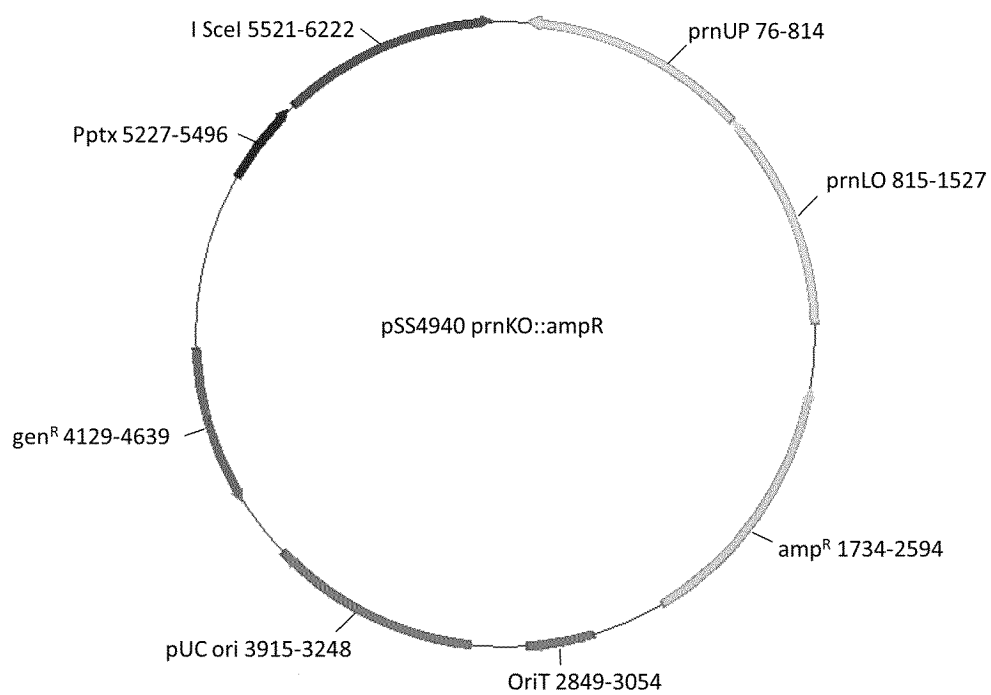
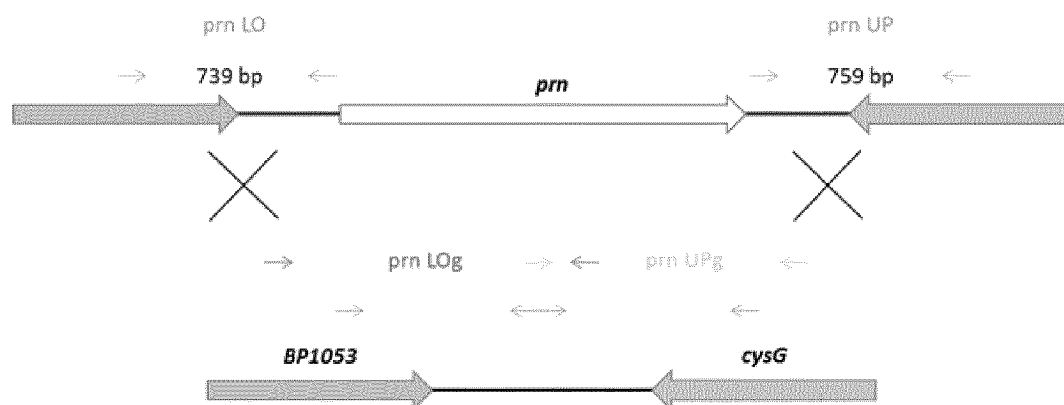
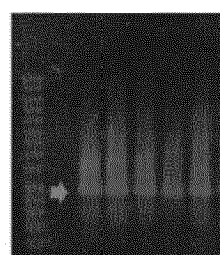


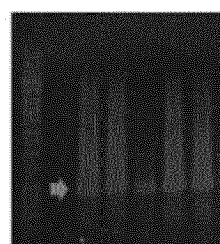
FIG. 1 A



BPZE1P construction PCR



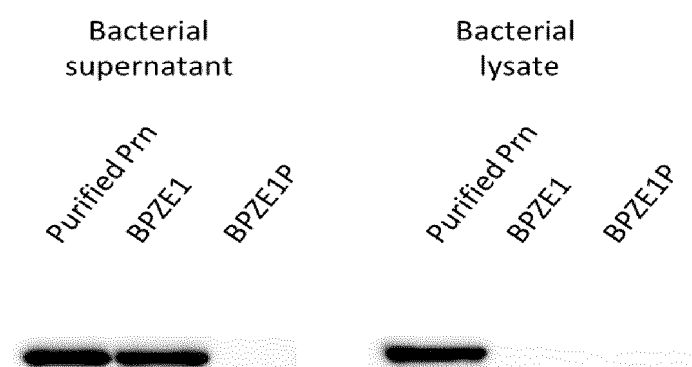
prn UPg PCR



prn LOg PCR

FIG. 1B

FIG. 1C



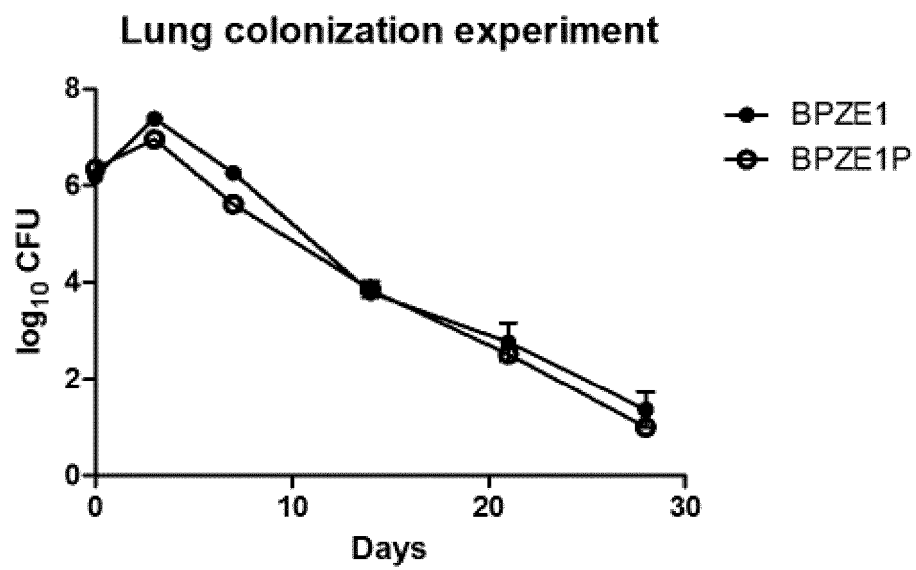


FIG. 2

Antibody titration against Bordetella total lysate

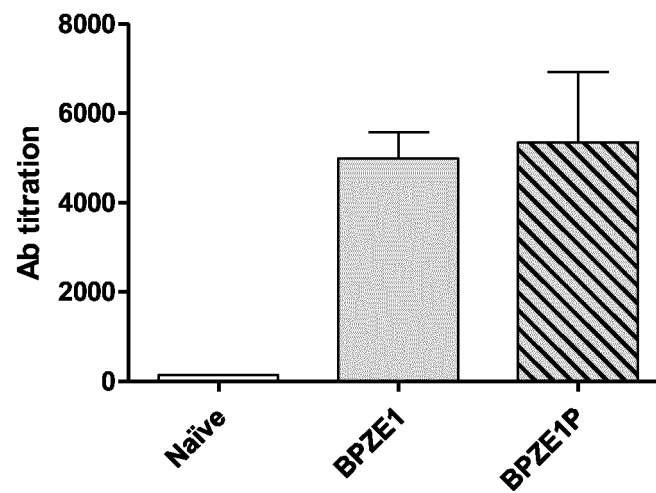


FIG. 3

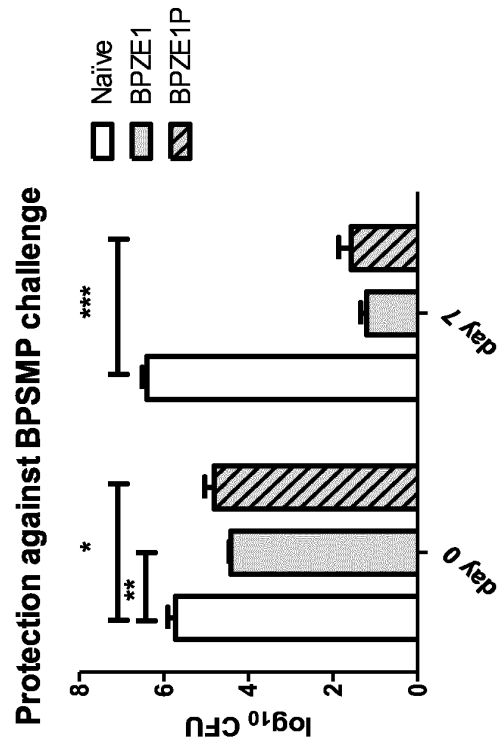


FIG. 4B

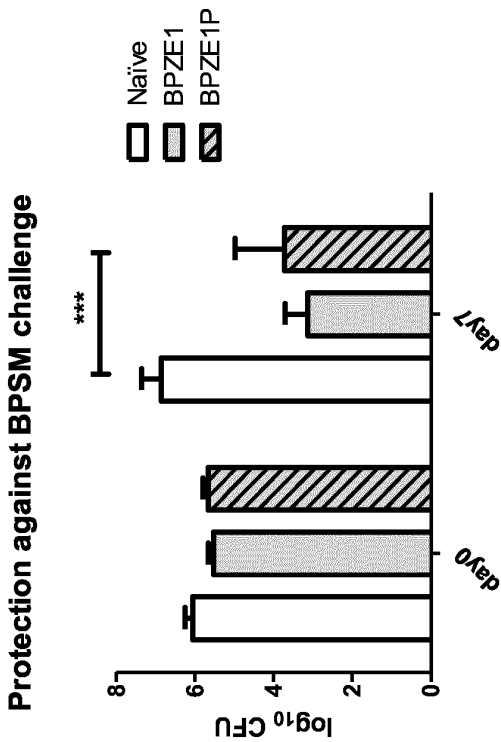


FIG. 4 A

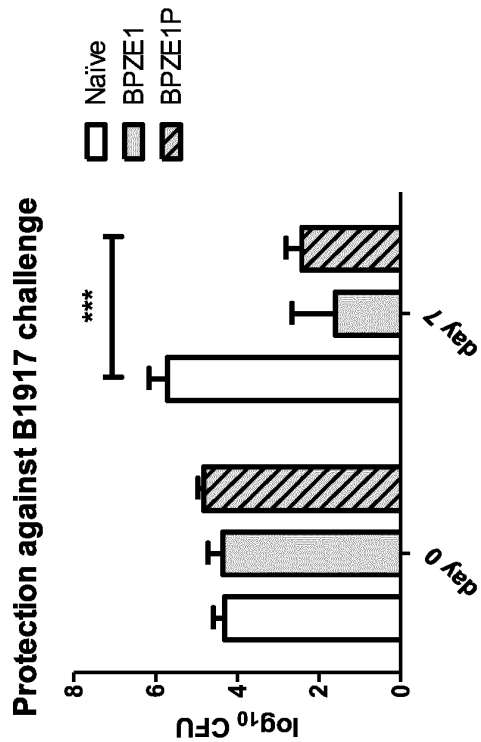


FIG. 4C

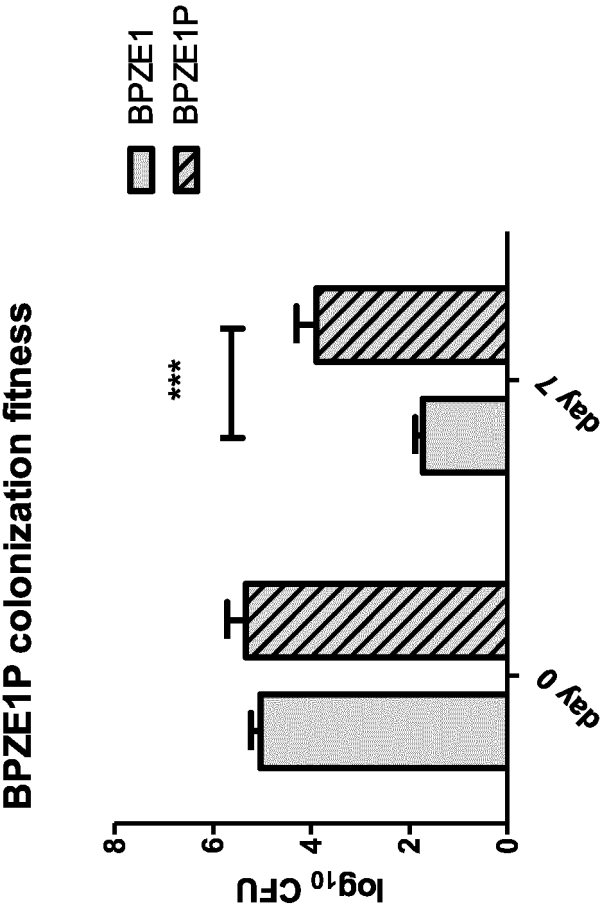


FIG. 5

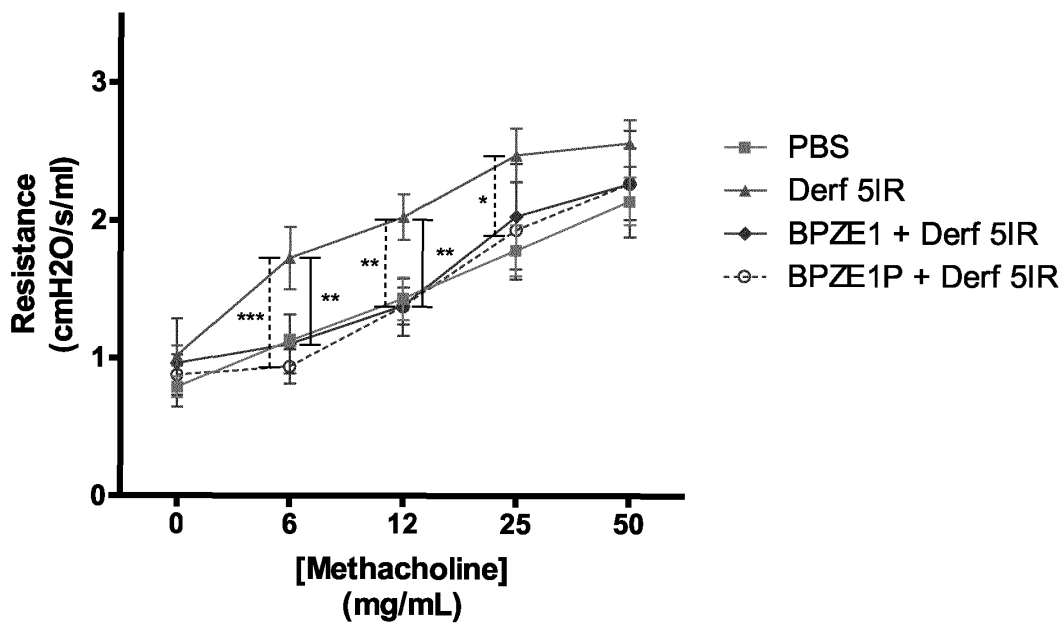
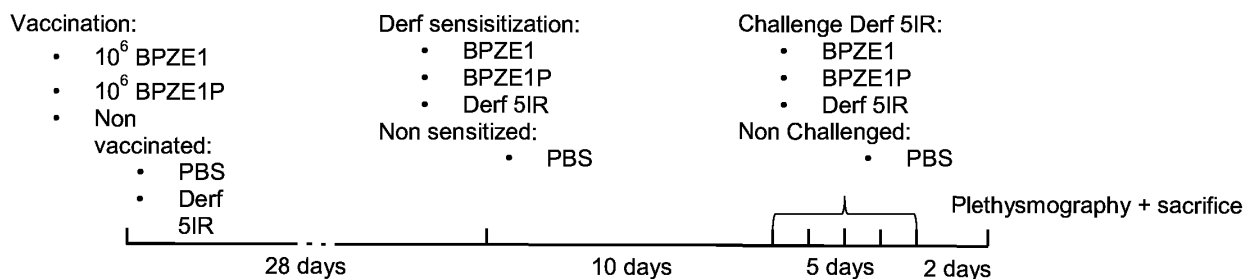


FIG. 6

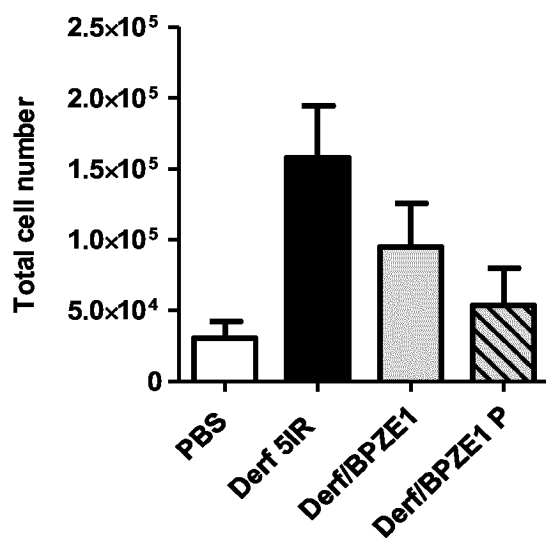


FIG. 7A

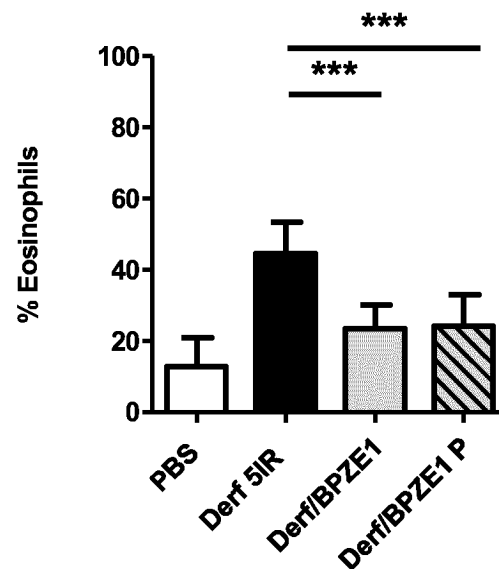


FIG. 7B

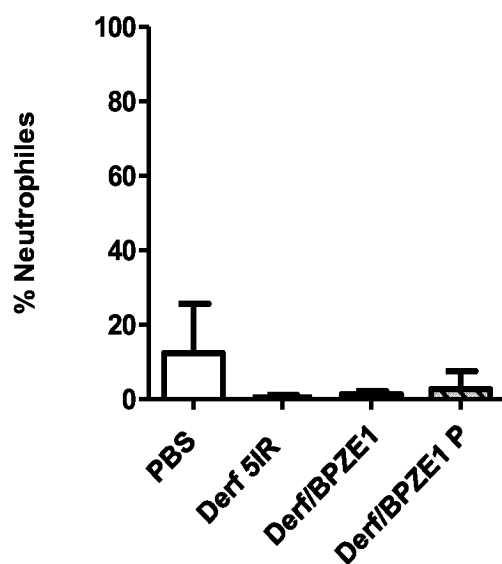


FIG. 7C

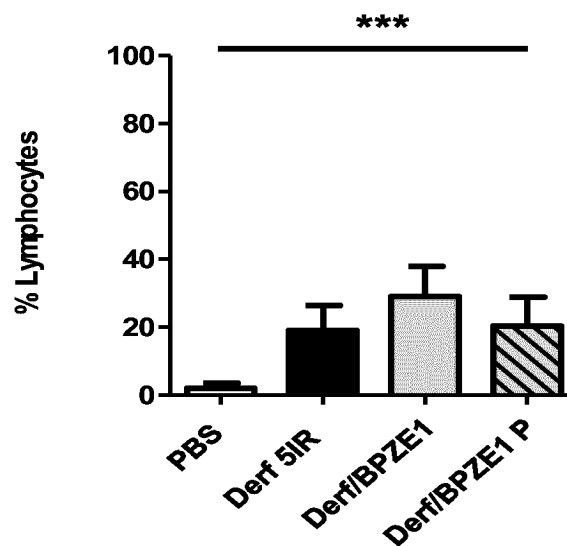


FIG. 7D

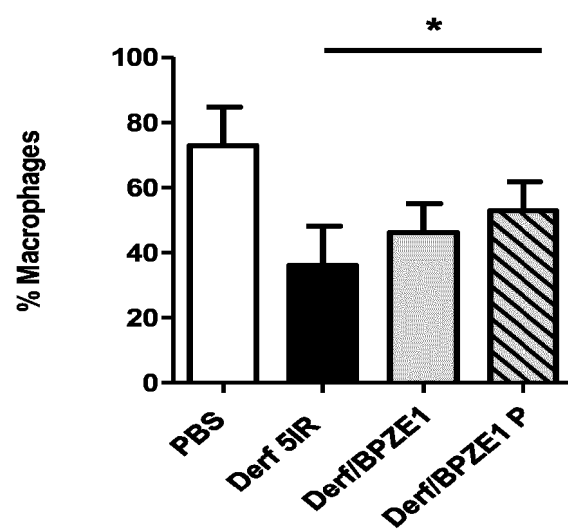


FIG. 7E

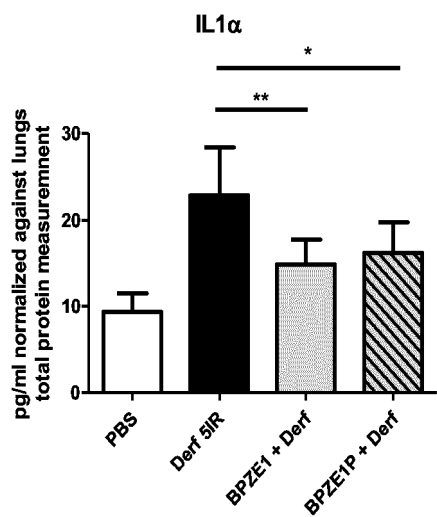


FIG. 8A

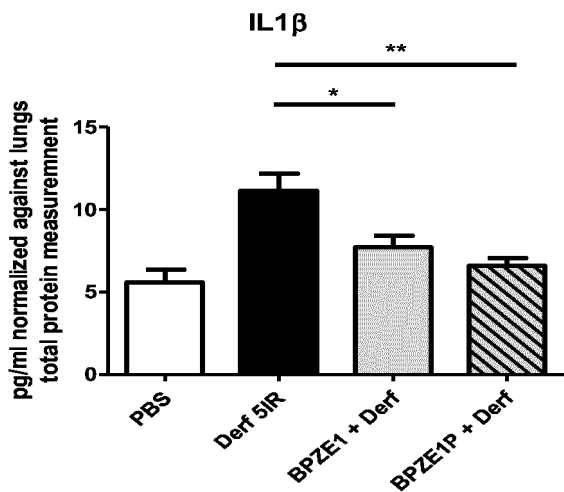


FIG. 8B

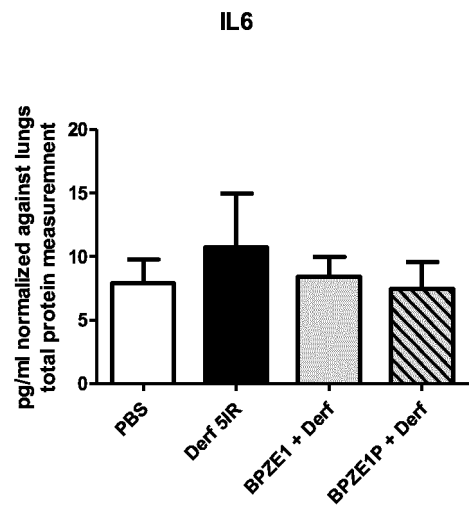


FIG. 8C

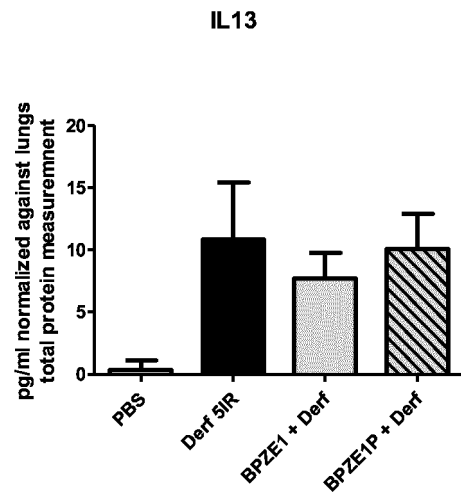


FIG. 8D

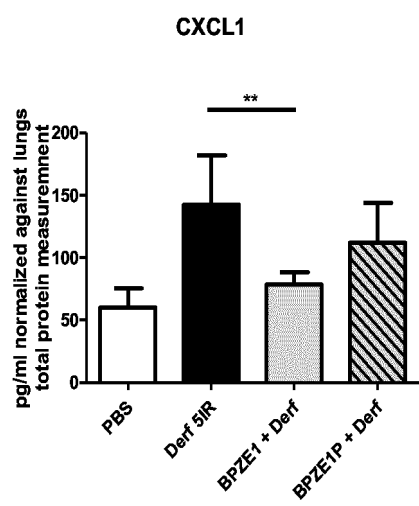


FIG.8E

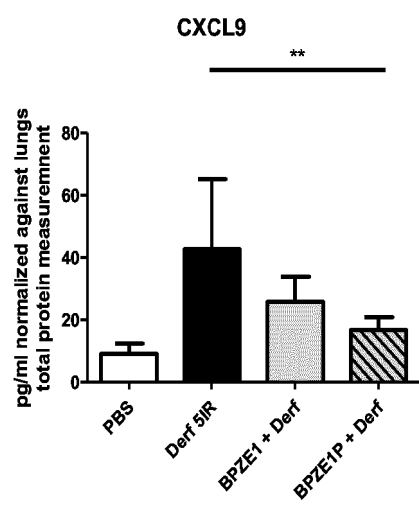


FIG. 8F

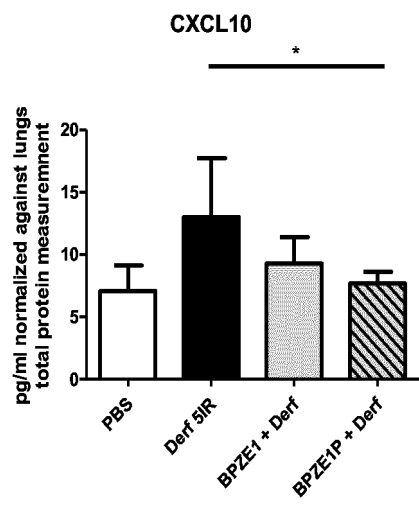


FIG. 8G

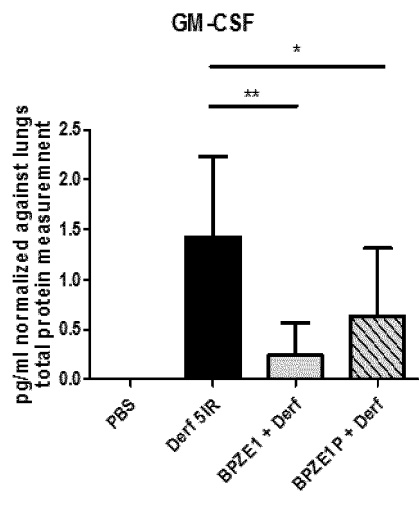


FIG. 8H

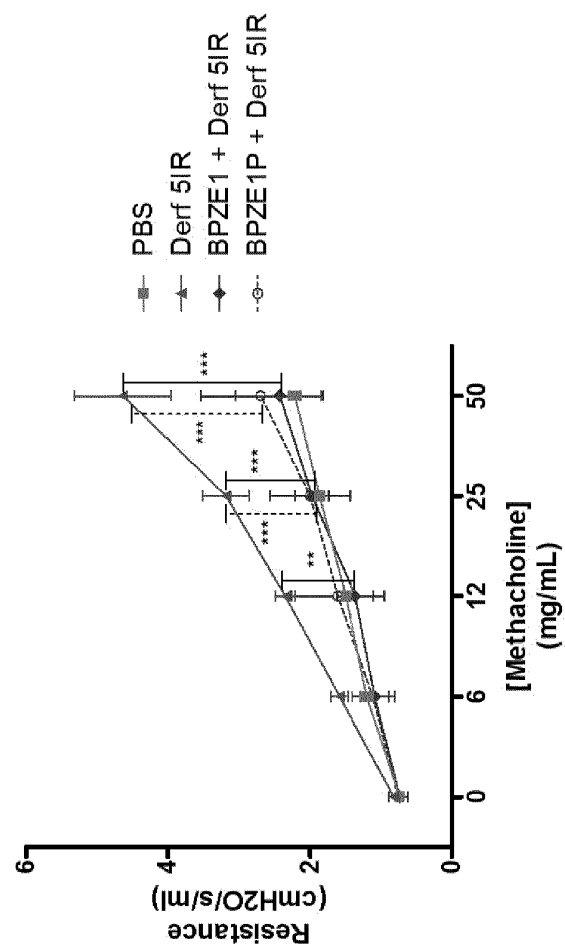
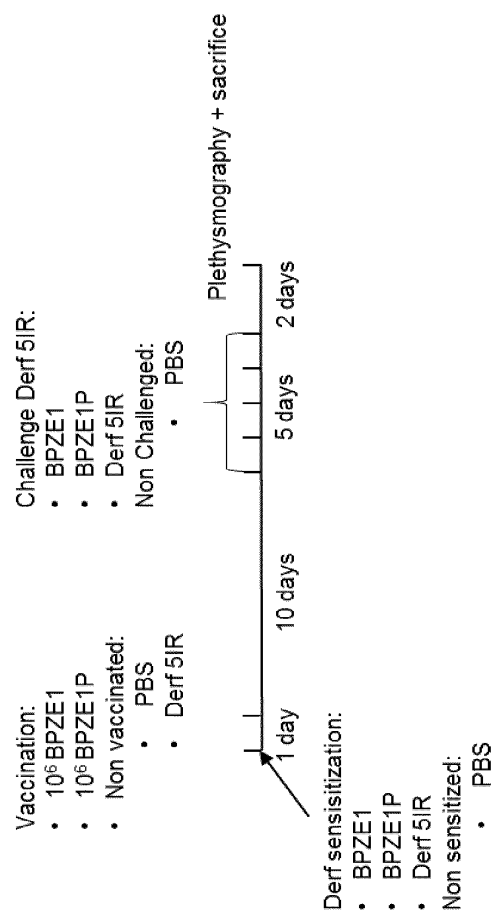


FIG. 9

REFERENCES CITED IN THE DESCRIPTION

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