

(12) STANDARD PATENT
(19) AUSTRALIAN PATENT OFFICE

(11) Application No. **AU 2001248314 B2**

(54) Title
Use of OprI lipoprotein from pseudomonas as a Th1 inducing natural adjuvant for heterologous antigens

(51)⁷ International Patent Classification(s)
A61K 039/39 A61P 031/06
C07K 014/21 A61P 037/00
C07K 014/44 A61K 039/008
A61P 033/02 A61K 039/00
A61P 031/10 A61K 048/00
A61P 031/08

(21) Application No: **2001248314** (22) Date of Filing: **2001.02.13**

(87) WIPO No: **WO01/60404**

(30) Priority Data

(31) Number (32) Date (33) Country
00200589.0 2000.02.18 EP

(43) Publication Date: **2001.08.27**

(43) Publication Journal Date: **2001.11.08**

(44) Accepted Journal Date: **2004.08.19**

(71) Applicant(s)
Vlaams Interuniversitair Instituut voor Biotechnologie VZW

(72) Inventor(s)
De Baetselier, Patrick; Cornelis, Pierre; Revets, Hilde

(74) Agent / Attorney
Davies Collison Cave, Level 15 1 Nicholson Street, MELBOURNE, VIC, 3000

(56) Related Art
WO 01/60404

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

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
23 August 2001 (23.08.2001)

PCT

(10) International Publication Number
WO 01/60404 A3

- (51) International Patent Classification⁷: **A61K 39/39**, 48/00, 39/00, 39/008, A61P 37/00, 31/06, 31/08, 31/10, 33/02 // C07K 14/44, 14/21
- (74) Common Representative: **VLAAMS INTERUNIVERSITAIR INSTITUUT VOOR BIOTECHNOLOGIE VZW**; Rijvisschestraat 120, B-9052 Zwijnaarde (BE).
- (21) International Application Number: PCT/EP01/01673
- (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
- (22) International Filing Date: 13 February 2001 (13.02.2001)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
00200589.0 18 February 2000 (18.02.2000) EP
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
- (71) Applicant (*for all designated States except US*): **VLAAMS INTERUNIVERSITAIR INSTITUUT VOOR BIOTECHNOLOGIE VZW** [BE/BE]; Rijvisschestraat 120, B-9052 Zwijnaarde (BE).
- Published:
— with international search report
- (72) Inventors; and
- (88) Date of publication of the international search report:
10 January 2002
- (75) Inventors/Applicants (*for US only*): **REVETS, Hilde** [BE/BE]; Kapellelaan 299, B-1860 Meise (BE). **CORNELIS, Pierre** [BE/BE]; Brillat Savarinstraat 60, B-1050 Elsene (BE). **DE BAETSELIER, Patrick** [BE/BE]; Mevr. Courtmanstraat 9 bus 6, B-2600 Berchem (BE).
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*



WO 01/60404 A3

(54) Title: USE OF OPRI LIPOPROTEIN FROM PSEUDOMONAS AS A TH1 INDUCING NATURAL ADJUVANT FOR HETEROLOGOUS ANTIGENS

(57) Abstract: The present invention relates to the use of the major OprI lipoprotein of *Pseudomonas aeruginosa* to elicit a Type-1 immune response towards a heterologous antigen. The invention relates specifically to the use of OprI - antigen fusion proteins to elicit said Type-1 response. More particularly, the present invention is directed to pharmaceutical formulations comprising OprI and/or OprI fusion proteins, optionally together with a suitable excipient, to stimulate the Th1 dependent, cellular immune response.

Th1 inducing natural adjuvant for heterologous antigens

The present invention relates to the use of the major OprI lipoprotein of *Pseudomonas aeruginosa* to elicit a Type-1 immune response towards a heterologous antigen. The invention relates specifically to the use of OprI – antigen fusion proteins to elicit said Type-1 response. More particularly, the present invention is directed to pharmaceutical formulations comprising OprI and/or OprI fusion proteins, optionally together with a suitable excipient, to stimulate the Th1 dependent, cellular immune response.

Upon T-Cell Receptor (TCR) – ligation, Th0 cells differentiate into distinct subsets characterized by their functions and cytokine production profiles (Mosmann and Coffman, 1989). Th1 lymphocytes, characterized by the production of IL-2, IFN- γ and TNF- β , contribute to cellular immunity whereas Th2 lymphocytes, producing IL-4, IL-5 and IL-10 are mainly involved in humoral immunity. The generation of cell-mediated immunity against many infectious pathogens relies on the induction of the innate immune system, which in turn generates appropriate signals for adaptive immune responses (Fearon and Locksley, 1996). Bacterial lipoproteins are among others, molecules that stimulate cells of the innate immune system to produce cytokines such as TNF- α (Radolf *et al.*, 1991; Vidal *et al.*, 1998) and IL-12 (Brightbill *et al.*, 1999). Hereby, bacterial lipoproteins activate innate immune cells via Toll-like receptors (Brightbill *et al.*, 1999; Aliprantis *et al.*, 1999) and their signaling activity resides in the NH₂-terminal triacylated lipopeptide region (Erdile *et al.*, 1993; Weis *et al.*, 1994). The potent capacity of bacterial lipoproteins and/or lipopeptides to induce the production of IL-12 (Brightbill *et al.*, 1999), a key signal of the innate immune system, may in turn trigger the development of adaptive immune responses.

Numerous examples of the consequences on disease outcome of skewed Th1 to Th2 ratios have been reported. Polarized Th2 responses have been implicated in pathological situations, such as *Leishmania major* infection (Heinzel *et al.*, 1991; Nabors *et al.*, 1995), TBC (de Jong *et al.*, 1997), human leprosy (Yamamura *et al.*, 1991) and mycotic infections (Murphy *et al.*, 1994). The contribution of Th1 cells relative to Th2 cells to the developing autoimmune response determines for a larger part whether or not this response leads to clinical disease (Racke *et al.*, 1994; Racke *et al.*, 1995; Leonard *et al.*, 1995). In allergic asthma, a predominant Th2 type response has been noted (Vogel,

1997). Also the chronic autoimmune graft-versus-host disease, which develops after the administration of mismatched lymphoid cells, can be prevented by switching a Th2 response to a Th1 response through administration of IFN- γ at the time of cellular transfer (Donckier *et al.*, 1994).

- 5 Several methods have been proposed to modulate the Th1/Th2 response. WO9726883 describes the use of ribavirin³ to treat imbalances in lymphokine expression. WO9848805 discloses chemical compounds that suppress the Th2 type response and can be used for treating or preventing a disease caused by abnormal activation of Th2 type immune response, such as asthma, allergic dermatitis, allergic rhinitis or systemic lupus
- 10 erythematosus. However, those chemical compounds may have unwanted side effects. WO9921968 describes the use of macrophages in the function of antigen presenting cells to redirect the balance of Th1/Th2 cell subsets during an immune response. Although the latter method is more specific, it is complicated because personalized immortalized macrophage clones should be made for each patient to be treated.
- 15 It has been demonstrated that bacterial lipoproteins may also be useful in modulating the Th1/Th2 immune response. The synthetic lipid moiety analogue of bacterial lipoproteins (i.e. the tripalmitoyl-s-glycerol-cysteine or Pam3Cys) was reported to increase the immunogenicity of heterologous antigens (Bessler *et al.*, 1985; Lex *et al.*, 1986; Deres *et al.*, 1989; BenMohamed *et al.*, 1997). Lipopeptides derived from the outer surface
- 20 lipoproteins of *Borrelia burgdorferi* were reported to induce Th1 phenotype development (Infante-Duarte and Kamradt, 1997). It has been reported that fusion proteins between the major Oprl lipoprotein of *Pseudomonas aeruginosa* and heterologous peptides or proteins were found to be highly immunogenic as evidenced by the induction of strong humoral and cytotoxic T cell responses without the need of adjuvants (WO9303762; Cornelis *et al.*,
- 25 1996; Leitao *et al.*, 1998). Although it is clear from these data that Oprl may be useful as an adjuvant, there is no indication that Oprl can modulate the immune response. Moreover, Ino *et al.* (1999) describes that Oprl can act as a strong inducer of cytokines in mouse bone marrow cells. When purified Oprl was added to mouse bone marrow cells, an induction of TNF α , IL-1 α , IL-1 β , IL-6 and granulocyte-macrophage colony stimulating
- 30 was seen. However, IL-2, IFN- γ and TNF- β , typical for a Th1 response, were not detected. Surprisingly it is demonstrated in this invention that the Oprl-antigen fusion elicits a

Type-1 immune response towards the heterologous antigen that is fused to Oprl, even in the case where the antigen on its own does not induce a Th1 type response, or induces the Th1 response only to a limited extent. It is especially unexpected that this response is not only directed towards Oprl itself, but also to the heterologous antigen, as is demonstrated by analysis of the antibody titers. The induction of the Type-1 immune response can be clearly allocated to the lipid tail of Oprl.

Therefore, one aspect of this invention is the use of Oprl, or functional fragments thereof, as an adjuvant to obtain a Th1 type immune response against a heterologous antigen. A preferred embodiment of the invention is said use, whereby Oprl or a functional fragment thereof is fused to the heterologous antigen. One particular embodiment of the invention is said use, whereby said antigen is gp63 of *Leishmania major* or a functional fragment thereof.

WO 9504079 describes the use of Oprl to expose proteins on the surface of host cells. In the present invention, it is unexpectedly demonstrated that host cells, presenting a heterologous antigen fused to Oprl, can stimulate the Th1 response towards the heterologous antigen in a similar way as if the purified Oprl-antigen fusion protein is used. Therefore, another aspect of the invention is the use of a host cell, expressing an Oprl-heterologous antigen fusion protein to obtain a Th1 type response against said heterologous antigen.

Another aspect of the invention is the use of Oprl and /or the use of an Oprl-heterologous antigen fusion protein and/or the use of a host cell, expressing an Oprl-heterologous antigen fusion protein to treat a disease in which the natural Th1 response is insufficient, and/or the response is polarized towards a Th2 response. Such diseases are well known to the people skilled in the art and include, but are not limited to, Leishmaniasis, TBC, leprosy and mycotic infections, allergic asthma, and several autoimmune diseases such as chronic autoimmune graft-versus-host disease.

Still another aspect of the invention is a process for the manufacture of a pharmaceutical composition characterized in the use of Oprl and/or Oprl fused to a heterologous antigen and/or a host cell expressing an Oprl-heterologous antigen fusion, according to the invention.

Still another aspect of the invention is a pharmaceutical composition to treat diseases in which the natural Th1 response is insufficient, comprising Oprl and/or Oprl fused to a

heterologous antigen and/or a host cell expressing an OprI-heterologous antigen fusion protein, optionally together with a suitable excipient.

Definitions

5

The following definitions are set forth to illustrate and define the meaning and scope of the various terms used to describe the invention herein:

Functional fragment of OprI means any fragment that has still the adjuvant capacity and Th1 inducing ability. Preferentially, the functional fragment is comprising at the least the 4
10 amino terminal amino acids of the sequence shown in SEQ.ID.N°1, including the lipid modification, more preferentially at the least the 10 amino terminal amino acids of the sequence shown in SEQ.ID.N°1, including the lipid modification and most preferentially the functional fragments is comprising the 57 amino terminal amino acids of the mature OprI protein, as shown in SEQ.ID.N°1, including the lipid modification.

15 *Functional fragment* of an antigen means a part of said antigen that still has antigenic activity and is containing at least one epitope.

Heterologous antigen means an antigen that has at least one epitope that differs from the epitopes of OprI.

Host cell means any host cell in which the OprI-heterologous antigen fusion protein can
20 be expressed and whereby the antigen is presented on the surface of said host cell. Preferentially, the host cell is a bacterium, more preferentially, the host cell is a gram negative bacterium, even more preferentially the host cell is *Escherichia coli*, *Alcaligenes eutrophus* or *Salmonella typhimurium*.

25 Brief description of the figures

Figure 1: Plasmid map of pVUB3.

Figure 2: Plasmid map of pC1MM2

30

Figure 3: Formulations of the three recombinant Gp63 preparations used in this study.

L-OprICOOHgp63: lipidated OprI/COOHgp63 fusion protein

NL-OprlCOOHgp63: non-lipidated Oprl/COOHgp63 fusion protein

COOHgp63: 6xhis-tagged COOHgp63

Figure 4: Release of IFN- γ and IL-10 from lymph nodes of L-OprlCOOHgp63, NL-OprlCOOHgp63 and COOHgp63 immunized-mice. Production of IFN- γ and IL-10 was quantified in the lymph nodes of BALB/c (a) and C57BL/6 (b) mice 7 days after immunization

Results show values of pooled sacral lymph nodes from five mice, representative of two similar experiments.

Figure 5: Anti-Gp63 antibody responses in mice immunized with L-OprlCOOHgp63, NL-OprlCOOHgp63 or COOHgp63. IgG antibody titers against COOHgp63 in sera from BALB/c (a) and C57BL/6 (b) mice 10 days after mice received the third injection of the mentioned protein. Results of end-point ELISA titers are from pooled sera of five mice.

The experiment was repeated twice and similar results were obtained.

Figure 6: The lipid tail of L-OprlCOOHgp63 is required to induce TNF- α release by peritoneal macrophages either activated or not with 100 units/ml IFN- γ . The data are representative for two independent experiments.

Figure 7: The lipoprotein-induced Type-1 immune response is affected in TNF- α knockout mice (TNF- $\alpha^{-/-}$). IFN- γ and IL-10 production in sacral lymph node (a, c) and spleen (b, d) cells from mice immunized with one (a, b) or three doses (c, d) of L-OprlCOOHgp63. IgG antibody titers against COOHgp63 in sera from BALB/c, C57BL/6 and C57BL/6 TNF- $\alpha^{-/-}$ mice, 10 days after mice received the third dose of L-OprlCOOHgp63 (e). Results show end-point ELISA titers from pooled sera samples of five mice. Similar results were obtained in a second independent experiment.

Figure 8: The Oprl-based COOHgp63 lipoprotein protects BALB/c mice against *Leishmania* challenge. Groups of 15 mice were vaccinated subcutaneously three times with the lipidated L-OprlCOOHgp63, the non-lipidated NL-OprlCOOHgp63 or COOHgp63.

Controls were injected with buffer. Mice were infected with 10^6 live promastigotes 10 days after the last immunization and lesion development was monitored weekly.

Figure 9: Plasmid map of pVUB3:3D15.

5

Figure 10: IFN- γ (A) and IL-10 (B) production in spleen cells from mice immunized once with SL3261(pVUB3:3D15). Splenic lymphocytes were restimulated with SL3261 lysate (SL3261), ovalbumin (OVA), non-lipidated Oprl (NL-Oprl) or 6x his-3D protein (3D).

10 Figure 11: Preimmune and immune humoral isotype responses in mice immunized once with SL3261(pVUB3:3D15). The abbreviations are the same as in Figure 9.

Figure 12: (A) Antibody response measured in serum and (B) Production of IFN- γ in spleen cells from BALB/c mice, immunised 3 times at a 10 days interval, 2 and 12 weeks after immunisation with L-OprlCOOHGp63 (indicated as Oprl-Cgp63).

Figure 13: (A) Antibody response measured in serum and (B) Production of IFN- γ in spleen cells from BALB/c mice, immunised 3 times at a 10 days interval, 2 and 12 weeks after immunisation with either the L-OprlCOOHGp63 fusion (indicated as Oprl-Cgp63), or a mixture of L-Oprl and COOHGp63 (indicated as Oprl + Cgp63).

Figure 14: Lysis of OVA₂₅₇₋₂₆₄ peptide-loaded RMA-S cells by cytotoxic T cell lymphocytes, induced against the OVA₂₅₇₋₂₆₄ (SIINFEKL) epitope, in the presence of various adjuvants.

Oprl + CTL: 1 μ g Oprl + 5 μ g OVA₂₅₇₋₂₆₄ MHC class I (K^b-restricted) peptide

25 Oprl + CTL + Th: 1 μ g Oprl + 5 μ g OVA₂₅₇₋₂₆₄ MHC class I (K^b-restricted) peptide + 5 μ g OVA₂₆₅₋₂₈₀ (MHC class II (I-Ab-restricted) T_h peptide

PBS + CTL: PBS + 5 μ g OVA₂₅₇₋₂₆₄ MHC class I (K^b-restricted) peptide

CFA + CTL: CFA + 5 μ g OVA₂₅₇₋₂₆₄ MHC class I (K^b-restricted) peptide

Oprl + OVA: 1 μ g L-Oprl + 1 μ g OVA protein

30 PBS + OVA: 1 μ g OVA in PBS

RMA-S + OVA: RMA-S cells loaded with OVA₂₅₇₋₂₆₄ peptide

RMA-S: non-loaded RMA-S cells

Figure 15: plasmid map of pVUB3:VP8

- 5 Figure 16: Rotavirus strain RF78 neutralisation assay using sera collected from mice immunized with *Salmonella typhimurium* χ 4046 (S.typhimurium), *S. typhimurium* χ 4046 transformed with pVUB3 (S.typh (pVUB3)) and *S. typhimurium* χ 4046 transformed with pVUB3-VP8 (S.typh (pVUB3-VP8)). Anti-RF78 represents the positive control using polyclonal anti-RF78 antibodies (obtained from Dr. Cohen, INRA, France).

10

Examples

Materials and methods to the examples

Mice.

- 15 Female BALB/c, C57BL/6 and LPS-resistant C3H/HeJ mice of 6-8 weeks of age were obtained from Harlan Nederland (Horst, The Netherlands). C57BL/6 TNF- α knockout (TNF- $\alpha^{-/-}$) mice were obtained from the National Institute of Animal Health, Tsukuba City, Japan (Taniguchi *et al.*, 1997) and maintained in our animal facility.

20 Construction of pVUB3.

The construction of the pVUB3 expression plasmid has been described in detail by Cote-Sierra *et al.* (1998). A plasmid map is depicted in Figure 1.

Construction of the expression vector pCIMM2.

- 25 The *P. aeruginosa* mature *oprI* gene sequence contained in plasmid pVUB3 (Cote-Sierra *et al.*, 1998) was amplified by PCR with the following primers 5'-GCGCGGATCCTGCAGCAGCCACTCCAAAGAAACCG-3' and 3'-CTTTTTCGGTCGGCGTTCATTATTCGAACGCG-5'. Amplified DNA was purified, digested with *Bam*HI and *Hind*III, and cloned downstream of a sequence encoding an
30 oligo-histidine peptide of six residues in the expression vector pQE-8 (Qiagen GmbH, Germany), devoided of its *Eco*RI site. The resulting construct, pCIMM2, was transformed

into JM109 competent cells. In pC1MM2, the *oprl* gene is devoided of its signal sequence and consequently, cannot be transported to the bacterial outer membrane. As such, the protein will remain in the cytosol as a non-lipidated protein (NL-OprI). Due to the 6xhis tail at its 5' end, the protein can be purified by Immobilized Metal Affinity Chromatography (IMAC). In addition, the expression plasmid can be used for further subcloning of heterologous antigens into the NL-OprI sequence in order to create non-lipidated OprI/heterologous antigen fusion proteins. A plasmid map of pC1MM2 is depicted in Figure 2.

10 Generation of lipidated (L-OprI) recombinant antigens.

The generation of the lipidated L-OprICOOHgp63 fusion construct was described in detail previously (Cote-Sierra *et al.*, 1998) (Fig.3). The ligation mixture was subsequently transformed into a chemocompetent *E. coli* host using standard procedures.

A lipidated L-OprI/3D-FMDV15 fusion antigen was constructed as follows: the plasmid P7.5/3D15 containing the chimaeric 3D-FMDV15 gene (a complex B-cell/T-cell construct consisting of the FMDV-15 peptide colineary linked with the T-cell immunodominant non-structural protein 3D) was kindly provided by Dr. M. Parkhouse (Institute for Animal Health, Pirbright, U.K.). The chimaeric gene was amplified as a *BclI*-*EcoRI* PCR fragment and directionally cloned into the pVUB3 expression vector restricted with *BglII*-*EcoRI* (see Fig. 9; SEQ. ID. N°2). The ligation mixture was subsequently transformed into a chemocompetent *E. coli* host using standard procedures.

The VP8 gene was amplified by PCR from the murine rotavirus strain EW (G3P17) and cloned into plasmid pGV4684 as fusion with *phoA*. Subsequently, the VP8-*phoA* fragment was ligated as a *StuI*-*HindIII* fragment into pVUB3, digested with *EcoRI*(fill-in)-*HindIII* (Fig. 15). The ligation mixture was subsequently transformed into a chemocompetent *E. coli* host using standard procedures.

Generation of non-lipidated (NL-OprI) recombinant antigens.

The recombinant vector producing the 6xhis-non-lipidated NL-OprICOOHgp63 protein (Fig.3) was constructed by introducing the *BglII*-*HindIII* COOHgp63 DNA fragment (generated by digesting vector pVUB3:COOHgp63, Cote-Sierra *et al.*, 1998) into the 6xhis-NL-OprI producing pC1MM2 plasmid (Fig. 2) using standard methods en the

resulting plasmid was subsequently transformed into chemocompetent *E.coli* cells.

Construction of 6xhis-tagged antigens.

The recombinant 6xhis-COOHgp63 protein (Indicated as COOHgp63; Fig. 3) was generated by directionally cloning the *BglIII-HindIII* COOHgp63 DNA fragment (generated by digesting vector pVUB3:COOHgp63) into the expression vector pQE32 (Qiagen GmbH, Germany) digested with *BamHI* and *HindIII*.

The recombinant His-tagged FMDV 3D protein was generated by directionally cloning a *BamHI-PstI* FMDV-3D amplicon (generated by PCR amplification from the plasmid p7.5/3D15 (provided by Dr. M. Parkhouse, IAH, Pirbright, U.K.) using 3D-specific primers containing the *BamHI* or *PstI* restriction site coding sequence, respectively) into the expression vector pQE30 (Qiagen GmbH, Germany), restricted with the same enzymes. The resulting ligation mixture was subsequently transformed into chemocompetent *E. coli* cells using standard procedures.

Expression and purification of recombinant antigens.

Induction of L-Opri, L-Opri fusion proteins, 6xhis-NL-Opri, 6xhis-NL-Opri fusion proteins and 6xhis-tagged proteins with IPTG and preparation of outer membrane fractions was performed as described previously (Cornelis *et al.*, 1996). Opri and Opri fusion proteins are purified from outer membrane fractions solubilized in a buffer containing 50mM Tris-HCl pH 8.0, 0.6% SDS, 10 mM β -mercaptoethanol. The outer membrane proteins were loaded onto a preparative SDS-polyacrylamide column and purified by continuous elution electrophoresis using the Bio-Rad Model 491 Prep Cell (Bio-Rad Laboratories, Hercules, California) according to the manufacturer's instruction. 6xhis-tagged proteins, 6xhis-NL-Opri and 6xhis-NL-Opri fusion proteins were purified by affinity chromatography (IMAC) under denaturing conditions using the Ni-NTA superflow resin (Qiagen GmbH, Germany) or TALON Metal Affinity resin (Clontech, Palo Alto, USA) and concentrated by using a VIVASPIN concentrator (VIVASCIENCE, Lincoln, UK), previously treated with 0.02% pluronic acid for 10 min (in the case of L-Opri and L-Opri fusion proteins). When necessary, IMAC-purified proteins were re-purified by continuous elution electrophoresis as mentioned above. Finally, proteins were subjected to two successive gel filtration chromatographies in the AKTA explorer (Amersham Pharmacia/Biotech, Sweden) using

Superdex-75 HR10/30 (Pharmacia Biotech, Sweden) in order to remove LPS (Hoekstra *et al.*, 1976), and eluted in a buffer containing 20 mM Tris-HCl, pH 8.0, 100mM NaCl, 20 mM glycine and 0.01% SDS. Protein concentration was determined using the Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, California). Lipopolysaccharide (LPS) in
5 the protein suspension was determined by the Limulus Amebocyte Lysate Assay (Biowhittaker, Inc. Walkersville, MD).

Transformation of recombinant plasmid into attenuated *Salmonella*.

Plasmid DNA was transformed into the respective *Salmonella* strains by electroporation
10 using standard procedures (2.5 kV, 400 Ω , 25 μ F). After electroporation, the bacterial cells were grown for 2 h at 37°C in LB medium. Aliquots of the transformation mix were grown on LB agar plates containing the appropriate antibiotic to select for recombinant bacteria.

15 Production of *Salmonella* lysates

Salmonella cultures were grown overnight in LB medium at 37°C. The bacteria were pelleted, resuspended in PBS supplemented with protease inhibitors (Boehringer Mannheim) and subjected to 3 freeze-thaw cycles followed by sonication. After removing debris by centrifugation, the lysates were aliquoted and stored at -80°C until use.

20

Production of *Salmonella* live oral vaccines

Cultures of recombinant *Salmonellae* were statically grown overnight at 37°C and used to seed fresh medium at a starting dilution of 1:50, and the subcultures were incubated at 37°C until the OD₆₀₀ reached between 0.7-0.75. Expression of the recombinant L-Opri
25 protein was then achieved by inducing the culture for 30 min with 1 mM IPTG. After induction, the bacteria were pelleted and resuspended to the appropriate cell density in PBS.

Immunizations with 6xhis-protein, L-Opri-, and NL-Opri formulations.

30 BALB/c, C57BL/6 or C57BL/6 TNF- α ^{-/-} mice were subcutaneously immunized three times at 10 days interval in the base of the tail with 1 μ g of either 6xhis-protein, L-Opri- or NL-Opri formulation. Preimmune sera were taken one day before the first immunization.

Seven or 10 days after the first or third immunization respectively, mice were killed. Sera, spleens and draining lymph nodes (sacral lymph nodes) were taken to analyze the immune response.

5 Cytokine assays.

Homogeneous lymph node and spleen cell suspensions from individual mice were prepared in supplemented RPMI 1640 medium (fetal calf serum 10%, penicillin-streptomycin 100 U and 100µg/ml respectively, L-glutamine 2 mM, 2-mercaptoethanol 5×10^{-5} M, MEM amino acid solution 1X and sodium pyruvate 1 mM). 2×10^6 cells were
10 separately stimulated with or without the appropriate antigen at 37°C in 24-well flat bottom tissue culture plates (Becton Dickinson, Franklin Lakes, NJ, USA). IFN-γ, IL-4, and IL-10 were determined in culture supernatants taken 24, 48, 72 and 96 hours after priming. The cytokine levels were analyzed by a sandwich enzyme-linked immunosorbent assay (ELISA) in accordance to the supplier's instructions (Pharmingen, San Diego, CA USA).
15 Data are represented as mean cytokine concentrations over 4 days.

Measurement of antibody titers.

Immunoglobulin isotype titers in the preimmune and immune sera were measured by using ELISA (Southern Biotechnology Associates, Inc. Birmingham, AL, USA). Briefly,
20 96-well Nunc-Immuno plates (Nalge Nunc International, Denmark) were coated with the appropriate antigen and after exposure to diluted preimmune or immune sera, bound antibodies were detected by HRP-labeled goat anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgA and IgM. ELISA titers were specified as the last dilution of the sample whose absorbance was above threefold the preimmune sera value.

25

Induction of TNF-α production in peritoneal exudate cells (PECs) after antigen stimulation.

PECs from LPS-resistant C3H/HeJ mice were harvested by washing the peritoneal cavity with 10 ml of ice-cold sucrose solution (0.34 M). The cells were washed in supplemented RPMI 1640 and left to adhere for 2 h. at 37°C in 24-well flat bottom tissue culture plates
30 (Becton Dickinson, Franklin Lakes, NJ, USA) at a concentration of 1×10^6 cells/ml. The peritoneal exudate cells were stimulated or not with recombinant murine IFN-γ (Life

Technologies, Ltd., Paisley, Scotland, 100 U/ml) in the presence of L- and NL-OprlCOOHgp63 or COOHgp63. After overnight incubation in a humidified atmosphere of 5% of CO₂, supernatants were collected to determine TNF- α concentrations by using the DuoSet ELISA development system (R&D systems, Abingdon, UK).

5

L. major challenge.

Groups of 15 BALB/c mice were subcutaneously immunized three times with 1 μ g of either L-OprlCOOHgp63, NL-OprlCOOHgp63 or COOHgp63. A control group was immunized with the buffer in which the proteins were dissolved. 10 days after the third
10 dose, mice were s.c. challenged with 10⁶ live virulent *L. major* promastigotes in the base of the tail. Progress of the disease was monitored weekly by scoring the lesion development.

CTL assay

15 CTLs were derived from spleens of mice that had been immunised with the respective OVA₂₅₇₋₂₆₄ peptide (SIINFEKL)/adjuvant or OVA protein/adjuvant formulations. Starting 10 days after the last immunisation, CTLs were restimulated in vitro by incubating (1-2) 10⁸ spleen cells with 10⁷ irradiated (7000 rad) RMA-S/B7 cells loaded with OVA₂₅₇₋₂₆₄ peptide (SIINFEKL), in 50 ml RPMI complete medium, supplemented with 1 mM non-essential
20 amino acids (Gibco), 1 mM sodium pyruvate (Gibco) and 20 μ M 2-mercaptoethanol (Merck) for 5 days at 37°C. After isolation of blasts on Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) gradient, the percentage specific lysis was determined in an ¹¹¹In release assay (Kupiec-Weglinski *et al.*, 1988), which is similar to the standard ⁵¹Cr-release assay. Briefly, target cells (RMA-S, RMA-S cells loaded with OVA₂₅₇₋₂₆₄ peptide
25 (SIINFEKL)) labeled with ¹¹¹In were incubated with CTL indifferent ratios for 4 h at 37°C and release of ¹¹¹In in the supernatant was measured. The percentage specific lysis was determined as [(release-spontaneous release)/(maximal release-spontaneous release)]. For maximal release, sodium dodecyl sulphate was added to the target cells (2% final concentration).

30

Example 1: The lipid moiety of OprI-COOHgp63 fusion protein is required for the induction of Type-1 immune responses.

To evaluate the potential adjuvant capacity of the lipoprotein I of *P. aeruginosa* to heterologous proteins and the contribution of its lipid moiety to the immunogenicity of the chimeric OprI-COOHgp63 lipoprotein, three different recombinant proteins were produced: the lipidated L-OprI-COOHgp63, the non-lipidated NL-OprI-COOHgp63 and the COOHgp63 (Fig 3). All three recombinant proteins contain the COOH-terminal domain of the glycoprotein Gp63 of *L. major*, which contains the host-protective T cell epitopes (Yang *et al.*, 1991). Mice (BALB/c, C57BL/6) were immunized subcutaneously once or three times with the recombinant proteins to respectively analyze the early cellular immune responses in the draining lymph nodes, and the secondary humoral immune responses, elicited against the heterologous COOHgp63 antigen. BALB/c is a highly susceptible mouse strain for *L. major* infection and an effective vaccine requires the induction of an IFN- γ -dependent Type-1 immune response (Reiner and Locksley, 1995; Milon *et al.*, 1995). *In vitro* restimulation with the COOHgp63 of lymph node cells from BALB/c mice immunized once with either type of lipoprotein construct or COOHgp63, resulted in a clear induction of IL-10 secretion (Fig 4a). In contrast, only lymph node cells from L-OprI-COOHgp63-immunized BALB/c mice secreted IFN- γ (Fig. 4a). Likewise, in the C57BL/6 strain, only lymph node cells from animals immunized with L-OprI-COOHgp63 produced very high levels of IFN- γ upon COOHgp63 restimulation (Fig 4b). The induction of IFN- γ production was sustained after three immunizations as evidenced by the production of high IFN- γ levels in the spleen compartment, whereas the induction of IL-10 production was completely abrogated (Fig. 7.d). When IL-4 was measured in the same culture supernatants, a secretion pattern similar to IL-10 was seen. However the levels of IL-4 production were either undetectable or much lower than the levels of IL-10.

Antibody isotype responses against the COOHgp63 protein were also analyzed in immunized animals. As shown for BALB/c (Fig. 5a) and C57BL/6 mice (Fig. 5b), three immunizations with the lipidated OprI-COOHgp63 induced a significant production of COOHgp63-specific IgG2a, IgG3, IgG2b and IgG1 antibodies. In sharp contrast, the non-

lipidated OprI-COOHgp63 and the COOHgp63 (the latter only shown for BALB/c mice) only induced significant levels of IgG1 anti-Gp63 antibodies and very low or undetectable levels of IgG2a, IgG3 and IgG2b in either mouse strain. There was no detectable IgA in the serum samples while the levels of IgM were marginal. Collectively, these immunization experiments demonstrate that the lipid tail of OprI-COOHgp63 chimeric proteins elicit potent cellular (IFN- γ) and humoral (IgG2a and IgG3 antibodies) Type-1 immune responses.

Comparative analysis of lipidated OprI-COOHgp63, the non-lipidated counterpart and COOHgp63 recombinant proteins in immunised mice demonstrated the crucial importance of the lipid tail of the *P. aeruginosa* lipoprotein I in inducing Type-1 immune responses against the heterologous antigen as evidenced by the cytokine pattern and profile of antibody subclass production. Indeed, a single immunization with the lipidated L-OprI-COOHgp63 biased the T-cell response towards IFN- γ production indicating a preferential induction of a Type-1 immune response. Besides the induction of IFN- γ -producing cells, our results also demonstrate that the lipid tail of OprI potentiates the induction of humoral responses against a heterologous antigen since immunizations with L-OprI-COOHgp63 increased or triggered IgG2a, IgG3 and IgG2b subclass responses against COOHgp63.

Example 2: The Type-1 inducing potential of L-OprI-COOHgp63 is TNF- α -dependent.

TNF- α , secreted by lipoprotein-activated macrophages (Radolf *et al.*, 1991; Vidal *et al.*, 1998), has been suggested to be a key molecule, together with IL-12, in the induction of IFN- γ production and amplification of Type-1 immune responses (Butler *et al.*, 1999; Tripp *et al.*, 1993). Therefore, it was of interest to test whether (i) OprI-based lipoproteins induce TNF- α production by macrophages and (ii) TNF- α contributes to the Type-1 adjuvant activity of OprI. Macrophages (the plastic adherent fraction of peritoneal exudate cells (PEC), unactivated or activated with 100 units/ml IFN- γ) from endotoxin-resistant C3H/HeJ mice were stimulated *in vitro* with either the lipidated COOHgp63, non-lipidated COOHgp63 or the COOHgp63 antigen. As shown in Fig. 6, a dose-dependent induction of TNF- α in unprimed macrophages was recorded with the lipidated L-OprI-COOHgp63.

Moreover, the TNF- α -inducing activity of L-OprlCOOHgp63 was strongly increased in IFN- γ -primed macrophages (Fig 6). In these experimental conditions, both the non-lipidated Oprl-COOHgp63 and the COOHgp63 elicited marginal levels of TNF- α synthesis.

- 5 To test whether the TNF- α -inducing capacity of L-OprlCOOHgp63 contributes to its Type-1 immune response-inducing potential, one and three immunizations with L-OprlCOOHgp63 were performed in C57BL/6 TNF- $\alpha^{-/-}$ mice. As shown in Fig. 7, both early and late priming of COOHgp63-specific IFN- γ production was markedly reduced in the culture supernatants of draining lymph node (Fig.7a) or spleen cells (Fig.7b) from L-
- 10 OprlCOOHgp63-immunized TNF- $\alpha^{-/-}$ mice as compared to immunized C57BL/6 wild-type mice. Likewise, decreased Type-1 responses were also recorded in the culture supernatants of draining lymph node (Fig. 7c) and spleen cells (Fig 7d) from TNF- $\alpha^{-/-}$ mice immunized three times with the antigen, and restimulated *in vitro* with COOHgp63. Analysis of the humoral responses elicited with L-OprlCOOHgp63 (after three
- 15 immunizations) in BALB/c, C57BL/6 wild type and C57BL/6 TNF- $\alpha^{-/-}$ mice, revealed that anti-COOHgp63 IgG3 and IgG2a responses were severely reduced in C57BL/6 TNF- $\alpha^{-/-}$ mice (Fig 7e). In contrast, the magnitude of IgG1 and IgG2b subclass responses were respectively unaffected or less impaired in immunized C57BL/6 TNF- $\alpha^{-/-}$ mice as compared to wild type C57BL/6 and BALB/c mice. Altogether, these data suggest that the
- 20 Type-1 immune response elicited by Oprl is strongly TNF- α -dependent.

The capacity of L-OprlCOOHgp63 to instruct acquired immune responses may reflect its potential to trigger innate immune cells. Corroborating other reports that bacterial lipoproteins are potent inducers of TNF- α production (Radolf *et al.*, 1991; Vidal *et al.*, 1998), our results show that only L-OprlCOOHgp63 was capable to stimulate significantly

25 TNF- α production by either naive or IFN- γ -primed macrophages. Local production of TNF- α may in turn signal the development of Type-1 acquired immune responses. Indeed, this cytokine was documented to induce the expression of B7-like costimulatory signals (Swallow *et al.*, 1999), IFN- γ production by T cells (Butler *et al.*, 1999; Darji *et al.*, 1996) and NK cells (Tripp *et al.*, 1993) and Type-1 antibody subclass responses (i.e.

30 IgG2a) (Pasparakis *et al.*, 1996). The involvement of TNF- α in the genesis and/or progression of cellular and humoral Type-1 acquired immune responses to leishmanial

antigens is herein further substantiated since both Type-1 cytokine (IFN- γ) and humoral subclass (IgG3 and IgG2a) responses against the heterologous antigen were severely compromised in L-OprlCOOHgp63-immunized TNF- $\alpha^{-/-}$ mice. Hereby, it should be emphasized that CFA-aided immunization did not reveal similar defects in TNF- $\alpha^{-/-}$ mice.

5 Hence, the defective induction of Type-1 responses recorded in L-OprlCOOHgp63 immunized TNF- $\alpha^{-/-}$ mice most probably reflects the TNF- α -inducing potential of Oprl by virtue of its lipid tail. According to our *in vivo* results TNF- α can be considered as a component of the innate immune system which, synergistically with or alternatively to IL-12, bridges the gap between innate and acquired immunity. Finally, since the TNF- α -

10 inducing capacity of Oprl is strongly increased upon macrophage-priming with IFN- γ , TNF- α -mediated induction of IFN- γ production by Oprl-based vaccines may further amplify ongoing or subsequent Oprl elicited immune responses.

Example 3: Vaccinations with Oprl-based COOHgp63 lipoproteins protect highly susceptible BALB/c mice against *Leishmania* challenge.

It is well established that during infection with *L. major*, resistant C57BL/6 mice mount a polarized Type-1 cellular immune response mediated by IFN- γ production (Reiner and Locksley, 1995; Milon *et al.*, 1995). In view of the capacity of the lipid-modified OprlCOOHgp63 to skew the immune response towards a IFN- γ -producing Type-1

20 immune response, it was of interest to test whether vaccinations with this lipoprotein could provide protection in highly susceptible BALB/c mice against *Leishmania* challenge. To this end, mice were vaccinated with the lipidated OprlCOOHgp63, the non-lipidated counterpart or COOHgp63 in order to compare the effect of immunization on lesion development. As shown in Fig. 8, a clear delay in the onset of skin lesions in mice

25 vaccinated with the lipid-modified protein was observed. In the groups vaccinated with the non-lipidated OprlCOOHgp63 and the COOHgp63, the pattern of disease appearance was similar to the control group although a slight delay was observed. After 14 weeks of infection, 73% of L-OprlCOOHgp63 vaccinated animals still remained healthy, indicating that vaccination with the lipid-modified protein delayed the appearance of the disease and

30 induced a protective immunity in the majority of the animals.

It is well established that immunological control of *L. major* infections depends on the

production of IFN- γ that activates macrophages to kill the parasites via induction of NO production (Milon *et al.*, 1995; Mossalayi *et al.*, 1999; Green *et al.*, 1990). Accordingly, the capacity of L-OpriCOOHgp63 to elicit COOHgp63-specific IFN- γ -producing memory cells is reflected by the induction of protective immunity against *L. major* infections in the highly susceptible BALB/c model. Taking into account that this type of immunization is highly TNF- α -dependent, it is worth mentioning that vaccination with leishmanial antigens together with TNF- α prevents disease enhancement and induces protective immunity against *L. major* infection in susceptible BALB/c mice (Liew *et al.*, 1991).

Example 4: induction of Type-1 immune responses against a heterologous antigen by immunization with a host cell expressing a Opri-heterologous antigen fusion protein

To see whether L-Opri, pathogen-derived antigens/peptides, presented in the context of L-Opri at the surface of live host cells, can induce a relevant immune response, a live vaccination experiment was carried out using a L-Opri/FMDV antigen presented at the surface of attenuated *Salmonella typhimurium* SL3261 (Hoiseth & Stocker, 1981).

BALB/c mice (8 weeks of age) were immunized internasally with 10^8 *S. typhimurium* SL3261(pVUB3:3D-15) in a 10 μ l volume (5 μ l per nostril). Three months after the intranasal immunization, mice were killed. Sera and spleens were taken to analyze the immune response.

Homogeneous spleen cell suspensions from individual mice were prepared in supplemented RPMI 1640 medium. 2×10^6 cells were restimulated with either 6 x his-3D, NL-Opri, ovalbumin (irrelevant antigen) or crude *Salmonella* SL3261 lysate. The cytokine levels were determined in the culture supernatants taken 24, 48, 72 and 96 h after restimulation. Antibody isotypes against recombinant 6xhis-3D protein, NL-Opri or crude *Salmonella* SL3261 lysate were determined in naive and immune sera by ELISA. Serum was applied to every separate antigen (2 μ g/ml) and detected with specific anti-isotype antibodies. Total anti-3D IgG titers are in the range of 1/10000.

A single immunization with SL3261(pVUB3:3D-15) biased the T-cell response against the heterologous 3D antigen towards IFN- γ production (Fig.10) indicating a preferential induction of a Type-1 immune response. Besides the induction of IFN- γ producing cells,

this immunization also elicited a selective humoral (IgG2a) type-1 immune response against 3D (Fig. 11).

Example 5: Oprl derived fusion proteins induce a long lasting Type-1 immune response

BALB/c mice were s.c. immunised 3 x at 10-days interval with 1 µg Oprl-COOHgp63 antigen. Spleen cells and sera were taken at 2 and 12 weeks after the last immunisation and analysed for the production of cytokines and antibodies resp. The results are shown in Fig. 12, indicating that both antibody response and IFN-γ production is hardly affected by the time.

Example 6: Oprl retains its Type-1 adjuvanticity when admixed with a heterologous antigen as demonstrated in the *Leishmania* model

BALB/c mice were s.c. immunised 3 x at 10-days interval with 1 µg Oprl-COOHgp63 antigen or L-Oprl + 6xhis-COOHgp63 at a same molar basis as the covalent formulation. Spleen cells and sera were taken 2 weeks after the last immunisation and analysed for the production of cytokines and antibodies resp. The results are shown in Fig.13. Although there are some differences in IgG3 response and IFN-γ production, it is clear that the response obtained by the Oprl / COOHgp63 mixture is mainly a Type-1 response.

Example 7: L-Oprl incytes cytolytic CD8 T cells towards MHC-class-I restricted T cell epitopes in a Th cell-independent way.

In view of the capacity of L-Oprl to induce Type-1 humoral and cellular responses against a heterologous antigen fused to its C-terminal or admixed with, it was of interest to look for the possible adjuvant capacity of L-Oprl to induce specific cytotoxic T lymphocytes against a heterologous antigen or peptide. To this end, we evaluated the CTL inducing capacity of L-Oprl in a OVA-model when admixed with free protein or peptides as compared to other adjuvants.

C57Bl/6 mice were immunised with i) 1 µg L-Opri + 1 µg OVA protein, ii) 1 µg Opri + 5 µg OVA₂₅₇₋₂₆₄ MHC class I (K^b-restricted) peptide, iii) 1 µg Opri + 5 µg OVA₂₅₇₋₂₆₄ MHC class I (K^b-restricted) peptide + 5 µg OVA₂₆₅₋₂₈₀ (MHC class II (I-Ab-restricted) T_h peptide, iv) PBS + 5 µg OVA₂₅₇₋₂₆₄ MHC class I (K^b-restricted) peptide, v) CFA + 5 µg 5 µg OVA₂₅₇₋₂₆₄ MHC class I (K^b-restricted) peptide and vi) 1 µg OVA in PBS).

Mice were immunised three times sub-cutaneously (s.c.) at the base of the tail, with 10 days interval. The CTL assay was set up 10 days after the last immunisation. Each mouse was analysed individually, each group consisted of 4 mice.

As shown in Fig. 14, splenocytes from all groups of mice immunized with Opri + antigen/peptide, lysed significantly OVA₂₅₇₋₂₆₄ peptide-loaded RMA-S target cells as compared to the unloaded RMA-S target cells. Immunisation with antigen/peptide in PBS did not induce a specific cytolytic activity. As compared to CFA, Opri seems to be a more potent adjuvant for the induction of CTLs against a minimal CTL epitope.

In conclusion, L-Opri was shown to incite cytolytic CD8 T cells toward MHC class I-restricted T cell epitopes in a Th cell-independent manner, which could be further potentiated by the addition of T-helper epitope.

Example 8: Live oral vaccination with recombinant *Salmonella* expressing an Opri-rotavirus recombinant antigen, elicits specific neutralizing antibodies against rotavirus

To see whether live oral vaccination using L-Opri as a carrier for the presentation of heterologous antigens on the surface of a host cell, can induce the appropriate immune response, immunisation experiments were performed with *S. typhimurium* χ 4064 (Curtiss & Kelly, 1987) harboring pVUB3:VP8 rotavirus antigen. BALB/c mice were immunised once intranasally with recombinant *S. typhimurium* (strain χ 4064) expressing i) L-Opri-rotavirus (VP8) recombinant antigen, ii) L-Opri or *S. typhimurium* χ 4064 alone. Subsequent analysis of the serum taken from all groups of mice revealed the presence of VP8-specific antibodies in the group immunised with *S. typhimurium* χ 4064 expressing L-Opri-rotavirus (VP8) recombinant antigen. To see whether the elicited VP8-specific antibodies could neutralize rotavirus strain RF78 (kindly provided by Dr. Cohen, INRA,

France), 100 pfu of rotavirus strain RF78 was mixed with different dilutions of sera collected from mice immunized with either *S.typhimurium* χ 4064, χ 4064(pVUB3), or χ 4064(pVUB3-VP8), and tested for plaque reduction. Polyclonal antibodies against RF78 (A polyclonal serum against rotavirus strain RF78 was prepared and provided by Dr. Cohen, INRA, France) were used as a positive control. As can be seen from Fig. 16, serum from χ 4064(pVUB3-VP8) immunised mice could partially neutralise the rotavirus. The titer of neutralization was determined as 60% of plaque reduction.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that that prior art forms part of the common general knowledge in Australia.

References

- Aliprantis, A.O., Yang R.B., Mark, M.R., Suggett, S., Devaux, B., Radolf, J.D., Klimpel, G.R., Godowski, P. & Zychlinsky, A. Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2. *Science* **285**, 736-739, (1999).
- BenMohamed, L. Gras-Masse, H., Tartar, A., Daubersies, P., Brahimi, K., Bossus, M., Thomas, A. & Druilhe P. Lipopeptide immunization without adjuvant induces potent and long- lasting B, T helper, and cytotoxic T lymphocyte responses against a malaria liver stage antigen in mice and chimpanzees. *Eur.J.Immunol.* **27**, 1242-1253, (1997).
- Bessler, W.G., Suhr, B., Buhning, H.J., Muller, C.P., Wiesmuller, K.H., Becker, G. & Jung, G. Specific antibodies elicited by antigen covalently linked to a synthetic adjuvant. *Immunobiology* **170**, 239-244, (1985).
- Brightbill, H.D., Libraty, D.H., Krutzik, S.R., Yang, R.B., Belisle, J.T., Bleharski, J.R., Maitland, M., Norgard, M.V., Plevy, S.E., Smale, S.T., Brennan, P.J., Bloom, B.R., Godowski, P.J. & Modlin, R.L.. Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. *Science* **285**, 732-736, (1999).
- Butler, D.M., Malfait, A.M., Maini, R.N., Brennan, F.M. & Feldmann, M. Anti-IL-12 and anti-TNF antibodies synergistically suppress the progression of murine collagen-induced arthritis. *Eur.J.Immunol.* **29**, 2205-2212, (1999).
- Cornelis, P. Sierra, J.C., Lim, A., Malur, A., Tungpradabkul, S., Tazka, H., Leitao, A., Martins, C.V., di Perna, C., Brys, L., De Baetseller, P. & Hamers, R. Development of new cloning vectors for the production of immunogenic outer membrane fusion proteins in *Escherichia coli*. *Biotechnology (N.Y.)* **14**, 203-208, (1996).
- Cote-Sierra, J., Jongert, E., Bredan, A., Gautam, D.C., Parkhouse, M., Cornelis, P., De Baetseller, P. & Revets, H. A new membrane-bound OprI lipoprotein expression vector. High production of heterologous fusion proteins in gram (-) bacteria and the implications for oral vaccination. *Gene* **221**, 25-34, (1998).

- Curtiss, R. & Kelly, S.M. *Salmonella typhimurium* deletion mutants lacking adenylate cyclase and cyclic AMP receptor protein are avirulent and immunogenic. *Infect.Immun.***55**, 3035-3043, (1987).
- 5 - Darji, A., Beschin, A., Sileghem, M., Heremans, H., Brys, L. & De Baetselier, P. In vitro simulation of immunosuppression caused by *Trypanosoma brucei*: active involvement of gamma interferon and tumor necrosis factor in the pathway of suppression. *Infect. Immun.* **64**, 1937-1943, (1996).
- Deres, K., Schild, H., Wiesmuller, K.H., Jung, G. & Rammensee, H.G. In vivo priming of virus-specific cytotoxic T lymphocytes with synthetic lipopeptide vaccine. *Nature* 10 **342**, 561-564, (1989).
- de Jong, R., Janson, A.A., Faber, W.R., Naafs, B. & Ottenhoff, T.H. IL-2 and IL-12 act in synergy to overcome antigen-specific T-cell unresponsiveness in mycobacterial disease. *J. Immunol.* **159**, 786-793, (1997).
- 15 - Donckier, V., Abramowicz, D., Bruyns, C., Florquin, S., Vanderhaeghen, M.L., Amraoui, Z., Dubois, C., Vandenabeele, P. and Goldman, M. IFN-gamma prevents Th2 cell-mediated pathology after neonatal injection of semiallogenic spleen cells in mice. *J. Immunol.* **153**, 2361-2368, (1994).
- Erdile, L.F., Brandt, M.A., Warakomski, D.J., Westrack, G.J., Sadziene, A., Barbour, A.G. & Mays, J.P. Role of attached lipid in immunogenicity of *Borrelia burgdorferi* 20 *OspA*. *Infect.Immun.* **61**, 81-90, (1993).
- Fearon, D.T. & Locksley, R.M. The instructive role of innate immunity in the acquired immune response. *Science* **272**, 50-53, (1996).
- Green, S.J., Crawford, R.M., Hockmeyer, J.T., Meltzer, M.S. & Nacy, C.A. *Leishmania major* amastigotes initiate the L-arginine-dependent killing mechanism in IFN- γ - 25 stimulated macrophages by induction of tumor necrosis factor-alpha. *J.Immunol.* **145**, 4290-4297, (1990).

- Heinzl, F.P., Sadick, M.D., Mutha, S.S. & Locksley, R.M. Production of IFN- γ , IL-2, IL-4 and IL-10 by CD4⁺ lymphocytes in vivo during healing and progressive murine leishmaniasis. *Proc. Natl. Acad. Sci. USA* **88**, 7011-7015, (1991).
- Hoekstra, D., van der Laan, J.W., de Leij, L. & Witholt, B. Release of outer membrane fragments from normally growing *Escherichia coli*. *Biochim.Biophys.Acta* **455**, 889-899, (1976).
- Hoiseth, S.K. & Stocker B.A.D. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. *Nature* **291**, 238-240, (1981).
- Infante-Duarte, C. & Kamradt, T. Lipopeptides of *Borrelia burgdorferi* outer surface proteins induce Th1 phenotype development in $\alpha\beta$ T-cell receptor transgenic mice. *Infect.Immun.* **65**, 4094-4099, (1997).
- Ino, M., Nagase, S., Nagasawa, T. Koyama, A. & Tachibana, S. The outer membrane protein I of *Pseudomonas aeruginosa* PAO1, a possible pollutant of dialysate in hemodialysis, induces cytokines in mouse bone marrow cells. *Nephron*, **82**, 324-330, (1999).
- Kupiec-Weglinski, J.W., Austyn, J.M. & Morris, P.J. Migration patterns of dendritic cells in the mouse. Traffic from blood, and T cell-dependent and -independent entry to lymphoid tissues. *J. Exp. Med.* **167**, 632-645, (1988).
- Leitao, A., Malur, A., Cornelis, P. & Martins, C.L. Identification of a 25-amino acid sequence from the major African swine fever virus structural protein VP72 recognised by porcine cytotoxic T lymphocytes using a lipoprotein based expression system. *J.Virol.Methods* **75**, 113-119, (1998).
- Leonard, J.P., Waldburger, K.E. & Goldman, S.J. Prevention of experimental autoimmune encephalomyelitis by antibodies against interleukin 12. *J. Exp. Med.* **181**, 381-386, (1995).
- Lex, A., Wiesmuller, K.H., Jung, G. & Bessler, W.G. A synthetic analogue of *Escherichia coli* lipoprotein, tripalmitoyl pentapeptide, constitutes a potent immune adjuvant. *J.Immunol.* **137**, 2676-2681, (1986).

- Liew, F.W., Li, Y., Yang, D.M., Severn, A. & Cox, F.E. TNF-alpha reverses the disease-exacerbating effect of subcutaneous immunization against murine cutaneous leishmaniasis. *Immunology* **74**, 304-309, (1991).
- Milon, G., Del Giudice, G. & Louis, J.A. Immunobiology of Experimental Cutaneous
5 Leishmaniasis. *Parasitol.Today* **11**, 244-247, (1995).
- Mosmann, T.R. & Coffman, R.C. Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.*, **7**, 145- (1989).
- Mossalayi, M.D., Arock, M., Mazier, D., Vincendeau, P. & Vouldoukis, I. The human
10 immune response during cutaneous leishmaniasis: NO problem. *Parasitol.Today* **15**,
342-345, (1999).
- Murphy, J.W., Wu-Hsieh, B.A., Singer-Vermes, L.M. Ferrante, A., Moser, S., Ruso, M., Vaz, S.A., Burger, E., Calich, V.L. & Kowanko, I.C. Cytokines in the host response to mycotic agents. *J. Med. Vet. Mycol.* **32 suppl 1**, 203, (1994).
- Nabors, G.S., Afonso, L.C.C., Farrell, J.P.O. & Scott, P. Switch from a type 2 to a type
15 1 T helper cell response and cure of established *Leishmania major* infection in mice is induced by combination therapy with interleukin 12 and pentosam. *Proc. Natl. Acad. Sci. USA*, **92**, 3142-3146 , (1995).
- Pasparakis, M., Alexopoulou, L., Episkopou, V. & Kollias, G. Immune and inflammatory responses in TNF alpha-deficient mice: a critical requirement for TNF
20 alpha in the formation of primary B cell follicles, follicular dendritic cell networks and germinal centers, and in the maturation of the humoral immune response [see comments]. *J.Exp.Med.* **184**, 1397-1411, (1996).
- Racke, M.K., Bonomo, A., Scott, D.E., Canella, B., Levine, A., Raine, C.S., Shevach, E.M. & Rocken, M. Cytikine induced immune deviation as a therapy for inflammatory
25 autoimmune disease. *J. Exp. Med.*, **180**, 1961-1966, (1994).
- Racke, M.K., Burnett, D., Pak, S.H., Albert, P.S., Cannella, B., Raine, C.S., McFarlin, D.E. & Scott, D.E. Retinoid treatment of experimental allergic encephalomyelitis. IL-4

- production correlates with improved disease course. *J. Immunol.*, **154**, 450-458, (1995).
- Radolf, J.D., Norgard, M.V., Brandt, M.E., Isaacs, R.D., Thompson, P.A. & Beutler, B. Lipoproteins of *Borrelia burgdorferi* and *Treponema pallidum* activate cachectin/tumor necrosis factor synthesis. Analysis using a CAT reporter construct. *J.Immunol.* **147**, 1968-1974, (1991).
 - Reiner, S.L. & Locksley, R.M. The regulation of immunity to *Leishmania major*. *Annu.Rev.Immunol.* **13**, 151-177, (1995).
 - Swallow, M.M., Wallin, J.J. & Sha, W.C. B7h, a novel costimulatory homolog of B7.1 and B7.2, is induced by TNFalpha. *Immunity.* **11**, 423-432, (1999).
 - Taniguchi, T., Takata, M., Ikeda, A., Momotani, E. & Sekikawa, K. Failure of germinal center formation and impairment of response to endotoxin in tumor necrosis factor alpha-deficient mice. *Lab.Invest.* **77**, 647-658, (1997).
 - Tripp, C.S., Wolf, S.F. & Unanue, E.R. Interleukin 12 and tumor necrosis factor α are costimulators of interferon γ production by natural killer cells in severe combined immunodeficiency mice with listeriosis, and interleukin 10 is a physiologic antagonist. *Proc. Natl. Acad. Sci. USA.* **90**, 3725-3729, (1993).
 - Vidal, V., Scragg, I.G., Cutler, S.J., Rockett, K.A., Fekade, D., Warrell, D.A., Wright, D.J. & Kwiatkowski, D. Variable major lipoprotein is a principal TNF-inducing factor of louse- borne relapsing fever. *Nat.Med.* **4**, 1416-1420, (1998).
 - Vogel, G. New clues to asthma therapies. *Science*, **276**, 1643-1646, (1997).
 - Weis, J.J., Ma, Y. & Erdile, L.F. Biological activities of native and recombinant *Borrelia burgdorferi* outer surface protein A: dependence on lipid modification. *Infect.Immun.* **62**, 4632-4636, (1994).
 - Yamamura, M., Uyemura, K. and Deans, R.J. Defining protective responses to pathogens: cytokine profiles in leprosy lesions. *Science*, **254**, 277-279, (1991).

- Yang, D.M., Rogers, M.V. & Liew, F.W. Identification and characterization of host-protective T-cell epitopes of a major surface glycoprotein (Gp63) from *Leishmania major*. *Immunology*. **72**, 3-9, (1991).

The Claims defining the invention are as follows:

1. The use of Oprl, or functional fragments thereof, as an adjuvant to obtain a Th1 type immune response against a heterologous antigen.
2. The use according to claim 1 in which Oprl is fused to the heterologous antigen
- 5 3. The use according to claim 1 or 2, in which the antigen is gp63 of *Leishmania major*, or a functional fragment thereof.
4. The use of a host cell expressing an Oprl-heterologous antigen fusion protein to obtain a Th1 type immune response against said heterologous antigen.
5. The use according to claim 1 - 4 to treat a disease in which the natural Th1 response is insufficient and/or in which the immune response is polarized towards a Th2 response.
- 10 6. The use according to claim 5 in which the disease is Leishmania, TBC, leprosy, a mycotic infection or allergic asthma.
7. The use according to claim 5 in which the disease is an autoimmune disease.
8. A method of obtaining a Th1 type immune response to a heterologous antigen in a subject, said method comprising administering to said subject said heterologous antigen with Oprl, or one or more functional fragments thereof, as an adjuvant.
- 15 9. The method of claim 8 wherein Oprl or one or more functional fragments thereof is fused to the heterologous antigen.
10. The method of claim 8 or 9 wherein said heterologous antigen is gp63 of *Leishmania major*, or a functional fragment thereof.
- 20 11. A method of obtaining a Th1 type immune response to a heterologous antigen in a subject, said method comprising administering to said subject one or more host cells which express an Oprl-heterologous antigen fusion protein.
12. The method of any one of claims 8 to 11, wherein said method is applied to treat a disease in which the natural Th1 response is insufficient and/or in which the immune response is polarized towards a Th2 response.
- 25 13. The method of claim 12 wherein said disease is selected from the list consisting of Leishmania, TBC, leprosy, a mycotic infection or allergic asthma.
14. The method of claim 12 wherein said disease is an autoimmune disease.

30 Dated this 5th day of July 2004.

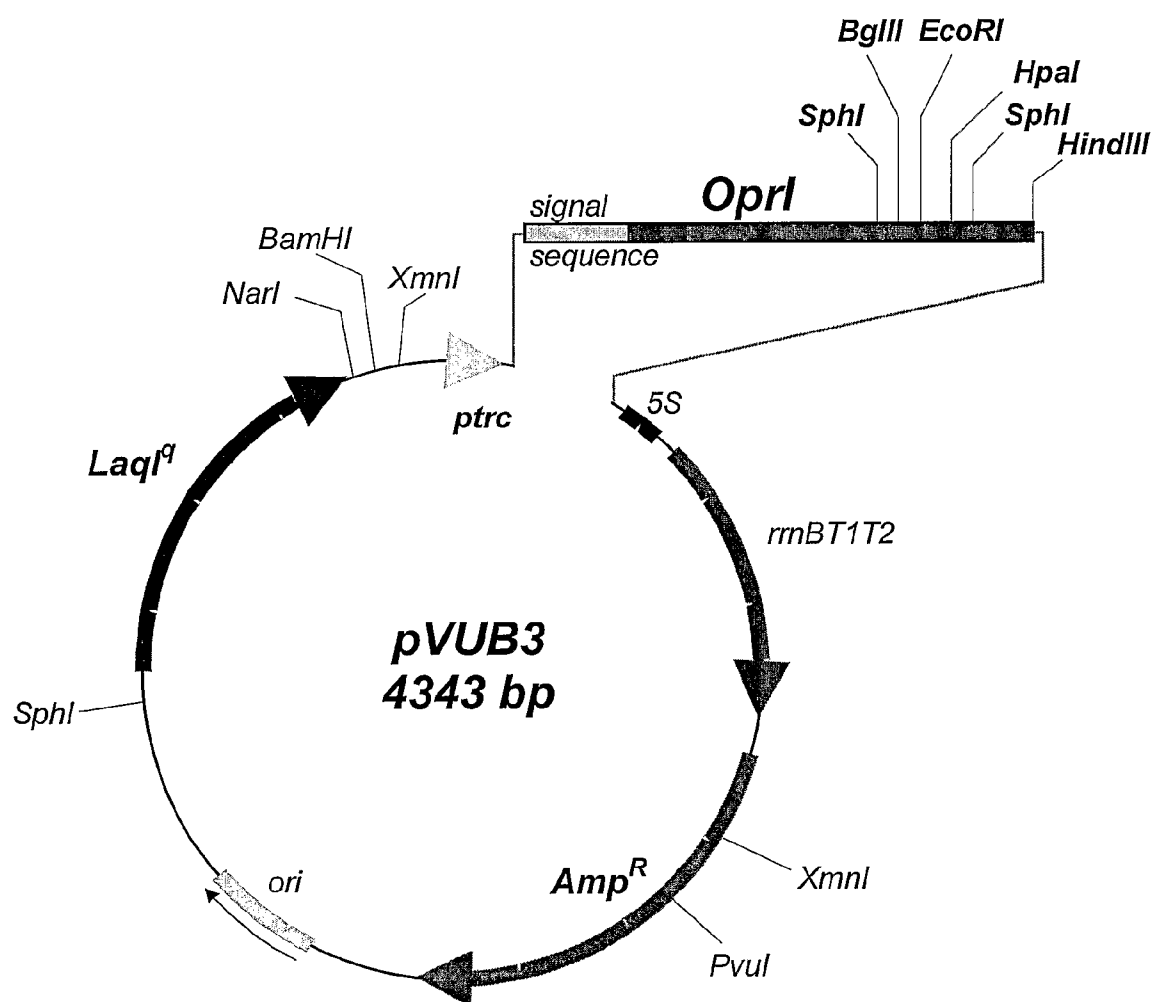
Vlaams Interuniversitair Instituut voor Biotechnologie vzw

By its Patent Attorneys

Davies Collison Cave

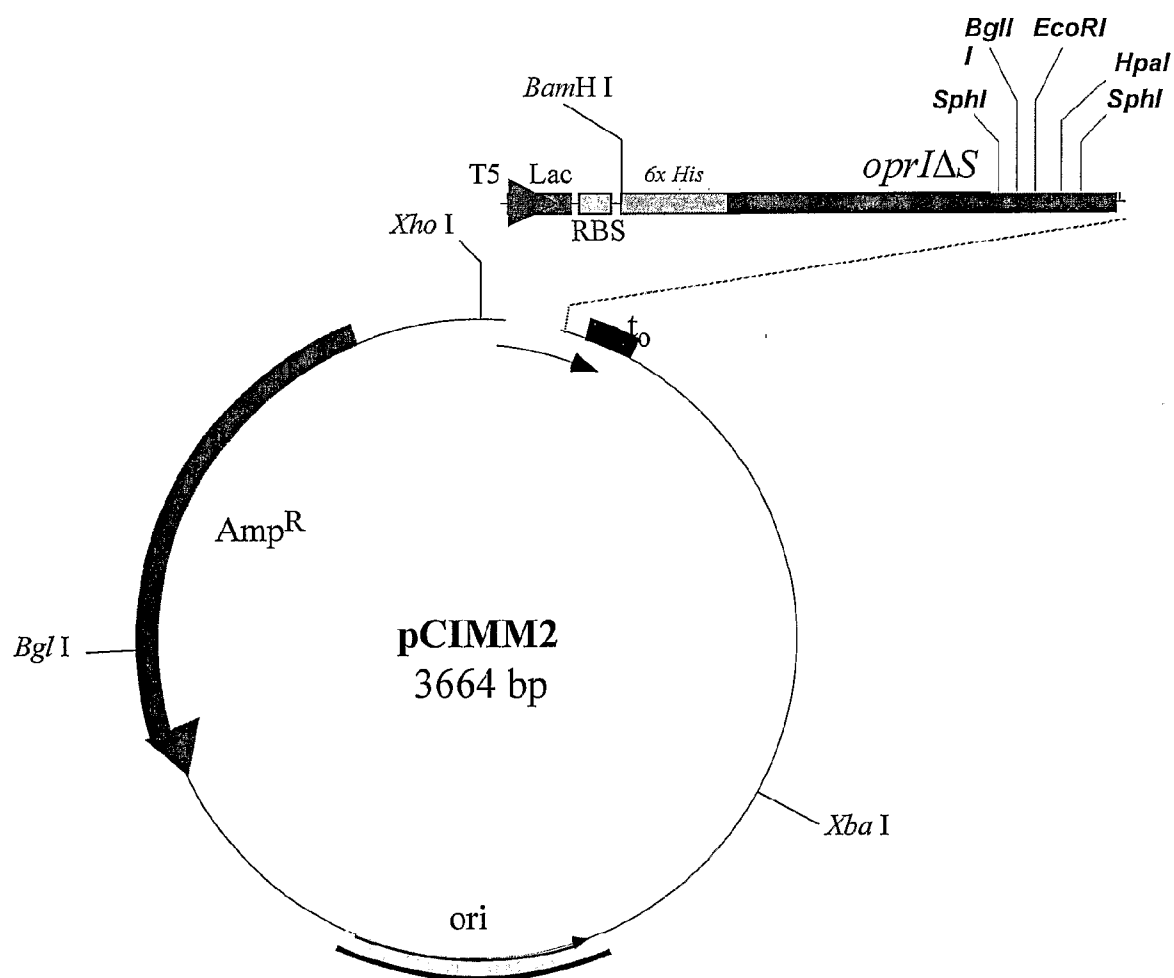
1/17

Fig. 1



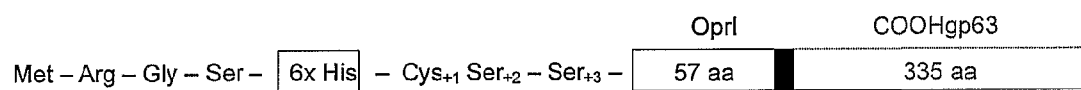
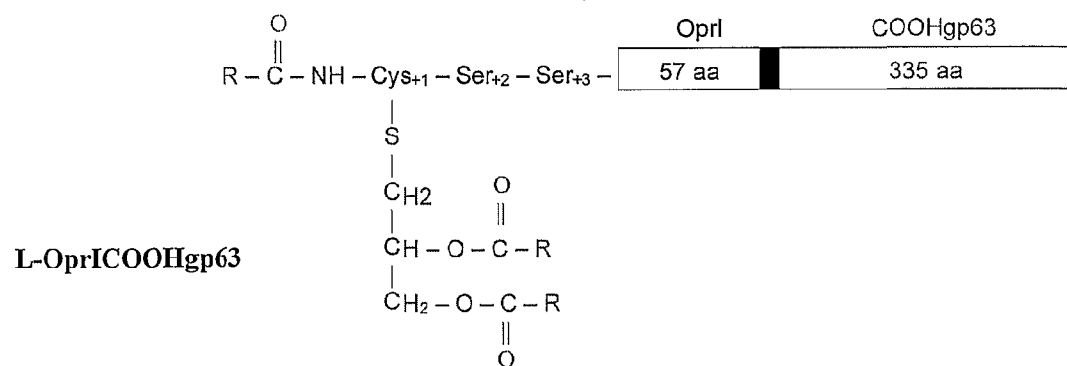
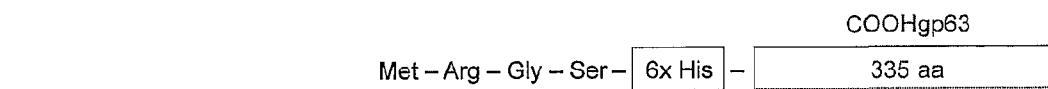
2/17

Fig. 2



3/17

Fig. 3

**NL-OprICOOHgp63**

4/17

Fig. 4a

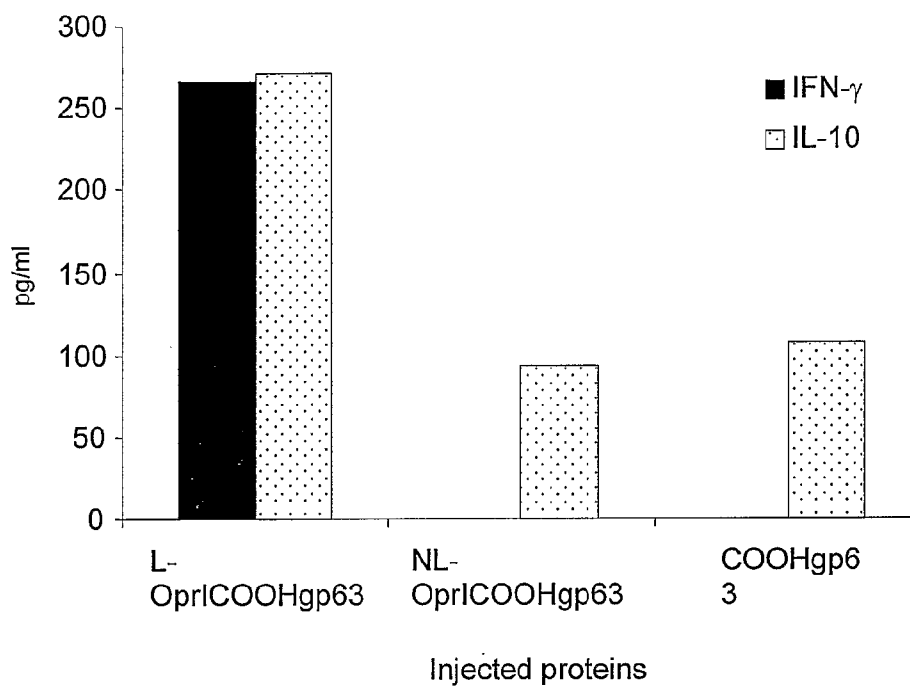
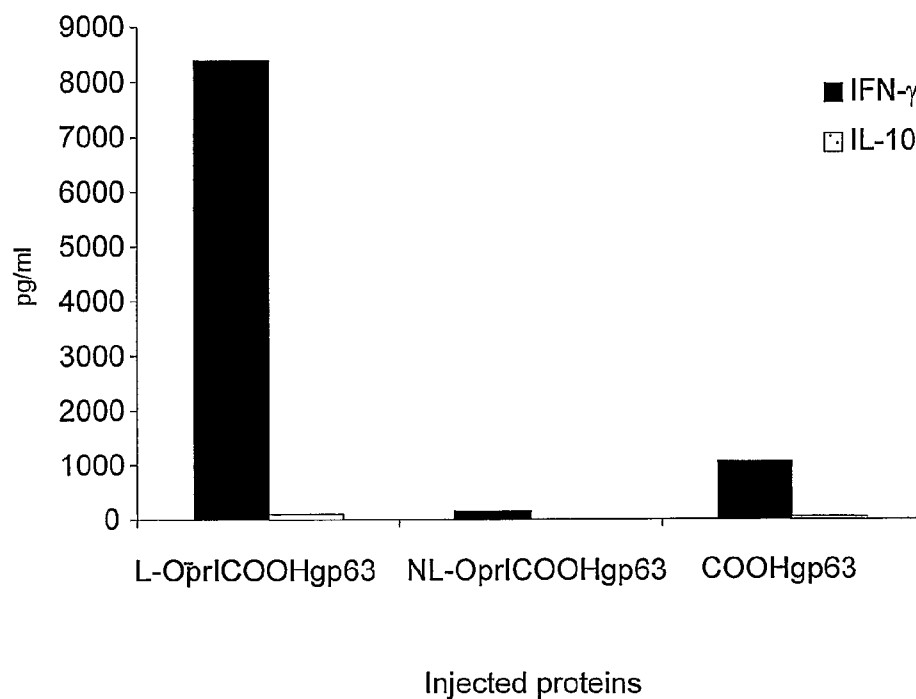


Fig. 4b



5/17

Fig. 5a

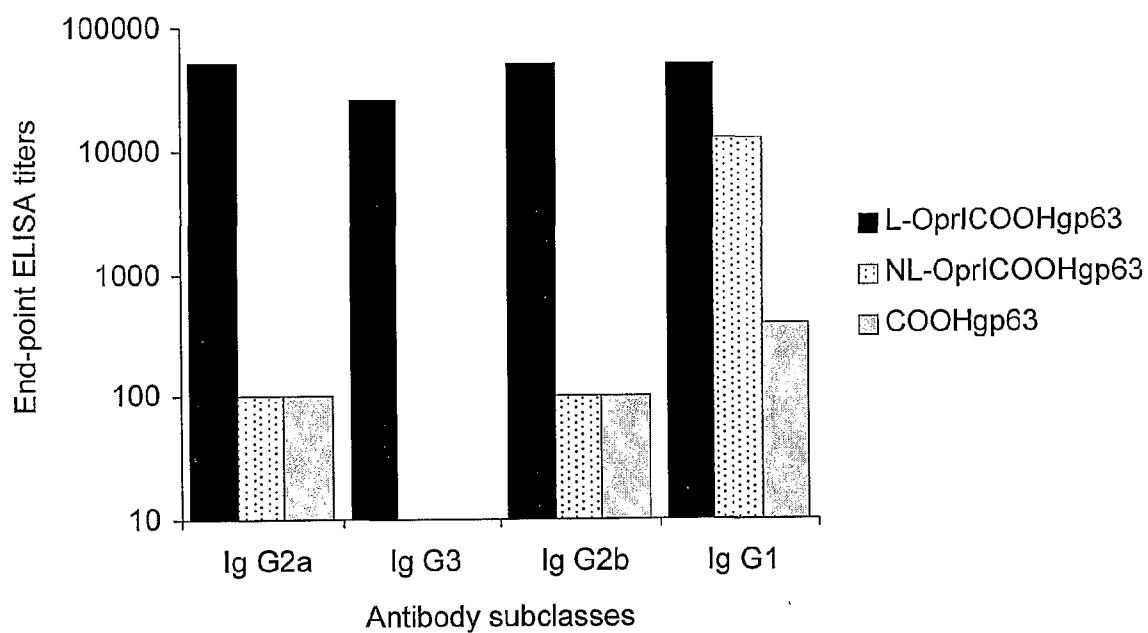
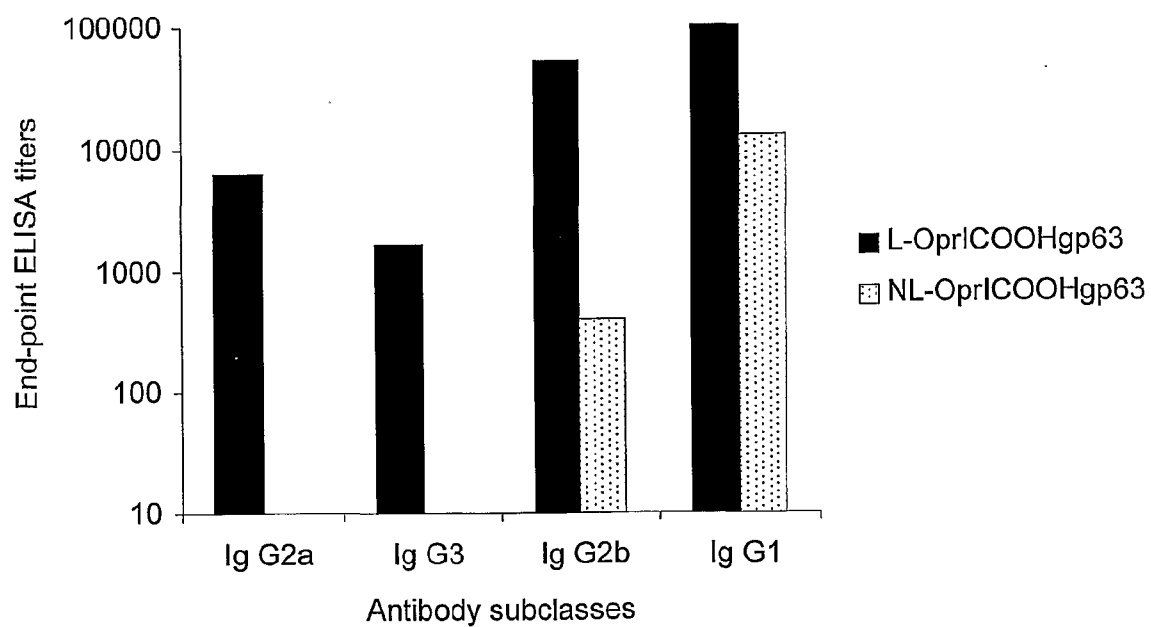
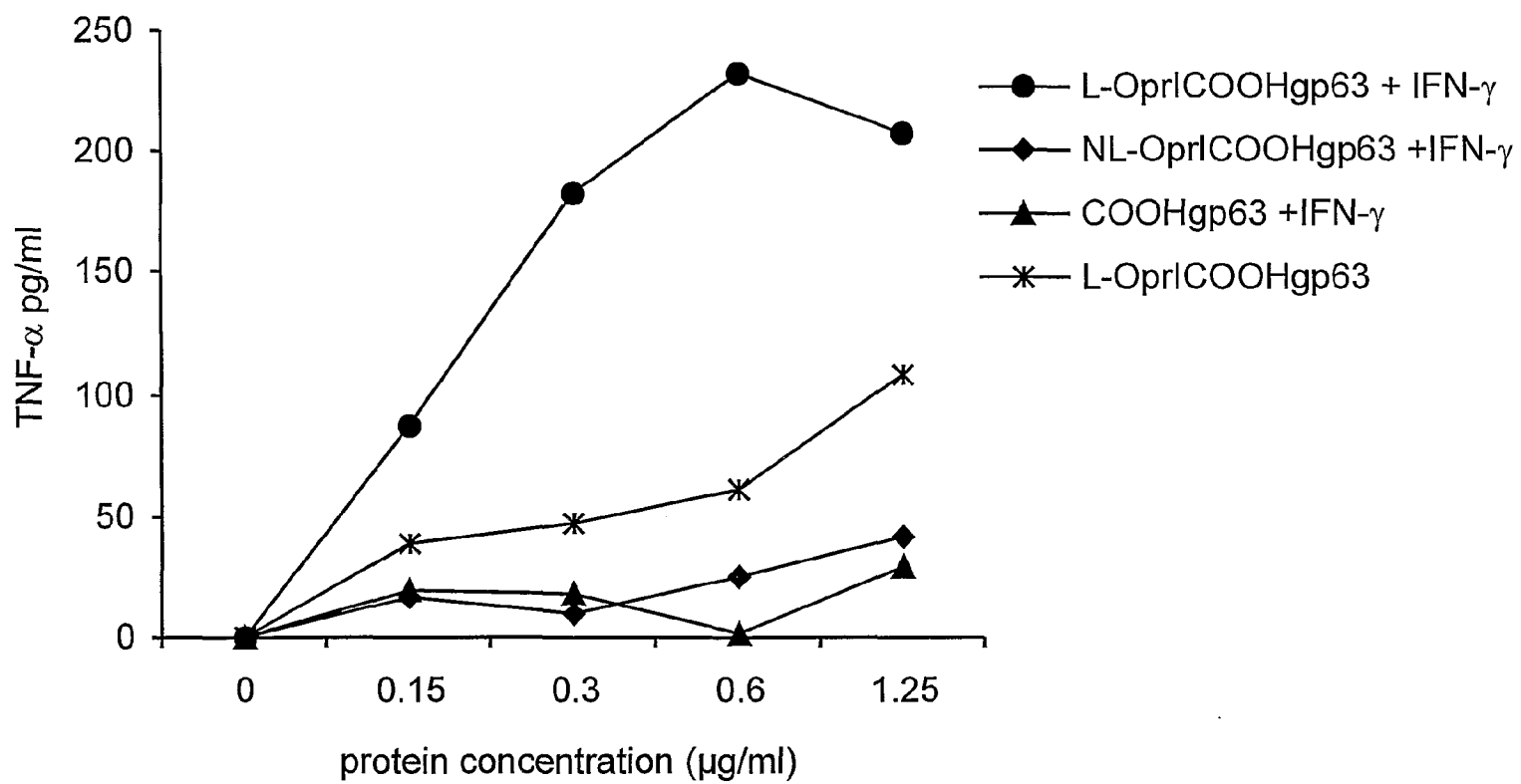


Fig. 5b



6/17

Fig. 6



7/17

Fig. 7a

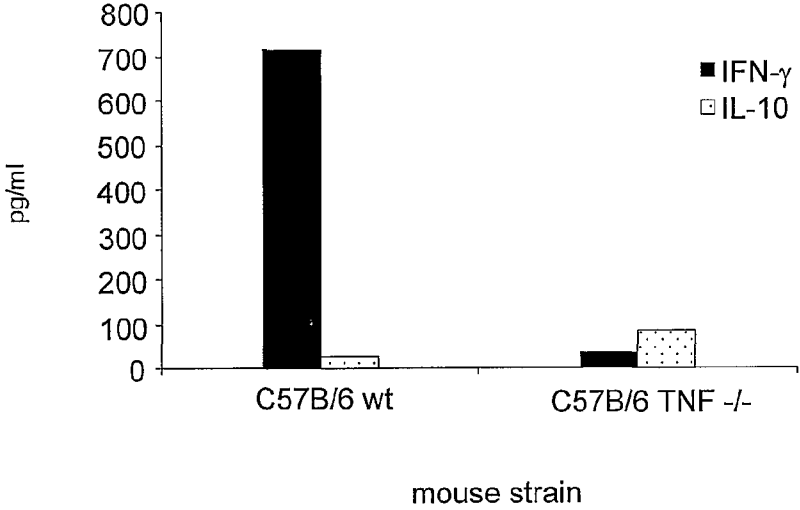


Fig. 7b

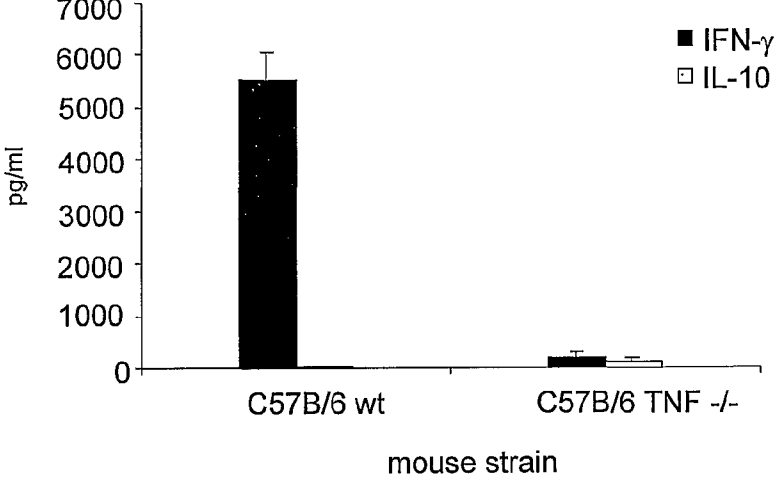
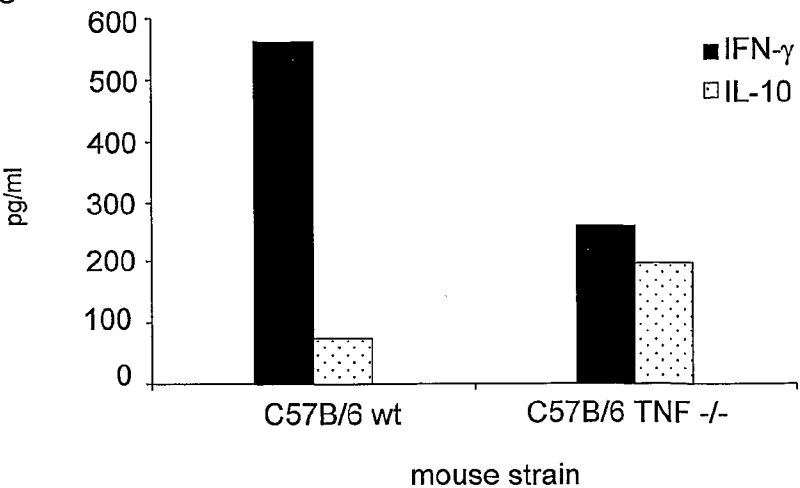


Fig. 7c



8/17

Fig. 7d

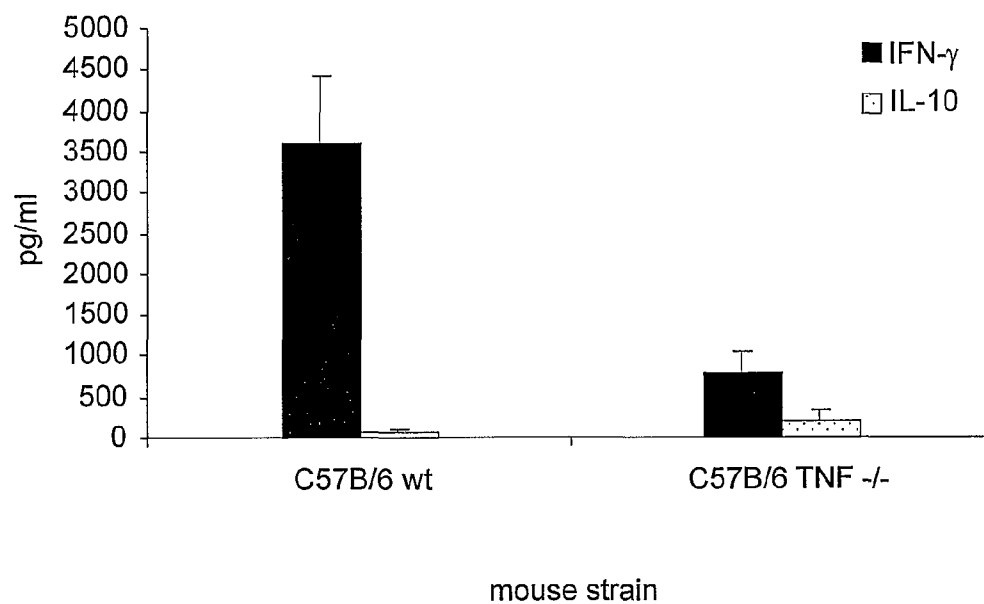
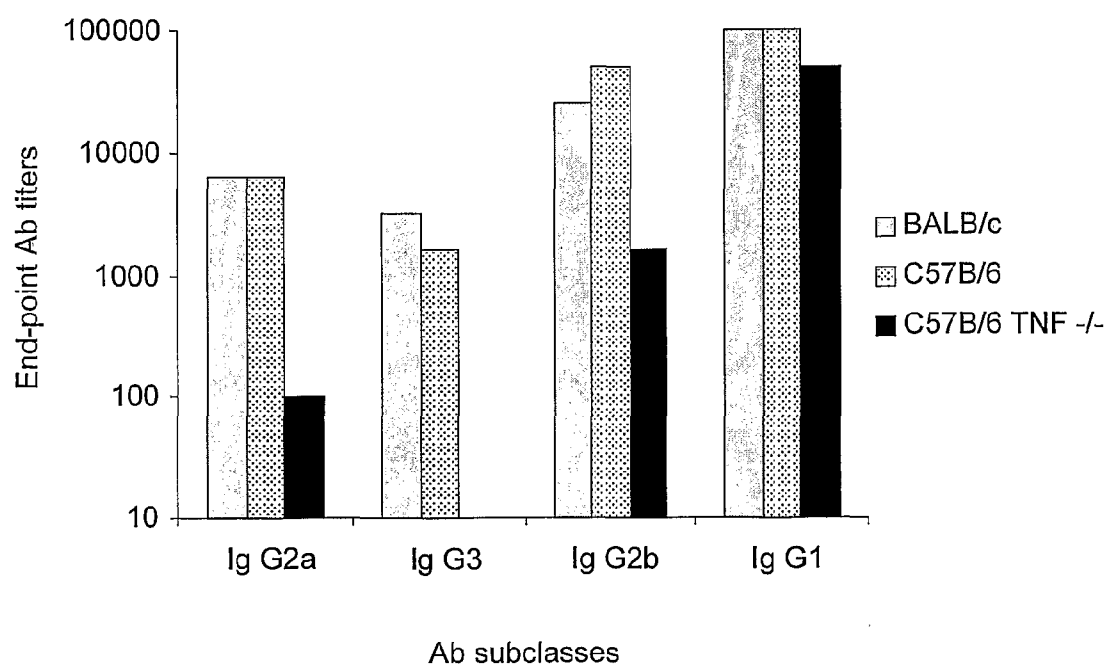
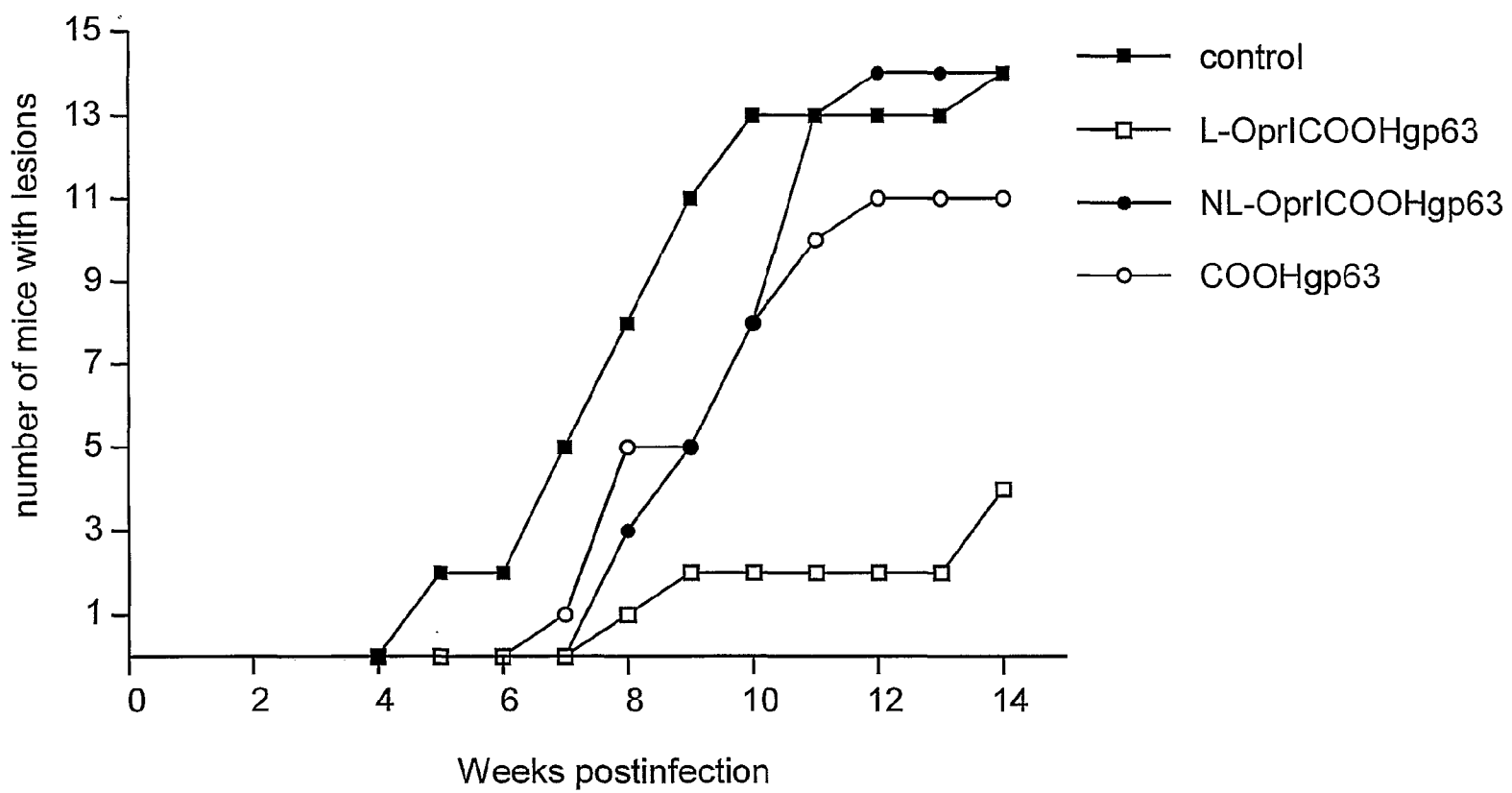


Fig. 7e



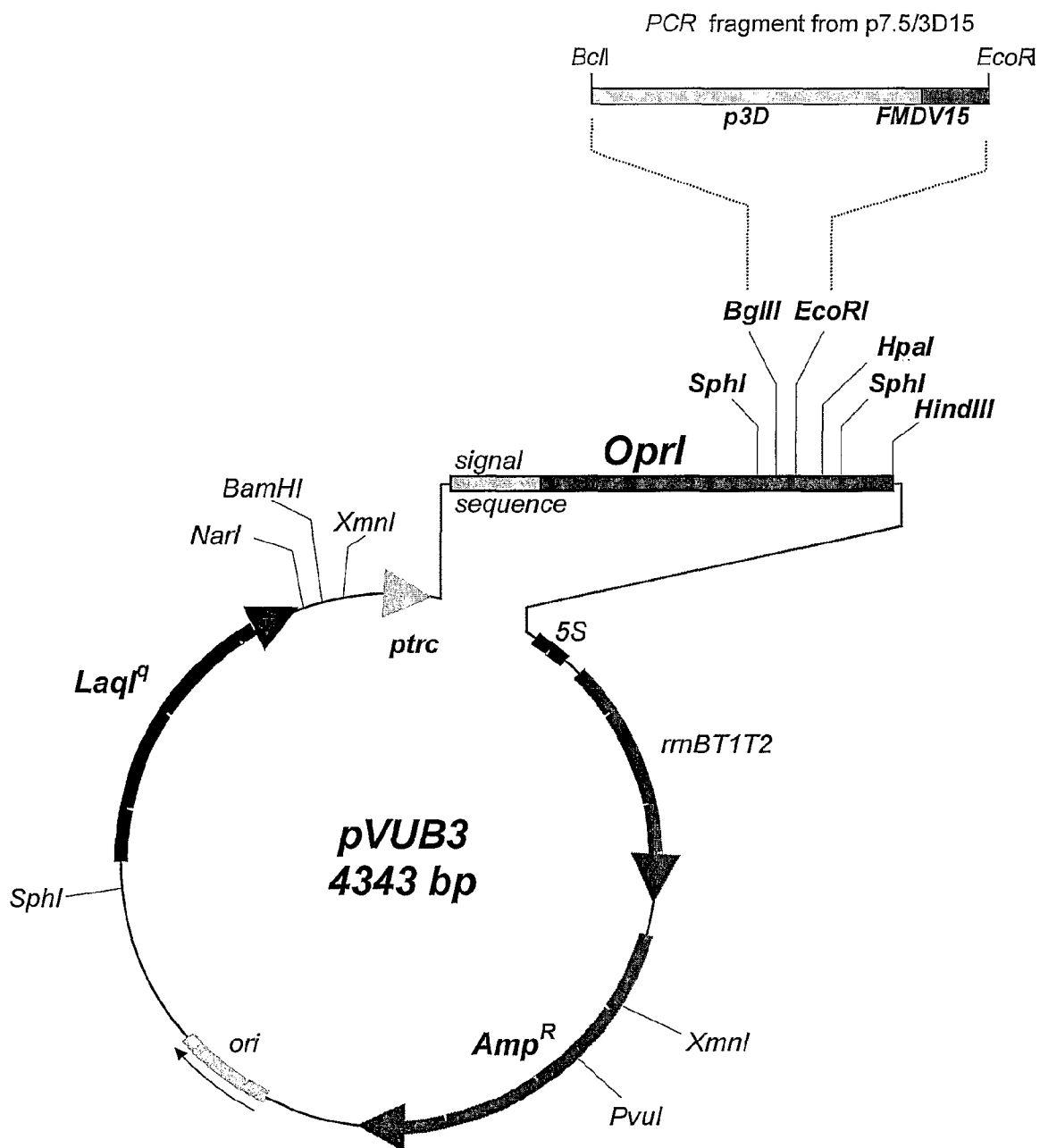
9/17

Fig. 8



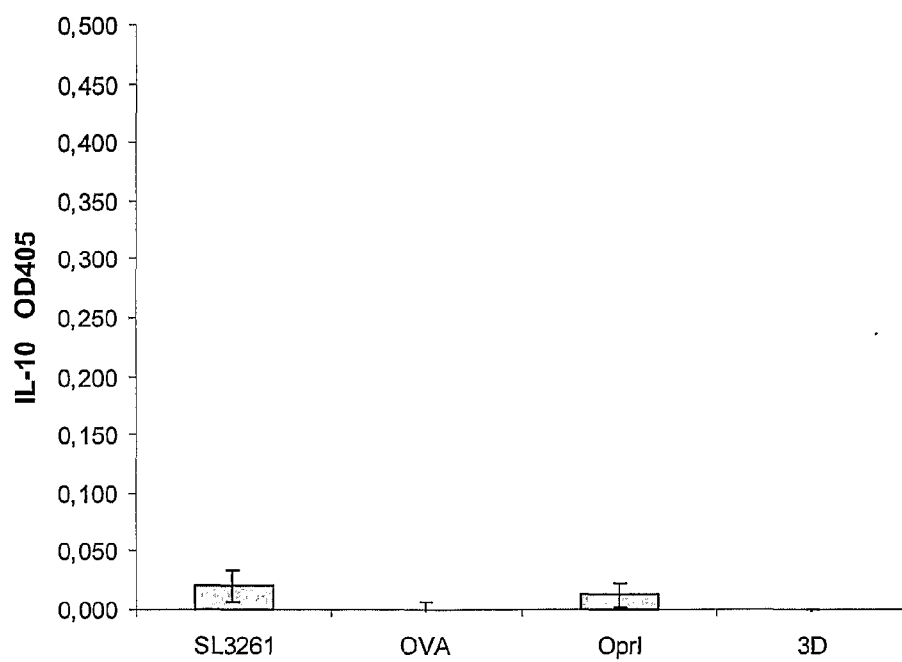
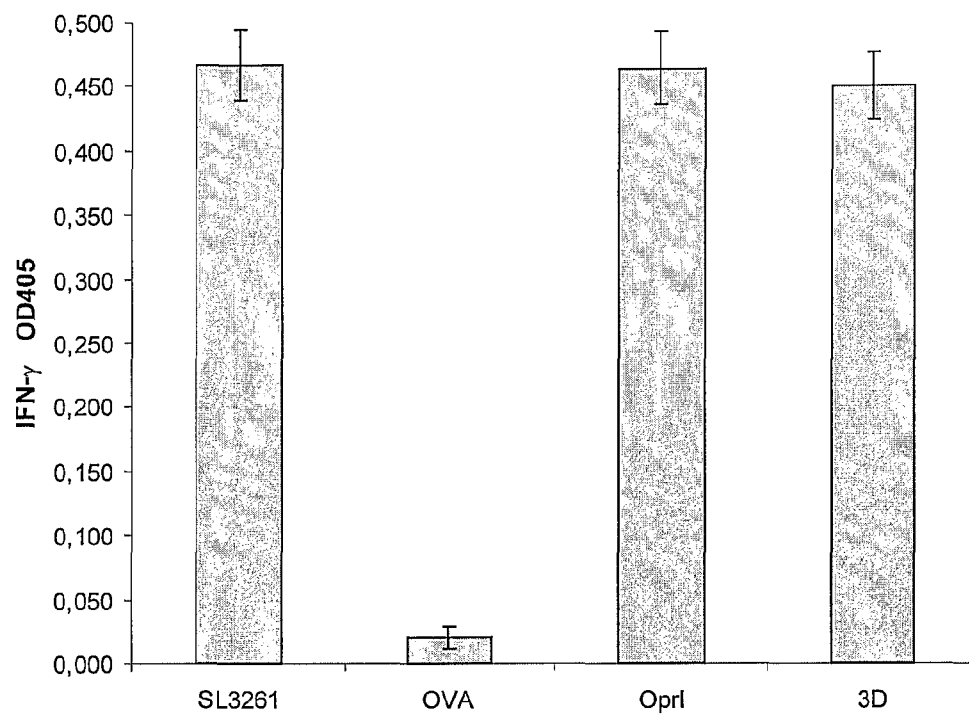
10/17

Fig. 9



11/17

Fig. 10



12/17

Fig. 11

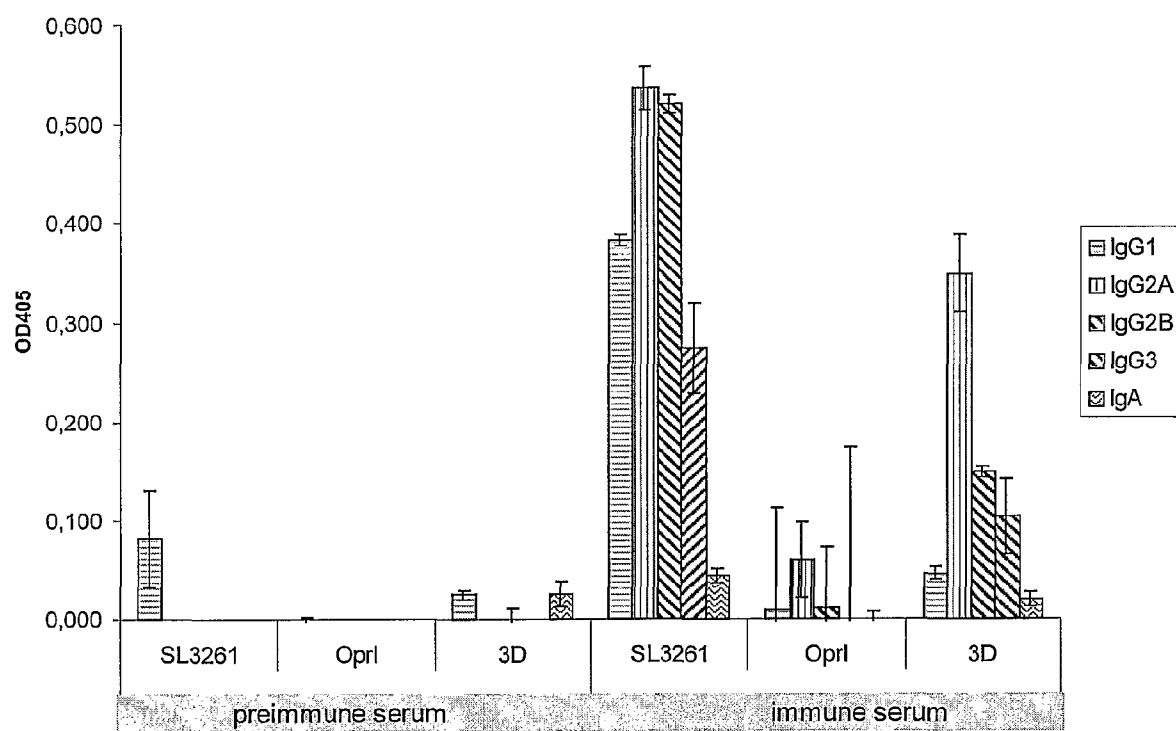


Fig. 12a

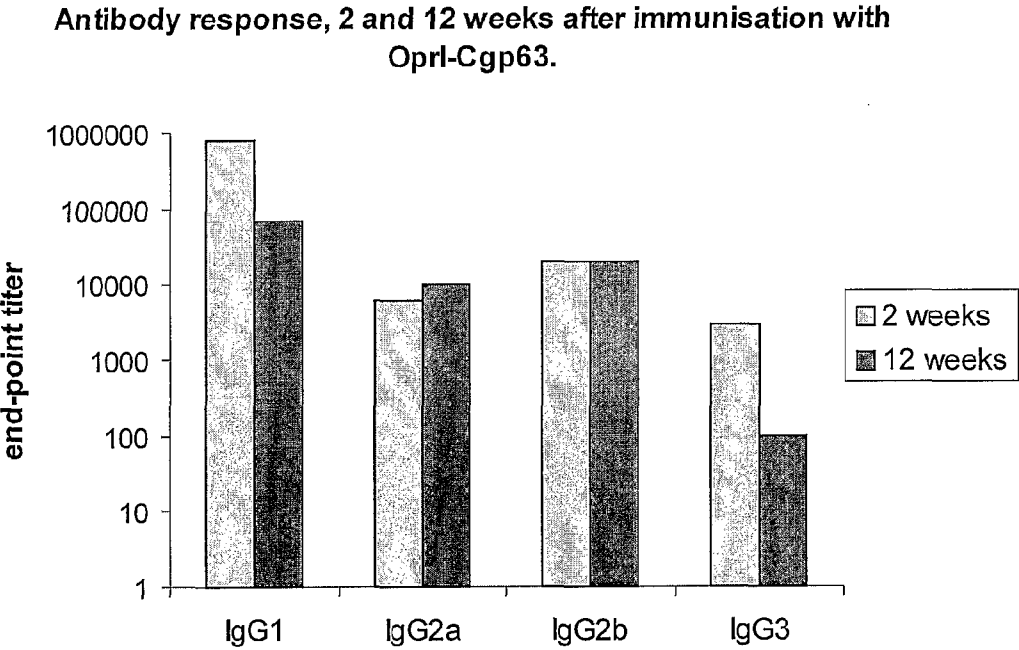
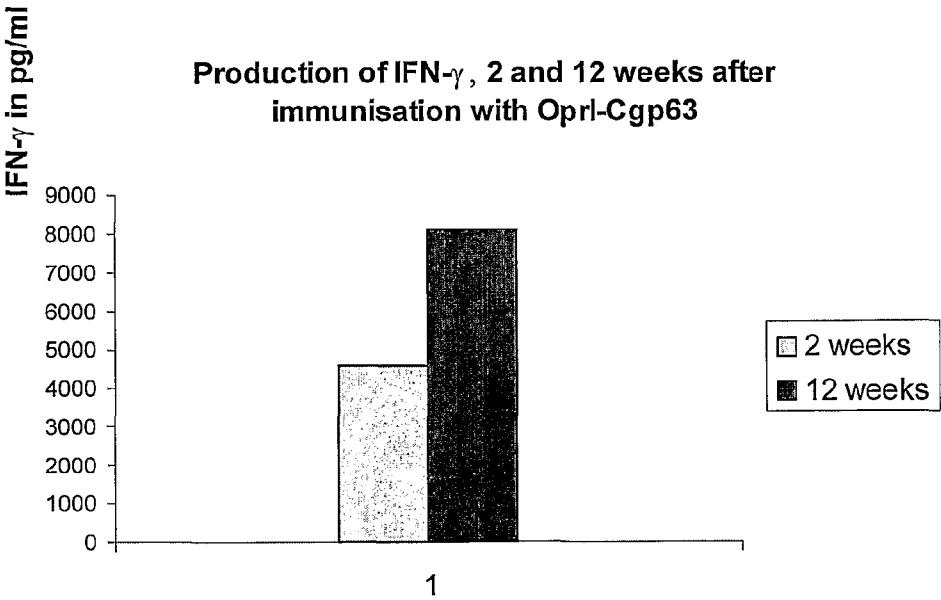


Fig. 12b



14/17

Fig. 13a

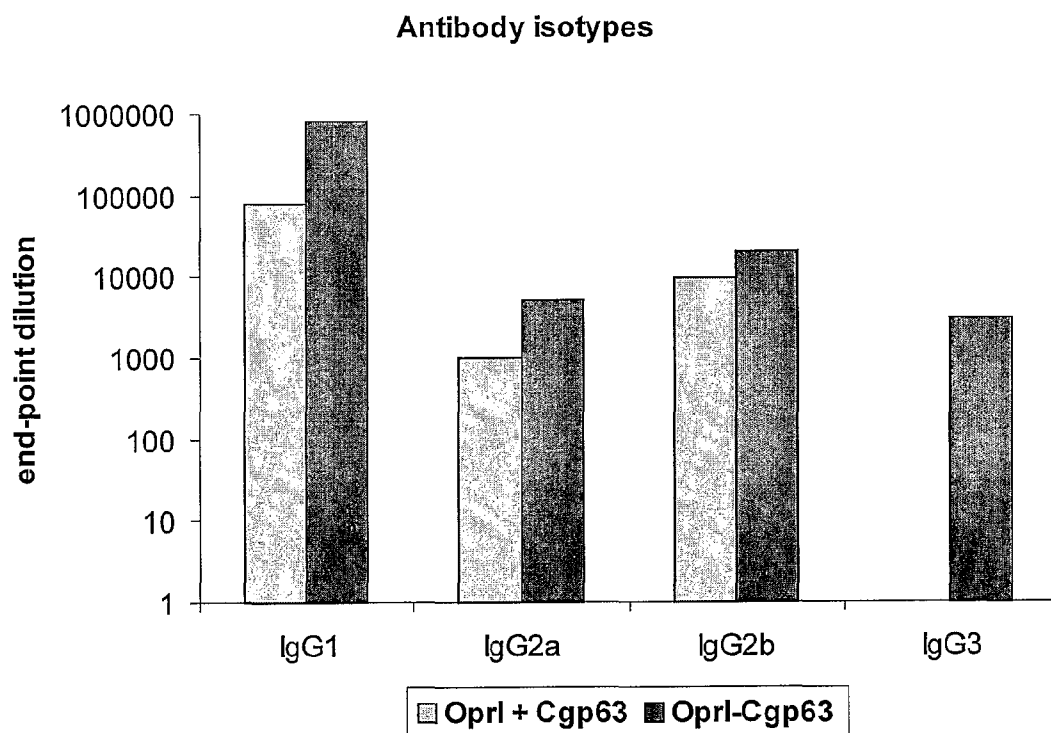
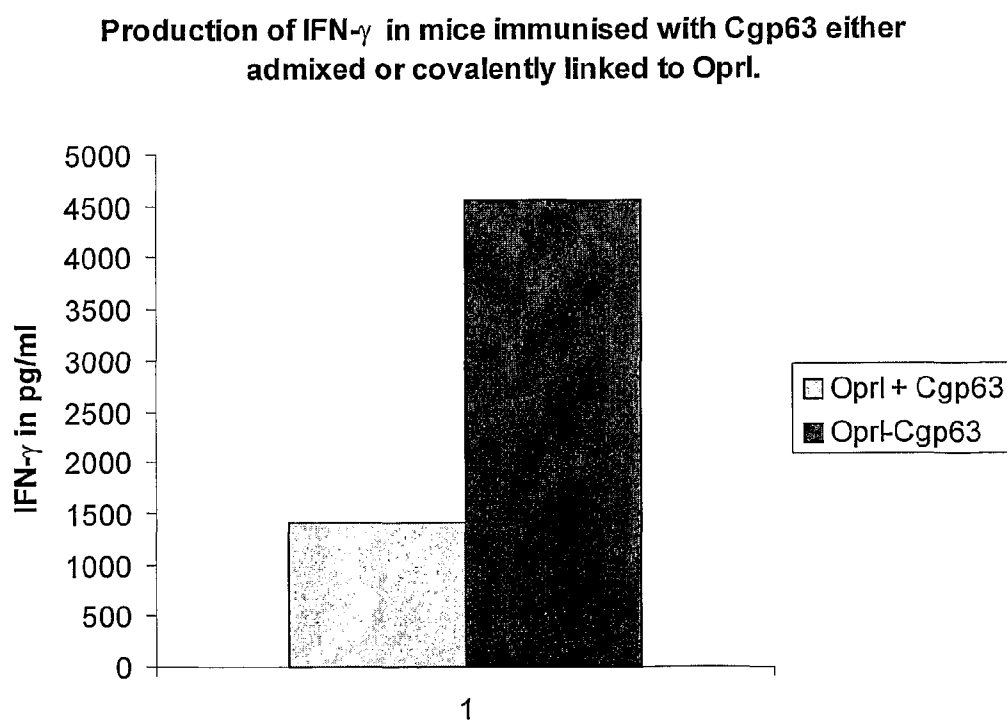
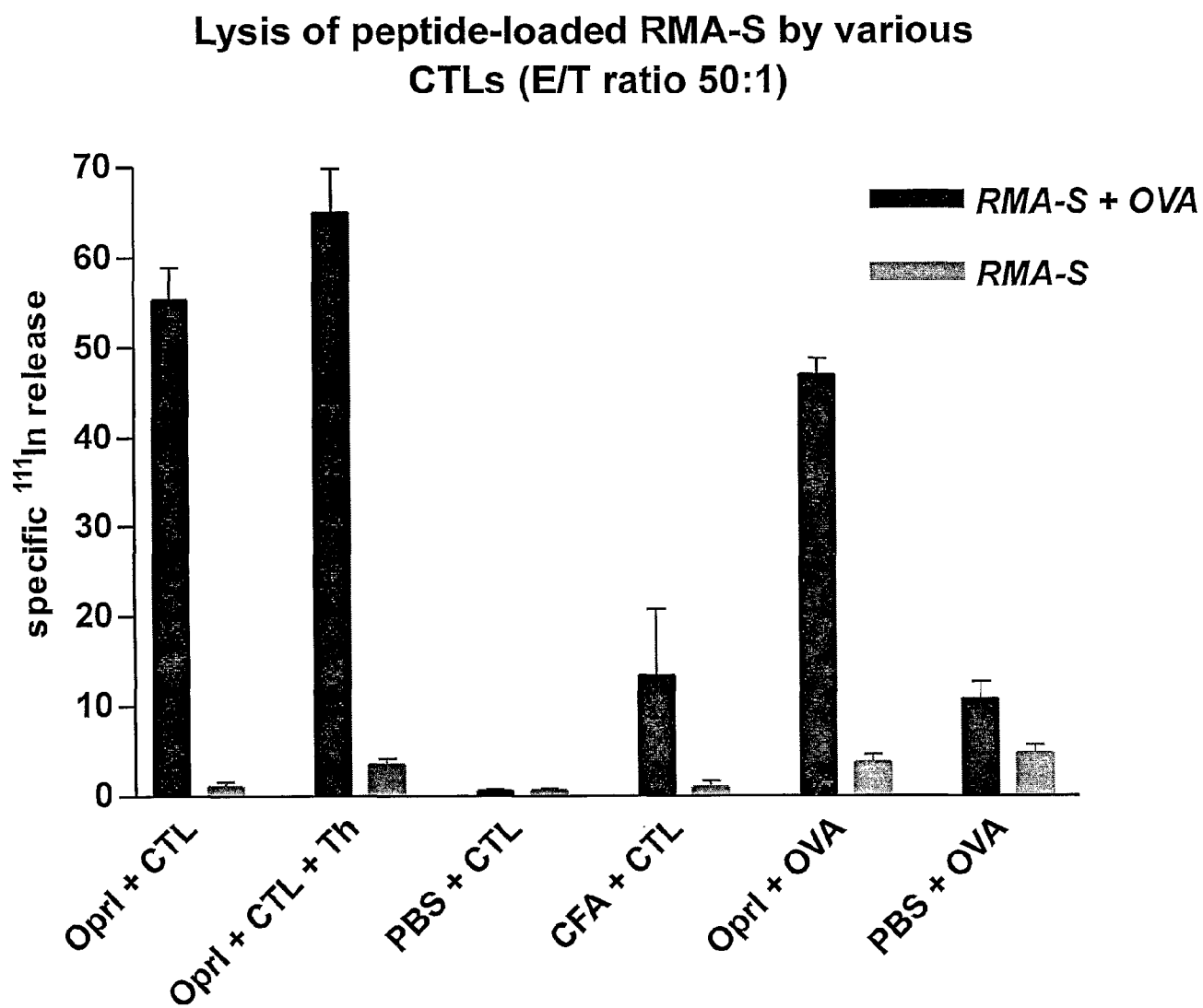


Fig. 13b



15/17

Fig. 14



16/17

Fig. 15

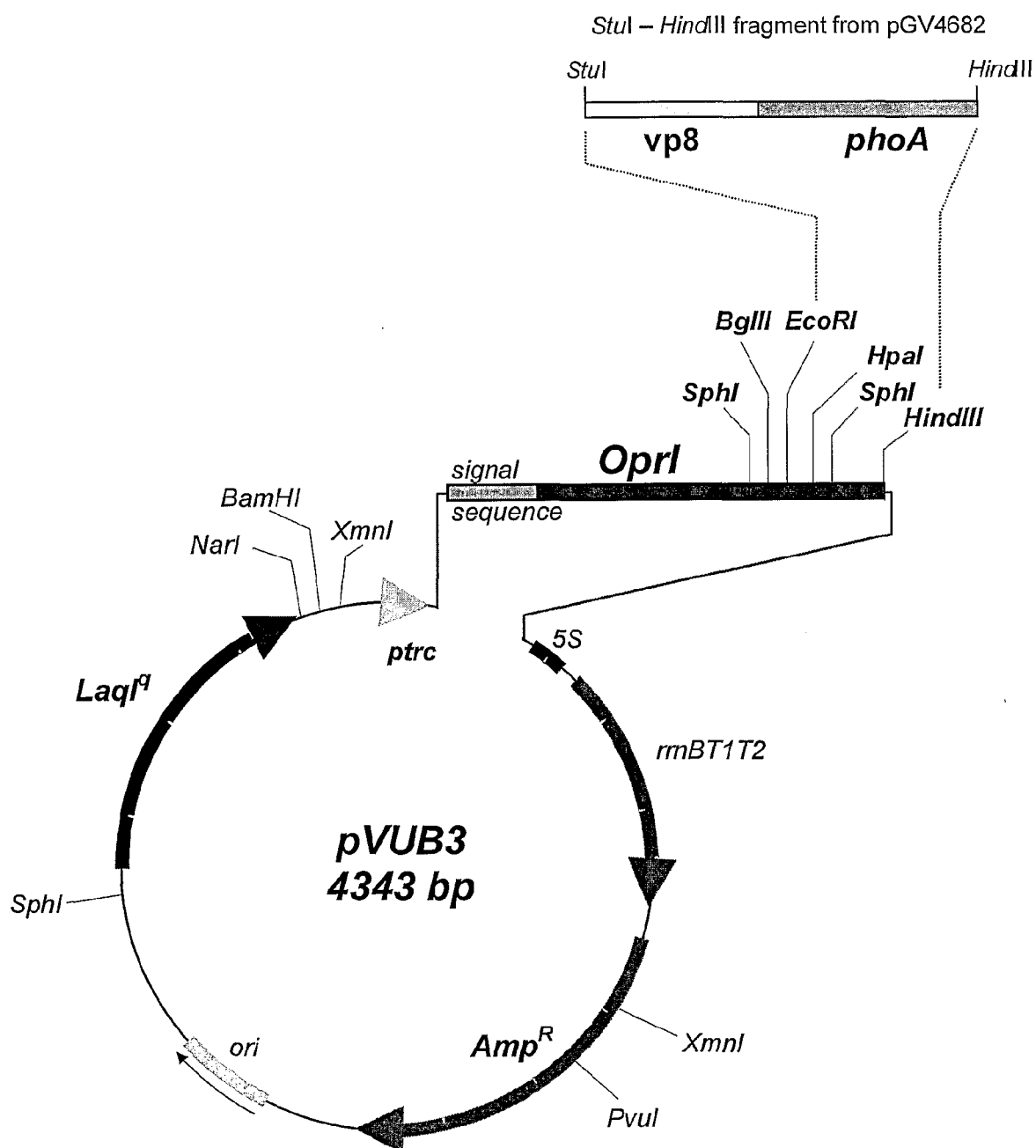


Fig. 16

