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Immunization with chlamydial plasmid protein pORF5 DNA vaccine induces protective immunity against genital chlamydial infection in mice

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To validate the immune protective efficacy of pORF5 DNA vaccine and to analyze potential mechanisms related to this protection. In this study, pORF5 DNA vaccine was constructed and evaluated for its protective immunity in a mouse model of genital chlamydial infection. Groups of BALB/c mice were immunized intranasally with pORF5 DNA vaccine. Humoral and cell mediated immune responses were evaluated. The clearance ability of chlamydial challenge from the genital tract and the *chlamydia*-induced upper genital tract gross pathology and histopathological characterization were also detected. The results showed that the total and the IgG2a anti-pORF5 antibody levels in serum were significantly elevated after pcDNA3.1-pORF5 vaccination, as were the total antibody and IgA levels in vaginal fluids. pcDNA3.1-pORF5 induced a significantly high level of Th1 response as measured by robust gamma interferon (IFN-γ). Minimal IL-4 was produced by immune T cells in response to the re-stimulation with pORF5 protein or the inactive elementary body *in vitro*. pcDNA3.1-pORF5-vaccinated mice displayed significantly reduced bacterial shedding upon a chlamydial challenge and an accelerated resolution of infection. 100% of pcDNA3.1-pORF5 vaccinated mice successfully resolved the infection by day 24. pcDNA3.1-pORF5-immunized mice also exhibited protection against pathological consequences of chlamydial infection. The stimulated index was significantly higher than that of mice immunized with pcDNA3.1 and PBS (P<0.05). Together, these results demonstrated that immunization with pORF5 DNA vaccine is a promising approach for eliciting a protective immunity against a genital chlamydial challenge.

Chlamydia trachomatis, pORF5, DNA vaccine, immune protection, Th1, immune response

Chlamydia trachomatis (*C. trachomatis*) is the most common cause of bacterial sexually transmitted disease worldwide[1]. Sexually transmitted *C. trachomatis* infections are a serious public-health concern. Despite the availability of potent antimicrobial drugs, the majority of genital chlamydial infections are initially asymptomatic, persistent, and recurring. Therefore, chlamydial infections induce immunopathology in the urogenital tract, causing inflammatory diseases and complications such as ectopic pregnancy and infertility. Meanwhile, genital chlamydial infections frequently increase the risk for HIV infection[2], as well as the risk for development of invasive cervical cancer[3–5].

Current strategies in chlamydial vaccine design and development include the identification of the elements of protective immunity, selection of a suitable vaccine candidate capable of inducing protective immunity and

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the development of effective delivery systems to boost immune responses. Progress in molecular immunology and biotechnology in the last two decades has led to substantial progress in chlamydial vaccine. However, because all attempts to induce chlamydial immunity have failed to produce a solid long-lasting immunity, development of a new vaccine capable of inducing protective immunity against chlamydial infection would be the most effective and promising strategy to control chlamydial disease.

After investigating the protective efficacy of intranasal immunization with pORF5 DNA vaccine against genital chlamydial infection in a mouse model, the results showed that intranasal immunization of pORF5 DNA vaccine induced robust cell-mediated and humoral immune responses and significantly accelerated resolution of genital infection with minimal development of oviduct pathology, suggesting that vaccination with pORF5 from *C. trachomatis* is a promising approach to produce protective immunity against chlamydial infection.

1 Materials and methods

1.1 Materials

C. trachomatis serovar D and *C. muridarum* EB were stored in Dr. Guangming Zhong's laboratory (University of Texas Health Science Center at San Antonio, UTHSCSA). pcDNA3.1 plasmid and restriction endoenzymes (*Bam*HI and *Not*I) were purchased from Invitrogen (Carlsbad, CA), and an mIL-4, mIFN-γ ELISA kit was purchased from R&D Systems (USA), Plasmid Mega Kit was purchased from Qiagen (USA), and BALB/c mice were provided and bred by UTHSCSA.

1.2 Methods

1.2.1 pORF5 gene clone and construction of pcDNA 3.1-pORF5. Specific primers were synthesized according to the pORF5 gene sequence of *C. trachomatis* seravar D as follows. Primers contained a *Bam*HI site (underlined) in the forward primer (5′-CGC*GGATCC*A TGGGAAATTCTGGTTTTTATTTG-3′), and in the *Not*I site (underlined) in the reverse primer (5′-TTTTCCTTT T*GCGGCCGC*TTAAGCGTTTGTTTGAGGTATTA-3′). The amplified pORF5 gene product was purified and ligated into a pcDNA3.1 eukaryotic vaccine vector. The recombinants were subsequently selected by restriction enzyme analysis and DNA sequencing to contain pORF5 gene.

1.2.2 Immunization procedure. Four-to-six week-old female BALB/c mice were divided into 3 groups (10 mice per group). Groups of mice were anesthetized and immunized intranasally with 50 μg (dissolved in 40 μL of PBS) pcDNA3.1-pORF5, pcDNA3.1 or 40 μL of sterile PBS (per mice per immunization) on days 0, 14, and 28 for a total of three times.

1.2.3 Detection of specific antibodies. To detect the pORF5 antibody level, sera and genital mucosal secretions from the mice were analyzed utilizing ELISA as follows. Microtiter plates were coated overnight with pORF5 antigen (10 μg/mL) in a sodium bicarbonate buffer (pH 9.5). After blocking with 1% BSA, mice sera and genital mucosal secretions were added in duplicate to wells followed by either horseradish peroxidash conjugated goat anti-mouse total immunoglobulin, or immunoglobulin G1 (IgG1), IgG2a, IgG2b, IgA (Jackson ImmunoResearch). Peroxidash substrate ABTS (Sigma) was used for color development, and the absorbance at 405 nm was measured using a microplate reader (Ramsey, MN).

1.2.4 Measurement of cytokines. Fourteen days after the last intranasal immunization, spleens were removed from each group, and single-cell suspensions were prepared to enrich T cells. Splenocytes were cultured in duplicate and stimulated for 96 h *in vitro* with pORF5 antigen (10 μg) or UV-inactivated *C. muridarum* (10^5 IFU/well) or as medium alone on culture plates. Supernatants from the culture wells were analyzed for IFN-γ and IL-4 production using ELISA kits according to a standard protocol.

1.2.5 Detection of lymphocyte proliferation. Lymphocyte proliferation was determined by MTT from splenic cells stimulated as described above. After coculturing for 96 h with pORF5 antigen or UV-inactivated *C. muridarum* or medium alone, 20 μL (5 mg/mL) of MTT (Sigma) was added to each well for 4 h, the cells were harvested by centrifugation, then resuspended in 100 μL of DMSO (Sigma). The results were expressed as optical densities at 570 nm.

1.2.6 Vaginal *C. muridarum* challenge and determination of bacterial shedding. One month following the final vaccination, mice were challenged intravaginally with 10^4 IFU of *C. muridarum* in 20 μL of SPG buffer. Each mouse was injected with 2.5 mg Depo-provera (Pharmacia Upjohn, Kalamazoo, MI) subcutaneously to

synchronize the menstrual cycle and to increase mouse susceptibility to chlamydial infection. To assess the infection level, vaginal swabs from each mouse were obtained once every three days after the challenge. Chlamydial inclusions were isolated in the cell culture with results expressed as the average number of inclusions per mice group.

1.2.7 Evaluating mouse genital tract tissue pathologies. Eighty days after infection, all mice were sacrificed and the mouse urogenital tract tissues were isolated. Gross examination was performed seeking evidence of hydrosalpinx formation and any other related abnormalities. The excised tissues were then fixed in 10% neutral formalin, embedded in paraffin and serially sectioned longitudinally. The sections were stained with hematoxylin and eosin for severity of inflammation and pathologies.

1.2.8 Statistical analysis. The student *t* test was used to analyze qualitative data. Values assigned to individual mice were calculated into means ± standard errors. Differences between groups were considered to be statistically significant if P values were <0.05.

2 Results

2.1 Construction of recombinant plasmids containing chlamydial pORF5 genes

To construct recombinant pcDNA3.1-pORF5, PCR was performed to amplify pORF5 genes using chlamydial genomic DNA as the template. The positive recombinant plasmid showed two fragments after digestion with *Bam*HI and *Not*I, one of which was the 5.4 kb fragment (blank plasmid pcDNA3.1), the other being an apporximately 800 bp fragment (pORF5 gene). The pORF5 gene was also amplified from the positive recombinant plasmid utilizing PCR (Figure 1).

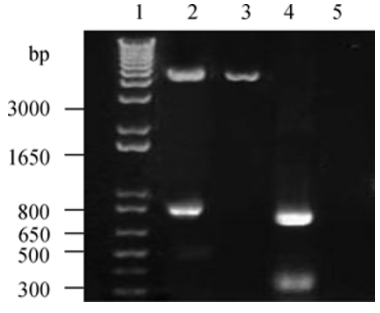

Figure 1 Identification of pcDNA3.1-pORF5 recombinant by PCR amplification and digestion. 1, DNA Marker; 2, pcDNA3.1-pORF5 digested by *BamH*I, *Not*I; 3, pcDNA3.1 digested by *Bam*HI, *Not*I; 4, PCR product from pcDNA3.1-pORF5; 5, PCR product from pcDNA3.1.

2.2 Humoral response after intranasal immunization

Intranasal vaccination induced a robust serum antibody response. The specific antibody titers were increased as the inoculation times accumulated 80 percent of the mice immunized with pcDNA3.1-pORF5 detected the specific antibody at three weeks after the first immunization, which subsequently increased markedly at six weeks. Antibody titers continued to rise until nine weeks, but there was almost no fluctuation in sera from the mice vaccinated with pcDNA3.1 or PBS (Figure 2). pcDNA3.1-pORF5 vaccination mice displayed high titers of serum anti-pORF5 IgG1, IgG2a and IgG2b. Specifically, they increased the level of IgG2a antibody (Figure 3), suggesting that the pcDNA3.1-pORF5 vaccine induced a superior Th1 immune response.

Because mucosal antibodies are important in protection against *C. trachomatis*, induction of an antibody response at local sites was measured in vaginal washes collected from different groups. pcDNA3.1-pORF5 vaccination mice displayed high titers of mucosal anti-pORF5 total antibody and IgA. As expected, there was no specific antibody in the vaginal washes of mice immunized with pcDNA3.1 or PBS (Figure 4).

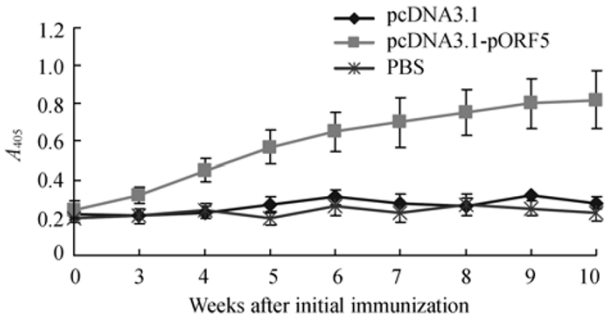

Figure 2 Intranasal immunization with pcDNA3.1-pORF5 induced robust antibody responses. Mice were bled on days 0 and 21, and at 1 week intervals after the 2nd immunization. Serum anti-pORF5 antibody levels were analyzed by ELISA using pORF5-coated microtiter plates. The results are representative of the results of two independent experiments.

2.3 Detection of IFN-γ and IL-4

The high concentration of IFN-γ in spleens of mice immunized with pcDNA3.1-pORF5 contrasted with the significantly lower concentration of that cytokine in spleens of mice immunized with pcDNA3.1 or PBS after being stimulated with pORF5 protein. The level of IFN-γ in mice inoculated with pcDNA3.1-pORF5, pcDNA3.1 or PBS each reached (1757.94 ± 400.45)

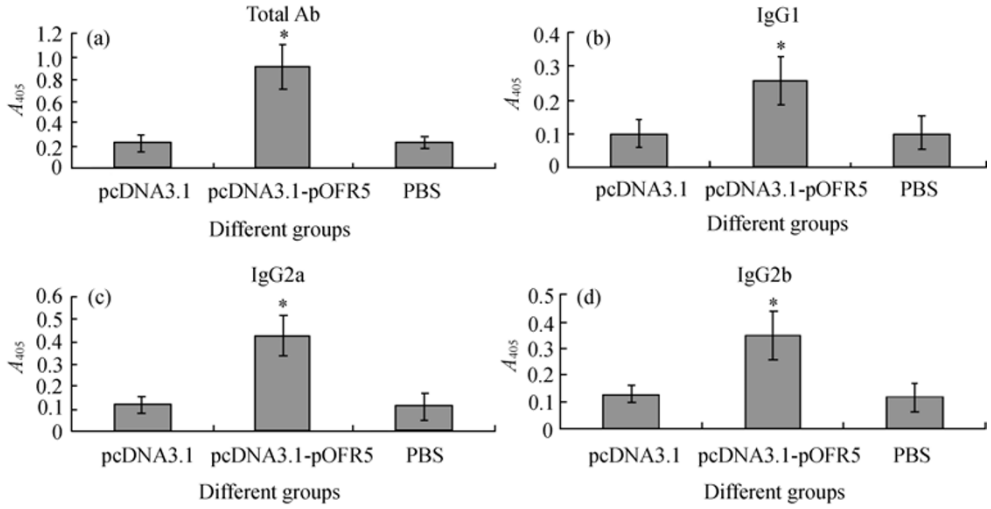

Figure 3 Systemic anti-pORF5 antibody responses after immunization.Mice were bled on day 14 after the 3rd immunization. Serum anti-pORF5 antibody levels were analyzed by ELISA using pORF5-coated microtiter plates. An asterisk indicates that there are significant differences between pORF5 and PBS or vector-immunized mice (P<0.05). The results are representative of the results of two independent experiments.

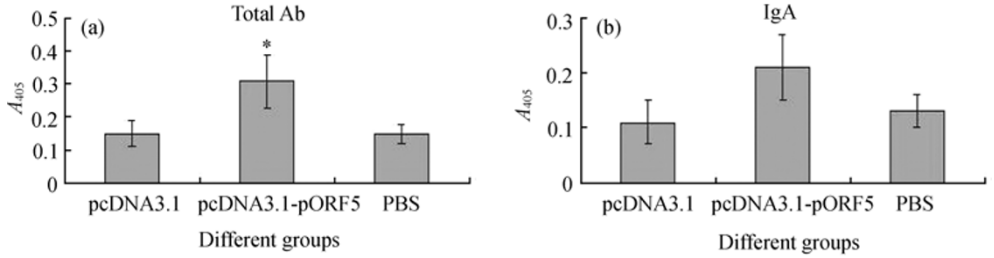

Figure 4 Mucosal anti-pORF5 antibody responses after immunization. Vaginal fluids were collected on day 14 after the 3rd immunization and analyzed by ELISA. An asterisk indicates that there are significant differences between pORF5 and PBS or vector-immunized mice (P<0.05). The results are representative of the results of two independent experiments.

pg/mL, (273.57 ± 61.22) pg/mL, (338.247 ± 87.38) pg/mL (Figure 5(a)). Inactive EB from *C. muridarum* also could stimulate production with a pattern similar to that of pORF5 protein. To assess the magnitude of Th2 response induced following immunization, the amount of pORF5-specific IL-4 was measured, wherein a very low level of IL-4 was detected in the spleens of the different inoculated groups of mice. No significant difference was observed between the vaccinated groups (Figure 5(b)).

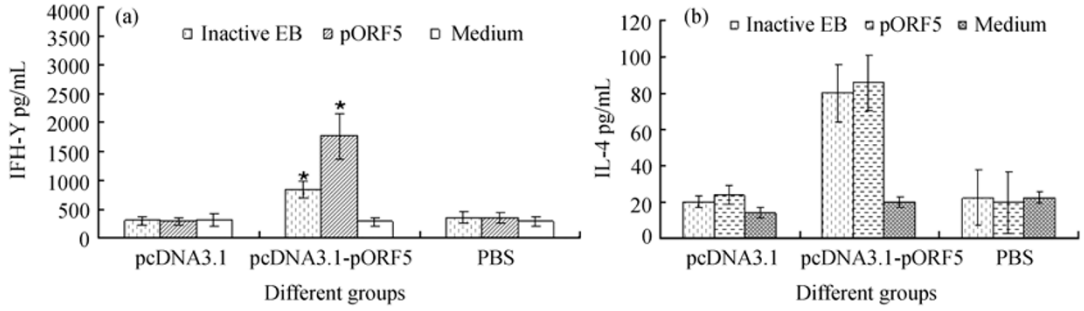

Figure 5 Cytokine recall responses after immunization. On day 14 after the 3rd immunization, mice were sacrificed, and spleens were tested for specific IFN-γ (a)and IL-4 (b) production by ELISA. The asterisks indicate that there are significant differences in IFN-γ secretion between pORF5 immunization and PBS or vector immunization (P<0.05). The results are representative of two independent experiments.

2.4 Antigen-specific proliferative response

Mononuclear cells from different groups were stimulated *in vitro* with recombinant pORF5 protein or inactive EB. Their capacity to proliferate was assessed by MTT. As shown in Table 1, an increase was detected in the mice immunized with pcDNA3.1-pORF5 after the pORF5 protein or EB stimulation compared to proliferation of cells isolated from the mice inoculated with pcDNA3.1, PBS (P<0.05).

Table 1 SI of spleen cells from different mice

Groups	pORF5	Inactive EB	Medium
pcDNA3.1 vector	1.05±0.21	1.15±0.22	1.42±0.34
pORF5 DNA vaccine	2.45±0.36*	2.25±0.47*	1.21±0.27
PBS	1.12±0.24	1.30±0.24	1.25±0.29

On day 14 after the 3rd immunization, the mice were sacrificed, and spleens were tested for a proliferation response by MTT. The asterisks indicate that there are significant differences in the proliferation response of spleen cells between pORF5 immunization and PBS or vector immunization (P<0.05). The results are representative of the results of two independent experiments.

2.5 *Chlamydia*-induced upper genital tract gross pathology and histopathological characterization in vaccinated mice

To determine the effect of pcDNA3.1-pORF5 vaccination on the development of inflammatory disease, gross pathology and histopathological characterization were analyzed on day 80 following the *C. muridarum* challenge. As shown in Figure 6(a)—(c), pcDNA3.1-pORF5 vaccination prevented the development of hydrosalpinx after the chlamydial challenge in the majority of the mice which was significantly lower than mice vaccinated with pcDNA3.1 or PBS. Detailed histopathological comparisons of the three different groups were also performed. While most of the pcDNA3.1-pORF5- immunized mice had nearly normal oviducts, pcDNA3.1 or PBS-vaccinated mice developed characteristic dilated oviducts. In addition, the incidence of fibrosis was significantly lower in vaccinated pcDNA3.1-pORF5 mice than that in vaccinated pcDNA3.1 or PBS mice (Figure 6(d)—(f)). These results demonstrate the efficacy of pcDNA3.1-pORF5 vaccination for reducing the pathology of a genital chlamydial infection in mice.

2.6 Chlamydial clearance after genital challenge in vaccinated mice

The efficacy of the different vaccination regimens against the genital *C. muridarum* challenge was examined by monitoring vaginal chlamydial shedding at 3-day intervals following the challenge. As shown in Figure 7, there was a significant reduction in the amount of chlamydia recovered from mice vaccinated with pcDNA3.1-pORF5 compared to the amount of chlamydia recovered from PBS, pcDNA3.1-treated mice. Vaccination with pcDNA3.1-pORF5 induced the resolution of infection in 20% of the mice as early as day 18, in 40% of the mice by day 21, and in all of the mice (100%) by day 24 following the challenge. By contrast, mice that received pcDNA3.1 or PBS were still shedding *Chlamydia* at day 24 after the challenge, and finally resolved the infection by day 30 (Figure 8).

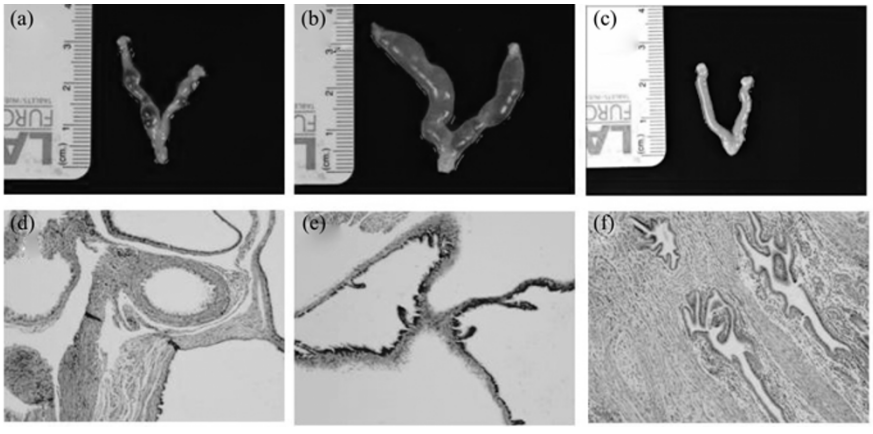

Figure 6 Representation of gross pathology and histopathological characterization of genital tract tissues from vaccinated mice after intravaginal infection with *C. muridarum*. (a)—(c) Gross pathology after genital chlamydial challenge. pcDNA3.1 (a) or PBS (b) immunized mice exhibit hydrosalpinx. pcDNA3.1-pORF5-immunized mice show almost normal oviduct (c). (d)—(f) Histopathological characterization of oviduct tissues: dilation of oviducts in control mice ((d) and (e)) and mild inflammation of the oviducts in pcDNA3.1-pORF5-immunized mice (f). The results are representative of the results of two independent experiments.

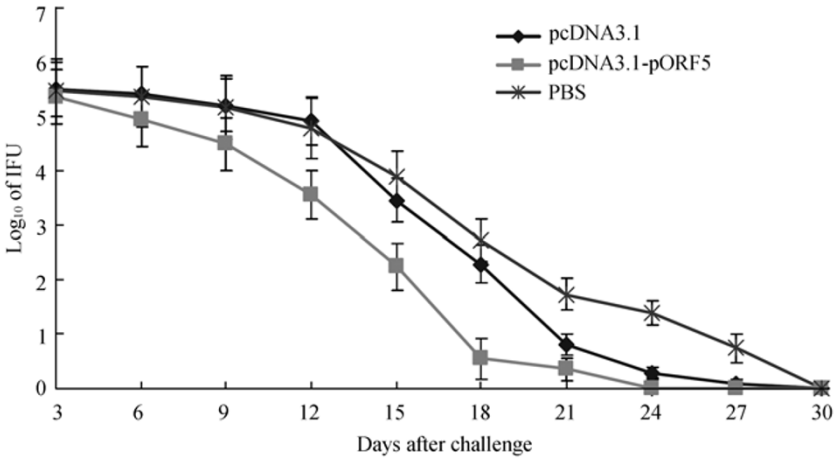

Figure 7 Chlamydial IFU in vaginal swabs from different groups. One month following the final vaccination, all mice were challenged intravaginally with 10^4 IFU of *C. muridarum*. On the days following the challenge indicated, chlamydial shedding was measured: The numbers of chlamydial IFU recovered from vaginal swabs were determined. Each symbol represents an individual mouse. The results are representative of the results of two independent experiments.

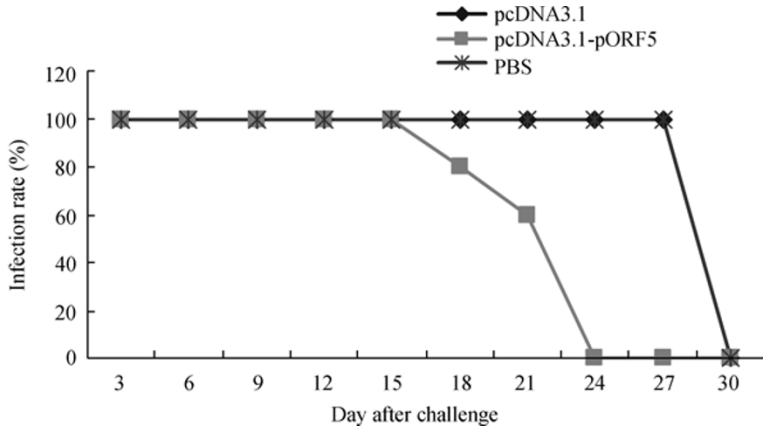

Figure 8 Percentage of immunized mice shedding Chlamydia after a genital challenge.One month following the final vaccination, mice were challenged intravaginally with 10^4 IFU of *C. muridarum*. On the days following the challenge indicated, chlamydial shedding was measured. There were significant differences in the time required for resolution of infection between pcDNA3.1-pORF5-immunized mice and two experimental groups (P<0.01). The results are representative of the results of two independent experiments.

3 Discussion

Because of the public health importance of chlamydial diseases, there has been long-standing interest in developing an effective vaccine. Early vaccine trials using inactivated *C. trachomatis* resulted in short-lived protection with possible enhanced inflammatory pathology during re-infection. Subunit vaccines such as the major outer membrane protein (MOMP) of *C. trachomatis* or peptide vaccine[6] have achieved only partial success. Based on these observations, recent efforts have focused on developing a DNA vaccine. For example, Zhang et al.[7,8] demonstrated that intramuscular immunization of mice with the MOMP DNA vaccine elicited an immune response including the Th1 response and cytikine production. Brunham et al.[9] and Donati et al[10] also showed similar results. Although immunization of mice with *C. trachomatis* MOMP DNA vaccine has had limited positive results, unfortunately, only partial protection was achieved which could not completely protect against any subsequent genital challenge. Thus, the selection of a suitable candidate capable of inducing the required immune effectors and the development of an efficacious chlamydial vaccine remains a high research priority. The plasmid encoded protein pORF5 of *C. trachomatis* is a secreted protein[11]. Li et al.[12] demonstrated that pORF5 was the most immunodominant antigen and was as dominant as CPAF. The immunologic and biochemical characterizations of pORF5 support its further evaluation as a potential vaccine candidate.

The role of humoral immunity in chlamydial genital infection has evolved after having been the focus of immune protection and vaccine development. The data presented in this study demonstrated that mice vaccinated with pcDNA3.1-pORF5 displayed high titers of serum anti-pORF5 total antibody, IgG1, IgG2a, IgG2b, specifically increasing the levels of anti-pORF5 IgG2a. The production of IgG2a is highly dependent on the IL-2, IFN-γ secreted by Th1 cells. However, the production of IgG1 is highly dependent on the IL-4, IL-5 secreted by Th2 cells, Taken together, pcDNA3.1-pORF5 vaccine could induce a strong humoral immune response and superior Th1 immune response. Intranasal pcDNA3.1-pORF5 vaccination also induced a robust humoral response in mucosal compartments. Vaginal fluids from the mice immunized with pcDNA3.1-pORF5 displayed high titers of total antibody and local IgA. The local antibody IgA has been shown to enhance resistance and to reduce the infection intensity[13]. Therefore, mucosal immunity plays an important role in protective immunity against chlamydial infection.

In this regard, T-cell proliferative responses from the mice vaccinated with pcDNA3.1-pORF5 were increased relative to those mice vaccinated with pcDNA3.1 or PBS. Because immunity against *C. trachomatis* is based primarily on the contribution of Th1 cells[14–18], $CD4^+$ Th1 cells and $CD4^+$ Th1-generated cytokines such as IFN-γ, IL-12 and TNF-α play critical roles in affording protective immunity against genital tract chlamydial infections[19–22]. Work using knockout mice also indicates that IFN-γ, IL-12, and TNF-α make a substantial contribution to resolving a chlamydial genital infection[19,23,24]. IFN-γ is an essential component of defenses against *Chlamydia* and is required for the full resolution of genital infection. The effect of IFN-γ on *Chlamydia*-infected cells *in vitro* has been demonstrated to occur involving indoleamine dioxygenase, which is possibly associated with chlamydial clearance[25]. Our data shows that splenocytes from pcDNA3.1-pORF5- immunized mice exhibited increased IFN-γ production along with concurrent minimal IL-4 production following *in vitro* pORF5 stimulation, indicating that the Th1 immune response was strongly induced in pcDNA3.1-pORF5-immunized mice.

C. trachomatis does not directly damage tissues, but induces an injurious host inflammatory response at the infected site. However, persistent inflammation may develop into pelvic inflammatory disease and complications such as ectopic pregnancy and infertility may result[26]. Resolution of infection is an important component of an effective anti-chlamydial vaccine. In our study, the incidence of hydrosalpinx of oviducts after intravaginal *C. muridarum* challenge was significantly reduced in pcDNA3.1-pORF5-vaccinated mice compared to pcDNA3.1 or PBS-immunized mice. The immunized and challenged mice had a significant decrease in the severity and length of vaginal shedding. Our data provided support for using pORF5 as a candidate vaccine against chlamydial infection.

In conclusion, our results suggest that immunization with pcDNA3.1-pORF5 may induce a protective response in mice against a genital challenge with *C. muridarum*. The establishment of this model would be of benefit in determining the vaccination protocols, vaccination candidates, and for understanding the mechanisms against chlamydial infections. In chlamydial infection, cell-mediated immunity is implicated as the major mechanism of clearance for *Chlamydia*, with antibodies being of lesser importance[27,28]. Therefore, an ideal vaccine to protect against *C. trachomatis* genital tract infection should induce both a strong Th1 response and antibody responses. Further investigation is required to extend these observations into immunization protocols, combined vaccines or adjuvant selection.

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