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ORIGINAL ARTICLE

Immunogenicity and protective efficacy of recombinant iron superoxide dismutase protein from *Bordetella pertussis* in mice models

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ABSTRACT

Whooping cough (pertussis) is a highly contagious respiratory infection caused by *Bordetella pertussis*. Although availability of effective pertussis vaccines reportedly decreases the incidence of the disease, *B. pertussis* circulation in populations has not been eliminated. Thus, it is necessary to find new protein candidates with greater immune protective capacities than the currently available acellular pertussis vaccines. In this study, iron superoxide dismutase (FeSOD) gene (*sodB*) was cloned, expressed in *Escherichia coli* and recombinant FeSOD protein thence purified. The recombinant protein (rFeSOD) was formulated with aluminum hydroxide (Alum) or monophosphoryl lipid A (MPLA) and injected intraperitoneally to immunize mice, after which IgG1, IgG2a and IFN-γ titers were measured to assess humoral and cellular responses, respectively, to these immunizations. The extent of bacterial colonization in lungs of intranasally challenged mice was determined 5, 8 and 14 days post-challenge. IgG1 and IgG2a responses were significantly stronger in mice that had been immunized with rFeSOD–MPLA than in those that had received rFeSOD-Alum ($P < 0.05$). Additionally, IgG2a titers were higher in mice vaccinated with recombinant protein FeSOD (rFeSOD) formulated with MPLA, especially after the second immunization. Immunization with rFeSOD–MPLA also provided a modest, but significant decrease in bacterial counts in lungs of mice ($P < 0.05$). Antigen specific-IFN-γ responses were significantly stronger in the group vaccinated with rFeSOD–MPLA, which could account for the lower bacterial counts. These findings suggest that rFeSOD protein formulated with MPLA has potential as an acellular pertussis vaccine candidate component.

Key words pertussis, superoxide dismutase, vaccine.

Pertussis is a highly contagious acute respiratory disease caused by *Bordetella pertussis*, which is a gram-negative, strictly human pathogen (1). The disease is among the ten infectious diseases with the highest morbidity and mortality worldwide (2). Globally, the number of reported disease cases is about 50 million and annual deaths from pertussis about 300,000 (3). Although pertussis predominantly affects infants and children aged one to five years, it

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List of Abbreviations: Alum, aluminum hydroxide; DSB, denaturing solubilization buffer; FeSOD, iron superoxide dismutase; FA, Freund's adjuvant; FHA, filamentous hemagglutinin; IPTG, isopropyl β-D-1-thiogalactopyranoside; MPLA, monophosphoryl lipid A; Pa vaccines, acellular pertussis vaccines; PRN, pertactin; Pw vaccines, whole cell pertussis vaccines; rBrkA, Bordetella Resistance to Killing Protein; rFESOD, recombinant iron superoxide dismutase; SOD, superoxide dismutase.

can also occur in adolescents and adults, which is a hallmark of waning vaccine-induced immunity (2). The incidence of pertussis decreased dramatically after introduction of childhood pertussis vaccines in the 1940s. However, the disease attracted renewed attention because of its resurgence in highly vaccinated populations (2, 4). The resurgence in question is associated with multiple factors, including increased awareness of the disease, waning vaccine-induced immunity, antigenic divergence between vaccine strains and clinical isolates and low efficiency of current vaccines (1, 5).

Although millions of deaths have been prevented by Pw vaccine, its reactogenicity and adverse effects led to the development of Pa vaccines, which contain a different combination of *B. pertussis* antigens, including pertussis toxin, PRN and FHA, with or without different fimbriae serotypes, that is, they have three to five components (6–8). Despite availability of effective vaccines and high vaccine coverage, whooping cough has re-emerged in recent years. It has been suggested that immunity provided by Pw and Pa vaccines has gradually decreased (9). Thus further studies to develop Pa vaccines with new immunogenic proteins are required for better protection and long term vaccine-induced immunity. In recent years, some reports on new immunogenic proteins from *B. pertussis* have been published, suggesting new protective antigens against pertussis (10–12). Moreover, in addition to searching for new antigenic proteins, the use of appropriate adjuvants in vaccine formulations is also important. Although aluminum salts have generally been preferred for pertussis vaccine studies (13, 14), some researchers employ other adjuvants because adjuvant choice can play a central role in determining the strength and type of immune responses (10, 11).

In a former study conducted in our laboratory, FeSOD was detected as an immunogenic protein in the surface immunoproteome of *B. pertussis* Tohama I and Saadet strains (15). SODs convert toxic superoxide (O_2^-) into O_2 and H_2O_2. Along with other SODs, the action of FeSOD as a virulence factor has been reported in *Francisella tularensis* (16). Moreover, the absence of FeSOD adversely affects the growth rate of *Escherichia coli* and *B. pertussis* (17, 18). In this context, the present study aimed to investigate humoral and cellular immune responses to rFeSOD from *B. pertussis* in mouse models by comparing two different adjuvants, a TLR 4 agonist, MPLA, and Alum.

MATERIAL AND METHODS

Bacterial strains and plasmids

B. pertussis Tohama I, the standard strain and Saadet, a local strain in Turkey, were used in this study. *E. coli* DH5α (ATCC, Manassas, VA, USA) and *E. coli* BL21 (DE3) (Novagen, Darmstadt, Germany) were the bacterial hosts for cloning and expression studies, respectively. The vectors pGEMT Easy (Promega, Madison, WI, USA) and pET28a(+) (Novagen, Madison, WI, USA) were used for PCR cloning and expression, respectively.

Molecular cloning of sodB gene and expression of recombinant FeSOD protein

The open reading frame of the FeSOD gene (*sodB*) was amplified from genomic DNA of *B. pertussis* Tohama I by PCR using specific primers designed with *Bgl*II and *BamH*I restriction sites (underlined). The primers for PCR cloning were as follows: forward primer of *sodB* 5′-GGATCCATGGCACACACTCTT-3′ and reverse primer of *sodB* 5′-AGATCTTTAGGCGAAATTCT TCG-3′. The PCR products of *sodB* were first cloned into pGEM-T Easy in *E. coli* DH5α (ATCC) and then into the *BamH*I restriction site of expression vector pET-28a(+) (pET28–FeSOD) in *E. coli* BL21 (DE3).

For expression of rFeSOD, *E. coli* BL21 (DE3) cells containing pET28–FeSOD were incubated in 10 mL LB broth (Merck, Darmstadt, Germany) containing 30 μg/mL kanamycin (Sigma, Munich, Germany) overnight with shaking at 37°C, after which then 3 mL of the culture was transferred into 200 mL LB containing kanamycin. When OD_{600} of the culture had reached 0.6, protein expression was induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma, Germany) at a final concentration of 1 mM. The culture was incubated for a further 5 hr after addition of IPTG to obtain the necessary amount of rFeSOD.

Purification of recombinant FeSOD protein

After expression of rFeSOD via IPTG induction in *E. coli* BL21 (DE3), the cells were harvested by centrifugation and resuspended in DSB containing 50 mM NaH_2PO_4, 1 M NaCl and 8 M urea (pH 8.0). The suspension was kept at −80°C for 15 min, thawed and vortexed twice and then lysated by sonication using a CP70T Ultrasonic Processor (Cole-Parmer, Vernon Hills, IL, USA) for 6 × 10 s at 60% amplitude. After centrifugation, the supernatant containing the protein of interest was collected. The recombinant protein was purified on Protino Ni-TED 2000 packed columns (Macherey-Nagel, Düren, Germany). The columns were equilibrated with 4 mL DSB and the supernatant loaded, after which the columns were washed three times with DSB. The protein was eluted with 3 mL denaturing elution buffer containing 50 mM NaH_2PO_4, 1 M NaCl, 8 M urea and 250 mM imidazole (pH 8.0). Eluted protein was dialyzed against a buffer containing

50 mM NaH_2PO_4, 500 mM NaCl and 4 M Urea (pH 8.0). To determine the total purified protein concentration, the modified Bradford assay described by Ramagli and Rodrigez was used (19). The purity and immunogenicity of the sample were determined by SDS-PAGE and western blotting, respectively.

Preparation of vaccine formulation

Forty micrograms/mL recombinant FeSOD protein was incorporated with an equal amount of MPLA (InvivoGen, San Diego, USA) or Alum (InvivoGen) to immunize the mice.

Antisera against *B. pertussis* and western blotting

Anti-sera against *B. pertussis* strain Tohama I and Saadet were prepared as previously described. The modified method described by Altindis *et al.* was used for western analysis with the anti-sera against Tohama I and Saadet (20).

Mice immunization and challenge

For each group, 15 female BALB/c mice (16–18 g) were immunized intraperitoneally with 100 μL of 40 μg/mL rFeSOD–MPLA or rFeSOD–Alum or PBS as negative control. A second immunization was carried out on Day 21 and the animals were intranasally challenged with 2.5×10^9 CFU of Saadet strain in 100 μL saline solution on Day 31. Blood was collected from the tail veins of mice on Days 20 and 30. The sera thus obtained was stored at −20°C until further use.

All animal experiments were performed with the approval of the Ethics Committee on Animal Experimentation, Middle East Technical University, Ankara, Turkey.

Quantitation of bacterial colonization in lungs

On post-challenge Days 5, 8 and 14, four mice from each group were killed and their lungs excised. The lungs were homogenized in 0.85% NaCl with 1% Casamino acid and serially diluted with the same solution. One hundred microliters of each diluted homogenate was plated onto Cohen–Wheeler plates supplemented with cephalexin at a concentration of 40 mg/L. The plates were incubated at 37 C for three days before counting the colonies. The $\log_{10}$ weighted mean number of CFU per lung and the SD were calculated for each day.

Measurement of antibody titers

Specific IgG responses were quantitated by ELISA using sera collected from the control and vaccinated groups. Purified rFeSOD protein was used as coating antigen at a concentration of 4 μg/well. Two-fold serial dilutions of the murine sera ranging from 1:100 to 1:102400 (when necessary) were used in duplicates as primary antibody. Rabbit anti-mouse IgG1 and IgG2a conjugated to alkaline phosphatase (Southern Biotech, Cambridge, UK) were used as secondary antibodies at a dilution of 1:2000. p-nitrophenyl phosphate disodium salt (Thermo Scientific, Waltham, MA, USA) was used for colorimetric detection, the plates being read at 405 nm.

Detection of INF-γ titers

One week after the second immunization, the spleens of three mice from each group were dissected under sterile conditions and transferred to 5 mL of RPMI 1640 medium containing 10% FBS and 1% penicillin/streptomycin (Biochrom, Cambourne, UK). They were then homogenized through a 70 μm nylon cell strainer (BD Bioscience, Franklin Lakes, NJ, USA) and cell counting carried out with a hemocytometer. Splenocytes from the samples were distributed on 96-well plates at a concentration of 1×10^6 cells/well in RPMI 1640 medium. The plates were incubated at 37°C in a CO_2 incubator with 5% CO_2. After 1 hr, splenocytes were stimulated with 30 μg/mL of rFeSOD or concavanalin A (1 μg/mL; Sigma). Culture media were collected on the third day (72 hr) and analyzed for IFN-γ using a Mouse IFN-γ Minikit (Thermo Scientific).

Statistical analysis

The results were analyzed by anova or Student's *t*-test with $P < 0.05$ considered to denote statistical significance. Results are shown as means and SD. Analyses were carried out with SPSS Statistic 20 for Windows.

RESULTS

Cloning, expression and purification of recombinant protein

The full-length s*odB* gene was amplified from genomic DNA of *B. pertussis* Tohama I and cloned into pET-28a(+) for expression of the recombinant protein with His-tags. After IPTG induction, rFeSOD (23 kDa) was purified and analyzed by SDS-PAGE after staining with Coomassie blue (Fig. 1a).

Immunogenicity of recombinant FeSOD protein

Two western blot assays were performed with sera obtained from mice that had been immunized s.c. with heat-inactivated *B. pertussis* Tohama I or Saadet strains. Uninduced and IPTG-induced whole culture lysates were

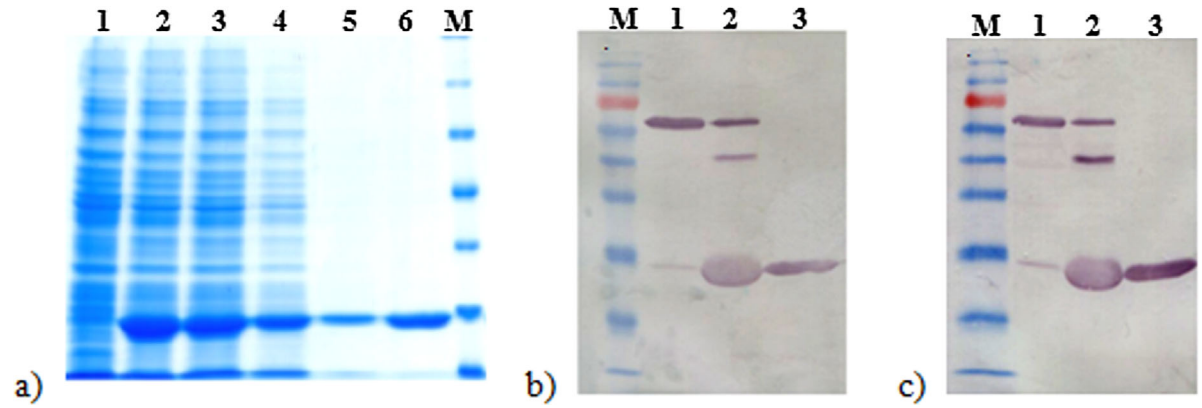

Fig. 1. Expression, purification, and western blot analyses of recombinant FeSOD. (a) SDS-PAGE of recombinant FeSOD expression. Lane 1, uninduced culture lysate; Lane 2, IPTG-induced culture lysate; Lane 3, flow through; Lanes 4–5, wash samples; Lane 6, purified recombinant FeSOD. (b) Western blot analysis with anti-Saadet serum. Lane 1, uninduced culture lysate; Lane 2, IPTG-induced culture lysate; Lane 3, purified recombinant FeSOD. (c) Western blot analysis with anti-Tohama serum. Lane 1, uninduced culture lysate; Lane 2, IPTG-induced culture lysate; Lane 3, purified recombinant FeSOD. (M, protein molecular weight marker).

included as controls. Results of the blots confirmed the immunogenicity of purified rFeSOD protein (Fig. 1b, c).

Immune responses against recombinant FeSOD protein

IgG2a is associated with Th1 cells and cellular immune responses, whereas IgG1 is linked to Th2 type humoral responses (21). To obtain IgG2a and IgG1 titers, sera from rFeSOD–MPLA and rFeSOD–Alum immunized mice and the PBS control group were collected prior to the second immunization and challenge. Figures 2 and 3 show serum IgG1 and IgG2a titers with SDs. Serum IgG1 titers in mice immunized with rFeSOD–MPLA and rFeSOD–Alum increased significantly after the first and second vaccinations ($P < 0.05$). The increase in serum IgG2a titers was significantly greater in mice vaccinated with rFeSOD–MPLA than in those vaccinated with rFeSOD–Alum ($P < 0.05$).

Some studies have shown an association between recovery from pertussis and induction of Th1 cells secreting IFN-γ, which is crucial for control of pertussis infection (22, 23). A mouse IFN-γ Minikit was used to measure IFN-γ titers in mice splenocytes stimulated with rFeSOD or ConA as positive control. rFeSOD–MPLA-immunized mice demonstrated significantly greater IFN-γ titers than a PBS negative control group ($P < 0.05$) (Fig. 4), whereas rFeSOD–Alum-immunized mice did not.

Protection of mice

Female BALB/c mice were immunized intraperitoneally twice with 40 μg rFeSOD formulated with MPLA or Alum and PBS as a negative control, after which they were intranasally challenged with the cells of *B. pertussis* Saadet strain. On Days 5, 8 and 14 after challenge, mice were killed and lung CFU quantitated to assess bacterial clearance as one of the indicators of protection against pertussis

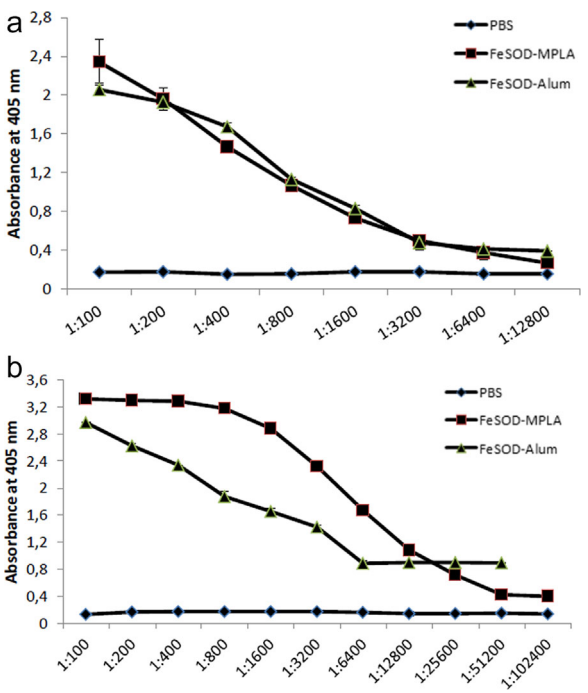

Fig. 2. Serum IgG1 titers in mice immunized with FeSOD-Alum, FeSOD-MPLA and PBS as a negative control. (a) Serum titers after first vaccination (Day 20) and (b) serum titers after second vaccination (Day 30) are presented with SDs. Higher SD values can be seen easily (e.g. Figure 2a). However, some SD values are quite small, so their bars cannot be seen clearly for some points.

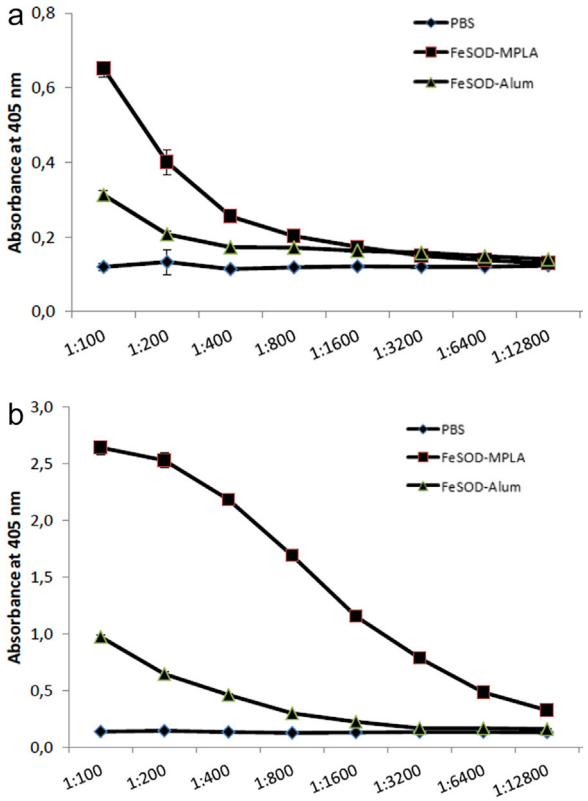

Fig. 3. Serum IgG2a titers in mice immunized with FeSOD-Alum, FeSOD-MPLA and PBS as a negative control. (a) Serum titers after first vaccination (Day 20) and (b) serum titers after second vaccination (Day 30) are presented with SDs. Higher SD values can be seen easily (e.g. Figure 2 a). However, some SD values are quite small, so their bars cannot be seen clearly for some points.

(13, 14, 24). As shown in Figure 5, mean numbers of CFU/mL/lung were significantly lower in mice vaccinated with rFeSOD–MPLA than in the control group ($P < 0.05$), whereas this was not the case in mice immunized with rFeSOD–Alum.

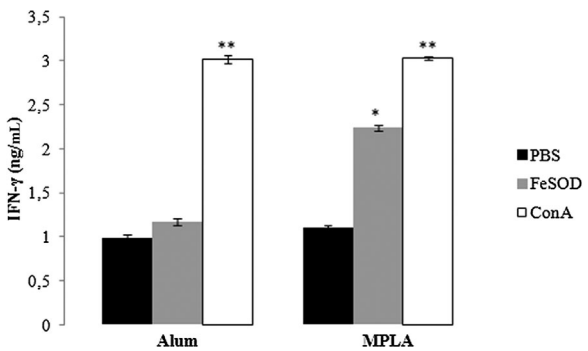

Fig. 4. IFN-γ production by spleen cells of mice immunized with FeSOD-Alum and FeSOD-MPLA. PBS was used as a negative control and Concanavalin A (ConA) as a positive control in parallel experiments. The results are presented as mean ± SD. (*$P < 0.05$; **$P < 0.01$).

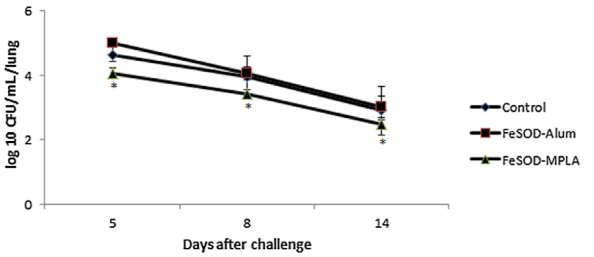

Fig. 5. Mean numbers of CFU/mL/lung of groups of mice intraperitoneally vaccinated with FeSOD-Alum, FeSOD-MPLA and PBS as a negative control after intranasal challenge with *B. pertussis* strain Saadet. The results are presented as mean CFU/mL/lung ± SD. *$P < 0.05$.

DISCUSSION

Whole cell pertussis vaccines containing inactivated *B. pertussis* were introduced in the 1940s (25). Although Pw vaccines have undoubtedly contributed to curbing morbidity and mortality rates of pertussis, mild to severe adverse effects led to the development of Pa vaccines that included certain virulence factors of *B. pertussis* (6). Despite high vaccination coverage, pertussis has reemerged in some countries including Australia, The Netherlands and Canada, which have highly vaccinated populations (26–28). The resurgence of pertussis has been linked to various factors including different vaccine types, pathogen adaptation and waning vaccine-induced immunity (5, 29). Some studies have shown antigenic divergence between vaccine strains and clinical isolates with respect to protective antigens. Moreover, polymorphisms have been found in FHA, Prn, fimbriae and pertussis toxin, which are the main components of Pa vaccines (2, 30, 31). With the aim of combatting pertussis more effectively, studies have focused on developing new and improved Pa vaccines using newly proposed antigens from *B. pertussis*. Marr *et al.* showed that the recombinant rBrkA alone does not confer protection against colonization by *B. pertussis* (14). However, when rBrkA was added to a two-pertussis-component diphtheria, tetanus, and pertussis acellular vaccine including PT and FHA, it was found to confer similar protection to Infanrix (GlaxoSmithKline Beecham Biologicals, Rixensart, Belgium), which contains PT, FHA and PRN (14). More recent studies have investigated the protective activities of new antigens named as IRP1-3 and AfuA from *B. pertussis* (10, 11). IRP1-3 is an outer membrane protein that probably has a role in iron uptake and AfuA is an iron binding protein. In these studies, mice were immunized with recombinant IRP1-3 mixed with Alum or complete FA, a strong adjuvant that is not used in human vaccines.

A booster dose was applied with the recombinant protein emulsified in Alum or incomplete FA. In addition to small, but significant decreases in bacterial colonization with the formulation containing FA, strong IgG1 and IgG2a antibody responses were detected. Moreover, higher titers of INF-γ and IL-10 were elicited with rIRP1-3/FA than with a PBS/FA control. In a subsequent study, the same group reported that recombinant rAfuA alone or in combination with rIRP1-3 and diphtheria, tetanus, and pertussis acellular vaccine (DTPa) including rIRP1-3-rAfuA induce protection against *B. pertussis* infection.

The function of SODs is to scavenge superoxide radicals to molecular oxygen or hydrogen peroxide in order to protect organisms against oxidative stress (32). Although most studies propose a cytoplasmic location for SODs, their secretion by some organisms, such as *Mycobacterium avium* and *M. bovis* BCG, has been reported (32, 33). In a former study, we found that FeSOD is immunogenic in the surface immunoproteome of *B. pertussis* (15). When the amino acid sequence of FeSOD is analyzed by BLAST, it displays 100% amino acid homology among different *B. pertussis* strains and 91–99% homology among different *Bordetella* species. The action of FeSOD B as a virulence factor has been reported previously (16). A mutant live vaccine strain of *F. tularensis* ($sodB_{Ft}$) with decreased FeSOD expression is less virulent in mice than its wild-type form. Additionally, *sodB* deletion in *Legionella pneumophila* reportedly results in loss of cell viability (34).

Moreover, the absence of FeSOD adversely affects the growth rate of *E. coli* and *B. pertussis* (17, 18), and a Δ*sodB* mutant of *B. pertussis* Tohama I strain reportedly has no detectable expression of two of the main virulence factors, namely adenylate cyclase-hemolysin and Prn (18). Unlike the parental strain, the-above-mentioned mutants reportedly fail to induce apoptosis in J774A.1 macrophages and are unable to colonize mouse lungs.

The preferred adjuvant in pertussis vaccine studies has usually been Alum, which was used in as much as 80% of all vaccines. Alum provokes a strong Th2 response, but elicits only weak antiviral and antibacterial Th1-cell-mediated responses (35). Th2 responses alone are insufficient to clear intracellular pathogens in particular. Th1 responses are needed because they can mediate immune responses against intracellular pathogens by activating macrophages and neutrophils as well as by facilitating formation of opsonizing antibodies (36). Moreover, several studies have shown that IFN-γ secreted by Th1 cells is associated with recovery of children from pertussis (22, 23). Thus, development of prospective adjuvants that induce Th1 responses in particular has an important place in pertussis vaccine development strategies. One desirable adjuvant is MPLA, which is a detoxified form of endotoxin LPS (37). It contributes to durable antibody and Th1-dependent cytotoxic T cell responses, which are required for clearance of pathogens such as *B. pertussis* (38). In addition, induction of Th1 cells by MPLA results in production of IFN-γ, which is required for bacterial clearance in pertussis (37). For this reason, we employed MPLA as an adjuvant in the present study to investigate the immunogenicity and protective efficacy of rFeSOD. According to the results of ELISA, rFeSOD–MPLA elicited a much stronger IgG2a response than rFeSOD–Alum, indicating that our formulation also induces a Th1-dependent immune response. While the present article was in preparation, the researchers of Novartis and GlaxoSmithKline Vaccines reported better-tailored immunological responses (strongly increased IgG2a antibody titers) against acellular pertussis-containing combination vaccine (TdaP) when 1 mg/mL MPLA is included in formulations prepared with 2 mg/mL Alum, thus supporting the present findings (39).

Th1-type responses mainly induce secretion of IFN-γ from Th1 cells. IFN-γ helps production of opsonizing antibody (IgG2a) and activation of macrophages and neutrophils (40). We found that there was a significant ($P < 0.05$) increment in antigen-specific-IFN-γ production in mice immunized with rFeSOD–MPLA; this was not the case for rFeSOD–Alum immunized mice. Bacterial clearance is one of the important indicators of recovery from pertussis. In an intranasal challenge experiment, when rFeSOD–Alum and rFeSOD–MPLA immunized groups were compared with respect to the clearance rate, a modest, but significant ($p < 0.05$) decrease in CFU/mL/lung was evident only with rFeSOD–MPLA. At this point, it is should be noted that a possible reason for cyclic pertussis outbreaks is the polymorphism of pertussis; accordingly, *B. pertussis* isolated from patients will be necessary for intranasal challenge studies in the future. The reduction in lung bacterial count can be attributed to increased IFN-γ production, which was detected in the present study. The protection obtained by using rFeSOD alone against *B. pertussis* colonization is noteworthy, especially considering the previous finding that rBrkA alone does not provide protection against *B. pertussis* colonization (14).

To summarize, we demonstrated that rFeSOD formulated with MPLA, but not with Alum, induces IgG2a and IFN-γ production and a modest decrease in

bacterial count, suggesting that this formulation is a promising candidate component for developing new generation combination vaccines against pertussis.

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DISCLOSURE

The authors declare that they have no conflicts of interest.

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