Immunobiology 224 (2019) 223–230

Contents lists available at ScienceDirect

Immunobiology

journal homepage: www.elsevier.com/locate/imbio

Vaccination with MIP or Pgp3 induces cross-serovar protection against chlamydial genital tract infection in mice

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ARTICLE INFO

Keywords:
Chlamydia trachomatis
MIP
Pgp3
Vaccination
Cross-serovar protection

ABSTRACT

Previously we reported that recombinant *Chlamydia muridarum* macrophage infectivity potentiator (MIP) provided partial protection against *C. muridarum* genital tract infection in mice. On the other hand, *Chlamydia trachomatis* plasmid encoded Pgp3could induce the protection against *C. muridarum* air way infection. This study aimed to evaluate the immunogenicity of MIP and Pgp3 from *C. trachomatis* serovar D and further investigate whether MIP and Pgp3 provide cross-serovar protection against *C. muridarum* genital tract infection in mice. Our results showed that vaccination by any regimen, including MIP alone, Pgp3 alone or MIP plus Pgp3, induced specific serum antibody production and Th1-dominant cellular responses in mice. Live chlamydial shedding from the vaginal and inflammatory pathologies in the oviduct markedly reduced. However, MIP + Pgp3 vaccination did not provide better protection than the single immunization. In conclusion, this study demonstrated that both MIP and Pgp3 can induce cross-serovar protective against chlamydial genital tract infection, and provided the guide for the development of optimal multisubunit vaccines against *C. trachomatis* infection.

1. Introduction

Chlamydia trachomatis is an obligate intracellular parasite consisted of multiple serovars. Different serovars may have different tissue tropism and cause different diseases. Serovars D-K mainly invade human reproductive tract, causing bacterial sexually transmitted diseases (Starnbach and Roan, 2008). The infection of chlamydia in the genital tract is often insidious and the clinical symptoms are not typical, eventually leading to serious complications such as pelvic inflammation and infertility (Budrys et al., 2012). *Chlamydia* urogenital infection may also increase the risk of secondary infection by other pathogens (Sperling et al., 2013). Therefore, it is important to develop vaccination for effective prevention and control of chlamydial disease.

However, early vaccines based on inactivated whole organisms in humans and non-human primates may aggravate subsequent *C. trachomatis* infections, and the approach was abandoned (Grayston et al., 1964, 1962). Instead, searching potential chlamydial antigens to develop subunit vaccine has been the trend. So far, many chlamydial antigens such as polymorphic membrane proteins (Pmps), major outer membrane protein (MOMP), macrophage infectivity potentiator (MIP) and chlamydia protease-like activity factor (CPAF) have been identified (Sun et al., 2009; Paes et al., 2016; Lu et al., 2013; Murthy et al., 2011). Unfortunately, none of the immunization based on these antigens could provide full protection against chlamydia genital tract infection.

All *C. trachomatis* serovars carry a 7.5 kb plasmid implicated in the pathogenicity of chlamydia, because the pathogenic ability of plasmid-free *C. trachomatis* is attenuated (Sigar et al., 2014; Kari et al., 2011). Pgp3 is an immunodominant antigen encoded by chlamydial plasmid, and it resembles receptor-binding domain of tumor necrosis factor (Galaleldeen et al., 2013). Human antibody recognized Pgp3 in a conformation dependent manner (Wang et al., 2010; Chen et al., 2010). in vitro, Pgp3 antigen is secreted into the cytoplasm of infected host cells and the secretion is conserved among all *C. trachomatis* serovars (Li et al., 2008a). Furthermore, vaccination with pgp3 DNA has been shown to provide the protection against chlamydial genital tract infection. In addition, *C. muridarum*-derived recombinant Pgp3 protein has been demonstrated to induce protection against *C. muridarum* air way infection (Donati et al., 2003; Li et al., 2008b; Mosolygo et al.,

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https://doi.org/10.1016/j.imbio.2018.11.009
Received 4 September 2018; Received in revised form 15 November 2018; Accepted 19 November 2018
Available online 05 December 2018
0171-2985/

2014). However, whether Pgp3 protein vaccination could provide cross-serovar protection on chlamydial genital tract infection has not been explored.

Our previous study reported that vaccination with *C. muridarum*-derived recombinant MIP partially protected mice from genital tract infection (Lu et al., 2013). This study aimed to evaluate the immunogenicity of MIP and Pgp3 from *C. trachomatis* serovar D and further investigate whether MIP and Pgp3 provide cross-serovar protection against *C. muridarum* genital tract infection in mice. We administrated MIP and Pgp3 derived from *C. trachomatis* serovar D to female BALB/c mice individually or in combination. We found that either MIP or Pgp3 vaccination induced strong Th1 response, accelerated lower genital tract chlamydial clearance and decreased the severity of upper genital tract tissue damage. However, the level of cross-serovar protection conferred by MIP and Pgp3 combination vaccination was no better than that conferred by MIP or Pgp3 vaccination alone.

2. Materials and methods

2.1. Chlamydia organisms

Both *C. trachomatis* serovar D and *C. muridarum* strains were grown on confluent HeLa 229 cells (ATCC, CCL-2.1). Cells were lysed by sonication, and Renograffin gradients centrifugation was used to purify chlamydial elementary bodies (EBs) as described previously (Cheng et al., 2008). Purified EBs were suspended in sucrose-phosphate-glutamine (SPG) buffer and stored at −80 °C.

2.2. Protein expression and purification

The two ORFs encoding *C. trachomatis* serovar D antigens MIP and Pgp3 (http://stdgen.northwestern.edu/) were amplified by PCR and cloned into pGEX6p. Then the recombinant plasmids pGEX6p/mip and pGEX6p/pgp3, as well as empty plasmid pGEX6p were transformed into *E. coli* XL-1 blue. GST fusion proteins or GST alone were expressed and purified with glutathione-conjugated agarose beads. GST tag was cleaved by using precision protease (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA) to get GST free MIP and Pgp3. The endotoxins were removed with polymyxin B cartridge (Sigma, St. Louis, MO, USA), and the concentrations of recombinant proteins rMIP or rPgp3 were titrated by bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA).

2.3. Mice and intranasal immunization

Female 4-week old BALB/c mice were purchased from Hunan SJA Laboratory Animal Co. Ltd, and divided into 5 groups (n = 11). Three groups of animals were immunized intranasally with 30 μg/dose of MIP, Pgp3, or MIP plus Pgp3 in 25 μl PBS containing 10 μg/dose CpG adjuvant. The other two groups of animals served as controls to receive 30 μg/dose of *Chlamydia* unrelated antigen GST plus 10 μg/dose CpG, or PBS (mock) alone. CpG adjuvant was purchased from Integrated DNA Technologies (Coralville, IA, USA) with the sequence of 5′-TCC·ATG·ACG·TTC·CTG·ACG·TT-3′. Each group of mice were administered 3 times on days 0, 20 and 30 with same dose.

2.4. Detection of specific IgG and IgG subclasses levels

Antigen specific antibody responses were detected by ELISA using methods described previously (Lu et al., 2013, 2012). Briefly, 3 mice/group were sacrificed and sera were separated at twenty days after final booster administration. 96-well microplates (Nunc, Roskilde, Denmark) were coated with either 1 μg/well of rMIP or rPgp3 in 100 μL PBS at 4 °C overnight, washed with PBS and blocked with 3% nonfat milk. Then serially diluted mice serum samples were added and incubated for 1.5 h at room temperature, and antigen specific IgG and IgG subclasses were detected with goat anti-mouse total IgG (H + L), IgG1 or IgG2a conjugated with HRP and ABTS (Sigma) as the substrate. The absorbance (OD) at 405 nm was read in a microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA).

2.5. Detection of antigen-specific cytokine production

Antigen-specific cytokine production were detected by ELISA using methods described previously (Lu et al., 2013, 2012). Briefly, 3 mice/group were sacrificed and spleens were collected at twenty days after final booster administration. Single-cell suspensions were cultured in 24-well plates (1×10^6 cells/well) and stimulated with UV-EBs, GST protein or medium. Supernatants were harvested 3 days after incubation and secreted IFN-γ and IL-5 were assayed by standard cytokine ELISA kits (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instruction.

2.6. Genital tract C. muridarum infection and monitor of live organisms shedding

Thirty days after final booster administration, each mouse was infected intravaginally with 2×10^4 IFUs of *C. muridarum* EBs. 2.5 mg Depo-provera were injected subcutaneously to each mouse at 5 days before infection to synchronize menstrual cycle. Vaginal live organisms shedding was monitored on the indicated days following infection, by plating the vaginal swab samples on HeLa cell monolayers cultured on coverslips in 24-well plates (Lu et al., 2013, 2012). After incubation at 37°C for 28 h, inclusion bodies were visualized by using rabbit anti-*C. muridarum* EB antiserum as primary antibody, and Cy2 conjugated goat anti-rabbit Ig (Jackson ImmunoResearch Laboratories) as second antibody. Five randomly selected fields from each coverslip were observed under fluorescence microscope, and total recovered IFUs number of each swabbed sample was converted into log10.

2.7. Histopathology

Animals were euthanized and genital tracts were removed on day 60 after infection. Both gross hydrosalpinx and histopathology in oviduct were evaluated as described previously (Lu et al., 2012; Peng et al., 2011). The presence of oviduct hydrosalpinx was scored for each mouse base on following criteria: No hydrosalpinx = 0; Hydrosalpinx is there but not obvious = 1; The diameter of hydrosalpinx less than ovary = 2; The diameter of hydrosalpinx similar to ovary = 3; The diameter of hydrosalpinx larger than ovary = 4. Then the genital tract tissues were embedded in paraffin and sectioned for H&E staining (Peng et al., 2011). Oviduct histopathology was scored for both dilation and inflammation. Dilation was scored as: no significant dilatation = 0; one single cross section mild dilation = 1; two to three cross sections dilation = 2; more than three cross sections dilation = 3; confluent pronounced dilation = 4. Inflammation was scored as: no significant inflammatory cell infiltration = 0; infiltration at a single focus = 1; infiltration at two to four foci = 2; infiltration at more than four foci = 3; confluent infiltration = 4.

2.8. Statistical analysis

Comparisons on antigen specific antibody production, cytokines level as well as chlamydial shedding in multiple groups were analyzed using one-way ANOVA method followed by Tukey's post hoc test. The incidences of histopathology were analyzed using Fisher's Exact test because of small sample size. All data were analyzed using SPSS version 12.0 software (SPSS Inc., Chicago, IL, USA) and p value < 0.05 was considered statistically significant.

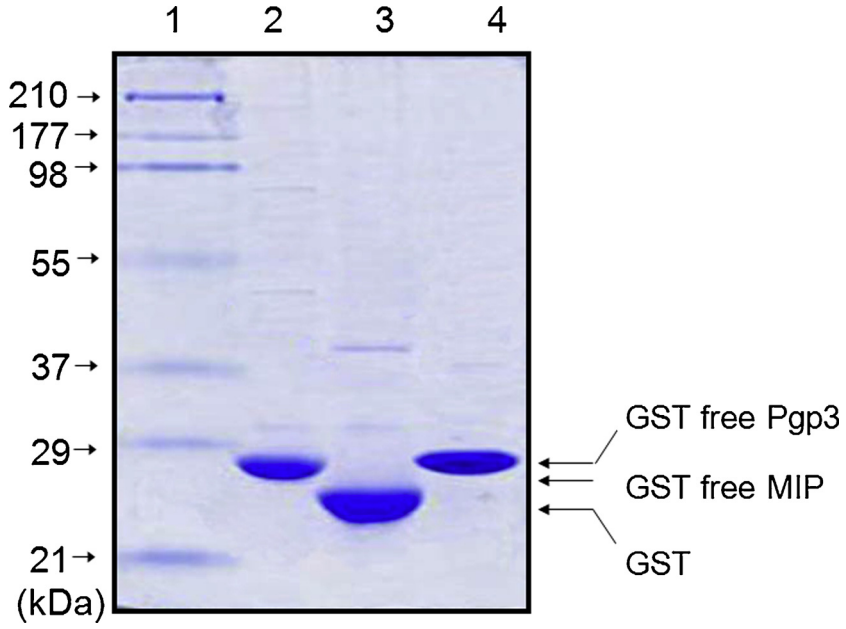

Fig. 1. Molecular weight of purified recombinant MIP, Pgp3 and GST. Lane 1, molecular mass standards; lane 2, MIP (27 kDa); lane 3, GST (26 kDa); lane 4, Pgp3 (28 kDa).

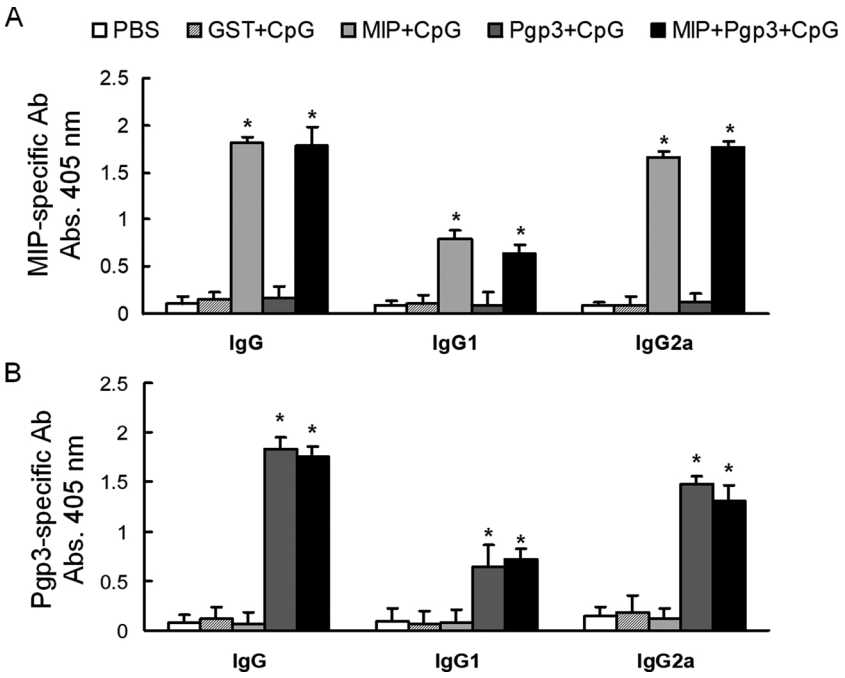

Fig. 2. MIP- and Pgp3- intranasal immunization induced robust humoral immune response. Five groups of BALB/c mice were intranasally immunized with MIP + CpG, Pgp3 + CpG, MIP + Pgp3 + CpG, PBS alone (mock control), or GST + CpG (unrelated antigen control). 3 mice/group were sacrificed and sera were separated at twenty days after final booster administration for specific antibody detection. (A) anti-MIP antibody level; (B) anti-Pgp3 antibody levels. MIP, Pgp3, or MIP + Pgp3 immunization preferentially induced more IgG2a than IgG1, indicating Th1-dominant response. *$p < 0.05$ vs. PBS analyzed by ANOVA.

3. Results

3.1. Purification of recombinant proteins by glutathione-conjugated agarose beads

GST-MIP, GST-Pgp3 and GST were expressed in prokaryotic system and purified by glutathione-conjugated agarose beads. Then precision protease was used to cleave GST fusion protein to get GST free rMIP (27 kDa) and rPgp3 (28 kDa). Reduced glutathione was used to elute GST (26 kDa). All three purified proteins were examined by SDS-PAGE and appeared at corresponding molecular weight position (Fig. 1).

3.2. Antigen specific humoral immune response by vaccination

Five groups of mice were immunized intranasally with Pgp3, MIP, MIP + Pgp3, GST (unrelated antigen control), or PBS (mock control) on days 0, 20 and 30, respectively. 20 days after the final booster immunization, three mice from each group were sacrificed and the antisera were used to titrate antigen-specific antibody level of IgG and IgG subclass (Fig. 2). Either MIP or Pgp3 immunization, but not GST, elicited significant ($p < 0.05$) antigen-specific IgG production compared to PBS control group. MIP + Pgp3 immunization induced both MIP- and Pgp3- specific IgG ($p < 0.05$) but the titer had no significant difference compared to MIP or Pgp3 alone. As for IgG subclass, the three means of immunization (MIP, Pgp3, or MIP + Pgp3) all induced more IgG2a than IgG1 ($p < 0.05$), indicating Th1-dominant response.

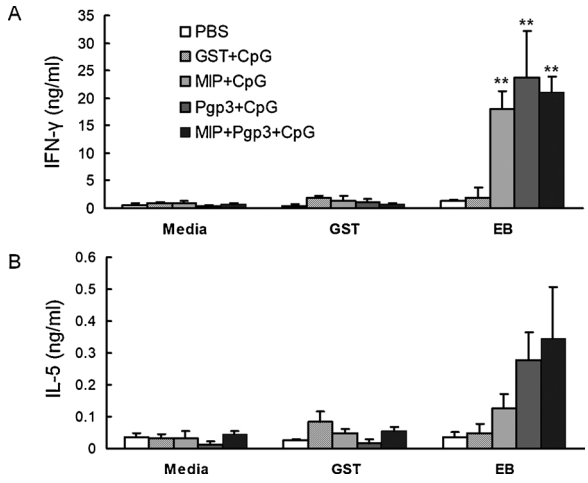

Fig. 3. MIP- and Pgp3- intranasal immunization induced robust cell-mediated immune response. The spleens collected from each group (3 mice per group) were used to prepare splenocytes suspension and stimulated with medium, GST, or purified *C. muridarum* EB. Then the supernatants were analyzed by ELISA for IFN-γ (A) and IL-5 (B) production. *p < 0.05 vs. PBS analyzed by ANOVA.

Interestingly, MIP + Pgp3 vaccination did not show higher ratio of IgG2a/IgG1 compared to MIP or Pgp3 vaccination alone.

3.3. Cellular response in the immunized mice

The splenocytes were re-stimulated in *vitro* with medium, GST, or purified *C. muridarum* EB (10^7 IFU/well). The levels of IFN-γ in MIP-, Pgp3-, or MIP + Pgp3-immunized mice were significantly higher compared to PBS- or GST-immunized group ($p < 0.01$), indicating Th1 biased cellular response. However, MIP + Pgp3 vaccination did not cause more IFN-γ production compared to individual vaccination (Fig. 3A). IL-5 production in various experimental groups was minimal even upon UV-EBs stimulation. Although IL-5 production increased in MIP + Pgp3 vaccination group compared to individual vaccination, the increase was not statistically significant (Fig. 3B). Taken together, these results suggest that MIP, Pgp3, or MIP + Pgp3 immunization elicits Th1 biased response.

Table 1
Percentage of mice shedding chlamydia.

Immunization	% mice shedding chlamydia from the vagina						
(n = 8/group)	Days after i.vag. challenge						
	D4	D8	D12	D16	D20	D24	D28
PBS	100	100	100	100	100	63	13
GST + CpG	100	100	100	100	100	63	13
MIP + CpG*	100	100	100	75	38	13	0
Pgp3 + CpG*	100	100	88	63	25	0	0
MIP + Pgp3 + CpG*	100	100	100	63	25	13	0

* $p < 0.05$ vs. PBS analyzed by ANOVA.

3.4. Live organism shedding after intravaginal C. muridarum challenge

To test the efficacy of Th1 biased response on the clearance of *C. muridarum* challenge, we monitored the vaginal chlamydia shedding at 4-days intervals after infection. Both MIP- and Pgp3-immunized group exhibited significant reduction in the shedding of chlamydia organism on day 16 and day 20 (Fig. 4). While animals immunized with MIP + Pgp3 had reduced chlamydia vaginal shedding as early as day 12 ($p < 0.05$), there was no greater reduction on day 16 and day 20. In Pgp3 immunized group, 12% of the mice resolved infection on day 12, but none of the mice resolved infection in the other four groups. On day 20, the percentage of mice shedding chlamydia was 38% in MIP group, 25% in Pgp3 group and 25% in MIP + Pgp3 group, compared to 100% in PBS group and GST control groups (Table 1). These results demonstrated that MIP, Pgp3, or MIP + Pgp3 vaccination accelerated chlamydial clearance in lower genital tract.

3.5. Gross hydrosalpinx and histopathology in upper genital tract

Sixty days after chlamydial intravaginal challenge, the intact urogenital tract (UGT) tissues were isolated. The representative images of gross pathology in each group were shown in Fig. 5A, and we found no

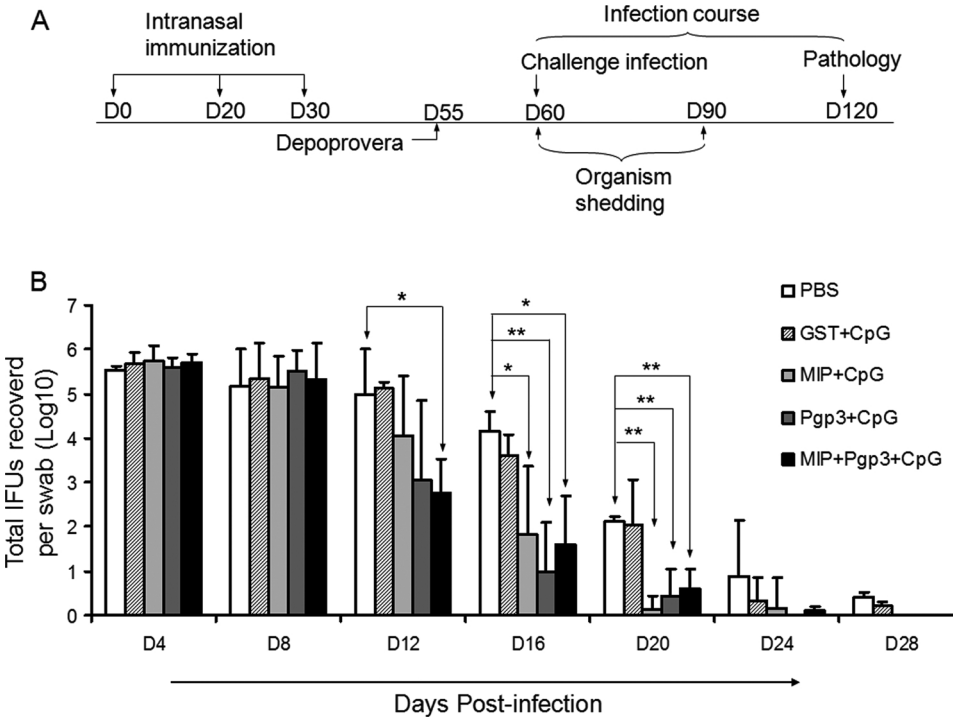

Fig. 4. MIP- and Pgp3- vaccine reduced chlamydial burden. (A) Experiment process. On day 0, 20 & 30, mice of each group were immunized. On day 60, mice were challenged i.v. with *C. muridarum* and vaginal chlamydia shedding was monitored at 4-days intervals. (B) The mean recoverable IFUs of each group were shown. ** $p < 0.01$, * $p < 0.05$, analyzed by ANOVA.

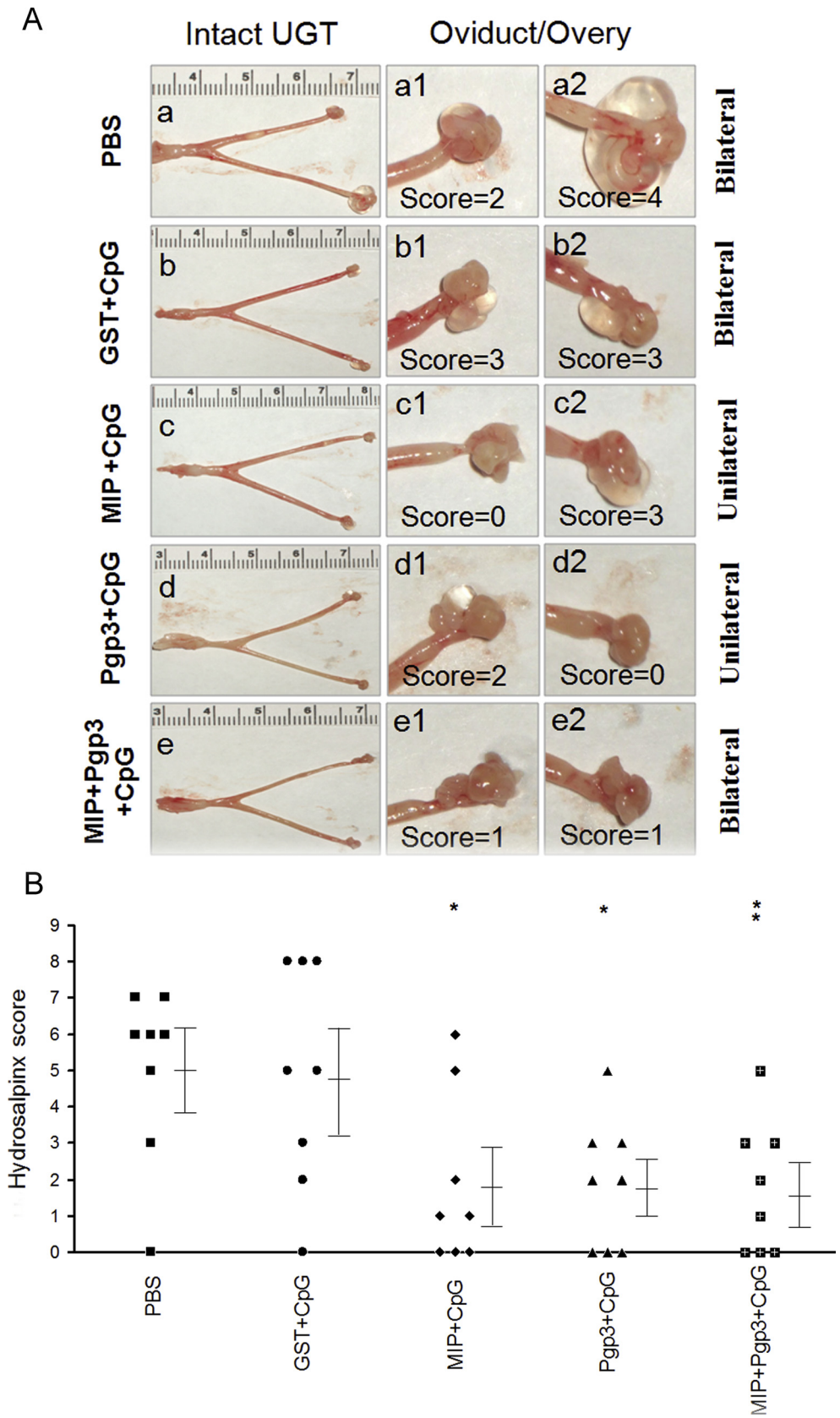

Fig. 5. MIP- and Pgp3- vaccine reduced *C. muridarum*-induced urogenital tract gross pathology. (**A**) Animals (eight mice/group) were sacrificed at 60 days post infection. Genital tracts were removed and evaluated under naked eyes for gross appearance. (**B**) The hydrosalpinx score for each mouse and the mean scores of each group were shown. ** $p < 0.01$, * $p < 0.05$, vs. PBS analyzed by ANOVA.

Table 2
Percentage of mice developing hydrosalpinx.

Immunization (n = 8/group)	% mice with hydrosalpinx	Bilateral	Unilateral
PBS	88	75	13
GST + CpG	88	63	25
MIP + CpG	63	25*	38
Pgp3 + CpG	63	13*	50
MIP + Pgp3 + CpG	63	25*	38

* $p < 0.05$ vs. PBS analyzed by ANOVA.

obvious dilation in the uterine horn of vaccinated mice. The severity and the bilateral incidence of hydrosalpinx in MIP, Pgp3, and MIP + Pgp3 vaccinated groups were significantly lower than control groups (Fig. 5B and Table 2). The representative images of oviduct histopathology in each group were shown in Fig. 6A. Both oviduct dilation and inflammation were significantly reduced in MIP, Pgp3, and MIP + Pgp3 vaccinated groups ($p < 0.05$, Fig. 6B). These results demonstrated that MIP, Pgp3, or MIP + Pgp3 vaccination protected the mice from chlamydial induced upper genital tract pathology.

4. Discussion

Globally, there are about 131 million new cases of chlamydia trachomatis infection each year, and genital infections are usually caused by multiple serotypes (Newman et al., 2015). Identification of antigens that provide cross-serovar protection is important to develop novel vaccine against *C. trachomatis* (Johnson and Brunham, 2016). In present study, two ORFs derived from *C. trachomatis* serovar D were selected for producing recombinant chlamydia proteins. MIP is a lipoprotein expressed on the surface of chlamydial EBs (Neff et al., 2007). Pgp3 is encoded by chlamydia plasmid and is secreted to host cell cytoplasm in the process of chlamydia infection (Li et al., 2008a). Both proteins are recognized by chlamydial urogenitally infected women antisera (Wang et al., 2010). These two recombinant proteins were administrated to mice individually or in combination to investigate the cross-serum protective effect against vaginal *C. muridarum* infection.

Both MIP and Pgp3 vaccination induced strong humoral immune

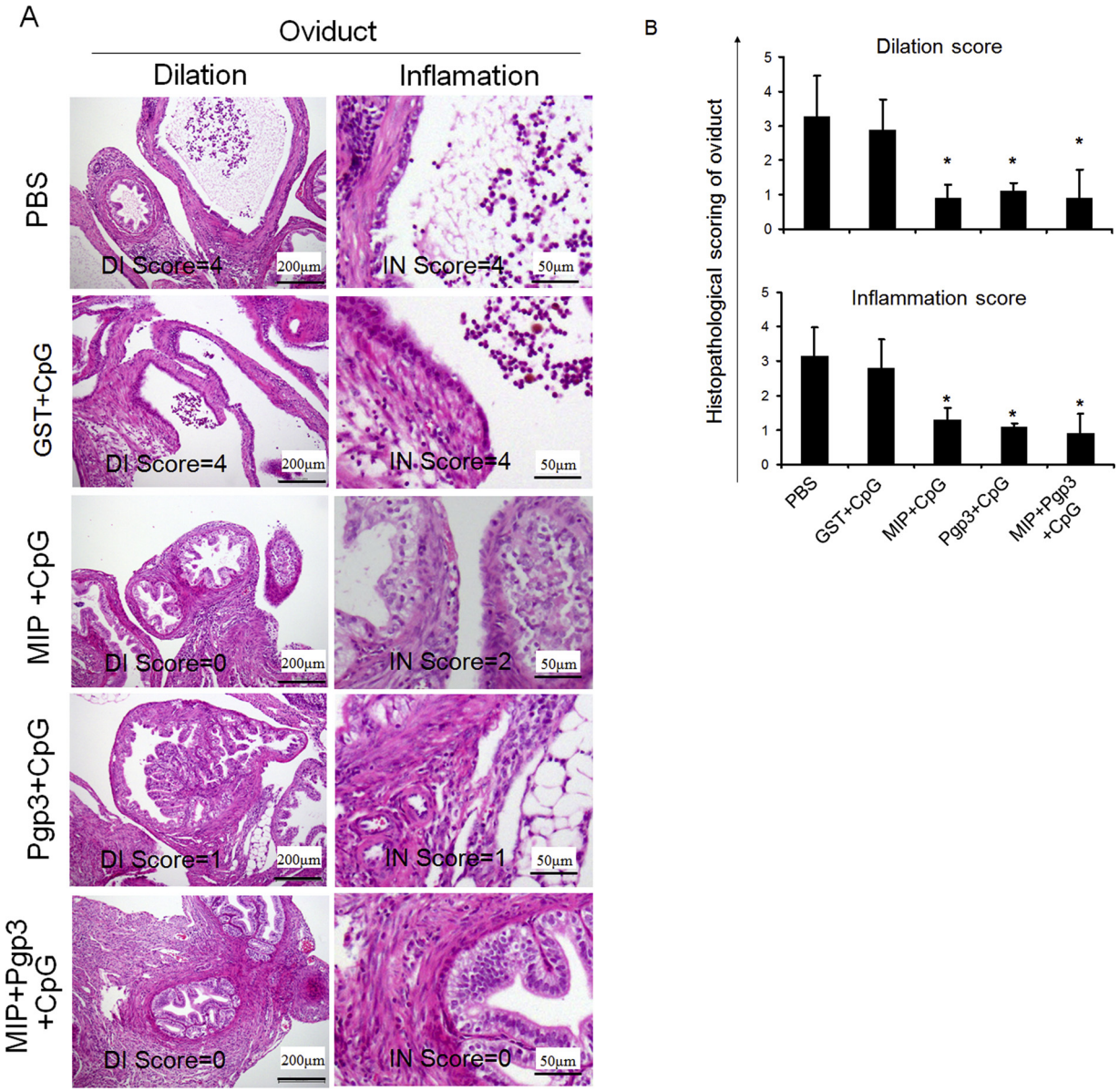

Fig. 6. MIP- and Pgp3- vaccine reduced *C. muridarum*-induced oviduct histopathology. (A) Oviduct histopathology was scored for both luminal dilatation (DI) and inflammatory infiltration (IN). (B) The mean dilation and inflammation scores of each group were shown. * $p < 0.05$, vs. PBS analyzed by ANOVA.

response and especially induced higher levels of IgG2a than IgG1. It was reported that anti-chlamydial IgG2a antibodies had effect on $FcR^{+/+}$ but not FcR-/- dendritic cells in *vitro*, indicating Th1 biased responses (Moore et al., 2003). IFN-γ production in vaccinated mice significantly elevated while IL-5 production was minimal, in accordance with the IgG subclasses pattern and Th1 response. Th1 reaction has been reported to be very important in preventing chlamydial urogenital primary infection (Johnson and Brunham, 2016; Morrison and Morrison, 2000; Steinbach et al., 2016). In this regard, both MIP and Pgp3 immunization significantly promoted the removal of chlamydial infection in the lower genital tract, and reduced the inflammatory pathology in the upper reproductive tract. In particular, the vaccination significantly decreased the incidence of bilateral oviduct hydrosalpinx, which will greatly reduce the occurrence of infertility.

MIP is a conserved chlamydial lipoprotein on the surfaces of various chlamydial species (Neff et al., 2007). Our previous study showed that mice intramuscularly immunized with *C. muridarum* derived MIP effectively resisted vaginal *C. muridarum* infection (Lu et al., 2013). In current study, we continued to evaluate the protection efficacy of chlamydial MIP protein with following changes: (i) Recombinant MIP protein was purified from *C. trachomatis* serovar D. (ii) Immunization route was intranasal but not intramuscular. (iii) CpG alone was used as adjuvant. Our results indicate that MIP is a promising vaccine candidate because it could provide cross-serovar protection and the protection efficacy is stable. Interestingly, intranasal immunization is known to induce antigen specific immune response in the genital mucosa (Murthy et al., 2007). It is worthy to examine antigen specific secretory IgA in the genital washes in our experimental system in further studies.

Mosolygó et al. reported that C57BL/6 N mice subcutaneously immunized with *C. muridarum* derived Pgp3 prevented *C. muridarum* lung infection, and the protective efficacy depended on Pgp3-specific CD4 + T cells but not antibodies (Mosolygo et al., 2014). In current study, BALB/c mice intranasally administrated with *C. trachomatis* serovar D derived rPgp3 induced cross-serotype protection against chlamydia genital infection. In fact, the cross-serovar protection was observed in other animal models of chlamydial infection. For example, in ocular model of *C. caviae* infection there was protection against infection in animals previously immunized with antigen derived from *C. trachomatis* (Inic-Kanada et al., 2015). It will be interesting to test whether immunization with Pgp3 would provide cross-serotype protection against chlamydial lung infection. Furthermore, Li et al. reported that vaccination with pgp3 DNA significantly accelerated the extinction of genital tract infection and minimized the pathologic changes of fallopian tubes (Li et al., 2008b). These results provide further support to using Pgp3 as chlamydial subunit vaccine.

Given the complex structure and infection biology of chlamydia, cocktail antigen vaccines usually offer better protection than the monocomponent subunit vaccine. Yu et al. found that after inoculation of PmpEFGH + MOMP in C57BL/6 mice, the cellular immune response and protection were stronger than those inoculated with individual protein antigen (Yu et al., 2014). Olsen et al. demonstrated that CTH1 (CT521 and CT443 fusion protein) vaccination provided better protection than single components against chlamydial intravaginal infection (Olsen et al., 2010). Li et al. showed that rCPAF inoculation accelerated the removal of *C. muridarum* and reduced the development of fallopian tube pathology. However, the protective efficacy of rCPAF + rMOMP + rIncA was comparable to that of rCPAF alone (Li et al., 2007). In this study, MIP and Pgp3 combined vaccination induced comparable protection efficacy as single vaccination. Further studies are needed to understand why combined vaccination could not achieve better protection efficacy.

In conclusion, vaccination with *C. trachomatis* serovar D derived Pgp3 and MIP provided cross-serovar protection against *C. muridarum* genital tract infection, and provided the guide to further development of optimal multisubunit vaccines against *C. trachomatis* infection.

Compliance with ethical standards

All animal procedures and treatments were approved by the Animal Welfare and Ethics Committee of the University of South China and were performed in accordance with the regulations of the institution.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This project was supported by the Natural Science Foundation of China (81471969, 81202374 & 31600150), the construct program of Hunan Provincial Key Laboratory for Special Pathogens Prevention and Control Foundation (Grant No. 2014-5), and Hunan Province Cooperative Innovation Center for Molecular Target New Drug Study (Grant No. 2015-351).

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