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Induction of protective immunity against *Chlamydia muridarum* intravaginal infection with the chlamydial immunodominant antigen macrophage infectivity potentiator

Chunxue Lu^a, Bo Peng^b, Zhihong Li^c, Lei Lei^d, Zhongyu Li^a, Lili Chen^a, Qingzhi He^a, Guangming Zhong^{a,d,**}, and Yimou Wu^{a,*}

^a Department of Microbiology and Immunology, University of South China, 28 West Changsheng Rd., Hengyang, Hunan 421001, China

^b Cancer Research Institute, University of South China, 28 West Changsheng Rd., Hengyang, Hunan 421001, China

^c Department of Surgery, Second Xiangya Hospital, Central South University, Changsha, Hunan 410000, China

^d Department of Microbiology and Immunology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78229, USA

Abstract

We previously reported that 5 *Chlamydia muridarum* antigens reacted with antisera from >90% mice urogenitally infected with *C. muridarum* and they are TC0660 (ABC transporter or ArtJ), TC0727 (outer membrane complex protein B or OmcB), TC0828 (macrophage infectivity potentiator or MIP), TC0726 (inclusion membrane protein or Inc) & TC0268 (hypothetical protein or HP). The orthologs of these antigens in *Chlamydia trachomatis* were also highly reactive with antisera from women urogenitally infected with *C. trachomatis*. In the current study, we evaluated these *C. muridarum* antigens for their ability to induce protection against a *C. muridarum* intravaginal challenge infection in mice. We found that only MIP induced the most pronounced protection against *C. muridarum* infection. The protection correlated well with robust *C. muridarum* MIP-specific antibody and Th1-dominant T cell responses. The MIP-immunized mice displayed significantly reduced live organism shedding from the lower genital tract and highly attenuated inflammatory pathologies in the upper genital tissues. These results demonstrate that MIP, an immunodominant antigen identified by both human and mouse antisera, may be considered a component of a multi-subunit chlamydial vaccine for inducing protective immunity.

Keywords

Chlamydia muridarum; Protective immunity; Macrophage infectivity potentiator; Vaccine

1. Introduction

Chlamydia trachomatis is the most common sexually transmitted bacterial pathogen and imposes serious health problems in humans. A major clinical challenge of *C. trachomatis* infection is that most acutely infected individuals do not seek treatment due to lack of obvious symptoms, thus potentially developing severe complications, such as pelvic inflammatory disease, ectopic pregnancy, and infertility [1–3]. Additionally, short-lasting natural immunity against chlamydial infections and non-treatment of affected sexual partners result in high reinfection rates [2]. Obviously, a most effective means to prevent *C. trachomatis*-induced complications is vaccination. However, there is still no licensed *C. trachomatis* vaccine [4].

To this end, multiple vaccination strategies for protection against genital *C. trachomatis* infection have been evaluated. The failed human trachoma trials more than 50 years ago [5,6] and the immunological studies in the past half-century [4,7–9] suggested that a subunit vaccine strategy is both necessary and feasible. So far, the major outer membrane protein (MOMP) in its native conformation appears to be the most effective antigen [10]. However, recombinant MOMP, MOMP synthetic peptides, DNA vaccines encoding MOMP, and the passive transfer of MOMP-specific monoclonal antibodies were only partially effective in protecting experimental animals from subsequent challenge [11–17]. Furthermore, immunization with chlamydial protease-like activity factor (CPAF) [18–20], translocated actin-recruiting phosphoprotein or Tarp [21], and other structural or secreted proteins all showed only partial protection against genital *C. trachomatis* challenge [22–26]. Given the complex chlamydial organism structures and chlamydial infection biology, it is likely that immunization with combinations of various antigens with each able to induce unique effector mechanisms to simultaneously attack multiple stages of the infection processes may be necessary to achieve a full protection against chlamydial infections and diseases. Therefore, more novel vaccine candidates are in urgent need.

Many previous studies on chlamydial pathogenic mechanisms and evaluating vaccine candidates have been carried out using mouse models intravaginally infected with *Chlamydia muridarum* (also called MoPn). Mice infected with *C. muridarum* can develop upper genital tract pathology that closely resembles the pathology in human fallopian tube following *C. trachomatis* infection [27,28]. In our previous studies with *C. trachomatis* whole genome array, we identified 38 ORFs dominantly recognized by >40% urogenital infected human antisera [29]. When their orthologs in *C. muridarum* were further profiled with antisera from mice intravaginally infected with *C. muridarum*, five antigens (ArtJ, OmcB, MIP, Inc & HP) were dominantly recognized by >90% of mice regardless of their strain background and H-2 haplotypes [30]. Thus, these five antigens were highly immunogenic in both human and mice, suggesting that chlamydial infection in both human and mice requires the expression of these antigens and these antigens may be important in chlamydial pathogenesis.

In the current study, we examined the protection efficacy of intramuscular (i.m.) immunization with these five antigens against genital *C. muridarum* challenge. We found that intramuscular vaccination with MIP plus CpG-IFA induced the most pronounced

protective immunity that partially protected mice from chlamydial live organism infection and infection-induced upper genital tract pathology. This protection correlates with a robust MIP- and MoPn-specific antibody and Th1-dominant T cell responses. Taken together, these results have demonstrated that an immunodominant chlamydial protein, MIP, can be considered a component of a multi-subunit vaccine against chlamydial infection and diseases.

2. Materials and methods

2.1. Chlamydial organisms

C. muridarum Nigg strain (also called MoPn) stocks were prepared as described previously [31,32]. Confluent mono-layers of HeLa cells were grown in Dulbecco's modification of Eagle's medium with 10% FBS and infected with *C. muridarum*. Cells were lysed using a sonicator and elementary bodies (EBs) were purified on Renograffin gradients as described previously. Stocks were stored at -80°C in sucrose–ephosphate–glutamine (SPG) buffer and diluted appropriately for the challenge.

2.2. Prokaryotic expression of chlamydial fusion proteins and protein purification

Five *C. muridarum* antigens, including ArtJ, OmcB, MIP, Inc & HP, were used for the current study (<http://www.ncbi.nlm.nih.gov/Sites/entrez>). These antigens are dominantly recognized by >90% of mice antisera regardless of their strain background and H-2 haplotypes and their counterparts in *C. trachomatis* were significantly recognized by *C. trachomatis*-infected women [29,30]. The 5 ORFs were cloned into pGEX vectors and expressed as fusion proteins with glutathione-S-transferase (GST) fused to the N-terminus. Expression of the fusion proteins was induced with isopropyl-beta-D-thiogalactoside (IPTG; Invitrogen, Carlsbad, CA) and the fusion proteins were extracted by lysing the bacteria via sonication in a Triton-X100 lysis buffer (1% Triton-X100, 1 mM PMSF, 75 units/ml of aprotinin, 20 mM leupeptin and 1.6 mM pepstatin). After a high-speed centrifugation to remove debris, the fusion protein-containing supernatants were further purified using glutathione-conjugated agarose beads (Pharmacia). Then the beads were used to purify soluble proteins by cleavage with a precision protease (Pharmacia). The freed chlamydial proteins were concentrated via centricon (Milli-pore, Billerica, MA) and used to immunize mice for evaluating protective immunity as described previously [21,32].

2.3. Mice

For the initial screening experiment, female Balb/c mice were purchased at the age of 3–4 weeks old from Charles River Laboratories, Inc. (Wilmington, MA) and divided into 7 different groups with 5 in each group. The MIP immunization experiment was repeated once with 15 mice per group. Mice were housed at the University of Texas Health Science Center at San Antonio and were given food and water ad libitum. Animal care and experimental procedures were approved by the University of Texas Health Science Center at San Antonio Animal Use Protocol committee.

2.4. Immunization procedure

All mice were immunized i.m. for a total of 3 times on day 0, day 20 and day 30 respectively. A total of 30 mg protein antigen or 10^6 IFUs of *C. muridarum* (or MoPn) EBs plus 10 µg of CpG in 50 µl PBS emulsified in 50 µl of IFA (incomplete Freund's Adjuvant, Sigma–Aldrich, St. Luis, MO) was given to each mouse each time. The first group was given GST protein as negative and the second group with EBs as positive control. The remaining 5 groups were each immunized with one of the 5 test antigens. The CpG with a sequence of 59-TCC.ATG.ACG.TTC.CTG.ACG.TT-39 (all nucleotides are phosphorothioate-modified at the 3' internucleotide linkage) was used (Integrated DNA Technologies, IDT, Coralville, IA). The CpG-IFA was used as adjuvant because it had been previously shown to induce Th1 dominant immune responses [21,31,32].

2.5. Vaginal *C. muridarum* challenge and determination of bacterial shedding

Mice were rested for one month after the final immunization and challenged intravaginally (i.vag.) with 2×10^4 inclusion forming units (IFUs) of *C. muridarum*, as described previously [31]. Subcutaneous (s.c.) depo-progesterone injections were given at 5 days prior to challenge to synchronize the estrous cycles of the mice. To monitor live organism shedding, vaginal swabs were collected every 3–7 days for a period of about 30 days after intravaginal infection. Each swab was titrated on HeLa cell monolayers in duplicates as described previously [30,33]. Briefly, serially diluted swab samples were inoculated onto HeLa cell monolayers grown on coverslips in 24 well plates. After incubation for 24 h, the cultures were fixed with 2% paraformaldehyde and stained with a rabbit anti-chlamydial organism pAb plus a goat anti-rabbit IgG conjugated with Cy2 (green; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Inclusions were counted in five random fields per coverslip. The total number of IFUs per swab was calculated based on the number of IFUs per field, number of fields per coverslip, dilution factors and inoculation and total sample volumes. The calculated total number of IFUs/swab was converted into log10 and the log10 IFUs were used to calculate means and standard deviation for each group at each time point.

2.6. Assessment of pathology in the upper genital tract

Mice were sacrificed 60 days after infection and the mouse urogenital tract tissues were isolated for gross pathology evaluation as described previously [21,31]. Both incidence and severity of hydrosalpinx were recorded for each mouse and compared between different groups. The severity of hydro-salpinx was scored based on the following criteria: No hydrosalpinx is assigned a score of zero (0); Hydrosalpinx is only visible after amplification (1); Hydrosalpinx is clearly visible with naked eye but the size is smaller than that of ovary (2); The size of hydrosalpinx is similar to that of ovary (3); If larger than ovary (4). The isolated tissues, after documenting the gross pathology with a digital camera, were fixed in 10% neutral formalin and embedded in paraffin and serially sectioned longitudinally (with 5 µm/each section). The sections were stained with hematoxylin and eosin (H&E) as described elsewhere [33]. The H&E stained sections were assessed for oviduct pathology, including both dilatation and inflammatory cell infiltrates were scored. Scoring for dilatation of oviduct: 0, no significant dilatation; 1, mild dilatation of a single cross section; 2, one to

three dilated cross sections; 3, more than three dilated cross sections; and 4, confluent pronounced dilation. Scoring for inflammatory cell infiltrates (at the chronic stage of infection, the infiltrates mainly contain mononuclear cells): 0, no significant infiltration; 1, infiltration at a single focus; 2, infiltration at two to four foci; 3, infiltration at more than four foci; and 4, confluent infiltration. Scores assigned to individual mice were calculated into means $\pm$ standard errors for each group of animals.

2.7. Detection of antibody and isotype levels

Two weeks after the last booster immunization, mice were bled and serum was separated to measure antibody levels as described previously [31,32]. ELISA microplates (Nunc, Rochester, NY) were coated with 1 μ g/well of recombinant MIP or UV-inactivated whole *C. muridarum* organisms, and incubated overnight at 4 °C. Serum was serially diluted and incubated for 2 h, followed by incubation with goat anti-mouse total Ig (H + L), IgG1 or IgG2a conjugated with HRP (Jackson ImmunoResearch Laboratories) in combination with the soluble substrate 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS; Sigma) and quantitated by reading the absorbance (OD) at 405 nm using a microplate reader (Molecular Devices Corporation, Sunnyvale, CA). To reduce background binding, all human serum samples were pre-absorbed with lysates made from XL-1blue bacteria expressing GST alone.

2.8. Antigen-specific splenocyte recall responses

Two weeks after the last booster immunization, mice (5 per group) were euthanized, spleens collected, and single cell suspensions made. Splenocytes (10^6 cells/well) were plated along with 0.1 μ g of recombinant MIP, UV-inactivated MoPn EBs, or the unrelated protein bovine serum albumin (BSA), and incubated for 72 h. Supernatants were collected and assayed for IFN- γ and IL-4 production by ELISA using standard cytokine ELISA kits [mouse IFN γ kit, (cat# DY485) & IL-4 (cat# DY404), both from (R&D Systems, Inc., Minneapolis, MN) as instructed by the manufacturer and described previously [31,33]. The levels of respective cytokines were quantified by measuring the absorbance at 405 nm using a microplate reader (Molecular Devices Corporation, Sunnyvale, CA).

2.9. Statistical analysis

ANOVA test (<http://www.physics.csbsju.edu/stats/anova.html>) was performed to analyze data from multiple groups and a two-tailed Student's t-test (Microsoft Excel) to compare the means between two groups. A Fisher's Exact test was used for comparing the incidences between two groups.

3. Result

3.1. Immunization with macrophage infectivity potentiator (MIP) reduced live organism shedding following an intravaginal infection

Our previous study has identified five *C. muridarum* immunodominant antigens (ArtJ, OmcB, MIP, Inc & HP) were recognized by >90% of mice antisera regardless of their strain background and H-2 haplotypes [30]. We screened all of them in a mouse intravaginal infection model for induction of protective immunity (Fig. 1). The initial screening

experiment included 5 mice in each group. The positive control group immunized with *C. muridarum* live organisms (MoPn EBs) completely cleared infection on day 21 ($p < 0.01$ when IFUs were compared, panel B; $p < 0.01$ when the number of mice with positive shedding was compared, panel C). More importantly, the mouse groups immunized with Inc, OmcB or MIP also displayed statistically significant reduction in shedding of live organisms on day 21 post infection. However, only MIP- and MoPn EB immunized animals can significantly reduce chlamydial shedding as early as day 14 after challenge. We then repeated the mouse protection experiment with MIP immunization, in which 10 mice were included in each group (Fig. 2). MIP immunization significantly reduced vaginal shedding of live organisms on day 15, 18 and 21 and most of the MIP-immunized mice cleared infection by day 21 post infection. Thus, MIP-induced immunity enhanced the resolution of MoPn infection from the mouse lower genital tracts although not as potently as the intact organism-induced immunity.

3.2. MIP-immunized mice displayed significantly less severe hydrosalpinx

Sixty days after infection, all immunized mice were sacrificed for evaluating inflammatory pathology in genital tract tissues (Fig. 3). Under naked eyes or a stereoscope, both uterine horn dilation and oviduct hydrosalpinx can be determined and semi-quantitatively scored [31,34,35]. The severity of hydrosalpinx largely correlates with the extent of oviduct blockage [35,36]. Due to large file size, only a representative image from each of the 7 mouse groups was displayed in Fig. 3A. The uterine horn dilation under naked eyes was not obvious in any of the Balb/c mice, which was consistent with previous observations [31,34]. Oviducts were scored for severity of hydrosalpinx based on the size of swollen oviducts relative to the size of ovary on the same side as described previously [21,31,34]. The overall incidences of hydrosalpinx were similar between all 7 groups although the EB- and MIP-immunized groups developed significantly lower rates of bilateral hydrosalpinx (Fig. 3B). When the severity of hydro-salpinx was compared, the MIP-immunized group (1.4 ± 1.5) developed significantly less severe hydrosalpinx comparing to the GST control group (5.4 ± 3.3 ; Fig. 3C). This reduced severity of hydrosalpinx in the MIP-immunized group correlated well with reduced inflammatory infiltration examined under microscope (data not shown). The MIP-induced protection was reproduced when 10 mice per group were used (Fig. 4). Thus, MIP immunization not only decreased the live organism shedding from the lower genital tract but also reduced pathologies in the upper genital tract following a challenge infection with *C. muridarum* organisms.

3.3. MIP- and MoPn-specific immune responses following MIP immunization

Groups of mice were vaccinated i.m. with MIP + CpG + IFA, MoPn EB + CpG + IFA, or with GST CpG IFA (Mock). Two weeks after final immunization, antisera were collected and used to react with recombinant MIP or purified EBs coated onto ELISA plates for titrating MIP- and MoPn-specific antibody titers (Fig. 5A). As expected, immunization with either MoPn or MIP induced high titers of antibodies that specifically recognized corresponding antigens. More interestingly, EB immunization induced high titers of MIP antigen-reactive antibodies and antibodies from MIP-immunized mice also reacted with purified EB organisms. We further isotyped the mouse IgG antibodies and found that immunization with either MIP or MoPn induced more IgG2a than IgG1 (Fig. 5B),

suggesting that both antigens provoked a Th1-dominant response, which is known to be required for clearing chlamydial urogenital infection in mice.

We evaluated chlamydial antigen-specific T cell responses in the immunized mice two weeks after final immunization. The mouse spleen cells were restimulated in vitro with MIP, purified EB, or the unrelated antigen BSA for 72 h. The supernatants were analyzed for IFN- γ and IL-4 levels using an indirect ELISA. As shown in Fig. 6, splenocytes from both MIP- and EB-immunized mice produced significant high levels of the Th1 cytokine IFN- γ upon in vitro re-stimulation with either EB or MIP antigens compared to GST immunized mice. No IL-4 was detected from the spleen cell culture supernatants of the immunized mice (data not shown). These results further strengthened the conclusion that immunization with MIP induced a Th1-dominant immune response.

4. Discussion

We have recently shown that intramuscular immunization of mice with purified chlamydial proteins can be used to identify vaccine candidate antigens for preventing *C. muridarum* infection and diseases [32]. In this study, we continue to adopt this immunization approach to screen chlamydial immunodominant antigens using the corresponding *C. muridarum* orthologs in mice and found that chlamydial MIP protein, when compared with the other four dominant antigens, developed the most pronounced protective immunity against *C. muridarum* intravaginal infection with a significant reduction in vaginal shedding of live organisms on day 14 after infection. The severity of oviduct hydrosalpinx, the infiltration of inflammatory cell and the incidence of bilateral disease were all significantly reduced in the MIP-immunized mice.

Since our long-term goal is to develop a vaccine for humans, the five *C. muridarum* antigens were selected based on the significant recognition of their counterparts in *C. trachomatis* by antibodies from women urogenitally infected with *C. trachomatis*. It is worth noting that although some *C. trachomatis* antigens are dominantly recognized by human sera, their orthologs in *C. muridarum* may not be necessarily immunogenic in mice. For example, pCT03 is one of the most dominantly recognized antigens by antisera from women diagnosed with *C. trachomatis* infection, but the *C. muridarum* ortholog can only be minimally recognized by antisera of mice intravaginally infected with *C. muridarum* [29,30]. In this case, the *C. muridarum* infection mouse model may not be able to provide useful information for human vaccine development. The five antigens tested in the current study can not only be recognized by >40% human sera but also, importantly, recognized by >90% of mice regardless of their strain background and H-2 haplotypes, suggesting that these antigens may play important roles in chlamydial pathologies and the *C. muridarum* infection mouse model can be used to provide information relevant for human vaccine development.

Among the 5 antigens tested, three are supposed to be surface-exposed proteins (ArtJ, OmcB, MIP), one is chlamydial inclusion membrane protein (Inc), and the remaining one is hypothetical protein (HP) with no known function. Some of these antigens, either alone or in combination with other chlamydial antigens and immunostimulatory adjuvants, have been

tested in various model systems and found to induce partial protection against chlamydial challenge infection [22,25,26,37–39]. However, the protection efficacy by some of these antigens against both lower genital tract infection and upper genital tract pathology has not been systematically evaluated using the *C. muridarum* urogenital infection mouse model. In the current study, we used the mouse urogenital infection model to evaluate and re-evaluate these antigens for their ability to induce protection against lower genital tract infection and upper genital tract pathology. We found that immunization with either Inc, OmcB or MIP significantly reduced vaginal live organism shedding at day 21 post infection. The OmcB-immunized group even resolved infection at day 21. However, the most pronounced protection was induced by MIP immunization. The MIP-immunized mice not only significantly decreased live organism shedding from the lower genital tract as early as day 14 post infection but also significantly reduced upper genital tract inflammatory pathology. Particularly, the reduction in bilateral oviduct hydrosalpinx incidence is medically significant since tubal factor infertility is a major concern in women infected with *Chlamydia* [40] and reducing the incidence of bilateral fallopian tube hydrosalpinx may lead to reduction in tubal infertility.

Vaccination with MIP induced high levels of anti-MIP and MoPn EB antibodies. Antibodies have been shown to play an important role in protective immunity especially against reinfection [7,41]. MIP is supposed to be exposed on the surface of elementary bodies. Anti-MIP antibodies that recognize surface-exposed epitopes of MIP may be able to block EB infection by neutralization or via Fc-receptor mediated mechanisms. The extensive immunological studies have led to the identification of a Th1-dominant host immune response as an essential element of the anti-*C. trachomatis* protective immunity [4,40]. We found that MIP immunization induced a Th1-dominant immunity, which should play a critical role in clearing infection. Although the precise molecular and cellular mechanisms of MIP-induced protective immunity need further investigations, the current study has shown that MIP can be considered a promising candidate vaccine antigen. Efforts are ongoing to combine MIP with other chlamydial antigens for inducing stronger protective immunity against chlamydial infection.

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Fig. 1.

Evaluation of 5 antigens for inducing protective immunity against *C. muridarum* intravaginal infection. (A) Experiment protocol. Groups of female Balb/c mice with 5/each were immunized intramuscularly with three doses of corresponding antigens plus adjuvant (CpG + IFA) as indicated in the figure. One month following the final immunization, mice were challenged intravaginally with 2×10^4 IFUs of *C. muridarum* organisms, then monitor lower genital tract chlamydia organism shedding and upper genital tract pathology outcome. (B) Vaginal swabs were taken weekly as indicated along the x-axis for measuring the number of live organisms (IFUs). The IFUs from each swab was converted into log₁₀, and the log₁₀ IFUs were used to calculate mean and standard deviation for each mouse group as displayed along the y-axis. The log₁₀ IFUs were compared between 7 groups of mice at each time point using ANOVA test, followed by a two-tailed Student's *t*-test for comparing between the GST control group and a test antigen group. **Indicates $p < 0.01$ while * $p < 0.05$. Although three antigen groups (Inc, OmcB & MIP) displayed significant reduction in live organism shedding on day 21 post infection, only the MIP-immunized mice exhibited

significant reductions on day 14 as compared to GST immunized group. (C) Number of mice with detectable infectious units (IFUs) from each group and at each time point was listed. The rates of IFU positivity were compared between the GST control and a test group using the Fisher's Exact test. *Indicates $p < 0.05$. All of the 5 mice immunized with OmcB cleared infection on day 21 post infection.

Fig. 2.

MIP vaccination enhances the resolution of a genital challenge. MIP along with two control groups (GST as negative and EB as positive controls) of mice (10 mice/group) were immunized and infected as described in Fig. 1 legend. At the indicated days following challenge, chlamydia shedding was measured. (A) Numbers of log₁₀ IFUs recovered from vaginal swabs at the indicated days after genital challenge. Results are expressed as means $\pm$ S.D. (B) Percentage of animals shedding chlamydia after genital challenge. Note that the MIP immunization significantly reduced the level of IFUs on day 15, 18 and 21 after infection although the MoPn organism immunization was much more robust in reducing the level of IFUs and the number of IFU positive mice. **Indicates $p < 0.01$ while * for $p < 0.05$. The quantitative IFU were analyzed with Student *t* test while number of mice with positive IFUs analyzed with chi-square test.

Fig. 3.

Effect of immunization on genital tract pathologies induced by intravaginal chlamydial infection. The 7 groups of mice described in Fig. 1 legend were sacrificed for evaluating pathologies of the mouse genital tract tissues. (A) Representative images of the gross appearance of mouse genital tracts were presented from each of the 7 groups of mice as listed on the left of the figure. The entire genital tract from vagina to ovary was displayed from left to right (left panels) and the oviduct and ovary regions were amplified from both sides (right panels). Although the uterine horn regions from all mice appeared to be normal, significant hydrosalpinx in the oviduct regions developed in different groups of mice. Both the incidence and severity of hydrosalpinx were scored under naked eyes and a numerical score was assigned for each oviduct as shown in the corresponding images. The scoring criteria were described in the method section. (B) The percent of mice with unilateral or bilateral hydrosalpinx from each group was summarized. Note that 80% of mice in GST while none in EB- and MIP-immunized groups developed bilateral hydrosalpinx. Fisher Exact test was used to compare the differences. (C) The hydrosalpinx severity scores from each group of mice (indicated along the X-axis) were displayed along the Y-axis. Note that both the EB- and MIP-immunized groups developed significantly reduced oviduct pathologies. The hydrosalpinx severity scores were compared between different groups using ANOVA followed by the two-tailed Student *t* test. *Indicates $p < 0.05$.

Fig. 4.

MIP vaccination reduces the development of oviduct pathology. The 3 groups of mice described in Fig. 2 legend were sacrificed for pathology observation. (A) Percentage of animals developing hydrosalpinx in different immunization groups was summarized. MIP immunization significantly reduced the rate of bilateral hydrosalpinx comparing to the GST group (20% vs. 70%, $p < 0.05$). Fisher Exact test was used to compare the differences. (B) The hydrosalpinx scores from each group were displayed along the Y-axis. (C) Dilation (top plot) and inflammation (lower plot) scores (derived from 5 different sections) assigned to individual mice were used to calculate the means and standard errors for each group as shown along the Y-axis. The mouse groups were indicated along the X-axis. The scores were compared between different groups using ANOVA followed by the two-tailed Student's *t*-test. Note that MIP immunization significantly reduced both the hydrosalpinx and inflammation and lumen dilatation scores in the oviduct tissues. *Indicates $p < 0.05$.

Fig. 5.

Induction of MIP- and MoPn-specific humoral immune responses with MIP immunization. Three groups of mice with 5 in each group immunized with GST, MoPn EB or MIP as described in Fig. 2 legend were sacrificed prior to challenge infection for collecting blood and splenocytes. (A) Individual antisera from the 3 groups of mice, after serial dilution, were reacted with recombinant MIP (top plot) or purified EBs (lower plot) coated onto ELISA plates. MIP immunization induced very high levels of MIP-specific Ab responses and the antisera significantly recognized the whole organisms although with much lower titers comparing to the EB-immunized mice. (B) The IgG antibodies from each individual mouse were further isotyped using the ELISA as described in the legend of panel (A) above. All mouse antisera were diluted 1:800 and the titers of the total IgG and each IgG isotype as indicated along the X-axis were measured as OD values at 405 nm as displayed along the Y-axis. The results were expressed as mean plus standard deviation. Both EB- and GlgP-immunized mice preferentially produced higher levels of IgG2a, indicating the dominance of Th1 cytokines in these mice.

Fig. 6.

Induction of MIP- and MoPn-specific IFN- γ responses with MIP immunization. Splenocytes harvested from three groups of mice as described in Fig. 5 legend were *in vitro* re-stimulated with the indicated antigens. Three days after the stimulation, the culture supernatants were collected for IFN- γ detection and the results were expressed as ng/ml as displayed along the Y-axis (mean $\pm$ SD). (A) MIP specific IFN- γ response. Splenic cells from each group were stimulated with 10 μ g/ml of MIP, unrelated antigen BSA, or cultured in media alone, and analyzed for IFN- γ production using an indirect ELISA. (B) MoPn EB-specific IFN- γ response. Cells were stimulated with UV-inactivated MoPn EBs at 1×10^6 IFUs per well, BSA, or cultured in media alone, and analyzed for IFN- γ production. Significant difference ($P < 0.05$; Student's *t* test) in cytokine secretion between GST-vaccinated mice versus MoPn EB or MIP groups.