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Identification of a new protective antigen of *Bordetella pertussis*

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ABSTRACT

Antigenic proteins whose expression is induced under iron starvation, an environmental condition that bacterial pathogens have to face during colonization, might be potential candidates for improved vaccine. By mean of immune proteomics we identified novel antigens of *Bordetella pertussis* maximally expressed under iron limitation. Among them, Bp1152 (named as IRP1-3) showed a particularly strong reaction with human IgG purified from pooled sera of pertussis-infected individuals. Computer analysis showed IRP1-3 as a dimeric membrane protein potentially involved in iron uptake. Experimental data revealed the surface-exposure of this protein and showed its increase under iron starvation to be independent of bacterial virulence phase. Immunization of mice with the recombinant IRP1-3 resulted in a strong antibody response. These antibodies not only recognized the native protein on bacterial surface but also promote effective bacterial phagocytosis by human PMN, a key protecting activity against this pathogen. Accordingly, IRP1-3 proved protective against *B. pertussis* infection in mouse model. Expression of IRP1-3 was found conserved among clinical isolates of *B. pertussis* and positively regulated by iron starvation in these strains. Taken together these results suggest that this protein might be an interesting novel vaccine candidate.

1. Introduction

Whooping cough (pertussis) is an acute respiratory illness in humans caused mainly by *Bordetella pertussis*, and less frequently by *Bordetella parapertussis* [1]. The disease continues to be a significant cause of morbidity and mortality worldwide, despite universal immunization [2]. The introduction of whole cell pertussis vaccines in the mid-1940s significantly reduced disease burden. Although generally effective, whole cell vaccines are reactogenic, causing fever and local reactions. Concerns about the safety of these vaccines led to the development and introduction of acellular (Pa) formulations, composed of some combination of *B. pertussis* antigens including pertussis toxin (PT), pertactin (Prn), filamentous hemaglutinin (FHA), and different fimbriae serotypes [3,4]. Over the last years, despite high vaccine coverage the incidence of whooping cough has been increasing [5–7]. Even in developed countries, pertussis had become the most commonly reported vaccine-preventable disease among young children. Efficacy of existing Pa vaccines might improve by the addition of further *B. pertussis* antigens. All immunogens included in current acellular formulations are proteins expressed in the virulent phase of the bacteria positively regulated by the BvgAS two-component phosphorelay system [8,9]. Recent studies raised some doubts about the expression of these antigens in the infective phenotype. Modulating conditions in intranasal cavity [10] might eventually induce the temporal lack of expression of one or more of these vaccine antigens in the infecting bacteria. On top of this, recently published data reported the existence of circulating strains of *B. pertussis* not expressing two of the main vaccine antigens [11]. An effective way to improve pertussis control may comprise updating current vaccines by including antigens that are expected to be present in the infective phenotype. Antigenic proteins whose expression is induced under physiological conditions, i.e. iron limitation, might be a good option. The lack of iron is an environmental stress that human pathogens have to face during infection. Successful microbial pathogens have developed mechanisms to overcome host iron restriction, including production and utilization of low-molecular-weight iron chelators (siderophores), capture of siderophores produced by other organisms, and direct removal of iron from host proteins via specific bacterial cell surface receptors [12]. Because these factors are essential for bacterial survival *in vivo* they gain importance as potential targets for the development of

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vaccines and therapeutic agents. In order to gain a better insight into the iron starved phenotype of *B. pertussis* and the immunogenicity of the differentially expressed proteins we used proteomic tools in combination with complete genome sequences to relate genome-wide expression response of this pathogen to iron starvation [13]. The results showed that iron restriction induces the expression of proteins of different functional classes, some of them immunogenic. Among the latter, Bp1152, a protein identified in the outer membrane subproteome, named IRP1-3 by us, showed a particularly strong reaction with human IgG purified from pooled sera of pertussis-infected individuals [13].

In this study we show that IRP1-3 is a surface-exposed highly immunogenic protein expressed under physiological conditions and protective against infection in mouse model. We further show that its gene is conserved among circulating strains and it is expressed under iron limitation irrespective of the virulence state of the bacteria. Altogether these results suggest that this protein is a good candidate for further study as vaccine antigen.

2. Materials and methods

2.1. Computational analysis

Sequence similarity searches were performed to identify close homologous proteins to IRP1-3 using BLAST. The proteins were retrieved with *E*-values below 1×10^{-4}, and aligned using CLUSTALX [14]. To search for templates to build structural models, fold assignment methods as HHpred [15] and FFAS03 [16] were applied. The best templates obtained were used to build models using the program Modeller [17]. The quality of the models was assessed using PROSA II [18]. TMHMM [19], and DAS [20] servers were used to estimate putative transmembrane regions.

2.2. Bacterial strains and growth conditions

B. pertussis strains BP536, a streptomycin-resistant derivative of Tohama I, and BP537, a Bvg-phase locked derivative of Tohama I [21] were used in this study. *B. pertussis* strain BP536 was transformed with plasmid pCW505 [22] (kindly supplied by Dr. Weiss, Cincinnati, OH) which induces cytoplasmic expression of GFP without affecting growth, or antigen expression [22]. The clinical strains used for PCR and western blot analysis were isolated from Argentinean patients in the period 2002–2007. *B. pertussis* strains were cultured as previously described [13] with a few modifications. Briefly, bacterial strains were grown in Bordet Gengou agar (BGA) plates and further subcultured in Stainer-Scholte (SS) for 24 h. Bacterial cells were harvested by centrifugation, washed with sterile iron-free saline solution. Equal volumes of bacterial cell suspensions were used to inoculate 100 ml of iron-replete SS (36 μM iron) (SS), and iron-depleted SS (without addition of $FeSO_4{\cdot}7H_2O$) (SS-Fe), with or without 50 mM of $MgSO_4$. Bacteria were cultured at 37 °C under shaking condition for 20 h, and subcultured twice in the respective culture media. Iron-depleted medium was prepared as previously described [13]. The chrome azurol S assay [23] was used to monitor the presence of siderophores in culture supernatants of *B. pertussis* grown in iron-depleted medium. *E. coli* BL21-CodonPlus (DE3)-RIL transformed with plasmid pET28/His6-IRP1-3 was grown in Luria Bertani (LB) medium at 37 °C supplemented with kanamycin at a final concentration of 50 μg/ml and used in expression experiments.

2.3. Cells

Peripheral blood neutrophils (PMN) were isolated from heparinized venous blood using Ficoll-Histopaque (Sigma, St. Louis, MO) gradient centrifugation. PMN were harvested and remaining erythrocytes removed by hypotonic lysis. PMN purity determined by cytospin preparations exceeded 95%, and cell viability was 99% as determined by trypan blue exclusion. Prior to functional assays, PMN were washed twice with DMEM supplemented with 0.2% of BSA (Sigma, St. Louis, MO), resuspended, and used immediately. All experiments described in this study were carried out with freshly isolated PMN lacking FcγRI (CD64) expression, as monitored by FACS analysis with anti-FcγRI mAb 22 (BD Biosciences, San Diego).

2.4. Sera

Immunoglobulin G (IgG) fractions from pooled sera of pertussis patients with high titers against *B. pertussis* (as measured by ELISA [24]) were obtained using protein G (Pharmacia Biotech, Uppsala, Sweden) chromatography as described before [25].

2.5. Molecular cloning of irp1-3 gene

The full-length coding region of *irp1-3* gene of *B. pertussis* was amplified from genomic DNA by polymerase chain reaction (PCR) using Platinum *Pfx* DNA polymerase (Invitrogen, Carlsbad, CA). The PCR reaction was carried out using the following conditions. Initial denaturation cycle at 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 56 °C for 30 s, 68 °C for 1 min, and a final extension of 5 min at 68 °C. The primers for PCR cloning were as follows: forward primer 5′-agc gtc gac tga tga aga aag cct tgc tac-3′ and reverse primer 5′-act aag ctt tca gta ccc gcc ctt ctt g-3′. The inserted restriction sites are underlined. The PCR product corresponding to the full-length gene sequence of *irp1-3* was cloned into the *Sal*I and *Hind*III restriction sites of the pET28a (Novagen, Madison, WI) expression vector to generate the recombinant protein with an N-terminal histidine tag. The cloned PCR fragment was sequenced using single primer extension to confirm that no PCR-induced mutations had been introduced (Macrogen Inc., Seoul, Korea).

2.6. Recombinant protein expression

To express the recombinant protein (rIRP1-3), transformed *E. coli* strain BL21-CodonPlus (DE3)-RIL cells were grown at 37 °C in LB medium (500 ml) to an A_{600} of 0.4. Protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG), to a final concentration of 0.5 mM and incubating for 3 h. The bacterial pellet was harvested and resuspended in denaturing buffer containing 50 mM Na_3PO_4, 300 mM NaCl, 10 mM imidazole and urea 8 M (pH 7.4). The extract was then clarified by centrifugation at 10,000 × *g* for 20 min and the recombinant protein in the supernatant was purified by cobalt-nitrilotriacetic acid-agarose affinity resin under denaturing conditions according to the supplier's instructions (Pierce Biotechnology, Rockford). After unbound proteins were washed from the column, the His-tagged recombinant protein elution was carried out in 150 mM imidazole and protein purity was analyzed on 12.5% (w/v) sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) stained with Coomassie blue. The purified protein (rIRP1-3) was dialyzed, lyophilized and reconstituted in phosphate buffered saline (PBS).

2.7. Mice immunization

Three to 6 weeks old female BALB/c mice were used in animal experiments. The mice were obtained from and bred in the specific pathogen-free breeding rooms of the animal facility of the Faculty of Veterinary, University of La Plata.

A group of 8 female BALB/c mice were immunized intraperitoneally (i.p.) with 4 μg of rIRP1-3 protein emulsified in complete Freund's adjuvant (FA), or Alum (Al). A booster dose was given on

the 21st day with 4 μg of rIRP1-3 emulsified in incomplete Freund's adjuvant or Alum, respectively. Another two groups of mice ($n = 8$ each group) were i.p. immunized with 5×10^6 cells of heat-inactivated Bp−Fe (wIDP) or Bp+Fe (wIRP) emulsified with Alum. As negative controls, two separated groups of 8 mice were i.p. immunized with an equal amount of adjuvant alone or PBS. Since there was no significant difference in immune responses between animals immunized with FA or PBS, the data of animals immunized with PBS are not shown here.

The mice were bled at days 0, 21 and 36 and the serum was separated by centrifugation and stored at −20 °C until analysis.

Animal handling and all the experimental procedures were carried out in compliance with the recommendations of the "Guide for the Care and Use of Laboratory Animals" of the National Research Council (Academy Press, 1996, Washington).

2.8. Challenge studies

Groups of mice ($n = 8$) i.p. immunized with rIRP1-3, wIDP (positive control), or Freund's adjuvant alone (negative control) were sedated and challenged by pipetting 50 μl of PBS containing 1.5×10^6 CFU of *B. pertussis* grown in SS-Fe onto the external nares as described [26] 15 days after the last immunization. For quantification of bacterial numbers, 3 and 8 days after challenge mice were sacrificed and the lungs were excised. The lungs were homogenized in PBS, serial diluted and plated onto BGA plates supplemented with 20 μg/ml streptomycin. The lower limit of detection was 10 CFU.

2.9. Determination of cytokines

Spleens were excised from groups of 4 BALB/c mice after i.p. vaccination with either rIRP1-3 emulsified in Freund's adjuvant or adjuvant alone. Splenocytes were isolated as previously described [27]. In brief, spleens were homogenized and red blood cells were lysed with 0.84% ammonium chloride. 2×10^6 cells were suspended in DMEM supplemented with 10% FCS, 1 mM sodium pyruvate, 100 μg/ml penicillin and streptomycin, and placed into each well of a 96-well tissue culture plate. Splenocytes were stimulated with 40 μg/ml of rIRP1-3, media alone, concanavalin A (Con A) (2.5 μg/ml), or media containing 10^7 heat inactivated Bp−Fe. After 3 days, the supernatants were collected and analyzed for IFN-γ and interleukin-10 (IL-10) production via Enzyme-linked immunosorbent assays (ELISA) according to the manufacturers' instructions (BD Biosciences, San Diego).

2.10. ELISAs

The antibody titers were determined by enzyme-linked immunosorbant assay (ELISA). Briefly, 100 μl of 10 μg/ml of rIRP1-3 in coating buffer (0.5 M carbonate buffer, pH 9.5), or 100 μl of either Bp+Fe or Bp−Fe (OD:1) in PBS were added to 96-well polystyrene microtiter plates and incubated overnight at 4 °C. The plates were then blocked with 5% skim milk and incubated with serially diluted serum samples. In whole cell ELISA coating control experiments 37F3 (IgG1) against *B. pertussis* FHA, or 36G3 (IgG1) against *B. pertussis* LPS (both monoclonal antibodies kindly provided by The Netherlands Vaccine Institute, Bilthoven, The Netherlands) were used. Bound IgG was detected after 2 h incubation (room temperature) with horseradish peroxidase (HRP)-conjugated goat anti-human IgG, or HRP-conjugated goat anti-mouse IgG (both from Jackson ImmunoResearch, Baltimore Pike). For measuring IgG isotypes, detection of bound antibody was determined using HRP-labeled subclass-specific anti-mouse IgG1 or IgG2a (Santa Cruz Biotechnology, Santa Cruz, CA). Antibody titers were measured for individual serum. A linear fit was made for optical density values of eight dilutions and the antibody level was calculated in arbitrary units, namely the reciprocal dilution that gave 50% of the maximum absorbance.

2.11. Phagocytosis assay

Phagocytosis of *B. pertussis* was evaluated as previously described [28] with a few modifications. Briefly, GFP-expressing Bp−Fe was opsonized by 30 min incubation at 37 °C with either rIRP1-3-induced or naïve serum in a final volume of 90 μl. Opsonization was evaluated by incubation of serum opsonized bacteria with phycoerythrin (PE)-labeled goat $F(ab')_2$ fragments of anti-mouse IgG (Southern Biotechnology, Birmingham, AL) for 30 min at 4 °C. FACS analysis was used to evaluate opsonization in each sample as described before [29]. Opsonized bacteria incubated with PMN for 10 min at 37 °C to allow interaction (multiplicity of infection (MOI): 200) were extensively washed at 4 °C to remove non-attached bacteria. An aliquot was maintained on ice to determine PMN surface-associated bacteria at this time point, while another aliquot was further incubated for 40 min at 37 °C. Phagocytosis was stopped by placing PMN on ice. PMN surface-bound bacteria in both samples were detected by antibody dependent labeling procedure as follows. PMN of both aliquots were incubated with PE-conjugated goat $F(ab')_2$ fragments of anti-mouse immunoglobulin (Southern Biotechnology, Birmingham, AL) for 30 min at 4 °C. Phagocytosis was analyzed by flow cytometry. Phagocytosis rates were calculated as previously described [28]. Bacterial phagocytosis by PMN was further evaluated by fluorescence microscopy using a confocal laser scanning microscope (FV300; Olympus, Tokyo, Japan).

To avoid eventual cytophilic binding of antibodies to FcγR, all incubations were done in the presence of 25% heat-inactivated human serum. All experiments were carried out at least three times in triplicate.

2.12. Western blot analysis

Purified rIRP1-3 or bacterial suspensions lyzed with Laemmli sample buffer were run on 12.5% SDS-PAGE. Proteins were transferred to polyvinylidene fluoride (Immobilon PVDF Millipore) sheets [30] and incubated with either mouse anti-rIRP1-3 or human IgG from *B. pertussis* infected patients. The immunochemical detection was performed using HRP-conjugated goat anti-mouse IgG or goat anti-human IgG antibodies (both from Jackson ImmunoResearch, Baltimore Pike). An ECL Western Blotting Detection Reagent (Pierce Biotechnology, Rockford) was used to develop the blot.

2.13. Statistical analysis

Differences between the results of the experiments were evaluated by mean of ANOVA or *t*-test. Significance was accepted at $P < 0.05$. Results are shown as means and SD.

3. Results

3.1. Computational analysis of the putative exported protein (IRP1-3)

Sequence comparison showed that *B. pertussis* IRP1-3 protein is encoded by a gene of 537 bp which is highly conserved between *B. pertussis* and *B. bronchiseptica,* and between *B. pertussis* and *B. parapertussis* (100% identity at protein level).

Sequence similarity searches showed that IRP1-3 belongs to a family of iron-binding proteins well conserved in bacteria. Using BLAST sequence similarity searches with an *E*-value below 1×10^{-4}, 193 sequences were obtained and retrieved. Most of them are proteins annotated as membrane antigens. Among them, two

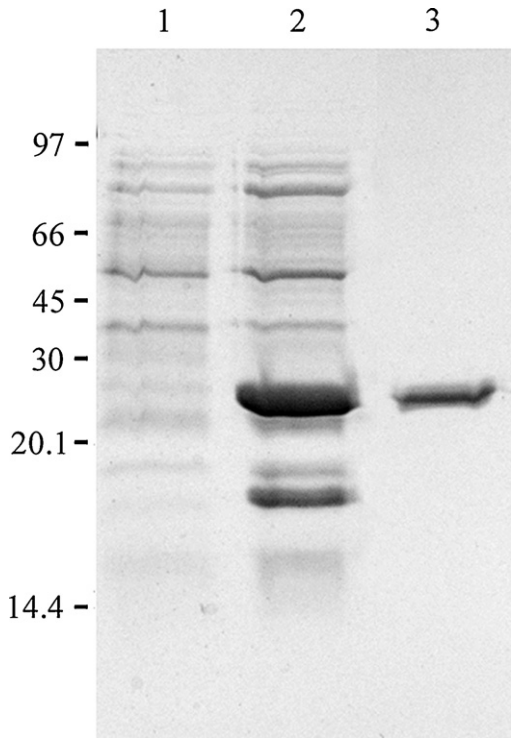

Fig. 1. Expression of *irp1-3* by *E. coli* BL21. Proteins of crude uninduced *E. coli* lysate (lane 1), crude induced *E. coli* lysate (lane 2), or purified rIRP1-3 (lane 3) were separated by SDS-PAGE (12.5%) and stained with Coomassie blue.

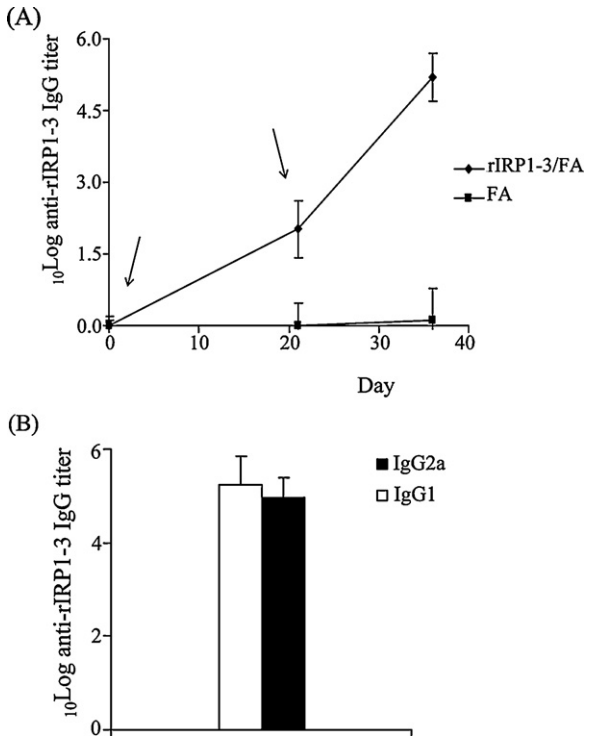

Fig. 2. Antibody response to rIRP1-3. (A) Time course of type-specific anti-rIRP1-3 IgG antibody response after priming and booster immunization with rIRP1-3. Groups of eight mice were immunized on days 0 and 21 (indicated by arrows). Serum samples were taken on days 0, 21, 36, and antibody response was determined by ELISA. Data represent mean antibody titers ± SD. (B) Anti-rIRP1-3 IgG1 and anti-rIRP1-3 IgG2a were determined by ELISA. The data show mean antibody titers ± SD at day 36.

are well-characterized iron-binding proteins, namely, P19 from *Campylobacter jejuni* [31] and Tp34 from *Treponema pallidum* [32]. Using P19 structure as template, structural models were built by MODELLER program [17]. The best model was selected according to the objective function of MODELLER and its quality was further assessed using PROSAII program. The high sequence identity between IRP1-3 and the template (50% identity and 66% similarity) along with the high Zscore derived from PROSAII analysis, support the quality of the model and the information derived from it. According to the structural model (Supplementary Fig. 1) IRP1-3 adopts the immunoglobuling-like fold built by a beta-helix sandwich found in P19 (PDB code 3LZL). Structural and sequence conservation showed that all the residues relevant for iron-binding are well conserved in IRP1-3. Most of these residues have been recently characterized in P19 protein. Supplementary Fig. 2 depicts the sequence alignment between *B. pertussis* IRP1-3 and iron-binding proteins from other bacteria. This figure shows the conservation of both binding sites described in P19, the iron-binding site and the cupper binding site [31]. The cupper binding site is built by the residues His 61,114 and 147 and Met 107, and supposedly located in the interface of the dimeric form of the protein. The iron-binding site formed by Glu 63 and Asp 111 is also predicted to be in the interface of the dimeric form of the protein. The evolutionary conservation of these interface residues supports the idea that IRP1-3 could adopt the dimeric form typically found in this iron-binding family.

3.2. Cloning and purification of recombinant IRP1-3

The full-length *irp1-3* gene was amplified from genomic DNA and cloned into pET28a expression vector. After IPTG induction whole cell lysates from *E. coli* were analyzed by SDS-PAGE (Fig. 1). The recombinant protein expressed in fusion with a six histidine tag at its N-terminus was purified by Co^{2+}-column chromatography yielding a single band of approximately 25 kDa on a Coomassie blue-stained SDS-PAGE gel (Fig. 1, lane 3). Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry analysis were done in order to identify the purified protein, which was confirmed as Bp1152 (data not shown).

3.3. IRP1-3 is immunogenic in mice

We examined the antibody response elicited by rIRP1-3 in mice immunized twice with rIRP1-3. Mice injected with the adjuvant alone or PBS served as control. Immunization with rIRP1-3 elicited an IgG specific response that was already detectable 21 days after the first immunization. Repeated immunization led to a booster response of specific IgG anti-rIRP1-3 (Fig. 2A).

As IgG1 and IgG2a are considered to be markers of Th2 and Th1 responses, respectively, anti-rIRP1-3 specific antibodies of both isotypes were also measured. Animals immunized with rIRP1-3/FA showed both IgG1 and IgG2a response against rIRP1-3 (Fig. 2B). Interestingly, similar IgG1/IgG2a profile was obtained using alum adjuvant (data not shown).

3.4. Immunization with rIRP1-3 induces both Th1 and Th2 immune responses

To get further information on the type of immune response induced by rIRP1-3, cytokine secretion was evaluated by ELISA in culture supernatants of spleen cells from rIRP1-3- or sham-vaccinated mice stimulated with medium alone, heat inactivated Bp−Fe, rIRP1-3, or Con A. No detectable levels of cytokines were produced by spleen cells of mice rIRP1-3- or sham-vaccinated when cultured in the presence of DMEM medium (data not shown).Splenocytes from mice immunized with rIRP1-3/FA or

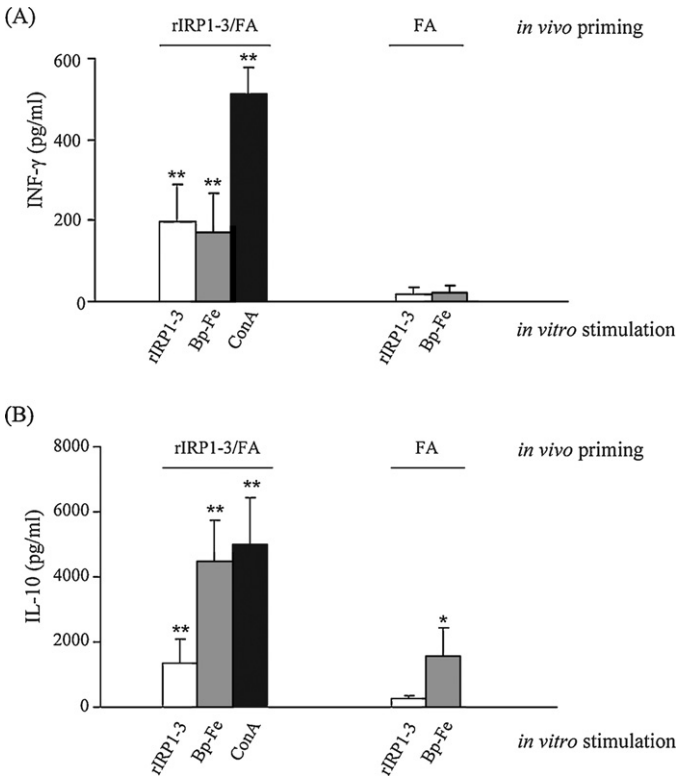

Fig. 3. Cytokine production by spleen cells of mice immunized with rIRP1-3/FA or adjuvant alone (FA). Spleen cells were incubated with 40 μg/ml of rIRP1-3, 1×10^7 cells of Bp−Fe, or 2.5 μg/ml concanavalin A (Con A). Supernatants were removed after 72 h to determine (A) IFN-γ and (B) IL-10. Data represent the mean ± SD of three independent experiments. Concentrations differed significantly from cells incubated with medium alone; $^*P < 0.01$, $^{**}P < 0.001$.

adjuvant alone produced high amount of IFN-γ and IL-10 upon stimulation with Con A, which served as positive control. Cells of rIRP1-3-immunized mice stimulated with either rIRP1-3 or whole cell Bp−Fe resulted in a significantly higher amount of INF-γ and IL-10 in comparison with cells incubated with medium alone (Fig. 3). These data suggest that rIRP1-3 is able to induce a mixed Th1/Th2 cytokine response.

3.5. Antibodies induced by rIRP1-3 recognize native IRP1-3 on bacterial surface

Mice antibodies raised against rIRP1-3 were investigated in their ability to recognize the native protein on bacterial surface. To this end, anti-rIRP1-3 antibody titer in mice sera was determined both in whole cell ELISA using cells of Bp+Fe as antigen and whole cell ELISA using cells of Bp−Fe. Sera from naïve mice were used as negative control. Fig. 4 shows that anti-rIRP1-3 anti-sera exhibited significantly higher anti-rIRP1-3 antibody titers when evaluated by ELISA performed using Bp−Fe cells as antigen. This result, while consistent with an over expression of IRP1-3 on the surface of iron starved *B. pertussis*, further demonstrated that antibodies induced by the recombinant IRP1-3 are able to recognize the native IRP1-3 on bacterial surface. For coating control of whole cell ELISAs two different monoclonal antibodies were used, anti-FHA and anti-LPS. Anti-FHA monoclonal antibodies showed the same level of antigen recognition in ELISAs performed with either Bp−Fe or Bp+Fe as antigens. The same results were obtained with anti-LPS monoclonal antibodies demonstrating similar coating level in both whole cell ELISAs (data not shown).

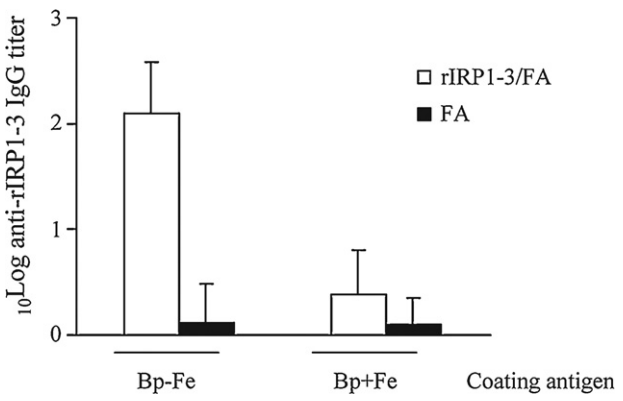

Fig. 4. Binding of rIRP1-3 induced antibodies to the native protein on bacterial surface. Anti-rIRP1-3 antibody titers induced in mice immunized with rIRP1-3/FA or FA alone were determined by whole cell ELISA in plates coated with either Bp−Fe or Bp+Fe as antigens. The Figure shows mean antibody titers ± SD.

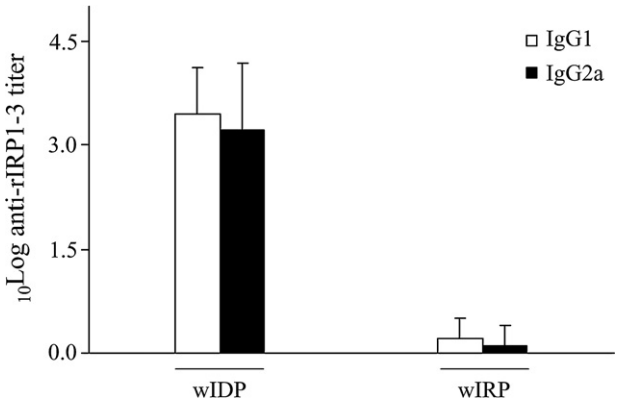

Fig. 5. Anti-rIRP1-3 IgG isotype distribution elicited by immunization with either wIDP (Bp−Fe cells) or wIRP (Bp+Fe cells). Antibodies titers were determined by ELISA in plates coated with rIRP1-3. Results are shown as mean titers ± SD.

3.6. Whole cell vaccine prepared with iron restricted bacteria induces high titers of anti-IRP1-3 antibodies

In order to evaluate whether IRP1-3 expressed on the bacteria is able to induce anti-IRP1-3 antibodies, naïve mice were immunized with vaccines formulated with cells of *B. pertussis* grown under different iron availability conditions, namely, wIDP (Bp−Fe cells) or wIRP (Bp+Fe cells). Anti-IRP1-3 antibody titers induced by each vaccine were then analyzed by ELISA using rIRP1-3 as antigen. In agreement with the reported higher expression level of this IRP1-3 under iron starvation [13], mice vaccination with wIDP induced significantly higher titer of specific anti-IRP1-3 antibodies. Isotype distribution of anti-IRP1-3 antibodies induced by wIDP vaccination showed similar to IgG isotype profile induced by rIRP1-3 immunization (Fig. 5).

3.7. Anti-IRP1-3 antibodies induce PMN phagocytosis of B. pertussis

Since whole cell ELISA results suggested that IRP1-3 is exposed on the surface of the bacteria we evaluated its potential as a target for opsonins, a key antibody function in *B. pertussis* immunity. Consistent with ELISA results, iron starved *B. pertussis* was efficiently opsonized by anti-rIRP1-3 antibodies (Fig. 6A). To examine if bacterial opsonization by antibodies directed against IRP1-3 is capable of inducing efficient bacterial uptake *B. pertussis* previously incubated with either rIRP1-3-induced or naïve serum were further incubated with PMN. A very low level of internalization of naïve serum treated

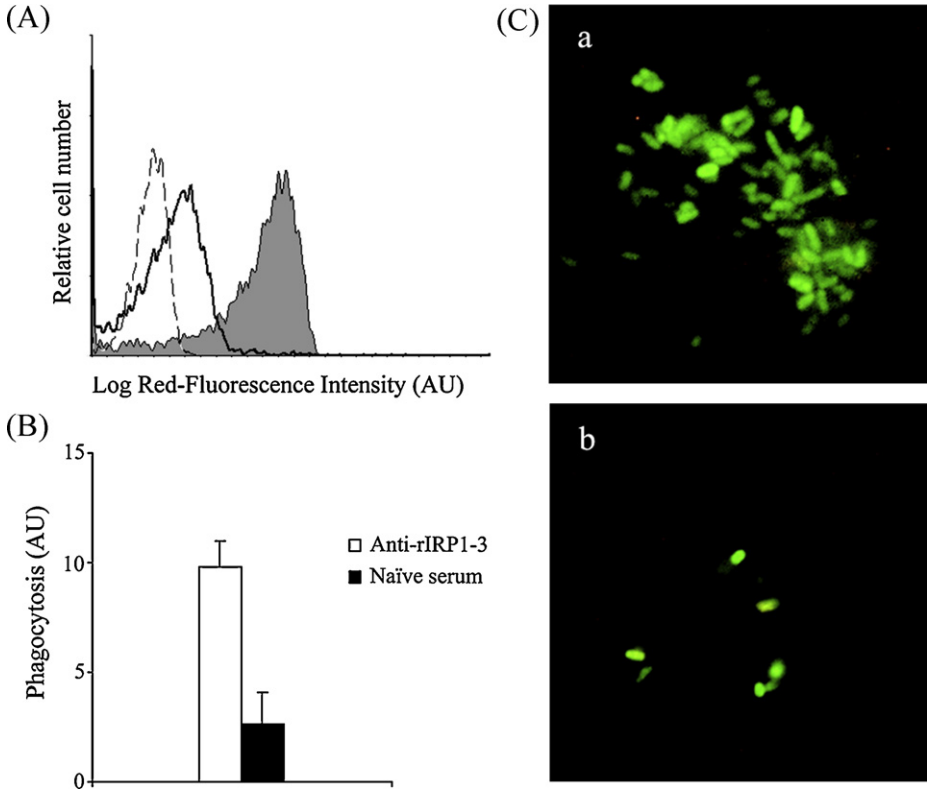

Fig. 6. *B. pertussis* osponophagocytosis. (A) *B. pertussis* opsonization. GFP Bp−Fe were incubated without (dashed line) or with anti-rIRP1-3 serum (grey filled) or naïve serum (black line), and further stained with PE-labeled goat $F(ab')_2$ fragments of anti-mouse IgG. The figure shows representative histograms of three independent studies. (B) Phagocytosis of *B. pertussis* opsonized with anti-rIRP1-3 or naïve serum. Data represent the mean ± SD of 3 independent experiments with PMN of different donors. AU, arbitrary units. (C) Confocal fluorescence microscopy of PMN incubated 40 min at 37 °C with GFP-bacteria opsonized with (a) anti-rIRP1-3, or (b) naïve serum. Representative panels of one out-of-three independent experiments are shown.

Table 1
Protection against *B. pertussis* infection induced by rIRP1-3 immunization.

Vaccine	Log CFU in lungs[a]	Units of protection[b]
PBS/FA	7.12 ± 0.10	–
rIRP1-3/FA	6.61 ± 0.10[c]	0.51
wIDP/Al	3.52 ± 0.54[c]	3.60

[a] The content of bacteria in lungs is represented as the mean log CFU ± SD per group.
[b] Units of protection were obtained by subtraction the mean log CFU of the vaccinated group from the mean log CFU of the control (PBS) immunized group.
[c] Significantly different from PBS-immunized mice $P < 0.05$.

bacteria was observed. In contrast, *B. pertussis* opsonized with anti-IRP1-3 antibodies was efficiently phagocytosed by PMNs, indicating that this protein is a good target for opsonophagocytosis (Fig. 6B and C).

3.8. IRP1-3 induces protection against B. pertussis infection

Given the type of immune response obtained we next investigated whether rIRP1-3 by itself is able to protect against *B. pertussis* infection. Mice vaccinated with rIRP1-3/FA were challenged intranasally with iron restricted virulent BP536. Mice immunized with wIDP or Freund's adjuvant alone were used as positive and negative controls, respectively. Bacterial clearance in the lungs was used to assess protective activity. Sham-vaccinated mice challenged with iron restricted *B. pertussis* had mean loads of 10^7 CFU in the lungs 3 days post challenge. Mice given rIRP1-3 plus adjuvant exhibited a small but significant ($P < 0.05$) degree of protection against *B. pertussis* when compared with controls receiving adjuvant alone (Table 1). The same level of protection was found 8 days post-challenge. Statistical analysis (ANOVA) of the data sets of CFU/lungs for each group of mice concluded that IRP1-3 by itself provided significant protection against bacterial infection.

3.9. B. pertussis infection induces anti-IRP1-3 antibodies in humans

To examine whether IRP1-3 is expressed during human infection we investigated the presence of anti-IRP1-3 antibodies in IgG fractions from pooled sera of pertussis patients both by ELISA and western blot. Sera from non-infected individuals were used as negative control. Sera from infected individuals but not healthy donors showed high titers of specific anti-IRP1-3 antibodies (960 ± 20) as determined by ELISA. Western blot analysis confirmed the presence of antibodies directed against IRP1-3 in these sera (Fig. 7).

3.10. IRP1-3 is conserved in clinical isolates and its expression is not regulated by the BvgAS system

In order to assess *irp1-3* gene conservation among circulating strains primers based on *B. pertussis* Tohama I were designed and used for PCR amplification from 14 randomly chosen clinical isolates that originate from a survey of *B. pertussis* in Argentine, from 2002–2007. The gene was found present in all the 14 clinical isolates tested (Fig. 8A). Each PCR product was the expected size indicating that the *irp1-3* gene was present in all strains and there was no variation in the length of the gene among the isolates.

To further evaluate whether *irp1-3* is transcribed and expressed under physiological conditions by circulating *B. pertussis*, a prerequisite to be included in vaccines, we performed SDS-PAGE and immunoblot analysis of whole cell lysates of a selection of recent

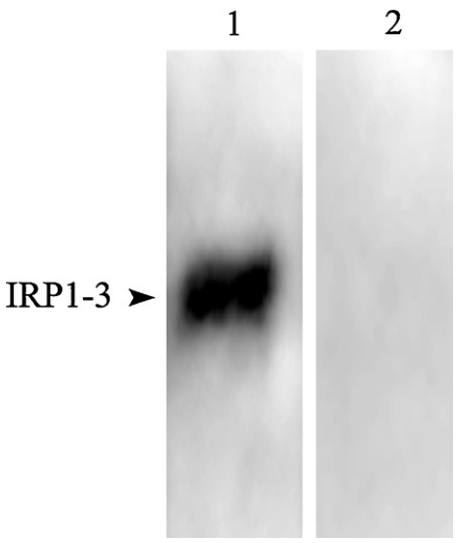

Fig. 7. Presence of anti-rIRP1-3 specific antibodies in sera from infected individuals. Purified rIRP1-3 was run in SDS-PAGE (12.5%), and transferred to PVDF membrane. Immunoblot analysis was performed with either IgG fractions from pooled sera of pertussis patients (lane 1) or sera from healthy donors (lane 2).

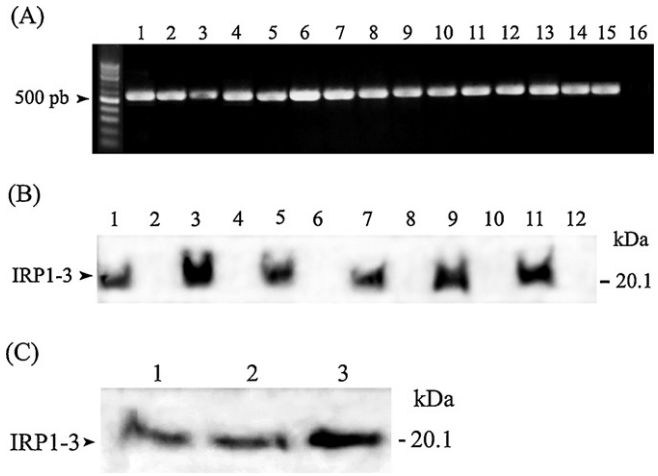

Fig. 8. IRP1-3 expression by *B. pertussis* clinical isolates. (A) Agarose gels showing amplicons from PCRs performed on clinical isolates (lanes 1–14) or BP536 (lane 15) with primers for *irp1-3*. PCR negative control was performed (lane 16). (B) Cell lysate of clinical isolates (five in total) grown under either iron starvation (lanes 1, 3, 5, 7, and 9) or iron excess (lanes 2, 4, 6, 8, and 10), and BP536 grown under either iron starvation (lane 11) or iron excess (lane 12) were subjected to SDS-PAGE (12.5%) and immunoblot analysis using anti-rIRP1-3 antibodies. The gel was loaded with the same amount of proteins of each sample. (C) Immunoblot analysis of BP536 grown in iron restricted media in the presence (lane 1) or the absence (lane 2) of 40 mM of $MgSO_4$, and BP537 (Bvg−) grown in iron restricted media (lane 3). Whole cell lysates were subjected to SDS-PAGE (12.5%) and immunoblot analysis using anti-rIRP1-3 antibodies.

clinical isolates. Whole cell lysates from five different randomly chosen clinical strains grown under either iron replete (SS) or low-iron (SS-Fe) growth conditions were analyzed by SDS-PAGE and immunoblotting using polyclonal mouse antiserum raised against rIRP1-3. Immunoblot analysis demonstrated that all five isolates cultured under iron restricted but not iron sufficient conditions expressed IRP1-3 (Fig. 8B). Finally, we investigated whether *irp1-3* gene expression is regulated by BvgAS. Western blot analysis of *B. pertussis* (BP536) grown under iron starvation with or without the addition of modulating concentrations of $MgSO_4$ [33] indicated that IRP1-3 is expressed both in Bvg+ and Bvg− *B. pertussis*. Bvg− mutant (BP537) was included as a control (Fig. 8C). Western blot analysis of Bvg+ and Bvg− *B. pertussis* using 37F3 (IgG1) monoclonal antibody against *B. pertussis* FHA (kindly provided by The Netherlands Vaccine Institute, Bilthoven, The Netherlands) were run in parallel to confirm bacterial modulation induced by the presence of $MgSO_4$ in the culture media (data not shown).

4. Discussion

Although pertussis is relatively controlled at present by extensive vaccination programs, it is evident that the circulation of *B. pertussis* throughout the world continues largely unabated. Recent surveys demonstrate a high frequency of yearly cases of pertussis worldwide despite vaccination. Pertussis whole cell (Pw) vaccine was replaced by acellular vaccines in most of the countries. In the last 10 years the majority of young children have been vaccinated with Pa vaccines, composed by a number of virulent factors that have been found polymorphic, with the currently circulating isolates being different from the vaccine. Although it is still unknown whether these variations have been vaccine-driven, the recent finding of French isolates lacking the expression of two of the main Pa components, PT or Prn, calls for attention [11]. The circulation of vaccine antigen-deficient *B. pertussis* isolates in a population with high Pa vaccine coverage suggests the need for new vaccine components. Several immunogens present in the virulent bacteria, other than those included in current acellular vaccines, have been proposed as new candidates for the next generation of pertussis vaccine, either in cellular formulations [34,35] or as purified antigens to be included in acellular vaccines [36,37]. Our approach was based on the hypothesis that immunogenic proteins whose expression is up-regulated under infecting conditions, irrespective of the bacterial BvgAS activation state, might represent an interesting option. Iron starvation is one of the main environmental stresses bacteria have to face during host colonization. In mammals, most of the iron content is maintained intracellularly in the form of heme and hemoproteins, while host transferrin and lactoferrin glycoproteins bind the iron in the extracellular environment [38]. *B. pertussis*, like other successful microbial pathogens, has mechanisms to overcome host iron restriction, including high affinity iron uptake systems via specific bacterial cell surface receptors [39–41]. *B. pertussis* is transmitted from host to host so infecting bacteria is expected to exhibit an iron starved phenotype. In this study, we searched for new pertussis vaccine candidates among the proteins induced under iron starvation as identified by comparative proteomics [13]. Previous studies had successfully used seroproteomics to the discovery of new bacterial vaccine candidates [42,43] eventually providing a proof-of-concept for the approach of searching for better antigens among proteins induced during infection. Similarly, by mean of comparative proteomics combined with serological proteome analysis we identified a number of proteins in the outer membrane subproteome of iron starved *B. pertussis* that were differentially recognized by antibodies induced by infection. Among them, Bp1152 (named as IRP1-3 [13]), whose expression was about 50-fold increased under iron starvation, showed highly reactive with sera from infected individuals. Bp1152 was annotated as a putative exported protein. Our computational analysis indicates that it has a putative transmembrane region between residues 5 and 20. The structural and sequence analysis further predicts that IRP1-3 is a dimeric protein with membrane localization bearing a highly conserved domain probably involved in iron uptake. Surface proteins of *B. pertussis* are attractive vaccine antigens particularly if they are able to induce opsonic antibodies, critical for cell mediated bacterial clearance [25,44]. The present study examined the surface exposure, immunogenicity and protective activity of IRP1-3.

We cloned, expressed, and purified IRP1-3 from *E. coli* to homogeneity. The identity of the recombinant protein was confirmed

both by sequencing and mass spectrometry. The purified protein was further used to evaluate the immunogenicity and protective activity in the intranasal challenge murine infection model of *B. pertussis*, an assay suggested as the most discriminatory test for preclinical evaluation [45]. The results showed that rIRP1-3 is able to elicit a strong IgG response with an IgG1 and IgG2a contribution. Interestingly, mice immunization with iron starved *B. pertussis* whole cell vaccine induced high IRP1-3 specific IgG response with similar IgG1/IgG2a distribution as the rIRP1-3/FA. In mice, IgG1 is usually associated with Th2 response, whereas IgG2a antibodies are though to reflect Th1 response. Several parameters influence the IgG subclass response to proteins. In addition to the modulatory effect of mouse strains and adjuvants on the Th1/Th2 balance of immune responses, there is accumulating evidence that intrinsic structures in the proteins themselves may also contribute to this balance. In this regard, it is interesting to note that even with the use of alum, known to selectively promote Th2 response, rIRP1-3 induced high titers of IgG2a specific antibodies. The induction of a mixed Th1/Th2 type of response by the rIRP1-3 was further confirmed by stimulation of splenocytes of immunized mice. Th1-type response has been found associated with a higher level of protection against *B. pertussis* infection [46–48]. In particular, INFγ has been shown to contribute to this protection. Our data showed that IRP1-3 immunization induced both IL-10 and INFγ in splenocytes of rIRP1-3 immunized mice stimulated with either rIRP1-3 or whole cell iron-starved *B. pertussis* supporting the Th1/Th2 mixed response and the protective potential of rIRP1-3.

Immune absorbent assays performed with whole cell bacteria showed that antibodies induced by the recombinant IRP1-3 bind to the native protein exposed on the surface of the bacteria. Importantly, the higher detection of cellular IRP1-3 in iron starved whole cell ELISA compared to iron replete whole cell ELISA reveals the over expression of this protein on the surface of *B. pertussis* grown under iron starvation.

The finding that IRP1-3 is a highly immunogenic surface exposed protein led us to evaluate whether the antibodies induced by the recombinant protein are able to induce opsonophagocytosis, one of the main antibody biological activities against *B. pertussis*. The results demonstrate that antibodies raised by the recombinant IRP1-3 are not only able to opsonize the bacteria but also to induce attachment to and efficient phagocytosis by human PMN. *B. pertussis* killing by immune cells critically relies on the presence of opsonic antibodies. In the absence of opsonins *B. pertussis* phagocytosis and bactericidal cellular activity are drastically impaired. Moreover, the small number of bacteria that are phagocytosed under non-opsonic conditions inhibits its own traffic to lysosomes and remain alive upon the encounter with neutrophils [25]. Similar behavior was found when *B. pertussis* interacts with human macrophages in the absence of opsonic antibodies [44]. According to recent findings those bacteria that remain alive inside the macrophage reside in phagosomes with early endosomes characteristics in which they have access to nutrients and eventually replicate inside the host cell [44]. Conversely, Ig-opsonization increases *B. pertussis* attachment, phagocytosis and bacterial killing via interaction with FcR [25,44]. Engagement of FcR transports *B. pertussis* to lysosomal compartments which ultimately leads to reduce the odds for intracellular bacterial survival underlining the importance of opsonic antibodies for immunity to *B. pertussis*. Among the antigen included in current Pa vaccines, solely Prn induces opsonic antibodies [49]. Prn is a polymorphic protein mainly expressed by the virulent phase of the bacteria whose expression has been found abolished by mutation in recent clinical isolates [11]. We here describe a new surface exposed antigen with high level of expression under physiological conditions and whose recombinant form is able to induce antibodies that bind the native protein on bacterial surface inducing efficient phagocytosis which might contribute to bacterial clearance *in vivo*. Accordingly, a significant degree of protection against *B. pertussis* infection was achieved by mice immunization with rIRP1-3 in a monovalent formulation. Taken together our results indicate that mechanism of IRP1-3-mediated immunity involves humoral and cellular response.

Importantly, IRP1-3 expression was found not dependent on the activation state of the BvgAS system Both the Bvg+ and Bvg− *B. pertussis*, as well as the Bvg− mutant (BP537) grown in iron limited media expressed IRP1-3 as assessed by western blot analysis. Accordingly, none of the reported variants of the heptameric BvgA consensus sequence TTT(C/C)NTA [50] was found upstream or downstream from the putative promoter region of IRP1-3 (data not shown) supporting the lack of BvgAS dependence of IRP1-3 expression. The virulence independence of IRP1-3 expression might add to its suitability as new vaccine component as it suggests that this antigen will be present under physiological conditions irrespective of the virulence phase of the infective phenotype.

Finally, we investigated IRP1-3 immunogenicity in humans. We had previously observed that sera from infected patients recognized IRP1-3 in dot blot assays [13]. The seroreactivity of the recombinant version of IRP1-3 with human infected sera was further confirmed by ELISA and western blot analysis. The development of a specific antibody response in infected patients demonstrates its immunogenicity in humans, and suggests that IRP1-3 is expressed during infection. Accordingly, the *irp1-3* gene was found conserved in clinical isolates of *B. pertussis,* and expressed under iron limitation which is in agreement with the presence of specific anti-IRP1-3 antibodies in infected individuals.

Taken together the data presented here show that IRP1-3 meets the characteristics of the good vaccine candidate, namely, surface exposure, conservation among circulating strains, high abundance under physiological conditions, and generation of protective immune response.

Further studies are needed to evaluate this antigen in the context of current acellular vaccines.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2011.07.143.

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