(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
19 March 2009 (19.03.2009)

PCT

(10) International Publication Number
WO 2009/036137 A1

(51) International Patent Classification:
C12N 15/74 (2006.01) *C12N 1/20* (2006.01)

(21) International Application Number:
PCT/US2008/075972

(22) International Filing Date:
11 September 2008 (11.09.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
11/854,027 12 September 2007 (12.09.2007) US

(71) Applicant *(for all designated States except US)*: **AERAS GLOBAL TB VACCINE FOUNDATION** [US/US]; 1405 Research Blvd, 3rd Floor, Rockville, MD 20850 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **SADOFF, Jerald, C.** [US/US]; 1622 Kalmia Road, Nw, Washington, DC 20012 (US). **FAKRUDDIN-JAMILUDDIN, Mohamad** [US/US]; 7107 Oberlin Circle, Frederick, MD 21703 (US). **ANANTHA, Ravi, P.** [US/US]; 205 Lee Street, Apt. 208, Gaithersburg, MD 20877 (US). **FULKERSON, John, F., Jr.** [US/US]; 207 Lexington Drive, Silver Spring, MD 20901 (US).

(74) Agents: **WHITHAM, Michael, E.** et al.; Whitham, Curtis, Christofferson & Cook, PC, 11491 Sunset Hills Road, Suite 340, Reston, VA 20190 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

[Continued on next page]

(54) **Title:** METHODS TO INCREASE TRANSGENE EXPRESSION FROM BACTERIAL-BASED DELIVERY SYSTEMS BY CO-EXPRESSING SUPPRESSORS OF THE EUKARYOTIC TYPE I INTERFERON RESPONSE

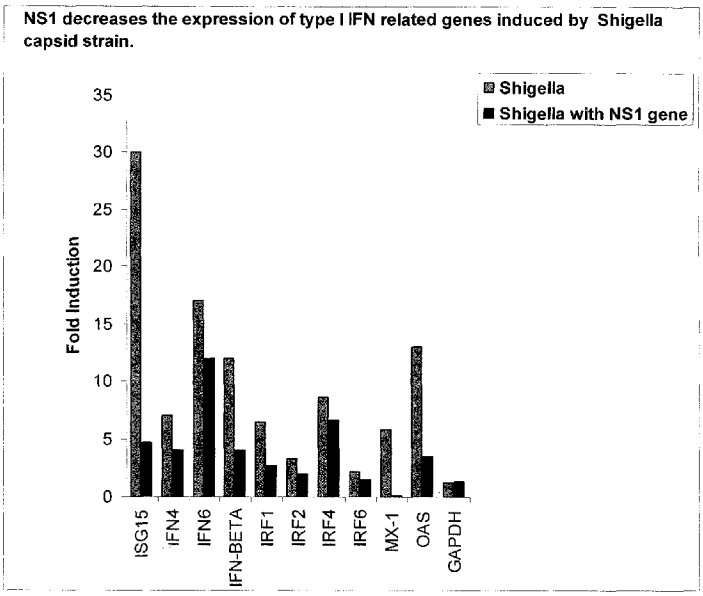

Figure 4.

(57) **Abstract:** Bacterial delivery systems with improved transgene expression are provided. The recombinant bacterial delivery systems deliver transgenes of interest and suppressors of the eukaryotic Type I interferon response to eukaryotic cells. Suppression of the eukaryotic Type I interferon response allows improved expression of the encoded transgene.

WO 2009/036137 A1

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

METHODS TO INCREASE TRANSGENE EXPRESSION FROM BACTERIAL-BASED DELIVERY SYSTEMS BY CO-EXPRESSING SUPPRESSORS OF THE EUKARYOTIC TYPE I INTERFERON RESPONSE

DESCRIPTION

BACKGROUND OF THE INVENTION

Field of the Invention

The invention generally relates to bacterial delivery systems that promote improved transgene expression in eukaryotic cells by inhibiting the innate type I interferon response. In particular, the invention provides recombinant bacterial delivery systems that deliver to eukaryotic cells: i) transgenes and ii) suppressors of the eukaryotic Type I interferon response.

Background of the Invention

Live attenuated mutants of several pathogenic bacteria have been exploited as potential vaccine vectors for heterologous antigen delivery by the mucosal route. Such live vectors offer the advantage of targeted delivery of macromolecules to mammalian cells and tissues in a single oral, intranasal or inhalational dose, thereby stimulating both systemic and mucosal immune responses. The great potential of bacteria-mediated transfer of plasmid DNA encoding vaccine antigens and/or therapeutic molecules has been demonstrated in experimental animal models of infectious diseases, tumors and gene deficiencies.

Unfortunately, bacterial vectored discharge of passenger RNA/DNA and other molecules for the expression of foreign proteins or inhibitory RNAs in mammals results in a type I interferon (IFN) response. A central component of the host's surveillance system for invading pathogens is an evolutionarily conserved family of pathogen recognition receptors (PRR) which bind patterned microbial/viral ligands ranging from cell wall components to nucleic acids. PRR signaling results in the activation of transcription factors such as Nuclear Factor - B (NF- B) and interferon regulatory factor 3 (IRF-3), which provide the inflammatory context for the rapid activation of host defenses. The NF- B pathway controls the expression of proinflammatory cytokines such as IL-1 and tumor necrosis factor-α,

whereas the IRF-3 pathway leads to the production of type I interferons (IFN-α and IFN-β). This initially produced "first wave" IFN triggers expression of a related factor, IRF-7, which is normally present in most cells at very low concentrations (Sato M et al., Immunity, 13(4)539-548; 2000). IRF-3 most likely cooperates with IRF-7 and is responsible for a positive feed back loop that initiates the synthesis of several IFN-α subtypes as the "second wave" IFNs (Marie et al., EMBO J 17(22), 6660-6669; 1998 and Sato M et al., FEBS Lett 441(1)106-110; 1998.). Type I IFNs activate several hundred IFN stimulated genes by autocrine and paracrine signaling (ISGs)(de Veer et al., J Leukocyte Biol 69(6) 912-920, 2001; Der et al., Proc. Natl. Acad. Sci. USA 95(26) 15623-15628; 1998), some of which code for antiviral proteins. To date, three IFN stimulated pathways have been firmly established. These include protein kinase R (PKR) (Williams, Oncogene 18(45) 6112-6120;1999), the 2'-5' oligoadenylate-synthetase (2'-5' OAS) (Silverman., J Interferon Res 14(3) 101-104;1994) and the Mx proteins (Haller and Kochs, Traffic 3(10) 710-714; 2002.). This type I IFN response limits the expression of foreign genes or inhibitory RNAs by means of PKR and 2'-5' OAS. Activated PKR blocks translation by phosphorylating the α subunit of eukaryotic initiation factor eIF2. On the other hand, 2-5A synthetases produce short, 2'-5' OAS associated oligoadenylates which activate RNase L, a single-stranded specific endoribonuclease that digests mRNA and ribosomal RNA. The importance of the Mx protein in host survival following infection with certain RNA viruses has been amply demonstrated (Hefti et al., J Virology 73(8) 6984-6991; 1999) but the exact mode of action is still unknown. This type I IFN response thus limits the expression of foreign nucleic acids by mechanisms which reduce RNA production and stability and also inhibits translation of message from passenger nucleic acids delivered by a bacterial vector.

Various components of bacterial vectors elicit the IFN response in host cells. The bacterium itself can trigger an IFN response through Toll-like receptors. Double stranded RNA produced by passenger nucleic acids during transcription not only induces type I IFNs but also directly activates PKR and 2'-5' OAS. Plasmid DNA, upon its delivery into the cytoplasm of mammalian cells, often contains cryptic promoters that generate anti-sense RNA which anneals with mRNA to form dsRNA. All these components of bacterial vectors thus diminish the efficacy of bacterial vectors as biomedical tools.

United States patent 6,525,029 (Falck-Perersen et al., Feb. 25, 2003) describes methods of inhibiting an immune response to a recombinant vector such as an adenoviral

vector. However, this technology is directed toward preventing humoral (e.g. antibody) responses to long-term expression of genes encoded by a vector and clearance of the vector by the immune system, and does not address prevention of a type I IFN response to a bacterial vector or its passenger nucleic acids.

The prior art has thus-far failed to provide bacterial vectors that eliminate or attenuate the type I IFN response of host cells.

SUMMARY OF THE INVENTION

The present invention provides recombinant bacterial expression vectors that successfully eliminate or attenuate the type I IFN response that is usually mounted by mammalian host cells in response to invasion by a bacterial expression vector. The recombinant bacterial expression vectors circumvent the usual IFN response by encoding factors that inhibit or suppress the type I IFN response in host cells. The IFN suppressor is expressed either i) in the bacterial cell for delivery as a protein or ii) in the eukaryotic cell from a nucleotide sequence that is delivered by the bacterial cell. Inhibition of the IFN response allows more robust expression of passenger genes delivered by the bacterial vector, and expression is enhanced only in a eukaryotic cell in which the type I IFN response has been suppressed. For example, when the recombinant bacterial expression vector of the invention delivers passenger nucleotide sequences encoding antigens to which an immune response is desired, production of those antigens by the mammalian cell is less impeded or not impeded by the host IFN system (or the degree of impeding is reduced), the antigens are expressed, and the desired immune response to the antigens may be produced. Alternatively, when the passenger nucleic acid sequence fro desired protein products such as enzymes, hormones or therapeutic or nutraceutic factors, production of these protein products by the mammalian cell is less impeded or not impeded by the host IFN system.

It is an object of this invention to provide a genetically engineered bacterium, comprising nucleic acid sequences encoding i) one or more passenger genes; and ii) one or more factors that inhibit a mammalian interferon response. The nucleic acid sequences encoding the one or more passenger genes are operably linked to a eukaryotic promoter, and the nucleic acid sequences encoding the one or more factors that inhibit a mammalian type I interferon response are operably linked to a eukaryotic promoter or a prokaryotic promoter. In yet another embodiment, the expressible nucleic acid sequences

encoding the one or more factors that inhibit a mammalian interferon response are present on a chromosome of the genetically engineered bacterium. In further embodiments, one or both of the: i) nucleic acid sequences encoding said one or more passenger genes, wherein the nucleic acid sequences are expressible in a eukaryotic cell; and ii) nucleic acid sequences encoding said one or more factors that inhibit a mammalian interferon response, are present on a plasmid. In addition, the one or more factors that inhibit a mammalian interferon response may be of viral origin. In some embodiments, the one or more passenger genes encode tuberculosis or malaria antigens. In further embodiments, the genetically engineered bacterium is a shigella bacterium or a myobacterium. Further, the passenger genes may be heterologous transgenes.

The invention further provides a method of increasing the production of one or more gene products of interest in a cell or tissue. The method comprises the step of administering to the cell or tissue a genetically engineered bacterium comprising nucleic acid sequences encoding: i) the one or more gene products of interest and ii) one or more factors that inhibit a mammalian interferon response. The nucleic acid sequences encoding the one or more passenger genes are operably linked to a eukaryotic promoter, and the nucleic acid sequences encoding the one or more factors that inhibit a mammalian type I interferon response are operably linked to a eukaryotic promoter or a prokaryotic promoter. The step of administering is carried out under conditions which allow the genetically engineered bacterium to invade the cell or tissue, and to produce the one or more gene products of interest and the one or more factors within the cell or tissue. In one embodiment, transcription of the expressible nucleic acid sequences is controlled by eukaryotic promoters. In another embodiment, transcription of the expressible nucleic acid sequences encoding the one or more factors that inhibit a mammalian interferon response is controlled by prokaryotic promoters. In yet another embodiment, the expressible nucleic acid sequences encoding the one or more factors that inhibit a mammalian interferon response are present on a chromosome of the genetically engineered bacterium. In further embodiments, one or both of: i) expressible nucleic acid sequences encoding the one or more gene products of interest, and ii) expressible nucleic acid sequences encoding the one or more factors that inhibit a mammalian interferon response, are present on a plasmid. In addition, the one or more factors that inhibit a mammalian interferon response may be of viral origin. In some embodiments, the one or more gene products of interest may be tuberculosis antigens. In

further embodiments, the genetically engineered bacterium is a shigella bacterium or a mycobacterium.

The invention further provides a method for inducing an immune response to an antigen of interest in a mammal. The method comprises the step of administering to the mammal a genetically engineered bacterium, comprising nucleic acid sequences encoding the antigen of interest; and nucleic acid sequences encoding one or more factors that inhibit a mammalian interferon response. The nucleic acid sequences encoding the one or more passenger genes are operably linked to a eukaryotic promoter, and the nucleic acid sequences encoding the one or more factors that inhibit a mammalian type I interferon response are operably linked to a eukaryotic promoter or a prokaryotic promoter. In one embodiment of the invention, the antigen of interest is a *Mycobacterium tuberculosis* antigen. In some embodiments, transcription of the expressible nucleic acid sequences is controlled by eukaryotic promoters. In other embodiments, transcription of the expressible nucleic acid sequences encoding the one or more factors that inhibit a mammalian interferon response is controlled by prokaryotic promoters. In yet other embodiments, the expressible nucleic acid sequences encoding the one or more factors that inhibit a mammalian interferon response are present on a chromosome of the genetically engineered bacterium. In some embodiments, one or both of: i) expressible nucleic acid sequences encoding the antigen of interest, and ii) expressible nucleic acid sequences encoding the one or more factors that inhibit a mammalian interferon response, are present on a plasmid. In further embodiments, the one or more factors that inhibit a mammalian interferon response are of viral origin.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Beta-galactosidase activity of cell lysates after invasion of HeLa or BHK-21 cells (IFN deficient) with *Shigella flexneri* NCD1 carrying a plasmid encoding eukaryotic expression of β-galactosidase. Black bar indicates β-galactosidase activity from cells post invasion with a bacterial strain harboring a plasmid encoding the *lacZ* gene; white bar indicates β-galactosidase activity from cells post invasion with a bacterial strain minus the *lacZ* plasmid.

Figure 2. β-galactosidase activity of lysates of HeLa cells after invasion with *Shigella flexneri* NCD1 harboring a plasmid encoding β-galactodisase and after co-invasion with *Shigella flexneri* NCD1 encoding an adenovirus derived inhibitor of PKR

(adenovirus-associated I, VAI).

Figure 3. Immunoblot showing transgene expression of green fluorescent protein (GFP) in HeLa cells post invasion with *Shigella flexneri* strain MPC51 which carries a eukaryotic GFP reporter gene only (lane 4) or GFP plus NS1(lane 5) or NSP1 (lane 2). Lane1: positive control; Lane 3: non-invaded HeLa cells.

Figure 4. Effect of NS1 protein IFN-α/β pathway in HeLa cells. NS1 suppresses (black bar) the expression of IFN associated genes induced by shigella (grey bar).

Figure 5. Effect of VAI RNA gene IFN-a/ß pathway in HeLa cells. VAI RNA gene suppresses (black bar) the expression of IFN associated genes induced by shigella (white bar).

Figure 6. Schematic map of IFN antagonist encoding reporter vector. Cloning of IFN antagonist gene in the back bone of GFP reporter vector. Single plasmid system for transcription of VAI RNA gene under the control of RNA polymerase III promoter and for expression of GFP gene by host cells via Pef-a promoter.

Figure 7. Invasion of HeLa cells with *Shigella flexneri* bearing a plasmid encoding GFP (pGFP) or a plasmid encoding GFP and VAI IFN-suppressor (pAdgfp). Expression of GFP was determined by immunoblot analysis using rabbit anti-GFP antiserum. Enhanced GFP protein expression was observed in the HeLa cells invaded by Shigella that contained both the GFP and VAI RNA gene (pAdgfp). The results of two independent experiments are shown.

Figure 8. Western blot of lysates of BHK21 cells invaded with Shigella carrying RNA encoding *Mtb* antigen 85A with and without a plasmid encoding IFN suppressor NS1. Lane 1, 51 MS85A 28; lane 2, 51 MS85A 37; lane 3, 51 MS85A pNS1 28; lane 4, 51 MS85A pNS1 37; lane 5, 51 MS85A pNS1 37; lane 6, *S. flexneri* NCD pcDNA-85A; lane 7, MPC51 pLM2653; lane 8, BHK21 cells; lane 9, BHK21 cells transfected with pcDNA-85A.

Figure 9A-C. Sequences of anti-viral immune response inhibitors. A, DNA sequence of NS1 from influenza virus (SEQ ID NO: 1); B, DNA sequence of NSP1 from rotavirus (SEQ ID NO: 2); C, RNA gene sequence of VAI from adenovirus (SEQ ID NO: 3).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS OF THE INVENTION

The recombinant bacterial expression vectors of the present invention are genetically

engineered to encode factors that eliminate, attenuate or suppress the type I interferon response that is usually mounted by mammalian host cells in response to invasion by a bacterium. These factors may be expressed by the bacterial vector cell or may be encoded in nucleic acids which are translated by the eukaryotic host cell. Attenuation or elimination of the IFN response in the eukaryotic host cell permits efficient transcription and translation of proteins and peptides of interest from vector introduced nucleic acids. Such vectored molecules may encode peptides and proteins that are necessary for the bacteria's reproduction and survival, as well as "passenger" molecules of interest contained within the bacterium. Examples of passenger nucleic acids of interest include but are not limited to, for example, antigens that the bacterium has been genetically engineered to encode. Because the eukaryotic host cell's type I IFN response is attenuated, the antigens are expressed persistently and at a level sufficient to cause the host cell to mount an immune response to the antigens. The bacterial expression vectors of the invention are thus ideal for use in vaccine preparations.

The bacterial expression vectors of the invention are genetically engineered to encode factors that eliminate, attenuate, inhibit or suppress the type I IFN response. Those of skill in the art will recognize that many viruses encode factors that target specific mediators of IFN responses. These factors can be referred to as IFN response antagonists. Among the best characterized viral targets are protein kinase R (PKR), RNaseL activating (2'-5') oligoadenylate synthetase and the Interferon Regulatory Factor (IRF) family of proteins.

By "inhibiting" or "suppressing" an immune response we mean that the typical or normal immune response that is elicited within a eukaryotic cell, is fully or partially inhibited, lessened, decreased, impeded, etc. Such inhibition may be detected and measured in any of several ways that will occur to those of skill in the art, including but not limited to: detection of a decrease in an amount, activity or attribute of a substance that is a hallmark of, is characteristic of or is associated with the immune response (e.g. IFNα, IFNβ, etc.). The level of inhibition is generally at least about 25%, preferably about 50% and more preferably about 60, 70, 80, 90 or 100%. A level of inhibition is typically measured by detecting a difference between an amount of one or more substances produced in a host cell that has been transfected with a vector of the invention (a vector that encodes one or more transgenes plus one or more immune system inhibitors), compared to the amount of the same substance produced in a control cell (a cell transfected with a vector that encodes the one or more

transgenes but does not encode an immune system inhibitor).

Similarly, "enhancing" or "increasing" expression of a transgene generally refers to an increase, augmentation, etc. in an amount of a transgene that is expressed (i.e. transcribed and translated) within a host cell transfected with a vector of the invention, compared to a control cell. Such enhancement may be measured by any of several methods that will occur to those of skill in the art, e.g. by detection of an increase in an amount, activity or attribute of a transgene product that is produced from the vector; by detection of an increase in an amount, activity or attribute of a substance associated with the transgene product that is produced (e.g. mRNA, substance or effect produced by the transgene product, antibodies to the transgene product, etc.). The level of enhancement is generally at least about 25%, preferably about 50% and more preferably about 60, 70, 80, 90 or 100%, or even more.

Immune response inhibiting proteins that are suitable for use in the present invention are encoded by a variety of viruses, examples of which include but are not limited to: rotavirus non structural protein1 (NSP1); influenza-A virus non structural protein 1 (NS1); adenovirus associated RNA I and II (VAI and II); vaccinia virus E3L or vIFN-α/β Rc protein; hepatitis C virus non structural protein 5A (NS5A) or NS3/4A protease; simian virus-V protein; Sendai virus C protein; ectromelia virus C12R protein; adenovirus E1A protein, C proteins of paramyxovirus, or human papillomavirus (HPV) E6 oncoprotein.

The fundamental importance of the IFN system as a host defense against viral infection is further illustrated by the finding that a number of viruses encode gene products that antagonize the IFN-induced antiviral response. Viruses utilize several different strategies to block the induction and action of IFN-inducible proteins. Both DNA and RNA viruses encode proteins that impair the activity of the IFN signaling pathway. Multiple mechanisms appear to be involved. Among these is mimicry. Several examples exist in which viruses encode products that mimic cellular components of the IFN signal transduction pathway. This molecular mimicry can lead to an antagonism of the IFN signaling process. Poxviruses, for example, encode soluble IFN receptor homologues (vIFN-Rc).These vIFN-Rc homologues are secreted from poxvirus-infected cells and bind IFNs, thereby preventing them from acting through their natural receptors to elicit an antiviral response. A vIFN-α/β Rc protein is secreted by vaccinia virus and several additional orthopoxviruses. The vIFN- α/β receptor homologue, the B18R gene product in the Western Reserve strain and the B19R product in the Copenhagen strain, binds several

different IFN-α subspecies as well as IFN-β and blocks IFN- α/β signaling activity. Three additional DNA viruses that affect IFN signaling are adenovirus, papillomavirus, and human herpesvirus 8 (HHV-8). The adenovirus E1A protein blocks IFN-mediated signaling at a point upstream of the activation of ISGF-3. The DNA binding activity of ISGF-3 is inhibited by E1A. The C proteins of SeV (SeV), a paramyxovirus that replicates in the cytoplasm of the host, circumvents the IFN-induced antiviral response by interfering with the transcriptional activation of IFN-inducible cellular genes. In the case of Sendai virus, the C proteins interfere with IFN action in at least two ways. C proteins prevent the synthesis of STAT-1 and they also induce an increased turnover of STAT-1. Human papillomavirus (HPV) E6 oncoprotein binds selectively to IRF-3 but only very poorly to other cellular IRFs including IRF-2 and IRF-9. Association of E6 with IRF-3 inhibits transactivation, thereby providing HPV with a mechanism to circumvent the IFN response. Adenovirus E1A protein also inhibits IRF-3-mediated transcriptional activation by a mechanism dependent on the ability of E1A to bind p300. HHV-8, a gamma herpes virus associated with Kaposi's sarcoma, synthesizes an IRF homologue (vIRF) that functions as a repressor of transcriptional activation induced by IFN- α/β. The HHV-8-encoded vIRF protein also represses IRF-1-mediated transcriptional activation. Two other herpesviruses, varicella-zoster virus (VZV) and cytomegalovirus (CMV), also disrupt the function of the IFN signal transduction pathway. VZV inhibits the expression of STAT-1 and JAK-2 proteins but has little effect on JAK-1. A different strategy of antagonism occurs in CMV-infected cells, where MHC class II expression also is inhibited. There is a specific decrease in the level of JAK-1 due to enhanced protein degradation in CMV-infected fibroblasts. Several nonsegmented negative-strand RNA viruses encode gene products that antagonize IFN receptor-mediated signaling from type I IFN receptors. For example, infection with simian virus 5 or mumps virus leads to an increased proteosome-mediated degradation of STAT-1 whereas in cells infected with parainfluenza virus type 2 there is a degradation of STAT-2. The VP35 protein of Ebola virus, a negative-strand RNA virus, functions as a type I IFN antagonist although the precise biochemical mechanism of the antagonism has not yet been defined. VP35 inhibits virus induction of the IFN- ß promoter and dsRNA- and virus- mediated activation of ISRE-driven gene expression. Nucleic acid sequences encoding three exemplary inhibitors (NS1 from influenza virus, NSP1 from rotavirus, and VAI from adenovirus) are presented in Figures 9A-C.

IFN suppressing factors may also be obtained from other non-viral sources, for example, from the host cell (e.g. suppressors of cytokine signaling (SOCS), dominant negative of PKR and dominant negative of RNaseL) and may be utilized in the practice of the present invention. Any factor that suppresses or attenuates the IFN response (e.g. siRNAs against Interferon stimulated genes) and which is encoded by a nucleic acid sequence that can be genetically engineered into and successfully expressed from a bacterial expression vector may be used in the practice of the present invention. Examples include but are not limited to those described above, as well as various autocrine IFN-induced effector and modulator proteins essential for the antiviral actions of type I IFNs such as RNA-dependent protein kinase (PKR); 2, 5'-oligoadenylate synthetase (OAS); RNase L; Mx protein GTPases; IFN-inducible RNA-specific adenosine deaminase (ADAR1); IFN regulatory factors such as IRF-5 and IRF-7; transcription factors of the (IRF) family such as TLR3, TLR4, TLR7 and TLR9; factors such as IRAK1/4 and TRAF6; RLR, MyD88, TAK1, TOLLIP, TIFA, etc.

By "bacterial expression vector" we mean a bacterial cell that has been genetically engineered to contain and deliver and/or express nucleic acid sequences of interest. Examples of bacteria which can be utilized in this manner include but are not limited to *Campylobacter spp*, *Neisseria spp.*, *Haemophilus spp*, *Aeromonas spp*, *Francisella spp*, *Yersinia spp*, *Klebsiella spp*, *Bordetella spp*, *Legionella spp*, *Corynebacterium spp*, *Citrobacter spp*, *Chlamydia spp*, *Brucella spp*, *Pseudomonas spp*, *Helicobacter spp*, or *Vibrio spp.*

The particular *Campylobacter* strain employed is not critical to the present invention. Examples of *Campylobacter* strains that can be employed in the present invention include but are not limited to: *C. jejuni* (ATCC Nos. 43436, 43437, 43438), *C. hyointestinalis* (ATCC No. 35217), *C. fetus* (ATCC No. 19438) *C. fecalis* (ATCC No. 33709) *C. doylei* (ATCC No. 49349) and *C. coli* (ATCC Nos. 33559, 43133).

The particular *Yersinia* strain employed is not critical to the present invention. Examples of *Yersinia* strains which can be employed in the present invention include: *Y. enterocolitica* (ATCC No. 9610) or *Y. pestis* (ATCC No.19428), *Y. enterocolitica* Ye03-R2 (al Hendy *et al.*, Infect. Immun., 60:870; 1992) or *Y. enterocolitica aro*A (O'Gaora *et al.*, Micro. Path., 9:105; 1990).

The particular *Klebsiella* strain employed is not critical to the present invention.

Examples of *Klebsiella* strains that can be employed in the present invention include *K. pneumoniae* (ATCC No. 13884).

The particular *Bordetella* strain employed is not critical to the present invention. Examples of *Bordetella* strains which can be employed in the present invention include *B. pertussis*, and *B. bronchiseptica* (ATCC No. 19395).

The particular *Neisseria* strain employed is not critical to the present invention. Examples of *Neisseria* strains that can be employed in the present invention include *N. meningitidis* (ATCC No. 13077) and *N. gonorrhoeae* (ATCC No. 19424), *N. gonorrhoeae* MS11 *aro* mutant (Chamberlain *et al.*, Micro. Path., 15:51-63; 1993).

The particular *Aeromonas* strain employed is not critical to the present invention. Examples of *Aeromonas* strains that can be employed in the present invention include *A. salminocida* (ATCC No. 33658), *A. schuberii* (ATCC No. 43700), *A. hydrophila*, *A. eucrenophila* (ATCC No. 23309).

The particular *Francisella* strain employed is not critical to the present invention. Examples of *Francisella* strains that can be employed in the present invention include *F. tularensis* (ATCC No. 15482).

The particular *Corynebacterium* strain employed is not critical to the present invention. Examples of *Corynebacterium* strains that can be employed in the present invention include *C. pseudotuberculosis* (ATCC No. 19410).

The particular *Citrobacter* strain employed is not critical to the present invention. Examples of *Citrobacter* strains that can be employed in the present invention include *C. freundii* (ATCC No. 8090).

The particular *Chlamydia* strain employed is not critical to the present invention. Examples of *Chlamydia* strains that can be employed in the present invention include *C. pneumoniae* (ATCC No. VR1310).

The particular *Haemophilus* strain employed is not critical to the present invention. Examples of *Haemophilus* strains that can be employed in the present invention include *H. influenzae* (Lee *et al.*, J. Biol. Chem. 270:27151; 1995), *H. somnus* (ATCC No. 43625).

The particular *Brucella* strain employed is not critical to the present invention. Examples of *Brucella* strains that can be employed in the present invention include *B. abortus* (ATCC No. 23448).

The particular *Legionella* strain employed is not critical to the present invention.

Examples of *Legionella* strains that can be employed in the present invention include *L. pneumophila* (ATCC No. 33156), or a *L. pneumophila mip* mutant (Ott, FEMS Micro. Rev., 14:161; 1994).

The particular *Pseudomonas* strain employed is not critical to the present invention. Examples of *Pseudomonas* strains that can be employed in the present invention include *P. aeruginosa* (ATCC No. 23267).

The particular *Helicobacter* strain employed is not critical to the present invention. Examples of *Helicobacter* strains that can be employed in the present invention include *H. pylori* (ATCC No. 43504), *H. mustelae* (ATCC No. 43772).

The particular *Vibrio* strain employed is not critical to the present invention. Examples of *Vibrio* strains that can be employed in the present invention include *Vibrio cholerae* (ATCC No. 14035), *Vibrio cincinnatiensis* (ATCC No. 35912), *V. cholerae* RSI virulence mutant (Taylor *et al.*, J. Infect. Dis., 170:1518-1523; 1994) and *V. cholerae ctx*A, *ace*, *zot*, *cep* mutant (Waldor J *et al.*, Infect. Dis.,170:278-283; 1994).

In a preferred embodiment, the bacterial strain from which the vector strain is developed in the present invention includes bacteria that possess the potential to serve both as a carrier and as a vaccine vectors, such as the *Enterobacteriaceae*, including but not limited to *Escherichia spp*, *Shigella spp*, and *Salmonella spp*. Gram-positive and acid-fast vector strains could similarly be constructed from *Listeria monocytogenes* or *Mycobacterium spp*.

The particular *Escherichia* strain employed is not critical to the present invention. Examples of *Escherichia* strains which can be employed in the present invention include *Escherichia coli* strains DH5α, HB 101, HS-4, 4608-58, 1184-68, 53638-C-17, 13-80, and 6-81 (See, e.g. Sambrook *et al.*, supra; Grant *et* al., supra; Sansonetti *et al.*, Ann. Microbiol. (Inst. Pasteur), 132A:351; 1982), enterotoxigenic *E. coli* (See, e.g. Evans *et al.*, Infect. Immun., 12:656; 1975), enteropathogenic *E. coli* (See, e.g. Donnenberg *et al.*, J. Infect. Dis., 169:831; 1994), enteroinvasive *E. coli* (See, e.g. Small *et al.*, Infect Immun., 55:1674; 1987) and enterohemorrhagic *E. coli* (See, e.g. McKee and O'Brien, Infect. Immun., 63:2070; 1995).

The particular *Salmonella* strain employed is not critical to the present invention. Examples of *Salmonella* strains that can be employed in the present invention include *S. typhi* (see, e.g. ATCC No. 7251), *S. typhimurium* (see, e.g. ATCC No. 13311), *Salmonella*

galinarum (ATCC No. 9184), *Salmonella enteriditis* (see, e.g. ATCC No. 4931) and *Salmonella typhimurium* (see, e.g. ATCC No. 6994). *S. typhi aroC, aroD* double mutant (see, e.g. Hone *et al.*, Vacc., 9:810-816; 1991), *S. typhimurium aro*A mutant (see, e.g. Mastroeni *et* al., Micro. Pathol., 13:477-491; 1992).

The particular *Shigella* strain employed is not critical to the present invention. Examples of *Shigella* strains that can be employed in the present invention include *Shigella flexneri* (see, e.g. ATCC No. 29903), *Shigella flexneri* CVD1203 (see, e.g. Noriega *et al.*, Infect. Immun. 62:5168; 1994), *Shigella flexneri* 15D (see, e.g. Sizemore *et al.*, Science 270:299; 1995), *Shigella sonnei* (see, e.g. ATCC No. 29930), and *Shigella dysenteriae* (see, e.g. ATCC No. 13313).

The particular *Mycobacterium* strain employed is not critical to the present invention. Examples of *Mycobacterium* strains that can be employed in the present invention include *M. tuberculosis* CDC1551 strain (See, e.g. Griffith *et al.*, Am. J. Respir. Crit. Care Med. Aug;152(2):808; 1995), *M. tuberculosis* Beijing strain (Soolingen *et al.*, 1995) H37Rv strain (ATCC#:25618), *M. tuberculosis* pantothenate auxotroph strain (Sambandamurthy, Nat. Med. 2002 8(10):1171; 2002), *M. tuberculosis rpoV* mutant strain (Collins *et* al., Proc Natl Acad Sci USA. 92(17):8036; 1995), *M. tuberculosis* leucine auxotroph strain (Hondalus *et* al., Infect. Immun. 68(5):2888; 2000), Bacille Calmette-Guérin (BCG) Danish strain (ATCC # 35733), BCG Japanese strain (ATCC # 35737), BCG, Chicago strain (ATCC # 27289), BCG Copenhagen strain (ATCC #: 27290), BCG Pasteur strain (ATCC #: 35734), BCG Glaxo strain (ATCC #: 35741), BCG Connaught strain (ATCC # 35745), BCG Montreal (ATCC # 35746).

The particular *Listeria monocytogenes* strain employed is not critical to the present invention. Examples of *Listeria monocytogenes* strains which can be employed in the present invention include *L. monocytogenes* strain 10403S (e.g. Stevens *et al.*, J .Virol 78:8210-8218; 2004) or mutant *L. monocytogenes* strains such as (i) *actA plcB* double mutant (Peters *et* al., FEMS Immunology and Medical Microbiology 35: 243-253; 2003); (Angelakopoulous *et al.*, Infect and Immunity 70: 3592-3601; 2002); (ii) *dal dat* double mutant for alanine racemase gene and D-amino acid aminotransferase gene (Thompson *et al.*, Infect and Immunity 66:3552-3561; 1998).

In some embodiments of the invention, the bacteria are, in particular, *Shigella* species, in particular attenuated invasive *Shigella flexneri* 2a. These strains, MPC51 and

NCD1 are derivatives of *S. flexneri* strain 2457T into which *asd* and *murI* deletion mutations have been introduced. The *asd* defect is complemented by the expression vector encoded *asd* allele and the *murI* mutation results in the inability of the strain to synthesize D-glutamate; hence, these strains are incapable of synthesizing a proper cell wall in the absence of diaminopimelic acid and D-glutamate, which promotes lysis of the bacterial cell after invasion of a eukaryotic cell. As measured by a gentamicin protection assay, the HeLa cell invasive behavior of the Δ*asd*, Δ*murI* double mutant MPC51 was similar to that of the parental strain and MPC51pYA3342 (plasmid encoding *asd*). The strain has been further modified by removal of the kanamycin resistance gene previously inserted in the chromosomal *asd* locus. The resultant strain, *Shigella flexneri* NCD1, is thus free of antibiotic resistance markers, still retains chromosomal deletions of the *asd* and *murI* genes, and is acceptable for pharmacologic use in humans under current regulatory requirements. NCD1 has also been shown to be invasive in HeLa and Caco-2 cells in a manner similar to the parent strain.

Generally, the bacterial expression vectors of the invention are genetically engineered to encode and deliver both the IFN inhibiting factors and one or more other genes of interest i.e. passenger genes. The passenger genes are typically heterologous transgenes that originate from another organism, such as another bacteria or pathogen, and may be from any organism. However, the "passenger gene" may also be a gene that naturally occurs in the bacterial vector itself (i.e. is derived from or originates from the bacteria that serves as a vector), but one or more additional copies are genetically engineered in the bacterial vector to be under the control of a promoter that, for example, increases the level of transcription above that which is typical for the bacteria, or a promoter that is specific for a particular type of host cell or tissue (e.g. lung, lymph node, dendritic cell, etc). Further, "passenger gene" is intended to refer not only to entire "genes" but to any sequence that encodes a peptide, polypeptide, protein, or nucleic acid of interest, i.e. an entire "gene" per se may not be included, but rather the portion of a gene that encodes a polypeptide or peptide of interest e.g. an antigenic peptide. Further, various other constructions may be encoded by passenger genes, e.g. chimeric proteins, or various mutant (either naturally occurring or genetically engineered) forms of an amino acid sequence. In addition, totally artificial amino acid sequences that do not appear in nature may also be encoded. The bacterial expression vector is genetically engineered to contain one or more of such "passenger genes", and may also

encode multiple copies of individual passenger genes. The recombinant bacterial expression vector functions as a vector to carry the passenger gene(s) into host cells that are invaded by the bacterium, where the gene product is expressed, i.e. the gene sequences are expressible and transcription and/or translation of the gene product occurs within the host cell that is invaded by the bacterium. The sequences encoding the passenger genes are operatively (operably) linked to expression control sequences, particularly expression control sequences that allow expression within the bacterium and/or the eukaryotic host cell. By "operably linked" we mean that the nucleic acid sequences encoding the passenger genes are amenable to successfully transcription and translation within a suitable host cell, such as a bacterial vector or a mammalian cell.

In particular, such passenger genes may encode one or more peptides or proteins that are antigens, and to which it is desired to elicit an immune response. Those of skill in the art will recognize that a wide variety of such antigens exists, including but not limited to those associated with infectious agents such as various viruses, bacteria, fungi, various parasites, etc. The viral pathogens, from which the viral antigens are derived, include, but are not limited to, Orthomyxoviruses, such as influenza virus (Taxonomy ID: 59771; Retroviruses, such as RSV, HTLV-1 (Taxonomy ID: 39015), and HTLV-II (Taxonomy ID: 11909), Papillomaviridae such as HPV (Taxonomy ID: 337043), Herpesviruses such as EBV Taxonomy ID: 10295); CMV (Taxonomy ID: 10358) or herpes simplex virus (ATCC #: VR-1487); Lentiviruses, such as HIV-1 (Taxonomy ID: 12721) and HIV-2 Taxonomy ID: 11709); Rhabdoviruses, such as rabies; Picornoviruses, such as Poliovirus (Taxonomy ID: 12080); Poxviruses, such as vaccinia (Taxonomy ID: 10245); Rotavirus (Taxonomy ID: 10912); and Parvoviruses, such as adeno-associated virus 1 (Taxonomy ID: 85106).

Examples of viral antigens can be found in the group including but not limited to the human immunodeficiency virus antigens Nef (National Institute of Allergy and Infectious Disease HIV Repository Cat. # 183; Genbank accession # AF238278), Gag, Env (National Institute of Allergy and Infectious Disease HIV Repository Cat. # 2433; Genbank accession # U39362), Tat (National Institute of Allergy and Infectious Disease HIV Repository Cat. # 827; Genbank accession # M13137), mutant derivatives of Tat, such as Tat-31-45 (Agwale et al., Proc. Natl. Acad. Sci. USA 99:10037; 2002), Rev (National Institute of Allergy and Infectious Disease HIV Repository Cat. # 2088; Genbank accession # L14572), and Pol (National Institute of Allergy and Infectious Disease HIV Repository Cat. # 238; Genbank

accession # AJ237568) and T and B cell epitopes of gp120 (Hanke and McMichael, AIDS Immunol Lett., 66:177; 1999); (Hanke, *et al.*, Vaccine, 17:589; 1999); (Palker *et al.*, J. Immunol., 142:3612 3619; 1989) chimeric derivatives of HIV-1 Env and gp120, such as but not restricted to fusion between gp120 and CD4 (Fouts *et al.*, J. Virol. 2000, 74:11427-11436; 2000); truncated or modified derivatives of HIV-1 env, such as but not restricted to gp140 (Stamatos *et al.*, J Virol, 72:9656-9667; 1998) or derivatives of HIV-1 Env and/or gp140 thereof (Binley, *et al.*, J Virol, 76:2606-2616; 2002); (Sanders, *et al.*, J Virol, 74:5091-5100 (2000); (Binley, *et al.* J Virol, 74:627-643; 2000), the hepatitis B surface antigen (Genbank accession # AF043578); (Wu *et al.*, Proc. Natl. Acad. Sci., USA, 86:4726 4730; 1989); rotavirus antigens, such as VP4 (Genbank accession # AJ293721); (Mackow *et al.*, Proc. Natl. Acad. Sci., USA, 87:518 522; 1990) and VP7 (GenBank accession # AY003871); (Green *et al.*, J. Virol., 62:1819 1823; 1988), influenza virus antigens such as hemagglutinin or (GenBank accession # AJ404627); (Pertmer and Robinson, Virology, 257:406; 1999); nucleoprotein (GenBank accession # AJ289872); (Lin *et al.*, Proc. Natl. Acad. Sci., 97: 9654-9658; 2000) herpes simplex virus antigens such as thymidine kinase (Genbank accession # AB047378; (Whitley *et al.*, In: New Generation Vaccines, pages 825-854).

The bacterial pathogens, from which the bacterial antigens are derived, include but are not limited to: *Mycobacterium spp.*, *Helicobacter pylori*, *Salmonella spp.*, *Shigella spp.*, *E. coli*, *Rickettsia spp.*, *Listeria spp.*, *Legionella pneumoniae*, *Pseudomonas spp.*, *Vibrio spp.*, *Bacillus anthracis* and *Borellia burgdorferi.*

Examples of protective antigens of bacterial pathogens include the somatic antigens of enterotoxigenic *E. coli*, such as the CFA/I fimbrial antigen (Yamamoto *et al.*, Infect. Immun., 50:925 928; 1985) and the nontoxic B subunit of the heat labile toxin (*et al.*, Infect. Immun., 40:888-893; 1983); pertactin of *Bordetella pertussis* (Roberts *et al.*, Vacc., 10:43-48; 1992), adenylate cyclase hemolysin of *B. pertussis* (Guiso *et al.*, Micro. Path., 11:423-431; 1991), fragment C of tetanus toxin of *Clostridium tetani* (Fairweather *et al.*, Infect. Immun., 58:1323 1326; 1990), OspA of *Borellia burgdorferi* (Sikand *et al.*, Pediatrics, 108:123-128; 2001); (Wallich *et al.*, Infect Immun, 69:2130-2136; 2001), protective paracrystalline-surface-layer proteins of *Rickettsia prowazekii* and *Rickettsia typhi* (Carl *et al.*, Proc Natl Acad Sci U S A, 87:8237-8241; 1990), the listeriolysin (also known as "Llo" and "Hly") and/or the superoxide dismutase (also know as "SOD" and "p60") of

Listeria monocytogenes (Hess, J., *et al.*, Infect. Immun. 65:1286-92; 1997); Hess, J., *et al.*, Proc. Natl. Acad. Sci. 93:1458-1463; 1996); (Bouwer *et al.*, J. Exp. Med. 175:1467-71; 1992), the urease of *Helicobacter pylori* (Gomez-Duarte *et al.*, Vaccine 16, 460-71; 1998); (Corthesy-Theulaz, *et al.*, Infection & Immunity 66, 581-6; 1998), and the *Bacillus anthracis* protective antigen and lethal factor receptor-binding domain (Price, *et al.*, Infect. Immun. 69, 4509-4515; 2001).

The parasitic pathogens, from which the parasitic antigens are derived, include but are not limited to: *Plasmodium spp.*, such as *Plasmodium falciparum* (ATCC#: 30145); *Trypanosome spp.*, such as *Trypanosoma cruzi* (ATCC#: 50797); *Giardia spp.*, such as *Giardia intestinalis* (ATCC#: 30888D); *Boophilus spp.*, *Babesia spp.*, such as *Babesia microti* (ATCC#: 30221); *Entamoeba spp.*, such as *Entamoeba histolytica* (ATCC#: 30015); *Eimeria spp.*, such as *Eimeria maxima* (ATCC# 40357); *Leishmania spp.* (Taxonomy ID: 38568); *Schistosome spp.*, *Brugia spp.*, *Fascida spp.*, *Dirofilaria spp.*, *Wuchereria spp.*, and *Onchocerea spp.*

Examples of protective antigens of parasitic pathogens include the circumsporozoite antigens of *Plasmodium spp.* (Sadoff *et al.*, Science, 240:336 337; 1988), such as the circumsporozoite antigen of *P. berghei* or the circumsporozoite antigen of *P. falciparum*; the merozoite surface antigen of *Plasmodium spp.* (Spetzler *et al.*, Int. J. Pept. Prot. Res., 43:351-358; 1994); the galactose specific lectin of *Entamoeba histolytica* (Mann *et al.*, Proc. Natl. Acad. Sci., USA, 88:3248-3252; 1991), gp63 of *Leishmania spp.* (Russell *et al.*, J. Immunol., 140:1274 1278; 1988); (Xu and Liew, Immunol., 84: 173-176; 1995), gp46 of *Leishmania major* (Handman *et al.*, Vaccine, 18:3011-3017; 2000) paramyosin of *Brugia malayi* (Li *et al.*, Mol. Biochem. Parasitol., 49:315-323; 1991), the triose-phosphate isomerase of *Schistosoma mansoni* (Shoemaker *et al.*, Proc. Natl. Acad. Sci., USA, 89:1842 1846; 1992); the secreted globin-like protein of *Trichostrongylus colubriformis* (Frenkel *et al.*, Mol. Biochem. Parasitol., 50:27-36; 1992); the glutathione-S-transferase's of *Frasciola hepatica* (Hillyer *et al.*, Exp. Parasitol., 75:176-186; 1992), *Schistosoma bovis* and *S. japonicum* (Bashir *et al.*, Trop. Geog. Med., 46:255-258; 1994); and KLH of *Schistosoma bovis* and *S. japonicum* (Bashir *et al.*, supra, 1994).

Alternatively, it may be desired to elicit an immune response to antigens that are not associated with infectious agents, for example, antigens associated with cancer cells, Alzheimer's disease, Type 1 diabetes, heart disease, Crohn's disease, multiple sclerosis, etc.

In addition, the passenger genes that are carried by the bacterium need not encode antigens, but may encode any peptide or protein of interest. For example, the methods of the invention can be used for the delivery of passenger molecules for correction of hereditary disorders. Such genes would include, for example, replacements of defective genes such as the cystic fibrosis transmembrane conductance regulator (CFTR) gene for cystic fibrosis; or the introduction of new genes such as the integrase antisense gene for the treatment of HIV; or genes to enhance Type I T cell responses such as interleukin-27 (IL-27); or genes to modulate the expression of certain receptors, metabolites or hormones such as cholesterol and cholesterol receptors or insulin and insulin receptors; or genes encoding products that can kill cancer cells such as tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL); or a naturally occurring protein osteoprotegerin (OPG) that inhibits bone resorption; or to efficiently express complete-length humanized antibodies, for example, humanized monoclonal antibody that acts on the HER2/neu (erbB2) receptor on cancer cells.

In addition, the passenger genes may encode inhibitory RNAs such as "small inhibitory" siRNAs. As is known in the art, such RNAs are complementary to an mRNA of interest and bind to and prevent translation of the mRNA, e.g. as a means of preventing the expression of a gene product.

Similar methods can be used for delivery of passenger molecules to down regulate the immune system in order to prevent or control autoimmune diseases or other diseases of immune system. Examples include the prevention or treatment of diabetes mellitus, multiple sclerosis, lupus erythematosis and Crohn's disease and inflammatory joint and skin diseases. Other examples include fine tuning of immune responses that hamper specific immune responses such as down regulation of immune responses that divert the therapeutic immune responses to cancer and other diseases. For example, down regulation of Th2 responses when Th1 responses are appropriate for prevention and treatment of cancer, Leishmaniasis, tuberculosis, and HIV. This can be achieved by means of the present technology through manipulation of the immunosuppressive nature of the immune system in combination with the ability to express the suitable cytokine milieu for stimulation of the proper immune response and inhibition of improper immune responses.

In a preferred embodiment, the present invention relates to a method for the introduction of IFN resistance genes into host cells. Such a method would comprise introduction of the desired IFN resistance genes, along with sequences encoding a gene or

nucleic acid sequence of interest, into a bacterial based delivery system such that the IFN resistance proteins and nucleic acid sequences of interest are expressed upon administering the bacteria to a host. The IFN inhibitor can be produced by the bacteria (e.g. shigella) or by the host cell. In other words, the IFN resistance genes can be expressed from a prokaryotic promoter or from a eukaryotic promoter. The gene or nucleic acid sequences of interest (passenger genes) are expressed within the host cell. Further, all genetic sequences may be either constitutively or transiently expressed or induced.

In yet another preferred embodiment, the present invention provides a method for the introduction of type I IFN resistance genes along with one or more genes of interest into cells *in vitro*. Such a method would comprise introduction of the genes encoding one or more proteins of interest along with one or more IFN resistance genes into, for example, attenuated or attenuated/inactivated shigella such that the desired proteins/peptides are produced upon administering the shigella to cells. Shigella infects several different cell types, such as BHK (baby hamster kidney cells), HeLa (human cervical epitheloid carcinoma), CaCo-2 (human colonic adenocarcinoma) and therefore is capable of delivering the desired passenger molecules into cells. Gene expression in the shigella-infected cells is enhanced by the inhibitor of the type I IFN response. Following nucleic acid delivery, the cells can be transplanted for therapeutic purposes, for gene therapy or used as reagents in diagnostic assays.

In some cases, the bacteria serve as "gene therapy" agents by delivering to the cell nucleic acid sequences that encode a desired substance and mediating its production in the cell. For example, delivering CXCR4/or CCR5 binding chemokine-encoding genes into the gut using shigella vectors could be considered for treatment for HIV-1 infection. Procedures for genetically engineering bacteria are well-known to those of skill in the art, and guidance for carrying out such procedures are well known. Methods to attenuate *E. coli*, *Salmonella*, *Mycobacteria*, *Shigella*, and *Listeria* are well known to those skilled in the art (Evans *et al.*, J. of Immuno., vol. 120, 1978, p. 1423.); (Noriega *et al.*, Infect. Immun., 62(11):5168-5172 1994); (Hone *et al.*, Vacc., 9:810-816; 1991). .

For example, a method for the delivery of a desired gene or genes into a cell may include introducing the gene of interest into a strain of bacteria. In accordance with the present invention, an anti-IFN response gene or genes can be introduced into the bacterial chromosome or virulence plasmid by methods well known to those of skill in the art or

alternatively can be carried in a replicating or nonreplicating plasmid. The vectors of interest can be introduced into the bacterium, for example, via transformation, electroporation, transfection, conjugation, etc. The recombinant DNA procedures used in the construction of the strains and bacterial vectors include but are not limited to: polymerase chain reaction (PCR), restriction endonuclease (herein referred to as "RE") digestions, DNA ligation, agarose gel electrophoresis, DNA purification, and dideoxynucleotide sequencing, which are described elsewhere (Miller, *A Short Course in Bacterial Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; 1992); (Bothwell *et al.*, Methods for Cloning and Analysis of Eukaryotic Genes, Eds., Jones and Bartlett Publishers Inc., Boston, Mass. 1990); and (Ausubel *et al.*, Current Protocols in Molecular Biology, vol. 2:10.8.1-10.8.13, 1992), bacteriophage-mediated transduction (de Boer *et al.*, Cell, 56:641-649; 1989); (Miller, supra, 1992) and (Ausubel et al., supra), or chemical (Bothwell et al., supra); (Ausubel et al., supra); (Felgner et al., supra); and (Farhood, supra), electroporation (Bothwell et al., supra); (Ausubel et al., supra); (Sambrook, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; 1992) and physical transformation techniques (Bothwell et al., supra). The genes can be incorporated in phage (de Boer et al., supra), plasmids vectors (Curtiss, In: New Generation Vaccines: The Molecular Approach, Ed., Marcel Dekker, Inc., New York, N.Y., pages 161-188 and 269-288 1989) or spliced into the chromosome (Hone *et al.*, supra) of the target strain.

Gene sequences can be made synthetically using, for example, an Applied Biosystems ABI™ 3900 High-Throughput DNA Synthesizer (Foster City, CA 94404 U.S.A.) using procedures provided by the manufacturer. To synthesize large sequences i.e. greater than about 200 bp, a series of segments of the full-length sequence are generated by PCR and ligated together to form the full-length sequence using procedures well know in the art. However, smaller sequences, i.e. those smaller than about 200 bp, can be made synthetically in a single round.

Recombinant plasmids may be introduced into bacterial strains by electroporation using, for example, a BioRad Gene-Pulser. Nucleotide sequencing to verify cDNA sequences may be accomplished by standard automated sequencing techniques (e.g. using an Applied Biosystems automated sequencer, model 373A). DNA primers for DNA sequencing and polymerase chain reaction (herein referred to as "PCR") may be produced synthetically.

In some embodiments of the invention, the bacteria that are genetically engineered are attenuated invasive *Shigella flexneri* and the genes that are introduced into the bacteria are the adenovirus VAI genes, NSP1 of rotavirus, and/or NS1 of influenzae virus which are cloned under the control of a eukaryotic promoter and are introduced into the bacterium by electroporation.

The present invention also provides preparations for administering the recombinant bacterial expression vectors of the invention. For example, vaccine preparations for use in eliciting immune responses are provided. The preparations include at least one genetically engineered bacterial strain as described herein, and a pharmacologically suitable carrier. The preparation of such compositions (e.g. for use as vaccines) is well known to those of skill in the art. Typically, such compositions are prepared either as liquid solutions or suspensions, however, solid forms such as tablets, pills, powders and the like are also contemplated. Solid forms suitable for solution in, or suspension in, liquids prior to administration may also be prepared. The preparation may also be emulsified. The active ingredients may be mixed with excipients that are pharmaceutically acceptable and compatible with the active ingredients. Suitable excipients are, for example, water, saline, dextrose, raffinose, glycerol, ethanol and the like, or combinations thereof. In addition, the composition may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and the like. The vaccine preparations of the present invention may further comprise an adjuvant, suitable examples of which include but are not limited to Seppic, Quil A, Alhydrogel, etc.

If it is desired to administer an oral form of the composition, various thickeners, flavorings, diluents, emulsifiers, dispersing aids or binders and the like may be added. The composition of the present invention may contain any such additional ingredients so as to provide the composition in a form suitable for administration. The final amount of recombinant bacteria in the formulations may vary. However, in general, the amount in the formulations will be from about 1-99 percent. Further, the preparations of the present invention may contain a single type of recombinant bacteria or more than one type of recombinant bacteria. Initially, the bacterial vector strains are administered at doses of about 10^2 - 10^9 cfu, and are administered by an appropriate route. The number of doses may vary, depending on the potency of the individual vector strain, and the valency of the encoded recombinant product of interest, the particular use, etc.

In the case of vaccine preparations, the present invention also provides methods of eliciting an immune response to antigens encoded by the bacterium, and methods of vaccinating a mammal against diseases or conditions associated with such antigens. By eliciting an immune response, we mean that administration of the vaccine preparation of the present invention causes the synthesis of specific antibodies (at a titer in the range of 1 to 1 x 10^6, preferably 1 x 10^3, more preferable in the range of about 1 x 10^3 to about 1 x 10^6, and most preferably greater than 1 x 10^6) and/or cellular proliferation, as measured, e.g. by ^3H thymidine incorporation. The methods involve administering a composition comprising a bacterial strain of the present invention in a pharmacologically acceptable carrier to a mammal. The vaccine preparations of the present invention may be administered by any of the many suitable means which are well known to those of skill in the art, including but not limited to by injection, orally, intranasally, by inhalation, by ingestion of a food product containing the recombinant bacteria, etc. In preferred embodiments, the mode of administration is oral, subcutaneous, intradermal or intramuscular.

The invention is further illustrated by the following non-limiting Examples.

EXAMPLES

EXAMPLE 1. Induction of type I interferon response in host cells by a recombinant shigella vector

The ability of bacteria to induce a type 1 interferon response in mammalian cells was tested and the nature of the response was analyzed. Experimental conditions were as follows: Semi-confluent monolayers of HeLa cells were exposed to *Shigella flexneri* carrying a RNA passenger molecule for 1 hour at a multiplicity of infection (MOI) of 100 in a 6 well plate at 37 °C. Cells were washed twice with Dulbecco's Modified Eagles's Medium (DMEM). Medium containing 150 μg/ml gentamicin was added to the cells for 1 hour to kill extracellular bacteria. Subsequently, cells were washed twice, and DMEM with 10% fetal bovine serum (FBS) was added and the infected cells were allowed to incubate for 20h. Cells were then washed twice with phosphate buffered saline (PBS) and total RNA was isolated using an RNeasy mini kit (Qiagen). The Human Interferons and Receptors RT²Profiler™ PCR Array (Superarray Biosciences) was utilized to identify up regulation or down regulation of the expression of 84 interferon related genes.

The results are presented in Table 1. As can be seen, invasion of the Shigella vector

into the human cells led to transcriptional induction of type I IFNs and IFN stimulated genes such 2'-5'-oligoadenylate synthetase (2'-5'-OAS). Of the 89 genes that were surveyed, 74 showed more than a 2-fold increase in transcription.

In addition, further experiments showed that expression of a reporter gene from a plasmid DNA passenger molecule delivered by Shigella into IFN-α/β deficient cells was enhanced compared to the cells having an intact IFN system (Figure 1).

These results show that IFN stimulated genes suppress the expression of genes from passenger molecules delivered to mammalian cells by bacterial vectors.

Table 1. Differential IFN associated gene expression: comparison of shigella-invaded HeLA cells vs non-invaded HeLa cells.

Gene	Fold Induction
ADAR (adenosine deaminase acting on RNA)	3.37
CNTFR (ciliary neurotrophic factor receptor)	3.54
CRLF2 (cytokine receptor-like factor 2)	3.10
CSF2RA (colony stimulating factor 2 receptor)	2.80
CSF3R (colony stimulating factor 3 receptor)	5.44
CXCL10 (chemokine (C-X-C motif) ligand 10)	649.87
EBI3 (Epstein-Barr virus induced gene 3)	4.30
F3 Coagulation factor III (thromboplastin, tissue factor)	2.90
IL20RB (interleukin 20 receptor beta)	1.35
ISG15 (interferon stimulated gene 15)	13.87
IFI6 (interferon, alpha-inducible protein 6)	18.69
IFI16 (interferon, gamma-inducible protein 16)	5.11
IFI27 (interferon, alpha-inducible protein 27)	52.13
IFI30 (interferon, gamma-inducible protein 30)	1.56
IFI35 (interferon-induced protein 35)	4.54
IFI44 (interferon-induced protein 44)	5.22
IFI44L (interferon-induced protein 44-like)	7.33
IFIH1 (interferon induced with helicase C domain 1)	65.53

IFIT1 (interferon-induced protein with tetratricopeptide repeats-1)	12.94
IFIT1L (interferon-induced protein with tetratricopeptide repeats-1-like)	13.21
IFIT2 (interferon-induced protein with tetratricopeptide repeats-2)	6.47
IFIT3 (interferon-induced protein with tetratricopeptide repeats-3)	19.08
IFITM1 (interferon induced transmembrane protein 1)	3.47
IFITM2 (interferon induced transmembrane protein 2)	0.85
IFNA1 (interferon, alpha 1)	2.34
IFNA14 (interferon, alpha 14)	3.02
IFNA2 (interferon, alpha 2)	19.48
IFNA21 (interferon, alpha 21)	14.66
IFNA4 (interferon, alpha 4)	7.86
IFNA5 (interferon, alpha 5)	37.90
IFNA6 (interferon, alpha 6)	3.28
IFNA8 (interferon, alpha 8)	3.77
IFNAR1 (interferon (alpha, beta and omega) receptor 1)	2.44
IFNAR2 (interferon (alpha, beta and omega) receptor 2)	3.72
IFNB1 (interferon, beta 1)	21.92
IFNE1 (interferon epsilon 1)	1.72
IFNG (interferon, gamma)	6.21
IFNGR1 (interferon-gamma receptor 1)	8.08
IFNGR2 (interferon-gamma receptor 2)	3.13
IFNK (interferon, kappa)	5.40
IFNW1 (interferon, omega 1)	18.18
IFRD1 (interferon-related developmental regulator 1)	8.36
IFRD2 (interferon-related developmental regulator 2)	1.07
IL10RA (interleukin 10 receptor, alpha)	9.67
IL10RB (interleukin 10 receptor, beta)	3.28
IL11RA (interleukin 11 receptor, alpha)	2.02
IL12B (interleukin 12, beta)	31.00

IL13RA1 (interleukin 13 receptor, alpha-1)	1.64
IL15 (interleukin 15)	2.59
IL20RA (interleukin 20 receptor, alpha)	2.82
IL21R (interleukin 21 receptor)	6.21
IL22RA2 (interleukin 22 receptor, alpha-2)	8.78
IL28A (interleukin 28, alpha)	5.26
IL28RA (interleukin 28 receptor, alpha)	1.94
IL29 (interleukin 29)	25.71
IL2RB (interleukin 2 receptor, beta)	9.47
IL2RG (interleukin 2 receptor, gamma)	26.61
IL31RA (interleukin 31 receptor, alpha)	5.22
IL3RA (interleukin 3 receptor, alpha)	12.85
IL4R (interleukin 4 receptor)	4.33
IL5RA (interleukin 5 receptor, alpha)	3.24
IL6 (interleukin 6)	42.34
IL6R (interleukin 6 receptor)	11.91
IL7R (interleukin 7 receptor)	22.38
IL9R (interleukin 9 receptor)	1.91
IRF1 (interferon regulatory factor 1)	20.03
IRF2 (interferon regulatory factor 2)	3.85
IRF2BP1 (interferon regulatory factor 2 binding protein 1)	2.32
IRF2BP2 (interferon regulatory factor 2 binding protein 2)	4.94
IRF3 (interferon regulatory factor 3)	1.88
IRF4 (interferon regulatory factor 4)	49.32
IRF5 (interferon regulatory factor 5)	5.75
IRF6 (interferon regulatory factor 6)	5.67
IRF7 (interferon regulatory factor 7)	3.02
IRF8 (interferon regulatory factor 8)	30.36
IRGM (immunity-related GTPase family, M)	350.68

LEPR (leptin receptor)	2.23
MPL (myeloproliferative leukemia protein)	4.64
MX1 (Myxovirus (influenza) resistance 1)	13.40
OAS1 (2'-5'-oligoadenylate synthetase)	8.66
PSME1 (proteasome (prosome, macropain) activator subunit 1)	1.13
PYHIN1 (pyrin and HIN domain)	2.63
SP110 (nuclear body protein)	1.82
TTN (encodes central sarcomeric protein, titin)	45.07
B2M (beta-2-microglobulin)	2.21
HPRT1 (hypoxanthine phosphoribosyltransferase 1)	0.68
RPL13A (ribosomal protein L13a)	0.49
GAPDH (glyceraldehyde-3-phosphate dehydrogenase)	0.98
ACTB (actin, beta)	1.39

EXAMPLE 2. Construction of bacterial delivery systems that suppress the type I IFN response on expression of passenger nucleic acids delivered by bacterial vectors

This example describes the construction and use of two bacterial delivery systems that suppress the type I IFN response and its effect on expression of passenger nucleic acids. In both cases, nucleic acids were genetically engineered into attenuated, invasive *Shigella flexneri* strains by electroporation. *Shigella flexneri* was selected because it is naturally invasive in many tissue culture cell lines and animal models. The *Shigella* strain carries introduced chromosomal mutations that cause it to lyse after invasion of eukaryotic cells and escape from the endocytic vesicle, enabling the release of passenger molecules into the eukaryotic cell cytoplasm.

In the first set of experiments, electro-competent *Shigella flexneri* strain NCD1 was prepared and electroporated with the commercially available *E. coli* beta-galactosidase-expressing reporter vector pcDNA3.1/His/lacZ (Invitrogen). Reporter vector pcDNA3.1/His/lacZ expresses *E.coli* beta-galactosidase under the control of the human cytomegalovirus (CMV) promoter in mammalian cells, permitting the ready analysis of mammalian-mediated gene expression after delivery of the vector. The interferon

resistance gene used in this experiment was the adenovirus-associated I (VAI) RNA gene. The adenovirus RNA gene is known to be transcribed by RNA polymerase III in large amounts after adenovirus infection (Reich *et al.*, J. Mol. Biol. 17, 428, 1966; Price *et al.*, J. Virol. 9, 62, 1972; Weinmann *et al.*, Proc. Nat. Acad. Sci. USA 71, 3426; Soderlund *et al.*, Cell 7, 585, 1976.) Adenoviruses use the virus-encoded virus-associated RNA as a defense against cellular antiviral responses by blocking the activation of the interferon-induced, double-stranded RNA-activated protein kinase PKR (Galabru J, Katze MG, Robert N, Hovanessian AG. Eur J Biochem. 1989 Jan 2;178(3):581-9). The pAdVAntage vector that contains the Adenovirus Virus-Associated I (VAI) RNA gene on a 1,724 bp insert was also electroporated into the *Shigella flexneri* NCD1 strain. Invasion of HeLa cells by electroporation with *Shigella flexneri* strains was carried out as described in Example 1. Briefly, to test the anti-interferon effect of the VAI gene, HeLa cells were infected with either 1) *Shigella flexneri* NCD1 containing the beta-galactosidase reporter vector (pcDNA3.1/His/lacZ) and *Shigella flexneri* NCD1 containing the pAdVAntage vector; or 2) with *Shigella flexneri* NCD1 strain containing only the beta-galactosidase reporter vector. After 24 hours, beta-galactosidase assay reagents (Stratagene) were used both for cell lysis and for the assay of beta-galactosidase activity in cell extracts. The results are presented in Figure 2. As can be seen, a large increase in beta-galactosidase activity was observed in the HeLa cells invaded by Shigella that contained both the beta-galactosidase reporter vector and the VAI anti-interferon vector.

Similarly, in the second set of experiments, a Shigella vector strain containing recombinant double-stranded RNA nucleocapsids (rdsRN) carrying the reporter gene Green Fluorescent Protein (GFP) was electroporated with sequences encoding either influenza-A NS1 or rotavirus NSP1 cloned into the eukaryotic expression vector pcDNA 3.1 zeo(+) (Invitrogen). The resulting *Shigella* strain thus contained both the GFP gene in the RNA nucleocapsid (rdsRN) and either NSP1 or NS1 in pcDNA. BHK-21 and HeLa cells were infected with the GFP and NSP1- or NS1-expression plasmid harboring *Shigella* strain, or with a *Shigella* strain harboring only a GFP-expression plasmid. After 16 hours, invaded HeLa cells were tested for green fluorescence and the HeLa cell lysate was analyzed for GFP protein by immunoblotting. The fluorescence results showed that expression of GFP protein was enhanced in the cells which were invaded with an NS1- or NSP1-expression plasmid harboring *Shigella* strain, compared to cells invaded by a *Shigella* strain with only a GFP

gene (data not shown). Immunoblotting of total protein produced by the eukaryotic cells confirmed higher GFP expression in cells invaded with a NS1 or NSP1-expression plasmid harboring *Shigella* strain (Figure 3).

These findings show that expression by a bacterial vector within a mammalian cell of a gene encoding an inhibitor of the type I interferon response (e.g. VAI, NS1 or NSP1) enhances the co-expression of a transgene encoding a protein of interest (e.g. beta-galactosidase or GFP). The results described in this Example are the first evidence showing that enhanced expression of a protein of interest can be obtained by attenuating the IFN response using a bacterial based delivery system.

EXAMPLE 3. Induction of type I interferon response in mammalian host cells by recombinant *Shigella* vector is suppressed by interferon antagonists.

This example describes the ability of interferon antagonists to suppress the expression of interferon stimulated genes (ISGs) induced by bacteria in mammalian cells. The *Shigella flexneri* strain used in these experiments carries chromosomal mutations that cause it to lyse after it invades eukaryotic cells. The shigella survives long enough in the host cell to escape the endocytic vesicle, enabling the release of plasmid molecules containing interferon antagonists into the mammalian cell cytoplasm. The *Shigella* strain contains and expresses recombinant nucleocapsids encoding the reporter gene green fluorescent protein (GFP). The *S. flexneri* strain was electroporated with either the influenza-A NS1 gene or the adenovirus VAI RNA gene which had been cloned into the plasmid vectors pcDNA3.1 zeo(+) and pCR-BluntII-TOPO (Invitrogen, Carlsbad, CA), respectively. These *S. flexneri* strains, harboring either the NS1 gene or the VAI RNA gene, were used to study gene expression in mammalian cells.

Experimental conditions were as follows: Semi-confluent monolayers of HeLa cells were exposed to *S. flexneri* carrying an IFN suppressor gene for 1 hour at a multiplicity of infection (MOI) of 100 in a 6 well plate at 37 °C. Cells were washed twice with Eagle's minimal essential medium (EMEM) and EMEM containing 150 μg/ml gentamicin was added to the cells for 1 hour to kill extra cellular bacteria. Subsequently, cells were washed twice, fresh EMEM was added and the invaded cells were allowed to incubate for 20h. Cells were then washed twice with phosphate buffered saline (PBS) and total RNA was isolated using an RNeasy mini kit (Qiagen). The Human Interferon's and Receptors RT²Profiler™ PCR Array (Superarray Biosciences) was utilized to identify up regulation or down

regulation of the expression of 84 interferon related genes.

The results are presented in Table 2. As can be seen, the invasion of *S. flexneri* into human cells led to transcriptional induction of type I IFNs and IFN stimulated genes such as 2'-5'-oligoadenylate synthetase (2'-5'-OAS). The induced genes are involved in the inhibition of transcription and translation of passenger nucleic acids vectored by *S. flexneri*. However, as shown in Figure 4, HeLa cells invaded with the *S. flexneri* strain carrying the interferon antagonist NS1 gene showed lower levels of expression of ISGs compared to cells invaded by S. flexneri alone. These results demonstrate that NS1 suppresses the bacterially induced expression of ISGs in mammalian cells.

EXAMPLE 4. Suppression of the expression of interferon stimulated genes (ISGs) by interferon antagonists

This example describes the ability of Interferon antagonists to suppress the expression of interferon stimulated genes (ISGs) induced by bacteria in mammalian cells. The *Shigella* strain used in these experiments carries chromosomal mutations that cause it to lyse after invasion of eukaryotic cells and escape from the endocytic vesicle, enabling the release of passenger plasmid molecules containing interferon antagonists into the mammalian cell cytoplasm. *Shigella flexneri* strain NCD was electroporated with the influenza-A NS1 gene or the adenovirus VAI RNA gene which were cloned into plasmid vectors pcDNA3.1 zeo(+) and pCR-BluntII-TOPO (Invitrogen, Carlsbad, CA), respectively. *Shigella* strains thus harboring the NS1 gene or VAI RNA gene were used to study induction and suppression of the Type I IFN response.

Experimental conditions were as follows: Semi-confluent monolayers of HeLa cells were exposed to *Shigella flexneri*, carrying the IFN suppressor gene (NS1 or VAI), for 1 hour at a multiplicity of infection (MOI) of 100 in a 6 well plate at 37°C. Cells were washed twice with Eagle's minimal essential medium (EMEM) and fresh EMEM containing 150 ug/ml gentamicin was added to the cells for 1 hour to kill extracellular bacteria. Subsequently, the HeLa cells were washed twice with EMEM, fresh EMEM was added and the cells were allowed to incubate for 20h post invasion. The HeLa cells were then washed twice with phosphate buffered saline (PBS) and total RNA was isolated using an RNeasy mini kit (Qiagen). The Human Interferon's and Receptors RT²Profiler™ PCR Array (Superarray Biosciences) was utilized to identify up regulation or down regulation of the expression of 84 interferon related genes. As can be seen in Table 2, invasion of the *Shigella*

into the human cells led to transcriptional induction of type I IFNs and IFN stimulated genes such 2'-5'-oligoadenylate synthetase (2'-5'-OAS) compared to un invaded cells. However, as shown in Figure 4, HeLa cells invaded with the *Shigella* strain carrying the type I interferon antagonist NS1 gene showed lower levels of expression of ISGs compared to *Shigella* alone. These results demonstrate that indeed NS1 suppresses the bacterially induced expression of ISGs. Similarly, qRT-PCR expression studies of HeLa cells infected with *Shigella* expressing the adenovirus VAI RNA gene indicate that expression of ISGs such as CXCL10 and MX2 were also repressed by interferon antagonists (Figure 5). The above results illustrate the underlying molecular mechanism of inhibition of the IFN response which allows more robust expression of heterologous passenger genes encoded by the bacterial vector.

These results show that expression of viral suppressors of the type I IFN response from a bacterial vector inhibit the IFN response of infected cells.

Table 2. Over expression of molecules involved in the inhibition of transcription and translation of passenger nucleic acids vectored by Shigella: comparison of Shigella-invaded HeLa cells vs non-invaded HeLa cells

Gene Symbol	Fold Induction
CXCL10	1937
ISG15	30
IF16	40
IF127	225
IFI44L	23
IFIH1	35
IFIT1	25
IFT2	28
IFIT3	30
IFN6	17
IFNB1	12
IFN12B	10

IL 12RG	32
IRF4	9
OAS	13

EXAMPLE 5. Construction of improved bacterial based transgene expression delivery system by co-expressing IFN antagonist.

A plasmid vector was constructed to express the reporter gene Green Fluorescent protein (GFP). The GFP gene was PCR-amplified using Accuprime DNA polymerase (Invitrogen, Carlsbad, CA) and primers including HpaI and NotI restriction enzyme sites. The size of the amplified sequence is verified by agarose gel electrophoresis, and was purified using a QIAquick PCR purification kit by following manufacturer's instructions (Qiagen, Cat. No. 28106, Valencia, CA). The GFP gene was cloned into the HincII and NotI sites (New England Biolabs, Beverly, MA,) of the mammalian expression plasmid pShooter (Invitrogen, Carlsbad, CA). Expression in pShooter vector is driven by the strong, constitutive human EF-1 α promoter. The interferon resistance gene used in this experiment was the adenovirus-associated I (VAI) RNA gene driven by RNA polymerase III promoter. The gene encoding the VAI RNA gene down stream of the RNA polymerase III promoter was PCR amplified and cloned into EcoRI site of the GFP pShooter vector. A map of the resulting construct is shown in Figure 6.

Recombinant plasmids harboring the appropriate inserts were identified and the novel plasmid was designated as pAdgfp. pAdgfp vector was electroporated into a *Shigella flexneri* NCD1 strain. Invasion of HeLa cells were carried out as described in Example 3. Briefly, to test the expression enhancing effect of the of the VAI gene, HeLa cells were invaded with *Shigella flexneri* NCD1 containing the pAdgfp plasmid (encoding GFP and VAI) or with *Shigella flexneri* NCD1 strain carrying the pShooter plasmid encoding GFP only (pGFP). After 24 hours, cells from each well were lysed and analyzed by immunoblotting with GFP specific antisera. The results are presented in Figure 7. As can be seen, an increase in GFP expression was observed in the HeLa cells invaded by *Shigella* that contained both the GFP and the anti-interferon VAI RNA gene.

EXAMPLE 6. Enhanced expression of plasmid encoded beta-galactosidase when delivered into eukaryotic cells by attenuated *Shigella flexneri* expressing the influenza interferon

inhibitor NS1

A plasmid encoding beta-galactosidase under the translational control of the CMV promoter (pcDNA-lacZ) was electroporated into *Shigella flexneri* NCD. A second plasmid encoding the influenza A NS1 gene (pEF NS1) was also electroporated into a separate *Shigella flexneri* NCD strain. In pEF NS1 vector, NS1 gene expression is driven by the strong, constitutive human EF-1 α promoter. Invasion of HeLa cells with *Shigella flexneri* strains was carried out as described in Example 1. Briefly, to test the anti-interferon effect of the NS1 gene, HeLa cells were co-invaded with *Shigella flexneri* NCD containing the beta-galactosidase reporter vector (pcDNA3.1/His/lacZ) and *Shigella flexneri* NCD containing the pEF NS1 vector, or with *Shigella flexneri* NCD strain alone (without a vector). After 18 hours, beta-galactosidase assay reagents (Stratagene) were used both for HeLa cell lysis and for the assay of beta-galactosidase activity in cell extracts. The results are presented in Table 3. As can be seen, a large increase in beta-galactosidase activity was observed in the HeLa cells co-invaded by *Shigella* that contained both the beta-galactosidase reporter vector and the NS1 gene.

Table 3. Beta-galactosidase activity in mammalian cells

Strain	Promoter type	β-galactosidase specific activity (OD)
HeLa control cells	NA	0.047
NCD	NA	0.03
NCD pcDNA3.1-lacZ	CMV promoter	0.46
NCD pEF-NS1	EF-1 α promoter	0.050
NCD pcDNA3.1-lacZ+pEFNS1	CMV EF-1 α promoter	1.8

EXAMPLE 7. Expression of capsid-encoded MTB antigen 85A when delivered by attenuated *Shigella flexneri* expressing the influenza interferon inhibitor NS1

A recombinant nucleocapsid was constructed to express *M. tuberculosis* antigen 85A on both the S and M RNA segments (51 MS85A). Invasion assays were performed using *Shigella flexneri* expressing these capsids ± the influenza NS1 gene and an *S. flexneri* strain expressing antigen 85A on a mammalian plasmid under the control of the CMV promoter

(pcDNA-85A). Bacteria were grown to early log phase at 28 °C or 37 °C and incubated at 37 °C for 1 hr without shaking to induce expression of *S. flexneri* virulence genes. The bacteria were resupended in Eagle's minimal essential media (EMEM) and incubated for 1 hr at 37 °C + 5% CO_2 at an MOI of 100 with BHK21 cells that had been seeded in 6 well plates at 10^6 cells/well. Cells were washed 3x with PBS and incubated with EMEM + gentamycin (150 μg/ml) to kill any extracellular bacteria. Cells were then washed 3x with PBS and incubated 20 hr in EMEM media. Cells were washed once with PBS and lysed with 250 μl MPER Mammalian Protein Extraction Reagent (Pierce). 30 μl of each cell extract was run on an SDS-PAGE gel, transferred to a nitrocellulose membrane and immunoblotted with antiserum against antigen 85A (Figure 8). Expression of antigen 85A was seen in BHK-21 cells only in the 85A capsid strain MS85A containing a plasmid expressing the NS1 gene (MS85A pNS1) grown at 28 °C (lane 3). No expression was seen from MS85A without pNS1 grown at 28 °C or 37 °C or from MS85A pNS1 grown at 37 °C (lanes 1,2, & 4). This shows that expression of the NS1 protein is required for the expression of antigens encoded by the recombinant nucleocapsids delivered by the *S. flexneri* strain.

EXAMPLE 8. Construction of an expression vector expressing an interferon resistance gene in both bacteria and mammalian cells and a protein of interest only in mammalian cells.

A plasmid vector is constructed to express the immunodominant Gag peptide of HIV-1. A 600bp fragment is PCR-amplified from a synthetic *gag* gene. The sequence is amplified using Accuprime DNA polymerase (Invitrogen, Carlsbad, CA) and primers including HpaI and NotI RE sites. The size of the amplified sequence is verified by agarose gel electrophoresis, and is purified using a QIAquick PCR purification kit by following manufacturer's instructions (Qiagen, Cat. No. 28106, Valencia, CA). The 600 bp *gag* gene is cloned into the EcoRV and NotI sites (New England Biolabs, Beverly, MA,) of the expression vector plasmid pcDNA3.1zeo(+) (Invitrogen, Carlsbad, CA). Recombinant plasmids harboring the appropriate inserts are identified and the novel plasmid is designated pGAG4X.

An interferon resistance gene (e.g.NS1 or NSP1) is cloned into the pGAG4X vector under the control of an appropriate eukaryotic promoter (e.g.SV40 promoter) or prokaryotic promoter (e.g. house keeping promoter of *arg1*), or both, generating a dual expression vector. (The particular eukaryotic and prokaryotic promoter sequences described herein are not critical to the construction of the vector and other suitable promoters will occur to those

of skill in the art.) Thus, this expression vector expresses an interferon resistance gene in both bacteria and mammalian cells; however the protein of interest (e.g. Gag of HIV-1) is expressed only in mammalian cells. This approach improves transcript stability an subsequent translation of passenger RNA/DNA and other molecules for the expression of foreign proteins of interest or inhibitory RNAs in mammalian cells.

EXAMPLE 9. Use of a recombinant bacterial expression vector that is genetically engineered to suppress the IFN response as a vaccine

The efficacy of any bacterial live-vector vaccine rests with its ability to present sufficient foreign antigen to the human immune system to initiate the desired protective immune response. However, passenger DNA/RNA molecules may become unstable *in vivo* due to the host defense system, namely the IFN response, resulting in the loss of foreign genes and a decrease in the intended immune response. This invention provides a solution for the synthesis of high levels of antigen within host cells by attenuating the IFN defense system.

Delivery and expression of genes encoding IFN resistance and an antigen of interest may be accomplished by the inoculation of targeted cells (tissue, organism, etc.) with a non-pathogenic or attenuated bacterial vaccine vector that carries nucleic acids encoding the transgene of interest and a suppressor of the type I IFN response. Biological responses of interest include, but are not limited to: protective or modulatory immune responses; therapeutic responses; and down-regulation of gene expression (e.g. siRNA) and up-regulation of gene expression (e.g. cytokine expression) of host proteins.

Once a non-pathogenic or attenuated bacterial vaccine vector strain has been selected, the strain is modified to serve as an interferon response suppressing strain. This is accomplished using the strategies described above that entail introducing one or more IFN resistance genes into the strain.

To generate strains that contain type I IFN resistance genes and antigens of interest, *in vitro* synthesized gene(s) are introduced into the strains by electroporation and transformants are isolated on solid media under conditions that only permit the growth of strains that harbor and express a positive selection allele in the recombinant plasmid (e.g. antibiotic resistance or complementation of auxotrophy). One method of enhancing the inheritance of expression plasmids by live vectors involves construction of a passenger nucleic acids designed to complement an introduced mutation in the bacterial chromosome.

In a plasmid-based complementation system, plasmids replicating in the cytoplasm of the bacterium express a critical protein required by the bacterium to grow and replicate; loss of such plasmids removes the ability of the bacterium to express the critical protein and results in cell death. (The phenomenon of plasmid loss during bacterial replication, which results in the death of any plasmid-less bacterium, is also referred to as "post segregational killing.") Such a system has been successfully employed in *Salmonella typhimurium* and is based on expression of the *asd* gene encoding aspartate ß-semialdehyde dehydrogenase (Asd) (Galen *et* al., Gene. 1990; 49:29-35). Asd is a critical enzyme involved in the synthesis of structural components essential for the formation of the cell wall in gram-negative bacteria. Therefore, loss of plasmids encoding such a critical enzyme would be lethal for any bacterium incapable of synthesizing Asd from the chromosome.

The amount of such recombinant bacteria to be administered varies depending on the species of the subject, as well as the disease or condition that is being treated. Generally, the dosage employed is about 10^3 to 10^{11} viable organisms, preferably about 10^3 to 10^9 viable organisms. The bacterial vector harboring the DNA/RNA passenger molecule is generally administered along with a pharmaceutically acceptable carrier or diluent. The particular pharmaceutically acceptable carrier or diluent employed is not critical to the present invention. Examples of diluents include a phosphate buffered saline, buffer for buffering against gastric acid in the stomach, such as citrate buffer (pH 7.0) containing sucrose, bicarbonate buffer (pH 10) alone (Levine et al., J. Clin. Invest., 79:888-902; 1987); (Black et al., J. Infect. Dis., 155:1260-1265; 1987), or bicarbonate buffer (pH 7.0) containing ascorbic acid, lactose, and optionally aspartame (Levine et al., Lancet, II: 467 470; 1988). Examples of carriers include proteins, e.g., as found in skim milk, sugars, e.g., sucrose, or polyvinylpyrrolidone. Typically these carriers would be used at a concentration of about 0.1-90% (w/v) but preferably at a range of 1-10% (w/v).

The biological activity of vector strains is assessed in an appropriate animal model (e.g. mice, rabbits, guinea pigs or Rhesus macaques). Initially, the bacterial vector strains are administered at doses of 10^2 - 10^9 cfu, and are administered by an appropriate route (e.g. *E. coli*, *Salmonella* and *Shigella* can be given intragastrically or intranasally). The number of doses will vary, depending on the potency of the individual vector strain, and the valency of the encoded recombinant product of interest.

Methods of measurement of immune and other biological responses to encoded

products in animal models are well known to those skilled in the art. To measure serum IgG and IgA responses to antigen, sera are collected before and 10, 20, 30, 40, 50, 60, 70, and 80 days after vaccination. About 400-500 µl of blood is collected into individual tubes and allowed to clot by incubating for 4 hr on ice. After centrifugation in a microfuge for five minutes, the sera are transferred to fresh tubes and stored at -80°C. Mucosal IgG and IgA responses to antigens expressed by the genes of interest are determined using fecal pellets and vaginal washes that will be harvested before and at regular intervals after vaccination (Srinivasan et al., Biol. Reprod. 53: 462; 1995); (Staats et al., J. Immunol. 157: 462; 1996). Standard ELISAs are used to quantitate the IgG and IgA responses to an antigen of interest in the sera and mucosal samples (Abacioglu et al., AIDS Res. Hum. Retrovir. 10: 371; 1994); (Pincus et al., AIDS Res. Hum. Retrovir. 12: 1041; 1996). Ovalbumin can be included in each ELISA as a negative control antigen. In addition, each ELISA can include a positive control serum, fecal pellet or vaginal wash sample, as appropriate. The positive control samples are harvested from animals vaccinated intranasally with 10 µg of the antigen expressed by the gene of interest mixed with 10 µg cholera toxin, as described (Yamamoto et al., Proc. Natl. Acad. Sci. 94: 5267; 1997). The end-point titers are calculated by taking the inverse of the last serum dilution that produced an increase in the absorbance at 490 nm that is greater than the mean of the negative control row plus three standard error values.

Cellular immunity may be measured by intracellular cytokine staining (also referred to as intracellular cytokine cytometry) or by ELISPOT (Letsch A. et al., Methods 31:143-49; 2003). Both methods allow the quantitation of antigen-specific immune responses, although ICS also adds the simultaneous capacity to phenotypically characterize antigen-specific CD4+ and CD8+ T-cells. Such assays can assess the numbers of antigen-specific T cells that secrete IL-2, IL-4, IL-5, IL-6, IL-10 and IFN- (Wu et al., AIDS Res. Hum. Retrovir. 13: 1187; 1997). ELISPOT assays are conducted using commercially-available capture and detection mAbs (R&D Systems and Pharmingen), as described (Wu et al., Infect. Immun. 63:4933; 1995) and used previously (Xu-Amano et al., J. Exp. Med. 178:1309; 1993); (Okahashi et al., Infect. Immun. 64:1516; 1996). Each assay includes mitogen (Con A) and ovalbumin controls. The anti-IFN bacterial based delivery system described herein has several advantages over delivery systems without IFN resistant genes. The antigen genes are expressed at higher levels and for longer periods of time, and therefore induce a more vigorous immune response. Bacterial vectors that display efficacy and are non-toxic in

animal models are further assessed in clinical trials.

EXAMPLE 10. Development of a tuberculosis vaccine

BCG bacteria are genetically engineered as described herein to contain nucleic acids encoding 1) one or more tuberculosis antigens as passenger genes, and 2) one or more factors that inhibit or interfere with a mammalian host cell type I interferon response. When administered to a mammalian host (e.g. a human), the genetically engineered BCG invade host cells, escape the endosome, and are lysed to release passenger genes to produce the one or more tuberculosis antigens. Further, the BCG also produce the one or more factors that inhibit the host cells IFN response. The factors attenuate the host cell IFN response, which would otherwise decrease the production of the TB antigen(s). As a result, sufficient TB antigen(s) is produced to result in a robust immune response to the TB antigen(s).

While the invention has been described in terms of its preferred embodiments, those skilled in the art will recognize that the invention can be practiced with modification within the spirit and scope of the appended claims. Accordingly, the present invention should not be limited to the embodiments as described above, but should further include all modifications and equivalents thereof within the spirit and scope of the description provided herein.

CLAIMS

We claim:

1. A genetically engineered bacterium, comprising

nucleic acid sequences encoding

one or more passenger genes; and

one or more factors that suppress a mammalian type I interferon response,

wherein said nucleic acid sequences encoding said one or more passenger genes are operably linked to a eukaryotic promoter, and said nucleic acid sequences encoding said one or more factors that suppress a mammalian type I interferon response are operably linked to a eukaryotic promoter or a prokaryotic promoter.

2. The genetically engineered bacterium of claim 1, wherein said nucleic acid sequences encoding said one or more factors that inhibit a mammalian type I interferon response are operably linked to a eukaryotic promoter.

3. The genetically engineered bacterium of claim 1, wherein said nucleic acid sequences encoding said one or more factors that inhibit a mammalian type I interferon response are operably linked to a prokaryotic promoter.

4. The genetically engineered bacterium of claim 1, wherein nucleic acid sequences encoding said one or more factors that inhibit a mammalian interferon response are present on a chromosome of said genetically engineered bacterium.

5. The genetically engineered bacterium of claim 1, wherein one or both of:

i) nucleic acid sequences encoding said one or more passenger genes,

and

ii) nucleic acid sequences encoding said one or more factors that inhibit a mammalian interferon response,

are present on a plasmid.

6. The genetically engineered bacterium of claim 1, wherein said one or more factors that inhibit a mammalian interferon response are of viral origin.

7. The genetically engineered bacterium of claim 1, wherein said one or more passenger genes encode tuberculosis antigens.

8. The genetically engineered bacterium of claim 1, wherein said genetically engineered bacterium is a Shigella bacterium.

9. The genetically engineered bacterium of claim 1, wherein said genetically engineered bacterium is a Mycobacterium.

10. The genetically engineered bacterium of claim 1, wherein said one or more passenger genes is a heterologous transgene.

11. A method of increasing the production of one or more gene products of interest in a cell or tissue, comprising the step of

administering to said cell or tissue a genetically engineered bacterium comprising nucleic acid sequences encoding: i) said one or more gene products of interest and ii) one or more factors that suppress a mammalian interferon response,

wherein said nucleic acid sequences encoding said one or more passenger genes are operably linked to a eukaryotic promoter, and said nucleic acid sequences encoding said one or more factors that suppress a mammalian type I interferon response are operably linked to a eukaryotic promoter or a prokaryotic promoter,

and wherein said step of administering is carried out under conditions which allow said genetically engineered bacterium to invade said cell or tissue; and

allowing said cell or tissue to produce said one or more gene products of interest.

12. The method of claim 11, wherein said nucleic acid sequences encoding said one or more factors that inhibit a mammalian type I interferon response are operably linked to a eukaryotic promoter.

13. The method of claim 11, wherein said nucleic acid sequences encoding said one or more factors that inhibit a mammalian type I interferon response are operably linked to a prokaryotic promoter.

14. The method of claim 11, wherein said nucleic acid sequences encoding said one or more factors that inhibit a mammalian interferon response are present on a chromosome of said genetically engineered bacterium.

15. The method of claim 11, wherein one or both of:

i) said nucleic acid sequences encoding said one or more gene products of interest, and

ii) said nucleic acid sequences encoding said one or more factors that inhibit a mammalian interferon response,

are present on a plasmid.

16. The method of claim 11, wherein said one or more factors that inhibit a mammalian interferon response are of viral origin.

17. The method of claim 11, wherein said one or more gene products of interest are *Mycobacterium tuberculosis* antigens.

18. The method of claim 11, wherein said genetically engineered bacterium is a bacterium selected from the group consisting of Shigella, Listeria, Salmonella, and Bacille Calmette-Guérin (BCG).

19. The method of claim 11, wherein said one or more passenger genes is a heterologous transgene.

20. A method for inducing an immune response to an antigen of interest in a mammal, comprising the step of

administering to said mammal a genetically engineered bacterium, comprising

nucleic acid sequences encoding said antigen of interest; and

nucleic acid sequences encoding one or more factors that suppress a mammalian interferon response.

wherein said nucleic acid sequences encoding said one or more passenger genes are operably linked to a eukaryotic promoter, and said nucleic acid sequences encoding said one

or more factors that inhibit a mammalian type I interferon response are operably linked to a eukaryotic promoter or a prokaryotic promoter; and

allowing cells or tissues in said mammal to produce said antigen of interest, whereby an immune response is raised against said antigen of interest.

21. The method of claim 20, wherein said antigen of interest is a *Mycobacterium tuberculosis* antigen.

22. The method of claim 20, wherein said nucleic acid sequences encoding said one or more factors that inhibit a mammalian type I interferon response are operably linked to a eukaryotic promoter.

23. The method of claim 20, wherein said nucleic acid sequences encoding said one or more factors that inhibit a mammalian type I interferon response are operably linked to a prokaryotic promoter.

24. The method of claim 20, wherein said nucleic acid sequences encoding said one or more factors that inhibit a mammalian interferon response are present on a chromosome of said genetically engineered bacterium.

25. The method of claim 20, wherein one or both of:

i) said nucleic acid sequences encoding said antigen of interest,

and

ii) said nucleic acid sequences encoding said one or more factors that inhibit a mammalian interferon response,

are present on a plasmid.

26. The method of claim 20, wherein said one or more factors that inhibit a mammalian interferon response are of viral origin.

27. A recombinant bacterial vector, comprising:

a bacterium genetically transformed with

one or more genetically engineered nucleic acid sequences coding for a host cell or tissue type 1 interferon (IFN) response suppressor factor, and

one or more genetically engineered nucleic acids coding for one or more host cell or tissue active amino acid sequences,

wherein said one or more genetically engineered nucleic acids coding for said one or more host cell or tissue active amino acid sequences are over expressed upon said bacterium invading said host cell or tissue.

28. The recombinant bacterial vector of claim 27 wherein said host cell or tissue type 1 IFN response suppressor factor is rotavirus NSP 1 or influenza virus NS1.

29. The recombinant bacterial vector of claim 27 wherein said one or more host cell or tissue active amino acid sequences are selected from tuberculosis antigens and malaria antigens.

30. The recombinant bacterial vector of claim 27 wherein said one or more host cell or tissue active amino acid sequences include one or more immunostimulatory amino acid sequences derived from one or more of rotavirus, influenza virus, ectromelia virus, hepatitis virus, vaccinia virus, adenovirus, paramyxovirus, HPV, HIV, HTLV, enteroviruses, herpesviruses, EEE, VEE, West Nile virus, Norwalk virus, parvoviruses, dengue virus, and hemorrhagic fever virus.

31. The recombinant bacterial vector of claim 27 wherein said one or more host cell or tissue active amino acid sequences are selected from hormone, enzymes, anticancer agents, and apoptotic factors.

32. The recombinant bacterial vector of claim 27 wherein said host cell or tissue type 1 IFN response suppressor factor is selected from the group consisting of rotavirus NSP1, influenza virus NS1, ectromelia virus C12R protein, hepatitis C virus NS3/4A protease, vaccinia virus vIFN-α/β Rc protein, adenovirus E1A protein, C proteins of paramyxoviruses, and human papillomavirus (HPV) E6 oncoprotein.

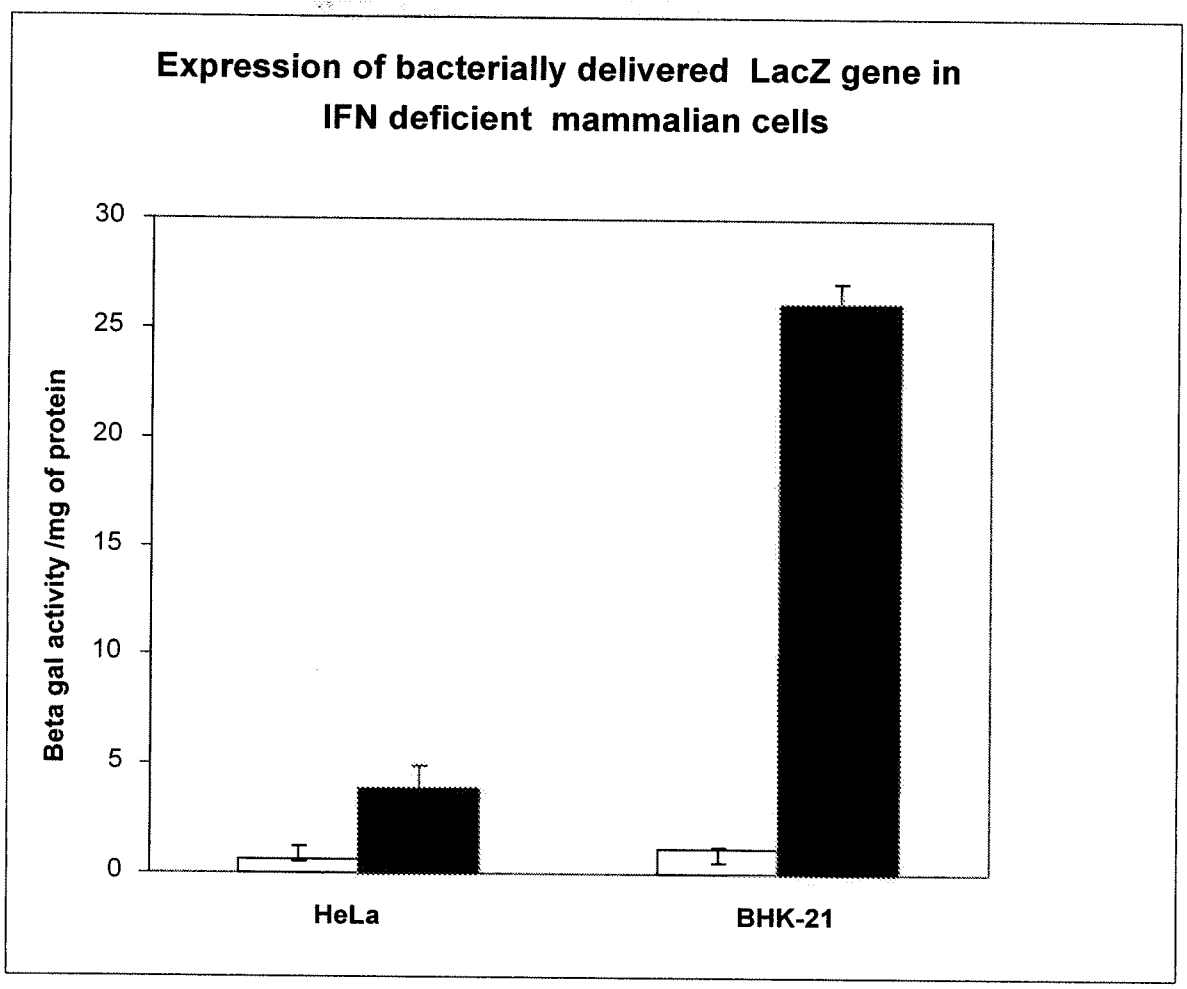
Expression of bacterially delivered LacZ gene in IFN deficient mammalian cells
Beta gal activity /mg of protein
30
25
20
15
10
5
0
HeLa
BHK-21

Figure 1.

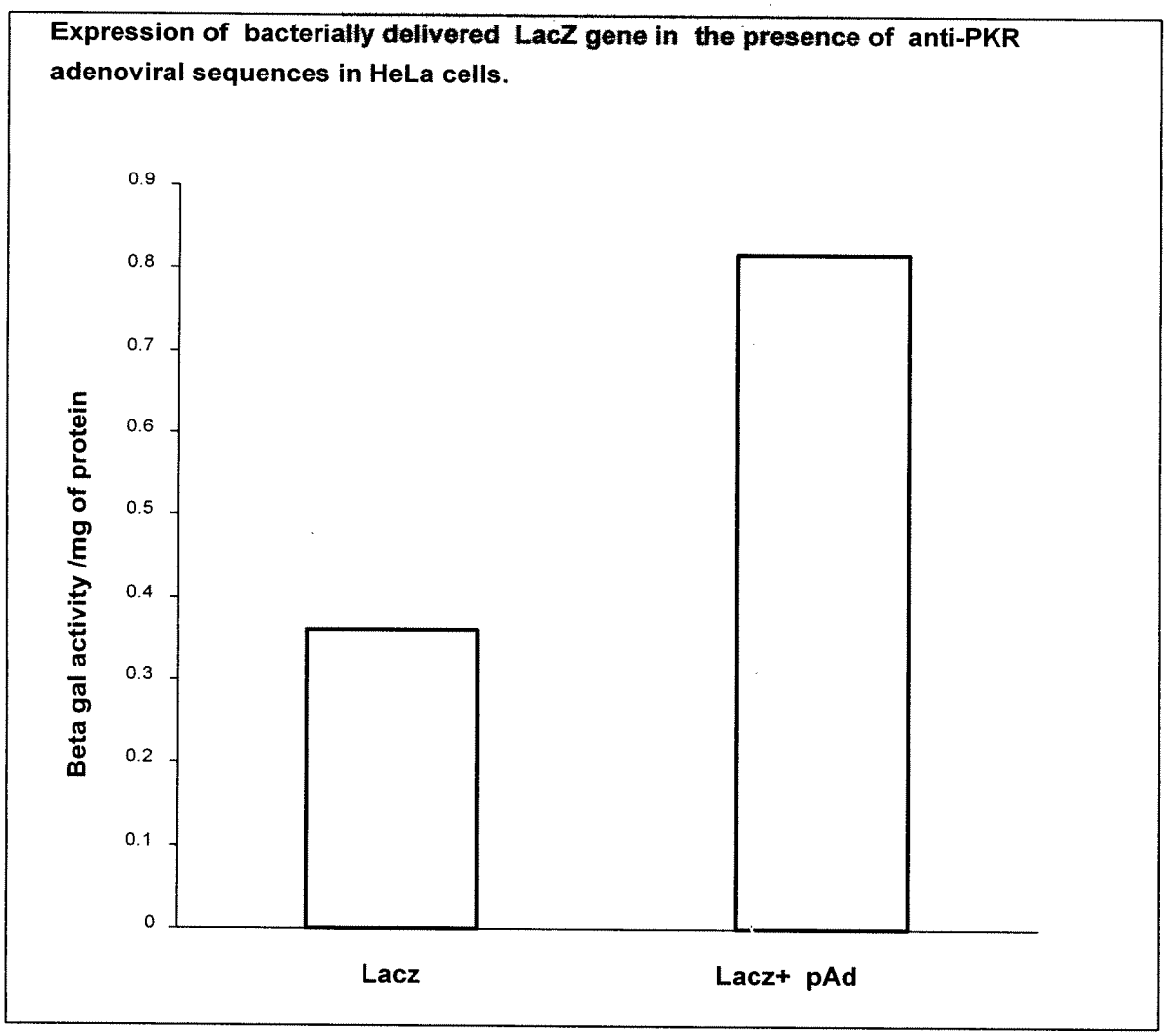
Expression of bacterially delivered LacZ gene in the presence of anti-PKR adenoviral sequences in HeLa cells.
Beta gal activity /mg of protein
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Lacz
Lacz+ pAd

Figure 2.

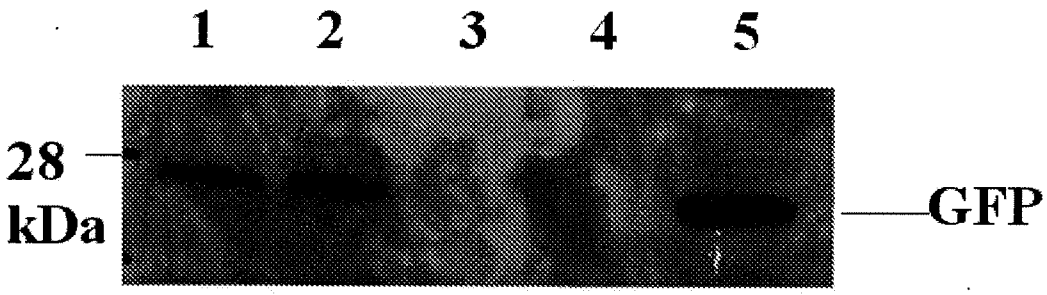

Figure 3.

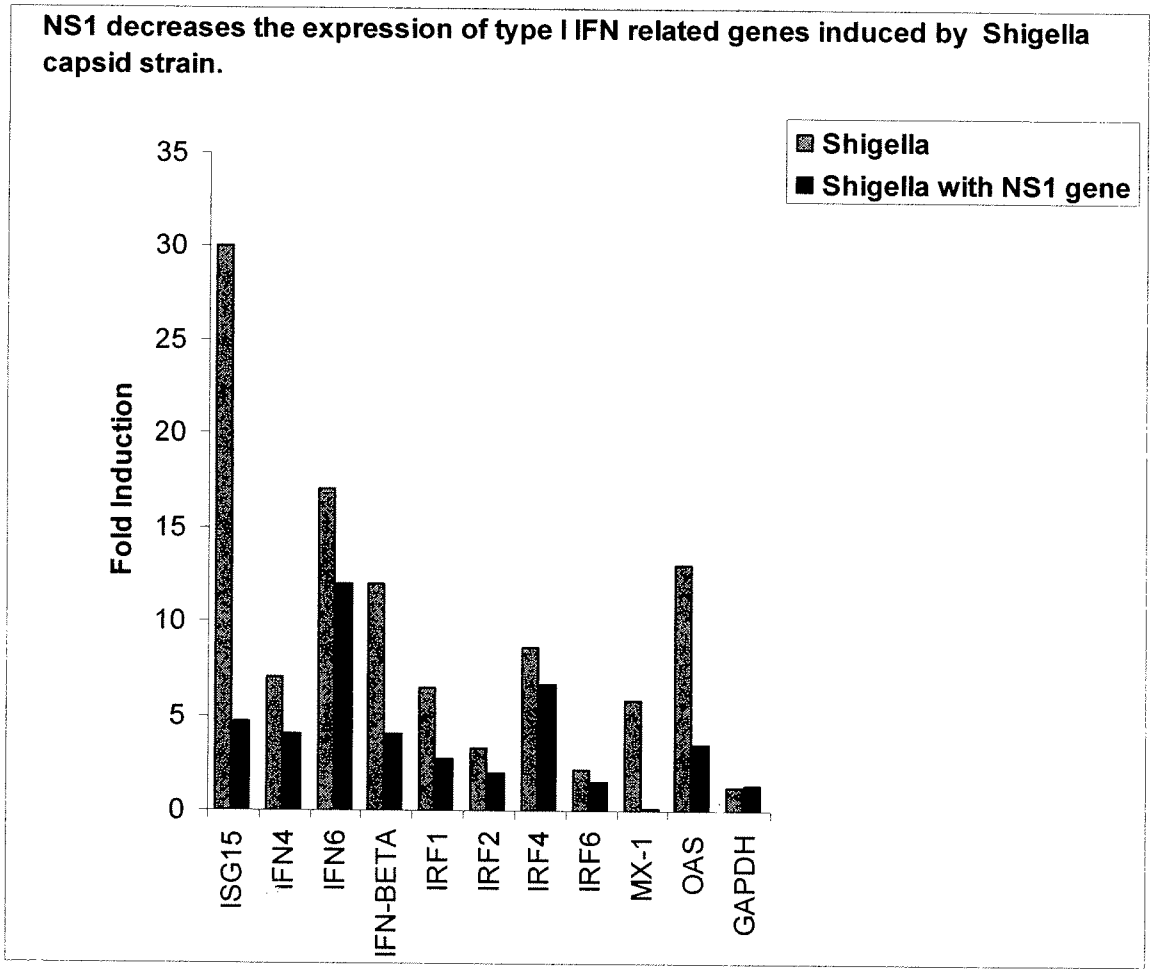
NS1 decreases the expression of type I IFN related genes induced by Shigella capsid strain.
Shigella
Shigella with NS1 gene
Fold Induction
35
30
25
20
15
10
5
0
ISG15
IFN4
IFN6
IFN-BETA
IRF1
IRF2
IRF4
IRF6
MX-1
OAS
GAPDH

Figure 4.

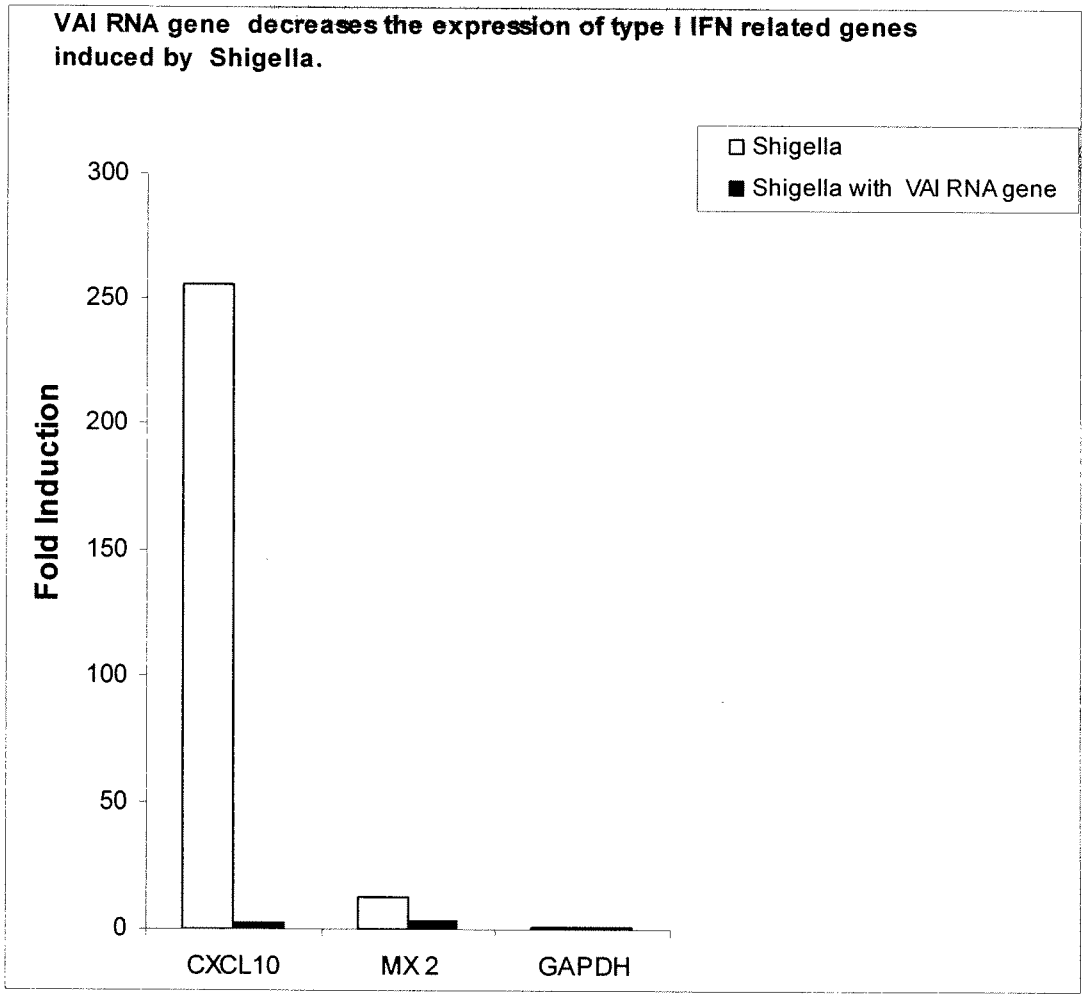
VAI RNA gene decreases the expression of type I IFN related genes induced by Shigella.
Shigella
Shigella with VAI RNA gene
Fold Induction
300
250
200
150
100
50
0
CXCL10
MX 2
GAPDH

Figure 5.

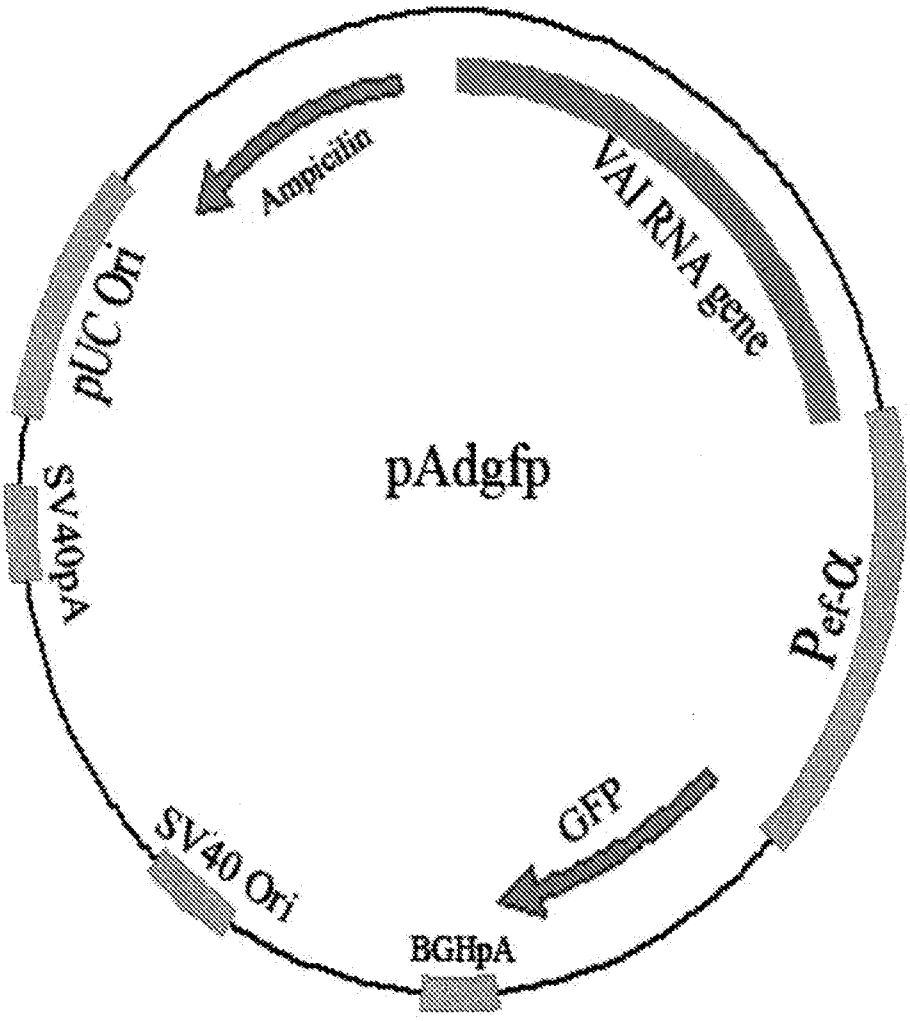
pAdgfp
Ampicilin
VAI RNA gene
pUC Ori
SV40pA
Pef-α
GFP
SV40 Ori
BGHpA

Figure 6.

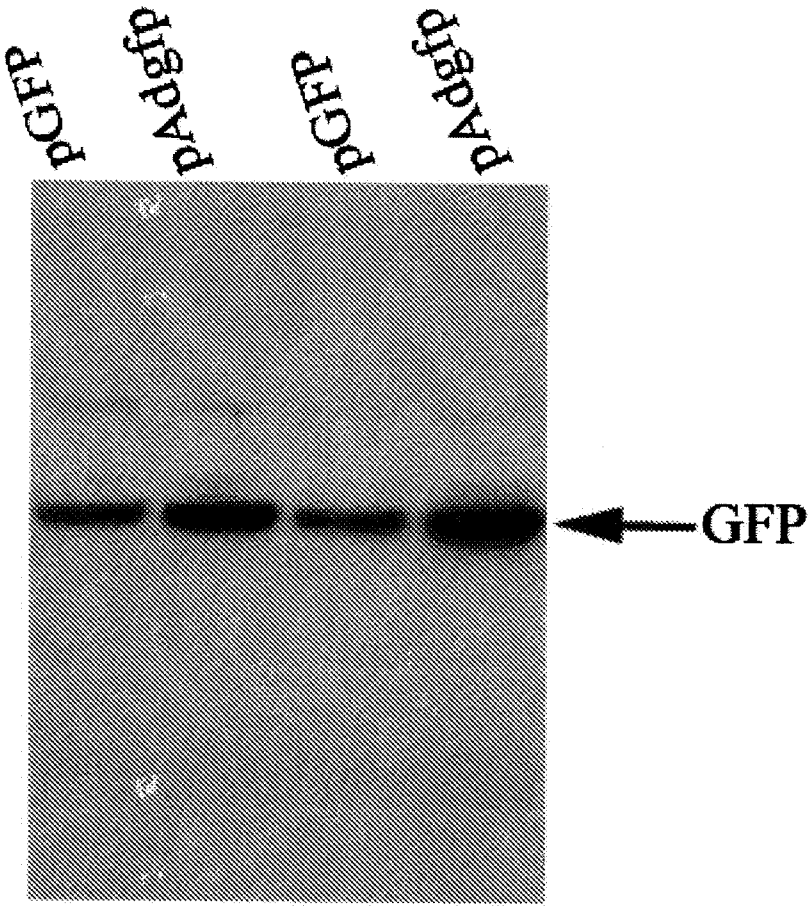

Figure 7.

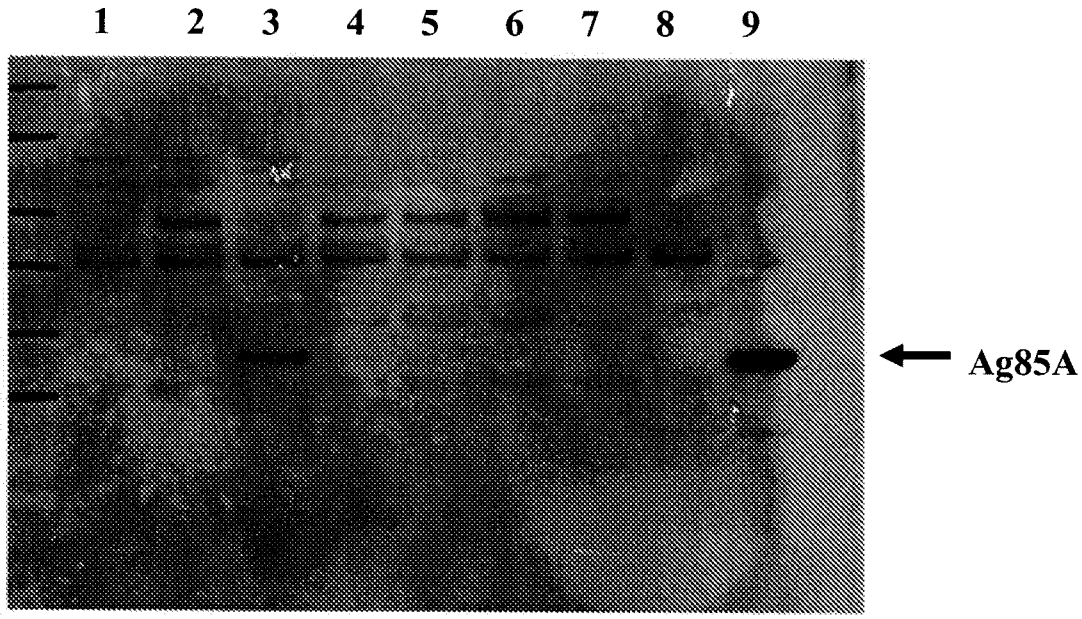

Figure 8.

A.

ATGGATCCAAACACTGTGTCAAGCTTTCAGGTAGATTGCTTTCTTTGGCATGTCCGCA
AACGAGTTGCAGACCAAGAACTAGGTGATGCCCCATTCCTTGATCGGCTTCGCCGAG
ATCAGAAATCCCTAAGAGGAAGGGGCAGCACTCTCGGTCTGGACATCGAGACAGCC
ACACGTGCTGGAAAGCAGATAGTGGAGCGGATTCTGAAAGAAGAATCCGATGAGGC
ACTTAAAATGACCATGGCCTCTGTACCTGCGTCGCGTTACCTAACTGACATGACTCTT
GAGGAAATGTCAAGGGACTGGTCCATGCTCATACCCAAGCAGAAAGTGGCAGGCCC
TCTTTGTATCAGAATGGACCAGGCGATCATGGATAAGAACATCATACTGAAAGCGAA
CTTCAGTGTGATTTTTGACCGGCTGGAGACTCTAATATTGCTAAGGGCTTTCACCGAA
GAGGGAGCAATTGTTGGCGAAATTTCACCATTGCCTTCTCTTCCAGGACATACTGCTG
AGGATGTCAAAAATGCAGTTGGAGTCCTCATCGGAGGACTTGAATGGAATGATAAC
ACAGTTCGAGTCTCTGAAACTCTACAGAGATTCGCTTGGAGAAGCAGTAATGAGAAT
GGGAGACCTCCACTCACTCCAAAACAGAAACGAGAAATGGCGGGAACAATTAGGTC
AGAAGTTTGA

B.

ATGGCAACCTTTAAGGATGCTTGTTTTCATTATAGAAGGATTACAAAACTAAACAGG
GAATTACTGAGAATTGGAGCAAACTCAGTATGGACTCCAGTTTCTTCAAATAAAATT
AGAGGATGGTGCATTGAGTGTTGTCAATTGACTGAATTGACCTTTTGTCATGGATGCT
CATTGGCTCACGTTTGTCAATGGTGCATTCAAAACAAACGTTGCTTCTTAGACAATGA
ACCACATCTTTTAAAGTTAAGAACCTTTGAATCTCCAATAACAAAGGAAAAATTGCA
GTGCATTATTGATTTATATAATCTACTATTTCCAATCAGCCCTGGTATCATAAATAGA
TTTAAAAAAGCAGTTAAACAAAGAAAGTGTAGAAATGAAAGTGATAAATCGTGGTA
TAATCAATTACTACTTCCAATTACTCTAAATGCCGCAGTTTTTAAATTTCATTCGAGG
GAAATTTACATTTTTGGATTTTATGAAGGATCATCAGCATGCATAGATTTACCATATA
GACTTGTAAATTGCATTGACTTATATGATAAATTGCTGTTAGATCAAATAAATTTTGA
AAGAATGAGTTGTCTTCCAGATAAGTTACAATCTATCTATGCAAATAATTATTTTAAA
TTGAGCAGACTTCCTTCAATGAAGTTGAAACAAGTTTATTATTCAGACTTCTCCAAAC
AAAATTTGATTAATAAGTATAAAACTAAGAGTCGCATAGTACTTAGAAATCTGACTG
AATTCACTTGGGATTCTCAAATTGATTTGCATCATGATCTAATCAATAACAAAGATA
AAATACTTGCAGCATTATCAACATCATCGTTAAAACAATTCGAAACACATGATTTAA
ATTTAGGAAGAGTAAAAGCTGACATTTTTGAACTTGGACATCATTGCAAACCAAATT
ATATTTCATCAAATCATTGGCAACCGGCATCAAAAATTTTCCAGTGTAAGTGGTGCA
ATGTAAAGTATGCATTTAGAGATATGGATTGGAAGATGGAATCAATGTATAATGAAC
TCTTAAGTTTTCTACAGTCCTGTTACAAAAGTAATGTTAATGTAGGGCATTGTAGTTC
AATTGAAAGTGTTTATCCACTAGTCAAAGATATGCTTTGGCATTCAATTACAAAATAT
ATTGATCAAACTATTGAGAAATTATTTGATGCAATGAATCCGGTGCAAGTGAATGAG
CAACTTGTAATTAACTTCTATTGGCAAATAGATATCGCTCTGTACACTCACATCAAAA
TGATACTAAAAACTGAAGCTCTTCCGTTTACTTTCACACTTAACCAGTTCAATTCTAT
AATTAAGGGAATCATTAACCAATGGTGTGACGTCGCTGAATTGGATCTTTTACCGTT
ATGCACTGAACAAACCGACAAATTGGTTAAACTGAAAGAAGAAGGGAAACTGTCTG
AAGAGTATGAGCTCCTAATCTCGGATTCCGAAGACGACGACTGA

Figure 9A and B

C.

GGCTCGACTC CGTGGCCTGG AGGCTAAGCG AACGGGTTGG GCTGCGCGTG
TACCCCGGTTCGAATCTCGA ATCAGGCTGG AGCCGCAGCT AACGTGGTAC
TGGCACTCCC GTCTCGACCCAGGCCTGCAC AAAACCTCCA GGATACGGAG
GCGGGTCG

Figure 9C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 08/75972

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 15/74; C12N 1/20 (2008.04)
USPC - 435/471, 252.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
USPC: 435/471, 252.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 435/471, 252.1 (see also text search below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Electronic Databases Searched: pubWEST, USPTO, Google, Answers.com, Google Patents
Search Terms Used: genetic, engineer, bacteria, plasmid, shigella, mycobacterium, heterologous, transgene, tuberculosis, antigen, IFN, viral, nucleic, acid, mammal, chromosome, suppressor, prokaryotic, eukaryotic, rotavirus, influenza

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2007/0160609 A1 (MAROUN) 12 July 2007 (12.07.2007) para [0028]-[0032], [0037]-[0039], [0042], [0052], [0058], [0069]-[0070], [0082], [0092]-[0093], [0101], [0103], [0118]-[0120], [0123], [0128] and [0133]	1-6, 11-16, 20, 22-27 and 31 --- 7-10, 17-19, 21, 28-30 and 32
Y	US 2004/0077090 A1 (SHORT) 22 April 2004 (22.04.2004) para [0093], [0096], [0103], [0155], [0178]-[0179], [0184]-[0185], [0200], [0203], [0237], [0399], [1063], [1459], [1921] and [2037]; Table 1	7-10, 17-19, 21 and 29-30
Y	US 2007/0207526 A1 (COIT et al.) 06 September 2007 (06.09.2007) para [0521]	28 and 32

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 December 2008 (17.12.2008)	12 JAN 2009

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (April 2007)