Immunization with recombinant FliD confers protection against *Helicobacter pylori* infection in mice

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Abstract

Nearly half of the world's population is infected with *Helicobacter pylori.* Clinical manifestations of this infection range from gastritis and peptic ulcers to gastric adenocarcinoma and lymphoma**.** Due to the emerging of antibiotic resistant strains and poor patient compliance of the antibiotic therapy, there is increasing interest in the development of a protective vaccine against *H. pylori* infection. The bacterial protein FliD forms a capping structure on the end of each flagellum which is critical to prevent depolymerization and structural degradation. In this study, the potential of FliD as a prospective *H. pylori* subunit vaccine was assessed. For this purpose, immunogenicity and protective efficacy of recombinant FliD (rFliD) from *H. pylori* was evaluated in C57BL/6 mice. Purified rFliD was formulated with different adjuvants and administered *via* subcutaneous or oral route. Subcutaneous immunization with rFliD elicited predominantly mixed Th1 and Th17 immune responses, with high titers of specific IgG_1 and IgG_{2a}. Splenocytes of immunized mice exhibited strong recall responses, resulting in the secretion of high amounts of IFN-γ and IL-17, and low levels of IL-4. Immunization with rFliD caused a significant reduction in *H. pylori* bacterial load relative to naïve control mice ($p < 0.001$), demonstrating a robust protective effect. Taken together, these results suggest that subcutaneous vaccination with rFliD formulated with CpG or Addavax could be considered as a potential candidate for the development of a subunit vaccine against *H. pylori* infection.

Key words*: Helicobacter pylori*, immunization, recombinant, adjuvant, protection.

1. Introduction

H. pylori is a spiral-shaped, extracellular Gram-negative, and microaerophilic bacterium that has colonized the stomach of approximately 50% of the worldwide human population [1]. In 1994, *H. pylori* was classified as a class I carcinogen by the World Health Organization [2]. Infection is strongly associated with the development of several gastrointestinal diseases, including duodenal and gastric ulcers, gastric adenocarcinoma and gastric mucosa-associated lymphoid tissue lymphoma (MALT) [3]. Antibiotic therapy, although effective, is associated with several disadvantages including low patient compliance due to the drugs' side effects, treatment failure as a result of the emergence of drug-resistant strains, high costs, and the failure to prevent reinfection [4-7]. Therefore, alternative approaches to combat *H. pylori* infection are currently being pursued, including vaccination. Several *H. pylori* proteins have been identified as immunogenic in preclinical models, including Urease B (UreB) [8], Vacuolating toxin A (VacA) [9], *H. pylori* adhesion A (HpaA) [10], neutrophil-activating protein A (NapA) [11], outer membrane protein (Omp) [12], cytotoxin-associated antigen (CagA) [9], heat-shock proteins (Hsp) [13, 14], OipA [15] catalase [16] and chimeric genes [17-19]. Each of these antigens has the ability to reduce the bacterial load in animal models, but the elicited protection provided by them is less than ideal. Current strategies to enhance vaccine efficacy include the identification of most suitable immune targets and the combination of immunodominant antigens into multivalent formulations. Khalifeh Gholi et al. [20] demonstrated that FliD, the flagellar hook-associated protein 2, reacts with approximately 97 percent of sera obtained from patients infected with *H. pylori*, but not with sera from uninfected individuals, and reacts very weakly with sera from patients whose infections had been eradicated, suggesting that this is a common immune target in the infected human host [20]. FliD plays a crucial role in flagella assembly. Flagellin is important

for bacterial motility and is essential for colonization and persistence of *H. pylori* in the stomach niche [21].

The objective of the current study was to investigate the protective efficacy and underlying mechanisms of recombinant FliD (rFliD) antigen. Owing to inherent safety and the low risk of adverse reactions [22], protein subunit immunization is an attractive vaccination approach. However, immune responses elicited by the administration of pure antigens are usually low. Therefore, we investigated whether formulations of FliD with different adjuvants, CpG, Cholera toxin subunit B (CTB), or Addavax, enhance protection of mice against *H. pylori* infection.

2. Materials and methods

2.1. Bacterial strains

E. coli strains BL21 and TOP10 were used for expression of FliD. Bacteria were routinely grown at 37°C in LB broth or agar. The *H. pylori* strain SS1 was grown on *Brucella* agar supplemented with 5% sheep blood, 5 µg/mL trimethoprim, 161.5 µg/mL polymyxin B (Sigma), 10 µg/mL vancomycin (sigma) and 2.5 µg/mL amphotericin B (Sigma), in an anaerobic jar with microaerophilic gas generating kit (Merck, Germany) for 3 days at 37°C. For infection experiments, bacteria were subcultured in brain heart infusion broth (BHI, Merck) containing 10 µg/mL vancomycin and supplemented with 10% Fetal Bovine Serum (FBS) (Sigma), and grown in microaerophilic conditions for 72 h at 37°C.

2.2. Cloning, expression and purification of recombinant FliD

Cloning and expression of FliD from *H. pylori* in BL21 (DE3) and its purification has been described previously [20]. Briefly, the *flid* gene was amplified from genomic DNA of *H. pylori* by PCR (forward primer: 5'-ATGGCAATAGGTTCATTAAGCT-3', reverse primer: 5'- ATTCTTTTTAGCCGCCGCTT-3'). The DNA fragment was directly inserted into pTZ57R (InsTAclone PCR Cloning Kit, ThermoFisher, USA) and then subcloned into the pET28a+ vector (Novagen, USA) to add a 6xHis tag. To express FliD, 1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) was used. Upon induction, rFliD was expressed in soluble form and purified under native conditions. Following SDS-PAGE separation, purity and identity of the recombinant protein was evaluated by Coomassie blue staining and Western blotting [23, 24]. For immunodetection, size-separated proteins were transferred to a PVDF membrane and rFliD detectedusing anti-6xHis peroxidase (Roche, Germany) (1/40,000). Finally, the membrane was developed using 3, 3′-Diaminobenzidine tetrahydrochloride

(Sigma). To preclude effects caused by residual endotoxin present in the protein preparation, only batches of purified protein with an endotoxin content of less than 0.05 endotoxin units per mg of protein (evaluated by Limulusamebocyte lysate analysis kit, Lonza, Basel, Switzerland) were used in further studies. The Bradford method was used to determine the concentration of the recombinant protein [25].

2.3. Mice

Six- to eight-week-old female C57BL/6 mice were purchased from the Pasteur Institute of Iran and maintained under specific pathogen-free conditions. Mice were handled under optimal conditions of temperature, hygiene, humidity and light (cycles of 12 h dark/light). All experimental animal procedures were approved by the ethical committee of Kashan University of Medical Science.

2.4. Immunization and *H. pylori* inoculation of mice

One hundred and forty four mice were randomly divided into 8 groups (n = 18 each). Five groups were immunized subcutaneously (s.c.) three times at 2-week intervals with either 30 μg rFliD formulated with 20 μg/mouse CpG (CpG, ODN1826 5′-TCCATGACGTTCCTGACGTT- 3′, synthesized by TAG Copenhagen, Denmark), 30 μg rFliD formulated with (1:1) Addavax (Invivogen, USA), or PBS, or 20 μg/mouse CpG, Addavax, and PBS alone. The two remaining groups were orally immunized with rFliD and 20 μg/mouse CTB (Sigma, Germany) or CTB alone (Table 1). To enable immunization in a small volume, the recombinant antigen was lyophilized and then reconstituted in a volume of 100 μl PBS containing the respective adjuvant.

Two weeks after the final immunization, eight mice from each group were challenged orally thrice in 2-day intervals with 5×10^8 CFU mouse-adapted *H. pylori* strain SS1 in 100 µl brain heart infusion broth. Five mice were bled to obtain sera at days 0, 15, 30, 45, and 75 after the first immunization. The remaining five mice were sacrificed to evaluate immune responses including cytokine production, sIgA secretion, and IgG immune responses at the day of challenge. For measuring sIgA secretion, gastric fluid was collected as described previously [26].

2.5. Humoral and mucosal immune responses

To measure rFliD-specific serum IgG_1, IgG_{2a} and gastric fluid sIgA titers in immunized mice, an enzyme-linked immunosorbent assay (ELISA) was used. 96-well polystyrene plates (Greiner Bio-One, Frickenhausen, Germany) were coated with the purified rFliD (1 µg/ml). After overnight incubation, TBST buffer (Tris-buffered saline, pH 7.4, and containing 0.05 % Tween 20) were used to wash the plates three times followed by blocking with 300 µl 10 % FBS in PBS for 2 h at 37°C. Thereafter, plates were incubated with serial dilutions of mouse sera or gastric and intestinal fluid for 2 h at room temperature, and then washed. HRP-conjugated goat-anti-mouse IgG_1, IgG_{2a} or sIgA antibodies (BD Pharmingen, USA) were added to the wells for another 90 min at 37°C. After the last washing step, specific reactivity was measured by the addition of 50 µl/well of the enzyme substrate TMB (Pishtaz Teb, Tehran, Iran). The reaction was stopped by adding 30 µl 20 % H_2SO_4 to each well. Optical density at 495 nm was measured using an ELISA plate reader (Bio-Tek Instruments, Winooski, USA).

2.6. Cellular immune responses

Assessment of cellular immune responses has been described elsewhere [22, 27]. Briefly, fifteen days after the last immunization, the mice were euthanized and spleens removed under sterile conditions. The spleens were minced and homogenized and then mononuclear cells (MNCs) were isolated by Ficoll–Paque (GE Healthcare, Uppsala, Sweden) density centrifugation. 2×10^6 splenocytes were plated in one well of a 24-well plate in 2 ml of complete medium (RPMI-1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10 % heat inactivated FBS) and 10 μg/ml rFliD were added to each well and the plates incubated at 37°C in 5 % CO_2 for 48 h. Control wells received PBS instead of the antigen. After 48 h, supernatants were collected and stored at -70°C for cytokine assay. Concentrations of interferon-gamma (IFN-γ), interleukin-17 (IL-17), and -4 (IL-4) were determined by ELISA according to the manufacturer's instructions (R&D Systems, USA).

2.7. Protection experiments

H. pylori CFU were quantified six weeks after the last immunization. For this purpose, the copy number of *H. pylori* 16s gene within mouse gastric tissues was measured by a quantitative PCR as described previously [28]. A recombinant plasmid carrying the *H. pylori* 16S gene was used as standard to measure copy numbers of the 16S gene in DNA extracted from half of a mouse stomach and the obtained Colony-Forming Units (CFU) calculated as CFU per stomach.

2.8. Statistical analysis

Statistical analysis was carried out using SPSS computer software, version 16.0. All data were represented as the mean ± standard deviation (SD). Differences among data of the five

groups were analyzed by one factor analysis of variance (ANOVA) and Tukey's post hoc test in SPSS. P-values <0.05 were considered as statistically significant.

Results

3.1. Expression and purification of the recombinant FliD protein

To obtain sufficient amounts of rFliD for protection experiments and immunological analyses, the *H. pylori flid* gene was amplified by PCR and fused to a 6x histidine tag in the pET28 bacterial expression vector. *E.coli* BL21 cells were transformed with the resulting plasmid and protein expression was induced with IPTG. Upon purification with Ni-NTA agarose, rFLiD expression product was verified *via* SDS-PAGE and Western blot [24]. Consistent with molecular weight prediction, recombinant rFliD had a size of approximately 84 kDa (Fig.1). From one liter of liquid culture, approximately 35 mg of rFliD protein was obtained.

3.2. Subcutaneous vaccination with rFliD formulated with adjuvants provides protection against *H. pylori* infection

To investigate whether immunization with rFliD provided protection against subsequent infection with *H. pylori*, groups of mice were immunized with rFliD formulated with different adjuvants, rFliD alone, or PBS. Two weeks after the last immunization, mice were challenged thrice at one day intervals with 5×10^8 *H. pylori* SS1. Bacterial DNA load was assessed by qPCR four weeks post infection.

The copy numbers of *H. pylori* DNA in groups immunized with rFliD formulated with adjuvants were significantly lower than in mice immunized with rFliD in PBS or in control mice. The highest degree of protection among animals vaccinated with rFliD plus adjuvants was observed in mice immunized with rFliD plus CpG, albeit without reaching statistical significance (Fig. 2). Compared to the negative control group, even mice immunized with rFliD in PBS showed a significant reduction in CFU, implicating a high immunogenicity of this antigen.

3.3. Vaccination with rFliD and adjuvants induces vigorous antibody responses

Specific antibody titers produced in mice immunized with rFliD formulated with different adjuvants were evaluated by ELISA in sera obtained at different time points after the first immunization. Vaccination with rFliD induced strong immunoglobulin G (IgG) responses, with IgG_1 titers usually exceeding those of the IgG_{2a} subtype (Fig. 3). The IgG1/IgG2a ratio was 1 to 2 and 1 to 3.44 in mice immunized subcutaneously and orally, respectively. These results suggested that CpG and Addavax induced a Th1-biased immune response.

Although different immune responses were observed for CpG and CTB, the total amount of IgG detected was similar for both adjuvants. Responses induced in mice orally immunized with rFliD formulated with CTB were markedly lower than in mice immunized subcutaneously with rFliD plus CpG or Addavax. However, the IgG responses of mice immunized with rFliD formulated with CTB were significantly increased 75 days after the initial immunization. Mice vaccinated with rFliD formulated with either CpG or Addavax demonstrated increased IgG_1 and IgG_{2a} titers commencing at the second week post-immunization, peaking at the sixth week and remaining elevated until the eleventh week after the first immunization. Compared to all adjuvant formulations, rFliD alone induced only low titers of both IgG_1 and IgG_{2a} antibodies in immunized mice (Fig. 3). To assess the mucosal immune responses elicited by immunization, gastric fluid IgA production was determined for each vaccine. As shown in Fig 3, the three groups immunized with rFliD plus adjuvants showed significantly elevated gastric mucosal IgA titers compared to the control group which had only received PBS ($P<0.0001$). The group immunized with rFliD and PBS exhibited no difference when compared to the PBS control group.

Taken together, these findings suggest that the combination of rFliD with CpG, Addavax, or CTB induced robust antibody responses.

3.4. Vaccination with rFliD and adjuvants elicits a mixed Th1 and Th17 type of immune response

To further characterize the induced immune responses, splenocytes from mice immunized with rFliD formulated with different adjuvants, or rFliD alone, or PBS were isolated 45 days after the first immunization. Following incubation with rFliD, cytokine production was assessed. Secretion of IFN-γ, IL-4, and IL-17 from splenocytes of immunized mice after re-stimulation was assessed two weeks after the final immunization boost. As shown in Fig 4, the concentrations of all tested cytokines were significantly increased in mice vaccinated with rFliD formulated with adjuvants compared to the PBS control group, yet the amount of IL-4 was comparable low. Splenocytes of mice immunized with rFliD without adjuvants secreted significantly higher amounts of IFN-γ and IL-17 than splenocytes from PBS control mice. These results indicate that rFliD alone is capable of eliciting cellular immune responses mostly of Th1 and Th17 type. Moreover, these immune responses were markedly enhanced by the use of adjuvants.

It is important to note that there were no discernable differences in the patterns of cytokines secreted by splenocytes from mice immunized with rFliD formulated with the three different adjuvants (Fig. 4).

4. Discussion

The development of an effective vaccine against *H. pylori* has been confounded by several aspects including choice of the antigen, route of vaccination, and selection of adjuvants to boost immunogenicity [29, 30]. Motility enables *H. pylori* to reach the gastric epithelium, attach *via* adhesion factors, and establish infection in the epithelium [31]. Polar flagella is considered an important virulence factor for *H. pylori*, as studies done with a non-motile *fliD* mutant demonstrated that FliD, and consequently a functional flagellum, is required for the colonization of mice [32]. Moreover, it has been shown that FliD promotes biofilm formation, thereby contributing toward environmental resilience [33]. The association between the presence of antibodies against FliD and gastric cancer is well documented. Based on these findings, it has been suggested that FliD can serve as biomarker in screening for GC patients [34]. We hypothesized that highly expressed proteins that are critical for *H. pylori* infection may constitute optimal candidates for subunit vaccines. In this regard, rFliD was administered orally or subcutaneously and formulated with Addavax, CpG, or CTB adjuvants. Addavax is a squalene-based oil-in-water emulsion capable of eliciting both cellular and humoral immune responses [35, 36]. MF59, which has similar composition as Addavax, has been approved for clinical use in the US with a seasonal flu vaccine [37]. By contrast, CpG oligodeoxynucleotides are toll-like receptor 9 (TLR9) agonists known to stimulate innate immune defenses and antigen-specific T cell responses [38]. CTB is a potent adjuvant which can stimulate innate and antigen-specific responses upon oral or intranasal administration [39].

We looked at the protective capability of rFliD by challenging vaccinated mice with live *H. pylori* SS1. Our results demonstrate that mice immunized with rFliD in combination with any of the tested adjuvants conferred significant protective immunity to mice as compared to the control group. Immunization with non-adjuvanted rFliD resulted in partial

protection against *H. pylori* but significant in comparison to the naïve control. Taken together, our results suggest that FliD is capable of eliciting strong and protective immune responses in mice when combined with adjuvants. Our results also demonstrated that the strongest immune responses were elicited by subcutaneous rather than oral immunization. Since both CpG and Addavax have been approved for human application, they may form the basis of future subunit vaccines against *H. pylori* [40, 41].

To investigate how immunity against *H. pylori* infection was established, cellular immune responses were assessed by measuring cytokine production of splenocytes from immunized mice after re-stimulation *in vitro*. These cells showed robust IFN-γ secretion, which is indicative of a Th1 type of response. Furthermore, high quantities of IL-17 were produced by splenocytes of immunized mice upon re-stimulation. These findings are consistent with earlier studies by other investigators who reported mixed Th1 and Th17 responses against a set of distinct *H. pylori* proteins [19, 26, 44-46]. In addition, increased amounts of IL-4 were produced by splenocytes of rFliD-immunized *versus* naïve mice. However, the amount of IL-4 produced by the cells was comparable low. Therefore, it remains unknown whether the vaccines also elicited a significant Th2 type of immune response. Adjuvants are able to modulate strength and type of the T cell response against a given antigen. For example, adjuvants like CTB can skew immune response from Th1 to Th2 whereas MF59® is a strong stimulator of both Th1 and Th2 immune responses [42, 43]. As determined by cytokine secretion assays, all adjuvants employed in this study induced very similar types of cellular immune responses against rFliD that encompassed different immune effector functions, which may explain their protective effect. Although the specific functions of the secreted cytokines in the control of *H. pylori* infection still need to be elucidated in more detail, Th1 and Th17 cells have been implicated in inducing local inflammation and in promoting protective immune responses [47]. Moreover, Th17 responses are involved in the

recruitment of neutrophils, the release of anti-microbial peptides and the induction of IL-17-driven Th1 immunity [48].

To further characterize the Th1 and Th2 profile of the elicited immune responses, production of antigen-specific IgG_1 and IgG_{2a} antibodies was assessed. Although rFliD-induced humoral immune responses were predominantly of IgG_1 isotype, significant levels of IgG_{2a} titers were also detected. Specific antibodies against rFliD of both isotypes were also produced in mice immunized with rFliD alone, but titers were significantly lower in comparison to mice immunized with rFliD and adjuvants. The elicited antibody responses in animals immunized with rFliD and adjuvants was more pronounced when applied subcutaneously than through the oral route. To assess the mucosal humoral immune response elicited by the different vaccines, gastric IgA production was evaluated. Immunizations with rFliD and Addavax, CpG, or CTB induced significant levels of antigen-specific gastric IgA compared to control mice. These findings suggest that immunization with rFliD and adjuvant, regardless of subcutaneous or oral route of delivery, efficiently induced gastric mucosal immune responses.

Although the immune mechanisms that provide protective immunity against *H. pylori* infection are still poorly defined, cellular immune responses seem to be critical and sufficient to provide protection even in the absence of B cells [54]. Which type of cellular immune response is most protective is still matter of debate. While Th1 type of responses were considered most effective in previous studies, Mohammadi et al. [55] recently showed that Th1 cells increased the severity of gastritis and Th2 cells were required for reducing bacterial load. Likewise, Th17 type of responses were shown to favor bacterial growth [56], while other studies emphasized the importance of Th-17 cells and IL-17 in reducing bacterial load. For example, DeLyria et al. [57] showed that mice vaccinated against *H. pylori* showed robust Th-17 responses and that protection was dependent on neutrophils. Moreover, the

secretion of IL-17 in mice vaccinated against *H. pylori* was shown to be critical for the reduction of the bacterial load [58]. In the present study, the ratio of IgG1/IgG2a and the cytokine profile of restimulated splenocytes from vaccinated mice are indicative of a mixed Th1 and Th17 type of response. How exactly protective immunity is established by the vaccine-induced immune responses is currently not known and requires analysis of the local recall response e.g. the production of cytokines, especially IFN-γ and IL-17, and antimicrobial peptides in the gastric tissue after challenge in immunized mice. Additional experiments are also needed to assess whether vaccine efficacy can be further increased by incorporating additional *H. pylori* antigens.

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Figures

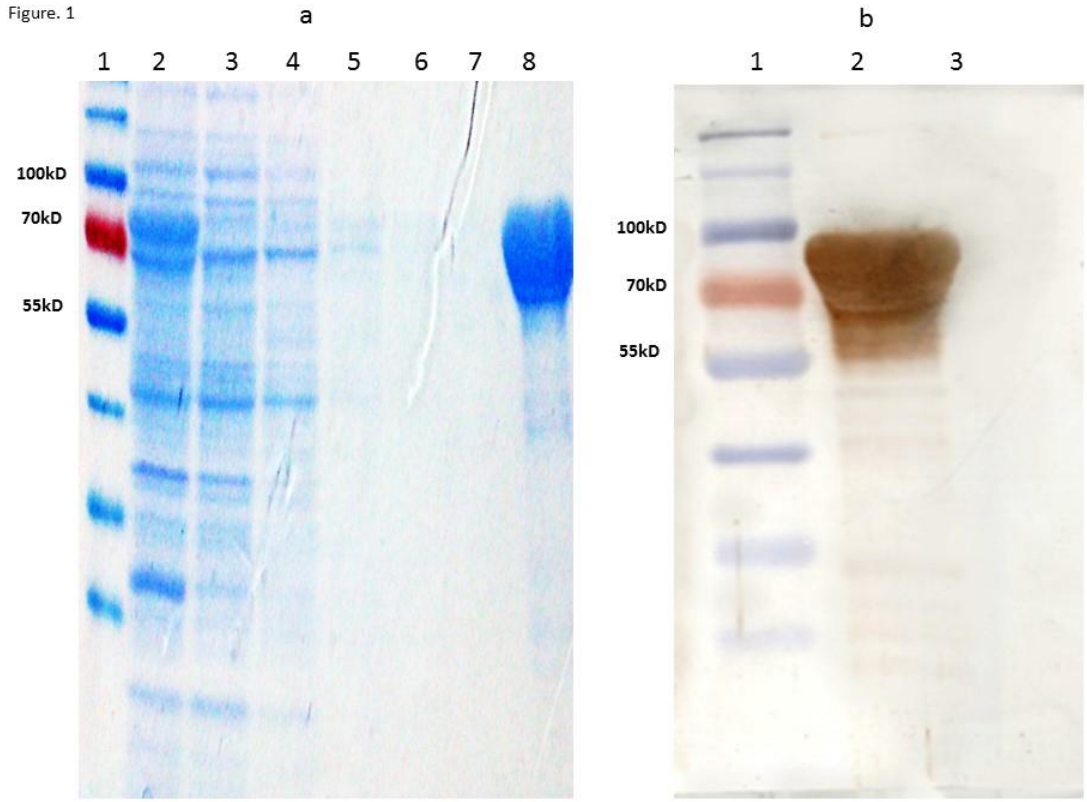

Fig. 1. Expression and purification of recombinant FliD. (a) Analysis of rFliD protein expression by SDS-PAGE. Protein expression in bacteria transformed with the *flid* gene was induced by the addition of IPTG to the media. Four hours after induction, the bacteria were harvested, the bacterial lysates run over Nickel-NTA columns and bound proteins eluted with Imidazole. Aliquots of the different fractions were size-separated by SDS-PAGE and the resulting gel stained with Coomassie-Blue. Lane 1: molecular weight marker, lane 2: bacterial lysate after IPTG induction, lane 3: flow through of Nickel-NTA column, lanes 4 and 5: column eluate with buffer containing 20 mM Imidazole, lanes 6 and 7: eluate with buffer containing 40 mM Imidazole, Lane 8: eluate with buffer containing 1 M Imidazole. (b) Western blot analysis of rFliD using a monoclonal antibody directed against the 6xHis-tag

attached to rFliD. Lane 1: Molecular weight marker, lane 2: purified rFliD, lane 3: lysate of untransformed bacteria. Expected size of rFliD: 84 kDa.

Figure. 2

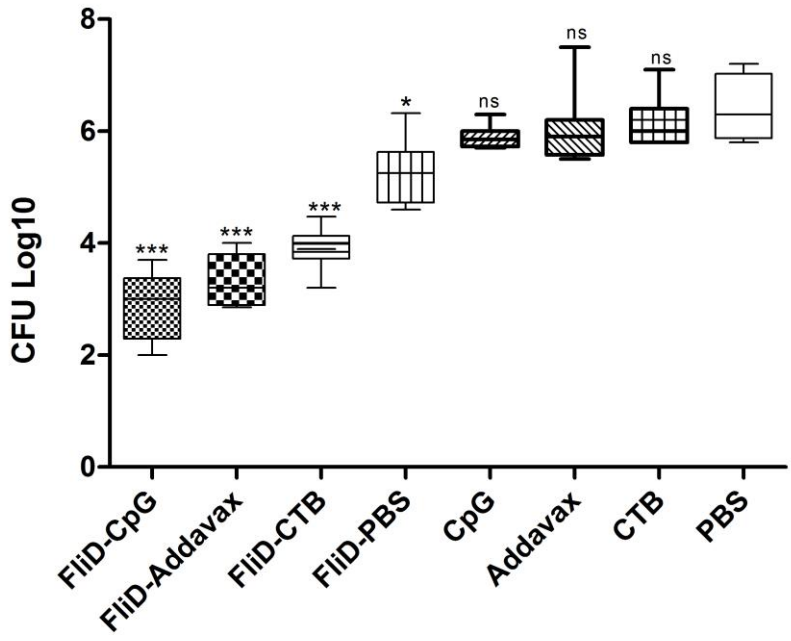

Fig. 2. *H. pylori* colonization of stomach tissue in immunized mice. C57BL/6 mice were immunized on day 0, 15 and 30 with three doses of 30 μg rFliD with CpG, Addavax, CTB or PBS. The control groups (8 mice per each group) only received adjuvants or PBS. Two weeks after final vaccination (on day 45), mice were challenged orally with *H. pylori*. Four weeks post challenge, levels of gastric *H. pylori* colonization were determined by real-time quantitative PCR. Significance (ANOVA) was evaluated with reference to the PBS control. ns: not significant, ***: $p < 0.001$.

Figure. 3

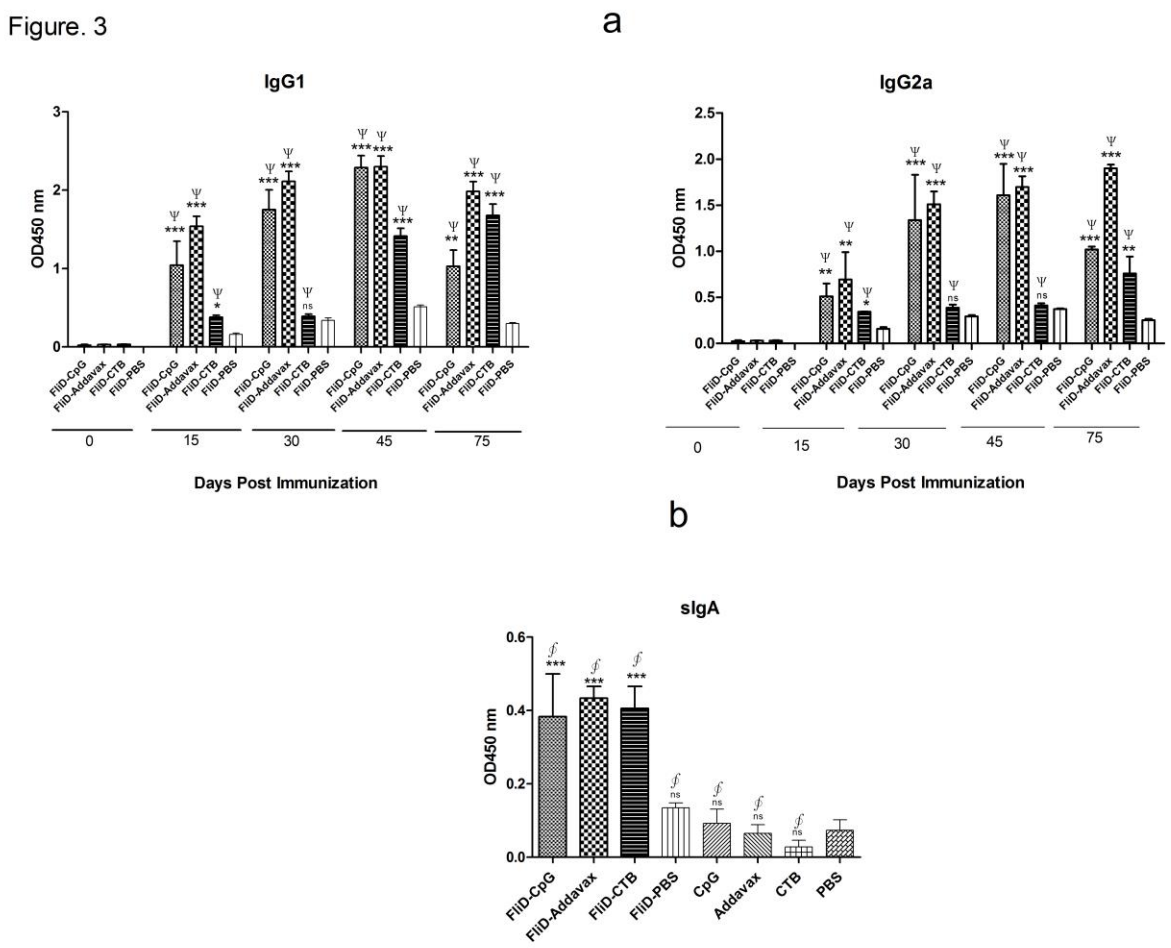

Fig. 3. Analysis of the rFliD-specific antibody response in immunized animals. (a) Kinetics of the IgG_1 and IgG_{2a} responses after immunization with rFliD plus different adjuvants. Animals (5 mice per each group) were bled retroorbitally on the indicated days and specific IgG_1 and IgG_{2a} antibody titers against rFliD were evaluated by ELISA. Titer values represent the mean ± SD of sera from three analyses of five animals each. (b) Gastric scrapings were collected 45 days post first immunization and rFliD-specific IgA titers analyzed by ELISA. Ψ: Comparison of antigen-specific anti-rFliD IgG_1 and IgG_{2a} in mice immunized with rFliD formulated with and without adjuvant. $\oint$: Comparison of anti-rFliD IgA in mice immunized with rFliD *versus* PBS. ns: not significant, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

Figure. 4

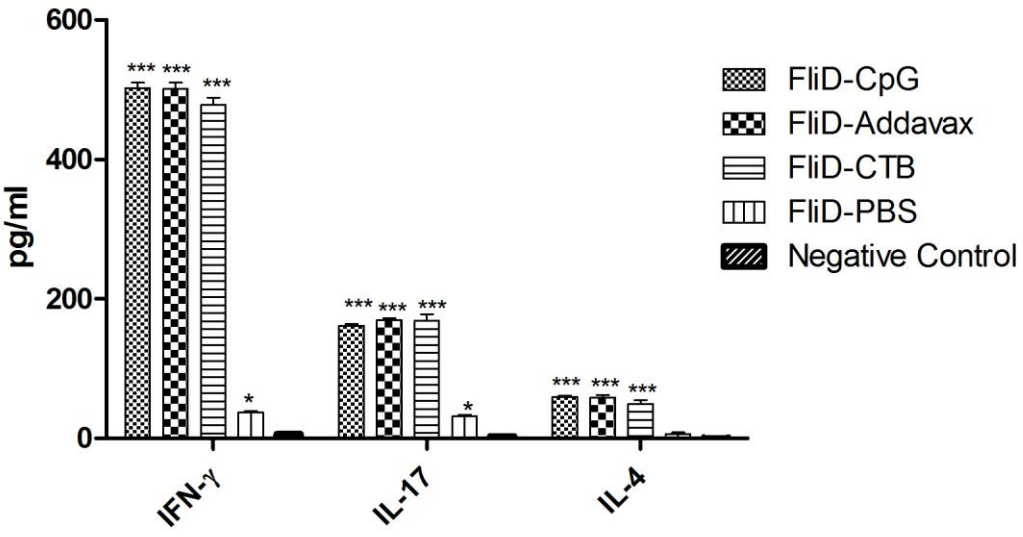

Fig. 4. Cytokine production by splenocytes from immunized mice after restimulation with rFliD. Spleen cells of mice from all five groups (5 mice per each group) were stimulated *in vitro* with 10 μg/ml rFliD for 48 h. Cytokine levels in culture supernatant were measured by sandwich ELISA. The data are the mean ±SD of five individual mice from each group with two repeats. Significance was assessed between mice immunized with rFliD +/- adjuvants and mice treated with PBS. ***: $p < 0.001$.