Immunization with a recombinant fusion protein protects mice against Helicobacter pylori infection

Amir Ghasemi a,b,⇑, Nazanin Mohammad a, Josef Mautner c, Mehrnaz Taghipour Karsabet a, Jafar Amani d, Abolfazl Ardjmand e, Zarieehr Vakili f

a Department of Microbiology and Immunology, Faculty of Medicine, Kashan University of Medical Sciences, Kashan, Iran
b Department of Infectious Disease and Immunology, College of Veterinary Medicine, University of Florida, FL, USA
c Technische Universität München & Helmholtz Zentrum München, Munich, Germany
d Applied Microbiology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran
e Physiology Research Center, Kashan University of Medical Sciences, Kashan, Iran
f Department of Pathology, Faculty of Medicine, Kashan University of Medical Sciences, Kashan, Iran

A R T I C L E   I N F O

Article history:
Received 13 October 2017
Received in revised form 10 July 2018
Accepted 16 July 2018
Available online 21 July 2018

Keywords:
Helicobacter pylori
Immunization
Chimeric
Adjuvant
Fusion protection

A B S T R A C T

More than 50% of the world’s population is infected with the bacterium Helicobacter pylori. If left untreated, infection with H. pylori can cause chronic gastritis and peptic ulcer disease, which may progress into gastric cancer. Owing to the limited efficacy of anti-H. pylori antibiotic therapy in clinical practice, the development of a protective vaccine to combat this pathogen has been a tempting goal for several years. In this study, a chimeric gene coding for the antigenic parts of H. pylori FliD, UreB, VacA, and CagL was generated and expressed in bacteria and the potential of the resulting fusion protein (rFUVL) to induce humoral and cellular immune responses and to provide protection against H. pylori infection was evaluated in mice. Three different immunization adjuvants were tested along with rFUVL: CpG oligodeoxynucleotides (CpG ODN), Addavax, and Cholera toxin subunit B. Compared to the control group that had received PBS, vaccinated mice showed significantly higher cellular recall responses and antigen-specific IgG2a, IgG1, and gastric IgA antibody titers. Importantly, rFUVL immunized mice exhibited a reduction of about three orders of magnitude in their stomach bacterial loads. Thus, adjuvanted rFUVL might be considered as a promising vaccine candidate for the control of H. pylori infection.

© 2018 Elsevier Ltd. All rights reserved.

1. Introduction

H. pylori is a spiral-shaped, microaerophilic, Gram-negative, flagellated bacterium that colonizes the stomach in more than 50% of the world’s human population [1,2]. Infection with this bacterium may cause chronic gastritis and peptic ulcers and is also associated with gastric adenocarcinoma and gastric mucosa-associated lymphoid tissue lymphoma (MALT) [3]. The strong epidemiological relation between H. pylori and distal (non-cardia) cancer led the World Health Organization Agency to classify H. pylori as a definite (type 1) carcinogen in 1994 [4]. Current therapies used to eradicate H. pylori include the use of three or four antibiotics in combination with a proton-pump inhibitor, an approach that has only been slightly changed over the three decades since H. pylori was identified [5,6]. Patient compliance, increasing antibiotic resistance (especially to clarithromycin and metronidazole), recurrence, and the high cost of the treatment have led to renewed interest in developing a vaccine to prevent against H. pylori infection [7–10].

Several H. pylori antigens have been investigated as vaccine candidates, but the protection provided by each antigen is less than ideal [11–19]. To improve efficacy, combinations of multiple protective antigens have been tested. For example, immunization with three different antigens derived from the vacuolating cytotoxin (VacA), cytotoxin-associated gene A (CagA), and H. pylori urease subunit B (UreB), was able to decrease H. pylori colonization in mice with pre-existing infection [20]. Because of the time-consuming and difficult process of producing three separate recombinant proteins, the simultaneous synthesis of antigenic polypeptides would be highly desirable. However, most of the reported H. pylori protective antigens are large and expressing them combined as a fusion protein is hardly feasible. Thus, a

⇑ Corresponding author at: Department of Microbiology and Immunology, Faculty of Medicine, Kashan University of Medical Sciences, Kashan, Iran.
E-mail address: amghasemi@ufl.edu (A. Ghasemi).

https://doi.org/10.1016/j.vaccine.2018.07.033
0264-410X/© 2018 Elsevier Ltd. All rights reserved.
chimeric antigen comprising various antigenic epitopes that have the potential to provoke robust cellular and humoral immune responses may be more effective and convenient than a single recombinant antigen [21–23].

We have recently reported an in silico study in which a chimeric antigen was designed using four antigenic parts of H. pylori flagellar hook-associated protein (FlId), UreB, VacA, and cytotoxin-associated gene L (CagL) proteins [24]. Also, we showed that vaccination of C57BL/6 mice with recombinant FlId combined with an adjuvant could stimulate protective immune responses against H. pylori infection [25]. Therefore, we sought to employ FlId as the main component of a polyvalent antigen by adding additional epitopes based on published findings. Since the UreB373–385 B-cell epitope from urease prompts antibody responses that nullify urease activity and UreB373–385 is considered a novel immunodominant CD4+ T-cell epitope [26,27], the region from UreB covering both epitopes (UreB327–385) was fused to FlId. In addition, amino acids 744–805 from VacA, which is deemed a major virulence factor of H. pylori type I strains [28], contain an MHC class-II binding peptide that consistently stimulates CD4+ T-cell responses [20]. Thus, this region was also incorporated into the chimeric antigen. Finally, we have previously shown through a bioinformatics approach that amino acids 51–100 of the CagL protein (CagL51–100), a versatile type IV secretion system (T4SS) surface protein that facilitates the translocation of CagA into host cells [29], contains numerous domains with antigenic activity [24] and was therefore also included into the potential vaccine candidate.

Following recombinant expression and purification, the chimeric polypeptide was used to immunize mice in combination with three different adjuvants: CpG ODN, Addavax, and Cholera toxin subunit B (CTB). The potential of the recombinant fusion protein to elicit immune responses in C57BL/6 mice and the prophylactic effect of the vaccine against subsequent challenge with H. pylori was investigated.

2. Materials and methods

2.1. Animals

Six- to eight-week-old female C57BL/6 mice were purchased from the Pasteur Institute of Iran (PI). All animal procedures were approved by the Experimental Animal Ethics Committee of the Kashan University of Medical Science. Mice were acclimated for one week after arrival before starting the experiments.

2.2. Bacterial strains and growth conditions

The H. pylori strain SS1 (a kind gift from Prof. James G. Fox, Massachusetts Institute of Technology) was grown on Brucella agar supplemented with 5% sheep blood, 5 μg/mL trimethoprim, 161.5 μg/mL polymixin B (Sigma-Aldrich, St. Louis, MO, USA), 10 μg/mL vancomycin (Sigma-Aldrich, St. Louis, MO, USA) and 2.5 μg/mL amphotericin B (Sigma-Aldrich, St. Louis, MO, USA), in an anaerobic jar with microaerophilic gas generating kit (Merck Group, Darmstadt, Germany) containing 10 μg/mL vancomycin and supplemented with 10% Fetal Bovine Serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA), and grown in microaerophilic conditions for 72 h at 37 °C.

2.3. Construction and expression of recombinant protein

The fuvl chimeric gene design was described previously [24]. Briefly, sequences encoding FlId (1–600), UreB (327–385), VacA (744–805) and CagL (51–100) polypeptides were obtained from GenBank. To facilitate epitope exposure, flexible glycine-serine (GS) linkers were inserted between the gene segments. In addition, the chimeric gene sequence was optimized for expression in Escherichia coli and a tag comprising six histidine amino acids was added at the C terminal of the gene to facilitate detection and purification of the recombinant protein. The fuvl gene synthesis was performed by Biomatik (Cambridge, Ontario, Canada) and subcloned into the pET26a vector using HindIII and EcoRI restriction enzymes. Subsequently, E. coli BL21 DE3 cells were transformed with the pET-fuvl plasmid and expression of the recombinant fusion protein induced by 1 mM isopropyl-d-1-thiogalactopyranoside (IPTG) for 4 h at 37 °C in the presence of 50 μg/mL kanamycin. rFUVL was expressed in soluble form and purified under native conditions. Identity and purity of the recombinant protein were evaluated by SDS-PAGE and visualized by Coomassie blue staining and Western blotting [30,31]. Briefly, purified rFUVL was size-separated by SDS-PAGE and transferred to a PVDF membrane. Next, the membrane was incubated with anti-6xHis peroxidase (Roche, Basel, Switzerland) (1/40,000) for 2 h. Finally, the membrane was developed using 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St. Louis, MO, USA). Contamination of the recombinant protein with endotoxin was prevented by the inclusion of 0.1% Triton X-114 in all wash buffers used during purification. Only purified recombinant protein containing an endotoxin content of less than 0.05 endotoxin units per mg of protein (evaluated by Limulus amebocyte lysate analysis kit, Lonza, Basel, Switzerland) was used for further studies [30,31]. The Bradford method was used to determine the concentration of recombinant protein [32].

2.4. Immunization and infection with H. pylori

One hundred and sixty mice were randomly divided into eight groups (n = 18 each). Five groups were immunized subcutaneously (SC) three times at fifteen-day intervals with 30 μg rFUVL formulated with 20 μg/mouse CpG ODN (CpG, ODN1826 5’-TCCAT GACGTTCCTGACGT-3’, synthesized by TAG Copenhagen, Fredriksberg, Denmark) or Addavax (Inovigen, San Diego, CA, USA), 30 μg rFUVL alone, 20 μg/mouse CpG ODN and Addavax alone, or only PBS. In addition, to evaluate the protection can be provided by the killed bacteria, eight mice were received 100 μg formalin-fixed H. pylori subcutaneously to evaluate the protection can be provided by the whole lystate of bacteria. To avoid immunizing with excessive volume, only 100 μl PBS comprising the antigen and the respective adjuvant was used for each vaccination. The two remaining groups were orally immunized with rFUVL and 5 μg/mouse CTB (Sigma-Aldrich, St. Louis, MO, USA) or CTB alone (Table 1). Distilled water was used to dissolve adjuvants.

Forty-five days post initial immunization, five mice from each group were challenged orally thrice in two-day intervals with 5 × 10^9 colony-forming units (CFUs) of mouse-adapted H. pylori strain SS1 in 100 μl brain heart infusion broth. In each group, five mice were bled to obtain sera at days 0, 15, 30, 45, and 75 after the initial immunization. Another five mice were sacrificed to obtain tissue and evaluate immune responses including cytokine production, IgA secretion, and humoral immune responses on the day of the challenge. To ensure the observed findings resulted from immunization treatment, the challenge experiment was repeated once with the same numbers of mice.

2.5. Protection experiment

To assess whether the vaccination of mice with adjuvanted rFUVL was able to reduce bacterial load in the stomachs of infected mice, H. pylori CFUs were quantified four weeks post-infection. For
this purpose, the quantitative bacterial culture of mouse stomach was used; a half section of the stomach from each euthanized mouse was homogenized, serially diluted, and then cultured as described above.

2.6. Evaluation of serum antibody responses by ELISA

To specifically evaluate serum IgG1 and IgG2a titers in immunized mice, an enzyme-linked immunosorbent assay (ELISA) was used. 96-well polystyrene plates (Greiner Bio-One, Frickenhausen, Germany) were coated with purified rFUVL (5 μg/mL). After overnight incubation, the plates were washed thrice with TBST buffer (Tris-buffered saline, pH 7.4, containing 0.05% Tween 20) followed by blocking with 300 μL PBS containing 10% FBS for 2 h at 37 °C. After the last washing step, specific reactivity was calculated by the addition of 50 μL/well of the enzyme substrate TMB (Pishbaz Teb, Tehran, Iran). The reaction was stopped by adding 15 μL of 2 M H2SO4. The absorbance at 450 nm was measured instead of the antigen. After 72 h, the supernatants were collected and stored at −70 °C for cytokine assays. Levels of interferon-gamma (IFN-γ), interleukin-17 (IL-17), and interleukin-4 (IL-4) were determined according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN, USA).

2.8. XTT assay

The MNCs obtained in 2.7 were rinsed in cold PBS-EDTA and re-suspended in DMEM without phenol red (Sigma) containing 10% FBS. The cell concentration was adjusted to 2 × 10^6 cells/mL, and 100 μL was added to each well of 96-well culture plates. Cells were then stimulated with PBS (negative control) or 10 μg/mL FUVL, rFUVL [25], synthetic UreB232-285, VcaA744,805 and CagI51,100 (Biomatik) or 3 μg/mL of concanavalin A (Con A). All measurements of cell proliferation were performed in triplicates. After 48 h of incubation at 37 °C in 5% CO2 atmosphere, 100 μL of 1 mg/mL 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium (XTT) (Sigma) containing 25 μL of 5 mM phenazinemethosulfate (PMS) (Sigma) were added to each well. The optical density was read at 492 nm (Bio-Tek Instruments). The stimulation index (SI) was calculated as the ratio between the optical density values of stimulated to unstimulated cells using the following formula:

\[
\text{SI} = \frac{\text{mean OD of stimulated culture} - \text{mean OD of blank control}}{\text{mean OD of unstimulated culture} - \text{mean OD of blank control}}
\]

2.9. Histology

Stomach tissues were first fixed in 10% neutral buffered formalin, then embedded in paraffin. The fixed tissues were sliced to 4 mm thickness and then stained with hematoxylin and eosin. Histopathological damage indexes were calculated by assessing atrophy grades and inflammation in the fundic and antral mucosa as described previously [36]. The degree of inflammation was scored on a scale of 0 to 6: grade 0, absence; grade 2, mild; grade 4, moderate; and grade 6, severe, based on the presence of polymorphonuclear (acute inflammation) and lymphocytic cells (chronic inflammation).

2.10. Statistical analysis

Statistical analysis was carried out using SPSS computer software, version 16.0. The statistical differences between two groups were analyzed by t-test and results among several groups were analyzed by one factor analysis of variance (ANOVA) and Turkey’s post hoc test. The statistical limit for accepting significance was p < 0.05. Data were visualized using the GraphPad Prism 7 program.
3. Results

3.1. Expression and purification of the rFUVL protein

To gain adequate amounts of rFUVL for the protection experiments and immunological analyses, the fuvl gene was designed and then synthesized commercially. The chimeric gene was subcloned into pET26a+ and E. coli BL21 DE3 cells transformed with the plasmid. Recombinant fusion protein synthesis was induced by IPTG and the product purified using Ni-NTA agarose. Identity and purity of rFUVL was confirmed by SDS-PAGE and Western blot [31]. Consistent with the molecular weight prediction, recombinant rFUVL had a size of approximately 86 kDa (Fig. 1).

3.2. Vaccination of mice with rFUVL induces protection against H. pylori

To assess whether immunization with the recombinant protein vaccine reduces bacterial load in the stomachs of infected mice, we determined CFUs of H. pylori using quantitative bacterial culture. Four weeks after challenge, the stomachs of euthanized mice were minced, homogenized, and cultured. As shown in Fig. 2, higher levels of protection were observed when an adjuvant, either CpG ODN, Addavax, or CTB, was used with rFUVL, compared to controls receiving PBS ($p < 0.001$), indicating that adjuvants have a crucial role in eliciting protective immunity in these experiments. Although sterile immunity was not elicited in any of the mice, the bacterial load in the rFUVL plus CpG ODN immunized group was three orders of magnitude lower than in the control group (Fig. 2). Even the immunization of mice with rFUVL alone showed a significant reduction in CFUs, indicating high immunogenicity of this chimeric antigen.

---

**Fig. 1.** Expression and purification of recombinant FUVL. (a) Analysis of rFUVL protein expression by SDS-PAGE. Protein expression in bacteria transformed with pET-fuvl 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 from the nickel-NTA column; lanes 4 and 5: column eluate with buffer containing 20 mM imidazole; lanes 6 to 8: eluate with buffer containing 40 mM Imidazole; lane 9: eluate with buffer containing 1 M imidazole, which was used as immunogen in subsequent experiments. (b) Western blot analysis of rFUVL using a monoclonal antibody directed against the C-terminal 6×His-tag. Lane 1: molecular weight marker; lane 2: purified rFUVL; lane 3: lysate from untransformed bacteria. The expected size of rFUVL: 86 kDa. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Fig. 2.** Colonization of stomach by H. pylori in immunized mice. C57BL/6 mice were immunized with three doses of 30 µg rFUVL (days 0, 15, and 30) with CpG ODN, Addavax, CTB, formalin-fixed H. pylori (FF-Hp) or PBS (eight mice per group). Control groups received only 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 quantitative bacterial culture. Significance (ANOVA) was evaluated with reference to the PBS control; ns: not significant, *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. 

---

A. Ghasemi et al. / Vaccine 36 (2018) 5124–5132
3.3. Immunization of mice with rFUVL and an adjuvant induces strong humoral and mucosal responses

Specific IgG1 and IgG2 antibody titers produced in mice immunized with rFUVL plus different adjuvants were measured by ELISA in sera obtained on different days after the initial immunization. Immunization with rFUVL in PBS induced strong immunoglobulin G (IgG) responses, in which the IgG1 titers were usually slightly above those of the IgG2a subtype (Fig. 3). In mice immunized with rFUVL and CpG ODN or Addavax, the IgG1 titer commenced increasing during the second week after the first immunization, peaked after six weeks, and remained elevated until the eleventh week. Remarkably, a sharp drop in both IgG1 and IgG2a titers was seen in mice immunized with rFUVL plus Addavax after 75 days. By contrast, high titers of both IgG1 and IgG2a were seen at day 75 after the first immunization in the group immunized with rFUVL plus CTB. The IgG1/IgG2a ratio was 1–1.7 and 1–2 in the groups that had received CpG ODN and Addavax, respectively, and 0.88–1.5 in mice immunized orally (CTB). These results indicated that rFUVL formulated with adjuvants elicited a Th1-biased immune response. To assess whether immunization with rFUVL also induced mucosal immune responses, gastric IgA production was evaluated for each vaccine candidate. As shown in Fig. 3, the groups immunized with rFUVL plus adjuvants showed significantly augmented gastric mucosal IgA titers compared to the control group that had only received PBS ($p < 0.0001$).

These findings indicated that combinations of rFUVL with CpG ODN, Addavax, and CTB adjuvants elicited systemic and mucosal humoral immune responses.

3.4. Immunization of mice with rFUVL plus adjuvant induces mixed Th1, Th2, and Th17-type immune responses

To further elucidate the provoked immune responses, splenocytes from mice immunized with rFUVL plus adjuvants, rFUVL alone, or controls were obtained 45 days after the first immunization. After re-stimulation of splenocytes with rFUVL, secretion of IFN-$\gamma$, IL-4, and IL-17 was measured by ELISA. As shown in Fig. 4, the levels of all analyzed cytokines were significantly increased in mice immunized with rFUVL together with adjuvants as compared to the PBS control group. Splenocytes of mice immunized with rFUVL alone secreted significantly higher amounts of IFN-$\gamma$,
IL-4, and IL-17 than splenocytes from PBS control mice. These results show that rFUVL is capable of provoking Th1, Th2, and Th17 type immune responses. Notably, there were no apparent differences in the patterns of cytokines secreted by splenocytes from mice immunized with rFUVL combined with any of the adjuvants (Fig. 4). To characterize the immune response against individual components of the fusion protein, splenocytes from mice immunized with rFUVL were isolated 45 days after the first immunization, incubated with rFUVL, rFliD, UreB 327–385, VacA 744–805 and CagL 51–100, and lymphocyte proliferation analyzed. In comparison to the PBS control, splenocytes from mice immunized with adjuvanted rFUVL showed proliferative responses against all antigen components (Fig. 5). Such recall responses were not observed in the mice immunized with adjuvants alone or PBS (data not shown). These results showed that all antigenic components of the fusion protein could induce cellular immune responses.

3.5. Immunization with rFUVL plus adjuvants induces stomach tissue inflammation in infected mice

To determine whether immunization with recombinant rFUVL induces inflammation in infected tissues, stomachs from mice were evaluated by histology 30 days after infection with H. pylori. Histological assessment showed a significant difference in the extent of tissue damage resulting from inflammation between mice immunized with rFUVL plus adjuvants in comparison to the control group. These results suggested that immunization with adjuvanted rFUVL leads to strong recall responses upon infection with H. pylori SS1 (Fig. 6).

4. Discussion

Vaccine efficacy depends on the immunogenicity of the antigen(s) and the appropriate activation of the immune system by adjuvants. In the present study, we used a recombinant fusion protein containing four antigenic fragments of different H. pylori proteins. In order to identify the most effective adjuvants and route of application, rFUVL was administered either orally or subcutaneously together with CpG ODN, Addavax, or CTB. It has been shown that CpG ODNs increase the efficacy of vaccines against infectious diseases and cancer [37], and CTB has been used as an adjuvant when an antigen is administered nasally or orally [38]. Addavax is a squalene-based oil-in-water nano-emulsion approved in Europe for use as an adjuvant; such emulsions are known to elicit both humoral and cellular immune responses. Addavax has a similar formulation as MF59® that has been approved for use with flu vaccines [39,40]. Compared to the control group, the recombinant fusion protein caused a significant reduction in bacterial load when

---

**Fig. 4.** Cytokine production by splenocytes from immunized mice after re-stimulation with rFUVL. Spleen cells of mice from all groups (five mice per group) were stimulated in vitro with 10 μg/ml rFUVL for 48 h. Cytokine levels in the culture supernatant were measured by sandwich ELISA. The data are the mean ± SD of five individual mice from each group tested in duplicates. Significance was assessed between mice immunized with rFUVL ± adjuvants and mice treated with PBS. ns: not significant, ***: p < 0.001.
combined with any of the tested adjuvants. Among these, CpG ODN caused the strongest reduction in bacterial load. Consistent with these findings, UreB formulated with CpG ODN has been shown to provide protection against *H. pylori* and to elicit cellular immune responses against certain epitopes of UreB [41].

Remarkably, both CpG ODN and Addavax based vaccines were administrated subcutaneously; how subcutaneous administration is able to elicit immune responses and subsequent protection in the gastric tissue remains to be addressed. That the subcutaneous route can be successfully used to immunize mice against *H. pylori* infection has also been demonstrated in some recent studies [22,41–44]. To gain insight into the underlying mechanism, we looked at the antibody response against rFUVL in immunized mice and found that both antigen-specific IgG1 and IgG2a antibodies were produced. Since IFN-γ and IgG2a are indicators of Th1 type of immune responses in mice, and synthesis of IgG1 can be controlled by Th2 clones, these findings suggested that the immune responses elicited by the vaccines were of mixed Th1/Th2 type [45]. Consistent with this, other investigators have observed mixed Th1/Th2 responses against a set of *H. pylori* antigens [13,20,23,46].

Notably, the IgG1/IgG2a ratio also showed that the response elicited in immunized mice had a strong Th1 bias. While C57BL/6 mice have been described as not having the IgG2a gene and thus being incapable of producing the IgG2a isotype [47], there are several strong reports that these mice are in fact able to produce IgG2a [48–51].

We next examined the mucosal immune responses elicited by immunization. To this end, the gastric IgA secretion into the gastric mucosa and immune mediated inflammation were measured for each vaccine formulation. Compared to control mice, immunization with rFUVL plus CpG ODN, Addavax, or CTB elicited significant levels of antigen-specific gastric IgA and inflammatory cell infiltrations and apoptotic cells in the stomach. These findings indicate that immunization with rFUVL plus adjuvant, regardless of whether delivered subcutaneously or orally, efficiently stimulated gastric mucosal immune responses.

To further characterize the Th1/Th2 profiles of the provoked immune responses, cell-mediated immune responses were measured by evaluating cytokine production after in vitro re-stimulation of immunized mice splenocytes. Our results showed that splenocytes from all mice immunized with rFUVL produced IFN-γ, IL-4, and IL-17 upon stimulation with antigen. However, higher amounts of cytokines were secreted in groups immunized with rFUVL plus adjuvants than PBS.

Cell-mediated immune responses were assessed in mice immunized with rFUVL formulated with different adjuvants by measuring antigen-specific proliferation and cytokine production of splenocytes in vitro. Splenocytes from mice immunized with rFUVL with different adjuvants showed significant levels of proliferation in vitro in response to stimulation with rFUVL, rFlID, UreB327–385, VacA744–805, CagL51–100 and 3 μg/ml ConA for 48 h and the extent of proliferation was assayed by XTT. Each bar represents the stimulation index (SI) calculated as the ratio between the obtained mean absorbance values of stimulated cells to the unstimulated cells. The data are the mean SI ± SD of five individual mice from each group with three repeats. *p < 0.05; **p < 0.01; ***p < 0.001.
Although the precise mechanism by which immunization provides protection against \textit{H. pylori} remains to be elucidated, the crucial role of cell-mediated immunity has been established in the past by showing that protection against \textit{H. pylori} infection can be established even in the absence of B cells [52]. Several groups have also demonstrated that protection depends on the induction of Th1- or Th2-biased responses [53–55]. In addition, Th17 cells and their effector cytokines appear to mediate immunity against several infections, particularly those caused by extracellular pathogens like \textit{H. pylori} [56]. Th17 immune responses comprise recruitment of neutrophils, secretion of antimicrobial peptides, and IL-17-driven Th1-mediated protection [57,58]. Although it has been shown that Th17 responses support bacterial growth [59], other studies provided strong evidence for the importance of Th17 cells and IL-17 in the reduction of bacterial load [36,60].

Notwithstanding the prominent relevance in eradicating or controlling several infectious diseases, vaccines are frequently related to questioning and condemnation about the side effect [61]. With regard to the toxicity of the vaccine, we didn’t notice any signs of fever, weight loss, or other systemic indications of toxicity after immunization with rFUVL formulated with different adjuvants.

Whether the decrease in bacterial load in our vaccinated animals was mediated by a single, or an interplay of different types of immune responses, is currently not known. A comprehensive analysis of cytokine secretion and production of the antimicrobial peptides in gastric tissue of mice after challenge with \textit{H. pylori} may shed some light on the mechanisms employed by the immune system to combat infection. Additional experiments are also needed to evaluate whether the vaccine efficacy can be further increased by incorporating additional \textit{H. pylori} antigens.

Taken together, our findings show that rFUVL is a highly immunogenic fusion protein that, when combined with adjuvants, especially CpG ODN, is capable of eliciting complex Th1/Th2/Th17 immune responses and protective immunity against \textit{H. pylori} infection.

Acknowledgments

We would like to thank Homayun Pourbabaee for technical support. The present study was supported by grants from the Iran National Science Foundation: INSF (grant No. 93034823).

References


Chen J, Li N, She F. Helicobacter pylori outer inflammatory protein DNA vaccine-loaded bacterial ghost enhances immune protective efficacy in C57BL/6 mice. Vaccine 2014;32:6054–60.


