Chlamydia muridarum major-outer membrane protein-specific antibodies inhibit *in vitro* infection but enhance pathology *in vivo*

Running head: Chlamydia antibodies accelerate infection pathology

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Abstract

Problem

Chlamydia trachomatis is a significant worldwide health problem, and the often-asymptomatic disease can result in infertility. In order to develop a successful vaccine, a complete understanding of the immune response to chlamydial infection and development of genital tract pathology is required.

Method of Study

We utilised the murine genital model of chlamydial infection. Mice were immunized with chlamydial Major Outer Membrane Protein (MOMP), and vaginal lavage was assessed for the presence of neutralising antibodies. These samples were then pre-incubated with *Chlamydia muridarum* and administered to the vaginal vaults of immune competent female BALB/c mice to determine the effect on infection.

Results

The administration of *C. muridarum* in conjunction with neutralizing antibodies reduced the numbers of mice infected, but a surprising finding was that this accelerated the development of severe oviduct pathology.

Conclusion

Antibodies play an under-recognized role in chlamydial infection and pathology development, which possibly involves interaction with Th1 immunity.

Key Words

Immunopathology, Hydrosalpinx, Oviduct

Introduction

Chlamydia trachomatis infections are a significant worldwide burden on healthcare, causing both ocular and genital disease. The true incidence of disease is uncertain, due to the highly asymptomatic nature of infections, with results of screening studies estimating prevalence between 1.5% and 13.3% within young adult populations [1, 2]. Untreated infections can become chronic over time resulting in infertility, thus we require a better understanding of the development of *Chlamydia*-induced pathology, and a successful vaccine needs to be developed for both men and women in order to curb this silent epidemic.

A successful vaccine will need to enhance or improve the immunity induced by a natural infection. Current literature suggests that the Th1 arm of the immune response plays the major role in the clearance of chlamydial genital infection (reviewed in [3]). Animal models of *Chlamydia* infection of the female genital tract have demonstrated that the development of a Th1 response, involving influx of IFN-γ secreting $CD4^+$ T cells, is crucial to resolution [4], which is confirmed by the fact that IFN-γ knockout mice cannot induce protective immunity despite high levels of vaginal IgA [5].

Conversely, the role of antibodies in clearance of *Chlamydia* from the vagina appears to be more significant following a secondary challenge [6]. B cell deficient mice are more susceptible to a secondary genital infection, having a greater number of mice re-infected than their wild type controls [6]. Mice

deficient in $CD4^+$T cells and B cells are highly susceptible to chlamydial genital challenge, but passive immunization with immune serum or monoclonal antibodies to chlamydial major outer membrane protein resulted in protection against re-infection [7]. Interestingly, mice deficient in B cells also demonstrate more significant pathology at day 80 following a primary chlamydial infection, suggesting broad-specificity antibodies are required to protect against tissue damage [8]. However, the role of antibodies in enhancing cell-mediated immunity in the clearance of a chlamydial infection is not well known. Furthermore, most studies assessing the relative impact of Th1 and Th2 immunity in clearance of a chlamydial infection have been performed in immune-compromised mice models without the capacity to investigate this interaction.

The determinants of pathology in a murine model have been investigated. One study suggested that upper tract pathology may be caused by a prematurely terminated Th1 response leading to increasing chronic infection, or an exaggerated proinflammatory Th1 response resulting in tissue damage [9]. Greater numbers of $CD4^+$ cells were found to infiltrate the oviduct tissue compared to cervico-vaginal tissue of infected BALB/c mice, despite having the same numbers of Th1 cells, thus the oviduct must recruit more non-Th1 cells. This study also demonstrated a key inverse relationship between the strength of the innate immune response induced in the lower reproductive tract, and the ability of chlamydiae to ascend to the upper genital tract [9]. However, the infectious dose is not related to the degree of inflammatory cell infiltration and hydrosalpinx development [10].

Experiments in knockout mice have demonstrated an important role of CD28 and CD80/86 in the development of hydrosalpinx, an indicator of subsequent infertility [11]. Pathology does not develop in TLR2-deficient mice [12]. TLR2 is required for MIP-2 and TNF-α expression, and subsequently neutrophil recruitment to the lungs during a *Chlamydia pneumoniae* infection [13]. Recent investigations in knockout mice have also indicated a crucial role of IL-1β in both clearance of Chlamydia and immunopathology [14].

The interactions between antibodies and cell-mediated immunity in an immune-competent model and the role in protective immunity and pathology development are unclear. There were two key aims of this study; 1) to measure the antibodies produced following immunization with chlamydial Major Outer Membrane Protein (MOMP) co-administered with the adjuvants cholera toxin and CpG, and 2) to determine if neutralising vaginal lavage (VL) antibodies are able to affect the course of infection *in vivo* in an immune-competent model.

Materials and Methods

Animals

Female BALB/c mice (6-8 weeks) were obtained from the Animal Resources Centre (Perth, Australia). Animals received food and water *ad libitum*. All procedures were approved by the Queensland University of Technology Animal Research Ethics Committee.

Reagents for immunization and chlamydial challenge

The transformed *Escherichia coli* (DH5α(pMMM3)) expressing the pMAL-c2 vector encoding recombinant maltose binding protein-MOMP fusion protein was a generous gift from Harlan Caldwell (Rocky Mountain Labs, Hamilton, MT, USA). MBP-MOMP was produced as previously described [15]. *Chlamydia muridarum* (Weiss; ATCC VR-123, Virginia, USA), formerly the mouse pneumonitis biovar of *C. trachomatis* (MoPn), was grown by inoculation of McCoy cell (ATCC CRL-1696, Virginia, USA) monolayers and elementary bodies (EBs) isolated as previously described [16]. *Chlamydia muridarum* is a suitable murine model for chlamydial genital tract infection in humans [17].

Immunization of mice

In two separate experiments, groups of five mice were immunized with MOMP (100μg), or cholera toxin (CT;5μg)/CpG(10μg), or a combination of protein and adjuvants. Negative controls were unimmunized naïve mice, and positive controls were mice receiving an initial genital inoculation with 5×10^4 inclusion forming units (ifu) of *C. muridarum*. Mice were immunized on Day 0,

and boosts were administered at Days 7,14 and 35. Vaginal lavage (VL) samples were collected at one week following the final boost by flushing the vaginal vault five times with a total volume of 40μl of sterile phosphate buffered saline.

Quantitation of MOMP-specific antibodies by ELISA

MOMP-specific IgA and IgG titres were determined for VL samples using previously described methods [18]. Greiner medium-binding 96-well ELISA plates (Greiner Bio-One, FL, USA) were coated with 2μg/well recombinant MBP-MOMP. VL was serially diluted two-fold from 1/10 to 1/1280 in PBS-Tween. Primary antibodies, biotin-conjugated anti-IgG and anti-IgA, and the secondary antibody Streptavidin-HRP conjugate were all used at a dilution of 1/1000 (Southern Biotechnology Associates, Birmingham AL, USA). The plates were developed using 0.1μg/ml Tetramethyl-benzidine (TMB, Sigma-Aldrich, North Ryde NSW Australia) in phosphate citrate buffer (PCB, Sigma-Aldrich), then stopped by the addition of 1M sulfuric acid. Absorbance of each sample was at 450nm and end-point titre (EPT) was defined as the mean dilution at which the absorbance reached that of negative wells (mean PBS sample absorbance + 2 SD).

VL neutralization of C. muridarum in vitro

To examine the *Chlamydia*-neutralizing capacity of these MOMP-specific antibodies, the VL samples were examined by *in vitro* neutralization assay on McCoy cells. *C. muridarum* (5×10^4ifu) was incubated with a 1/5 dilution of VL (containing 10μl of sample) for 1h at 37°C before addition to McCoy cell

monolayers in 48-well plates. The plates were then incubated for 4h before rinsing the monolayers with PBS and replacing with 500μl Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% FCS and 1μg/ml cycloheximide (Sigma-Aldrich). Infection was stopped 24h post-inoculation. Chlamydial inclusions were stained using the *Chlamydia* Cel LPS staining kit (CelLabs, Brookvale NSW, Australia). Percent neutralization was determined relative to *C. muridarum* only control wells.

Statistics

ELISA and *in vitro* neutralization data are presented as means and SEM. Statistical analyses were performed using GraphPad Prism version 4.0b for Windows (GraphPad Software, San Diego CA, USA). Differences between treatment groups and naïve mice were examined by unpaired two-tailed t-test, with significance determined as $p<0.05$.

Preparation and intra-vaginal inoculation of antibody-bound EBs

Groups of five naïve mice were given an injection containing 2.5mg of medroxyprogesterone acetate (Depo-Provera, Pfizer, West Ryde NSW, Australia) subcutaneously, seven days prior to experimentation. For the second aim of this study, the characterized VL samples from the first mouse experiment were used. As with the *in vitro* neutralization assay described above, 5 x 10^4ifu *C. muridarum* was pre-incubated with a 1/5 dilution of VL (containing 10μl sample) for 1h at 37°C. Recipient mice were anaesthetized intraperitoneally using ketamine (Parnell Laboratory, Australia) and xylazine (Bayer, Australia), and the antibody-bound EB solutions were then

administered to the vaginal vaults of these mice. An additional control group receiving *C. muridarum* but no VL was included. Infection was monitored by collecting cervico-vaginal swabs (Copan, Murrieta, CA, USA) every three days post infection. Swabs were placed in tubes containing 500μl SPG and glass beads and were stored at -80°C. To monitor infection levels, swabs were cultured as described elsewhere [10]. The inclusions were visualised by staining with rabbit anti-*C. trachomatis* antibody (Pierce/Progen, Richlands NSW, Australia) and Immunopure ABC/DAB Staining Kit (Pierce/Progen, Richlands NSW, Australia), as described elsewhere [15]. Two separate experiments were conducted with 5 mice per group for post-infection sample evaluation, and results are tabulated as numbers of mice positive for infection (defined as >10 inclusions per high power field).

Sacrifice and pathology scoring

At Day 22 post-infection mice were sacrificed using a lethal injection of Sodium pentobarbitone. Gross pathology of the oviducts at sacrifice was evaluated *in situ*. The degree of pathology development was recorded and graded as described previously [10]. Briefly, absence of oviduct dilation was given a score of 0; between 2-3mm dilation was given a score of 1 (mild); 3-5mm was scored 2 (moderate); and >5mm was scored 3 (severe). Furthermore, oviducts were ethanol fixed, sectioned and stained with haematoxylin and eosin, then numbers of infiltrating immune cells (polymorphonuclear leukocytes and lymphocytes) counted per high power field. Data presented are representative of two separate experiments.

Results

Immunization with MOMP and CT/CpG results in high titres of neutralising antibodies in VL

Immunized mice were assessed for levels of VL MOMP-specific antibodies by ELISA. MOMP-specific IgA was present at an EPT of ~200 in the mice receiving intranasal (IN) immunization with MOMP and CT/CpG+MOMP, and also in the group given an intra-vaginal live inoculation (LIC) with *C. muridarum* (Fig.1A). MOMP-specific IgG was seen in both immunized groups receiving MOMP, with an EPT of ~150 for IN-MOMP, and ~320 for IN-CT/CpG+MOMP (Fig.1B). Levels of IgG in the serum were also high in these two groups, and these MOMP-specific antibodies (VL and serum) dropped significantly from 1-8 weeks post-infection (data not shown).

When the VL sample was obtained from LIC mice, approximately 75% *in vitro* neutralization of *C. muridarum* infection of McCoy cells was observed. The VL from mice immunized with CT/CpG+MOMP demonstrated neutralization of approximately 65% (Fig. 2). Little to no neutralization was seen with VL samples collected from immunized mice at 8 weeks post-boost (data not shown). The VL samples collected at one-week post-boost were thus selected for subsequent investigation. It is interesting to note that although low levels of MOMP-specific IgG were present in the VL of LIC mice, these samples showed significant neutralization.

Neutralizing MOMP-specific antibodies slightly enhance clearance of C. muridarum

Due to variations in levels of initial infection achieved between mice, the data are presented as numbers of mice positive for infection (defined as >10 ifu per high power field) every three days post-inoculation up to Day21 (Table 1). Mice receiving *C. muridarum* pre-incubated with VL from all negative control groups (No VL, Naïve VL, MOMP only, adjuvants only) showed little difference in clearance rates. The mice receiving anti-*C. muridarum* VL (from LIC mice) in conjunction with *C. muridarum* demonstrated the best clearance, which was not unexpected. The VL from CT/CpG+MOMP immunized mice which contained high levels of *in vitro* neutralizing antibodies, was able to slightly enhance the clearance of infection in recipient mice. From Days 9-15, this group shows fewer mice positive for infection than the negative control groups (with the singular exception of the naïve VL group at Day15 only). Beyond this point, Th1-mediated processes would be mobilized [10], likely abrogating any potential differences effected by the presence of early-administered antibodies.

MOMP-specific antibody-bound EB administration results in the development of accelerated oviduct pathology

Despite enhancing chlamydial clearance from the vagina, administering VL MOMP-specific antibodies and *C. muridarum* into the vaginal vault of naïve mice has actually generated the presence of moderate to severe hydrosalpinx 22 days post-inoculation. The groups receiving VL from mice with high titres of MOMP-specific IgG showed the greatest pathology scores at day 22,

particularly in the left oviduct (Fig.3). This phenomenon of greater pathology in the left oviduct has also been observed previously (Carey A., unpublished results). Furthermore, the groups displaying greater oviduct dilation were assessed for numbers of infiltrating immune cells. There was a trend for higher numbers of polymorphonuclear leukocytes in the mice receiving VL from the MOMP+CT/CpG immunized animals, and there was a significantly greater number of lymphocytes in these mice ($p=0.0159$).

Discussion

Intranasal immunization with MOMP has resulted in the generation of MOMP-specific mucosal IgA and IgG antibodies in genital tract secretions. This is in concordance with previous studies demonstrating that IN immunization can effectively target immunity to reproductive tracts of both females and males [18-20]. The abundance of these MOMP-specific antibodies did not directly correlate with the ability of the VL samples to neutralize chlamydial infection *in vitro*. The VL from challenged mice, despite possessing no MOMP-specific IgG, demonstrated the greatest *in vitro* neutralization. This is likely due to the presence of additional antibodies present in the VL sample, which are directed against chlamydial antigens other than MOMP, thus not detected by this ELISA assay. Another interesting finding is that the VL from mice receiving IN immunization with MOMP alone demonstrated high levels of MOMP-specific antibodies, but these were not able to neutralize *in vitro* infection of McCoy cells. CpG adjuvant induces signalling via toll-like receptor (TLR) 9, while CT enhances B cell activity [21]. The presentation of MOMP antigen may follow a different pathway when co-administered with these adjuvants, thus possibly resulting in the differences in neutralizing capacity of VL antibodies seen in this study.

We aimed to determine what effect the *in vitro* neutralizing antibodies would have on the course of a *C. muridarum* infection *in vivo*. Thus, VL samples were pre-incubated with *C. muridarum*, which were then administered to the vaginal vaults of naïve mice. The groups receiving antibody-bound EBs

derived from LIC and CT/CpG+MOMP immunized mice, had slightly fewer mice positive for infection from Days 9-15 than mice receiving *C. muridarum* with either no VL or non-immune VL. Similarly, in another study, when *C. trachomatis* elementary bodies (EBs) were pre-coated with IgG2b monoclonal antibodies and administered intravaginally to C3H/HeJ mice there was a reduced infection [22]. In our experiment, *in vitro* and *in vivo* neutralization capacity was closely related. This is not always the case, as another study shows a lack of correlation [22].

C. muridarum EBs in complex with IgG antibody are translocated by Fc-receptors (FcR) into cells more efficiently than EBs alone. In fact, six times more mucosal T cells are initiated in wild type mice than in FcR knockout mice in the first week of inoculation with *C. muridarum* immune complexes [23]. This does not require active replication. By this mechanism, enhanced presentation of MOMP antigen to T cells via FcR would explain the reduction in early levels of chlamydial infection observed in this model. Further investigations are underway to examine this process. This would also explain why other studies in T cell deficient mice have shown limited ability of antibodies to clear infections [24], if the primary role of antibodies is to enhance or accelerate the activation/activity of cell-mediated immunity.

While some protection was afforded at early time points, perhaps the most interesting finding from this investigation was that the presence of MOMP-specific antibodies accelerated the development of oviduct pathology, namely hydrosalpinx. While this is normally observed at or beyond 29 days post-

infection [10, 25], we observed development of severe hydrosalpinx at Day 22 post-infection, which appeared to correlate with levels of MOMP-specific IgG in the VL samples. In mice, oviduct occlusion correlates 100% with infertility [25].

There are a number of possible mechanisms by which this accelerated pathology occurred. The first possibility is that inoculation with antibody-bound EBs has effectively reduced the chlamydial dose, resulting in reduced immune recognition in the lower reproductive tract and an increased ascending infection. Similar phenomena have been observed in female mice where genital inoculation with a low dose of *C. muridarum* has resulted in greater chlamydial load in the oviducts [9].

A second possibility is that the presence of antibodies actually induces greater tissue infection at an earlier time point than *C. muridarum* alone, or recruits complement, ultimately causing tissue damage in an earlier time frame. Interestingly, preliminary experiments have indicated that lower levels of MOMP-specific antibodies can actually facilitate, rather than neutralize, chlamydial uptake *in vitro* (data not shown), a phenomenon also previously demonstrated with antibodies against chlamydial NrdB [26]. The implications for these findings have great significance for *in vivo* studies. It would be necessary to determine tissue load throughout the course of the infection, with a range of IgG:EB ratios to investigate this possibility.

Endogenous IgG is taken up into *Chlamydia* infected cells and is co-localised with the inclusion, seen in early and late stages of chlamydial development both *in vitro* and in human blood smears from infected patients. This requires the Fc region of the immunoglobulins [27]. However, Moore et al. [23] demonstrated that FcR I, II and III knockout mice can still develop hydrosalpinx, and thus while possibly still involved in increased antigen presentation, the FcRs are not the means by which we observe accelerated pathology in this model.

A likely candidate is the neonatal Fc receptor (FcRn). FcRn in human and murine dendritic cells enhances uptake of immune complexes [28, 29]. In a recent study, it was found that cytomegalovirus (CMV) virions were transcytosed across the placental surface, co-localized with IgG [30]. In villus explant studies, low avidity antibodies were predictive of congenital infection with CMV, while high avidity IgG resulted in internalized nucleocapsids without replication, which were then captured by macrophages [30]. FcRn saturation with the Fc fragment of human IgG resulted in a decrease in CMV virion uptake, thus implicating this receptor in CMV-IgG immune complex uptake. FcRn is implicated in other inflammatory pathologies including rheumatoid arthritis and bullous pemphigoid [31, 32]. Simple interfering RNA studies are underway to determine if FcRn is involved in the pathology of chlamydial infection. There is strong expression of FcRn in the efferent ducts and caput epididymis of the male rat, consistent with regions of IgG reabsorption from lumen into epithelial cells in these regions [33], and we have

determined the presence of FcRn in McCoy cells used in these *in vitro* studies (data not shown).

A third possible explanation for antibody-bound EB-mediated acceleration of pathology development is an early interaction of intracellular *C. muridarum* and TLR2. Whether rapid ascension of EBs or early uptake into epithelial cells occurs, this may result in the early induction of TLR2-signalling events that are essential for pathology [12]. Interaction of EBs and TLR2 likely occurs at the phagosome [34], and downstream signaling requires active replication and the presence of the EB plasmid [12, 35]. This results in the secretion of TNF-α [35] and MIP-2 [36], and subsequent neutrophil recruitment, therefore this step is essential for the development of pathology.

Future studies will examine these hypothesized mechanisms of accelerated pathology development observed in this model. This will improve our understanding of the pathophysiology of oviduct occlusion *in vivo*, and hopefully provide us with targets to inhibit such pathology in response to future vaccination protocols.

Conclusions

The findings of this investigation and future studies have strong implications, not only for determining mechanisms of reproductive disease and infertility of women, but also for vaccine development. While the vast majority of vaccine experiments report on reduction of bacterial shedding, the potential for experimental vaccines to induce damaging immunity resulting in organ disease is largely ignored, and rarely reported in the literature. The potential for immunity to induce pathology must always be considered in rational vaccine design.

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Tables

Table 1: Numbers of mice positive for chlamydial infection (>10ifu per HPF)

Day	No VL	Naïve VL	LIC	MOMP	CT/CpG	MOMP +CT/CpG
6	10	10	9	10	10	10
9	9	9	7	10	10	8
12	10	7	6	7	8	5
15	7	5	3	8	7	5
18	1	3	1	4	2	2
21	0	0	1	1	0	1

VL: vaginal lavage, LIC: live inoculation control, MOMP: major outer-membrane protein
CT/CpG: cholera toxin/CpG oligodeoxynucleotides

Figure captions

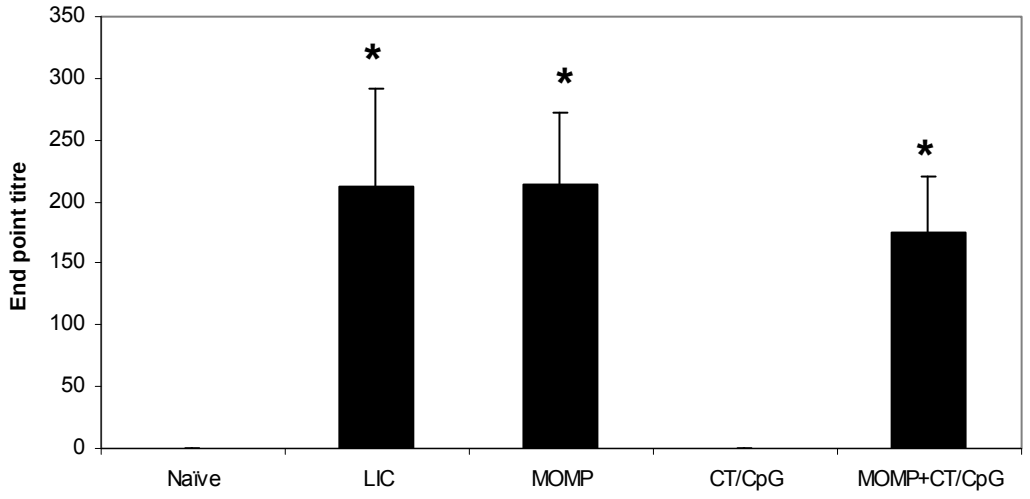
Figure 1. Vaginal lavage antibodies following intranasal immunization. One week post-boost vaginal lavage samples were collected from five mice per group, and ELISA conducted to determine levels of MOMP-specific IgA (A) and IgG (B). Absorbance at 450nm was measured and end point titre determined. Significance (*) denotes a p-value of <0.05 by Student's t-test compared to naïve mice. Data presented are pooled from two separate experiments.

Figure 2. *In vitro* neutralization of *C. muridarum* with vaginal lavage samples from immunized mice. McCoy cell monolayers were inoculated with 5 x 10^4 ifu *C. muridarum* pre-incubated with a 1/5 dilution of vaginal lavage. Infection proceeded for 24h before cells were fixed, then DAB-stained for inclusion counting. Number of inclusion forming units per mL of inoculating fluid were determined and t-tests conducted on each sample compared to *C. muridarum* only control for significance (* $p<0.05$).

Figure 3. Pathology assessment. Naïve mice were administered antibody-bound EBs via the vaginal vault. At day 22 post-infection, mice were sacrificed and degree of pathology of the oviducts graded (A); no oviduct dilation = 0; mild dilation = 1; moderate = 2; severe = 3. Also, tissues were fixed, sectioned and H&E stained and assessed for numbers of polymorphonuclear leukocytes (B), and lymphocytes (C). Results presented are from five recipient mice per group.

Figure 1

A)


End point titre
350
300
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*
Naïve
LIC
MOMP
CT/CpG
MOMP+CT/CpG

B)

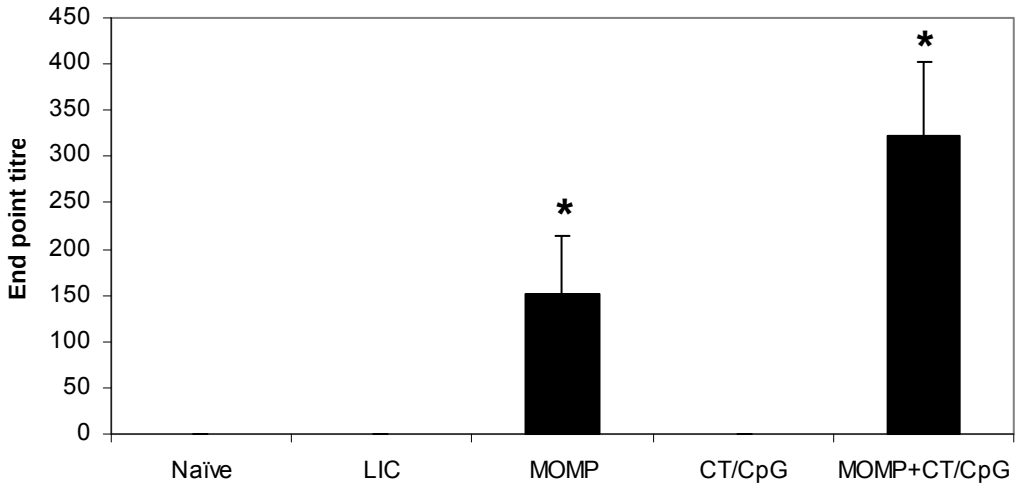

End point titre
450
400
350
300
250
200
150
100
50
0
*
*
Naïve
LIC
MOMP
CT/CpG
MOMP+CT/CpG

Figure 2

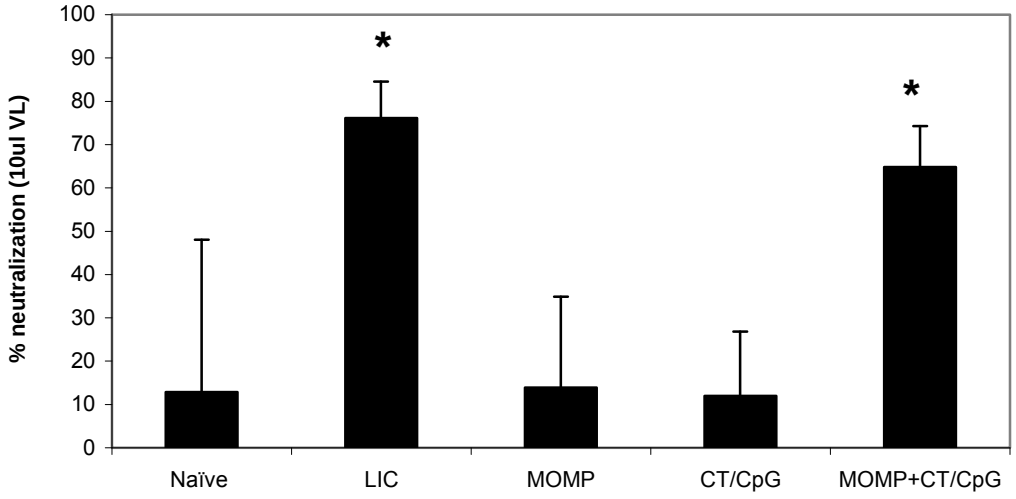
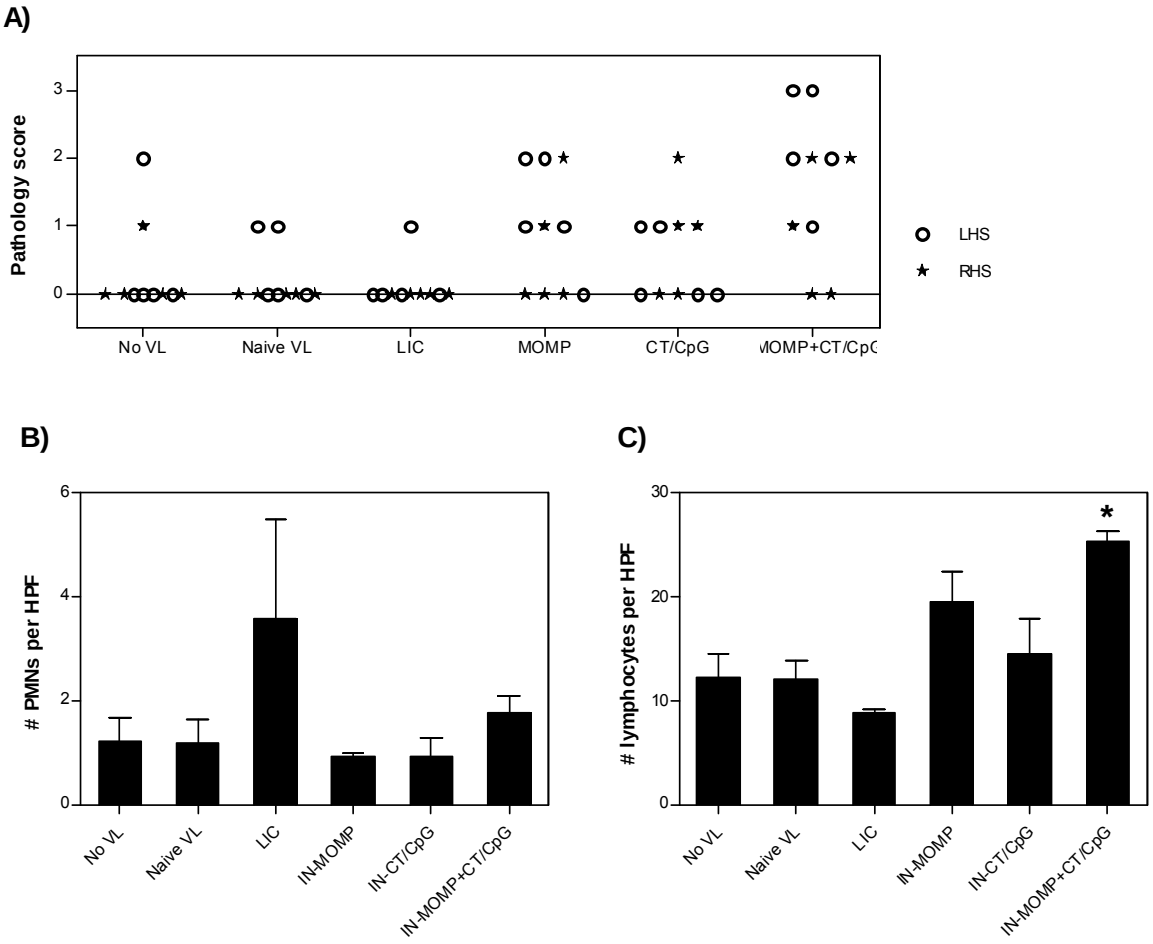

% neutralization (10ul VL)
100
90
80
70
60
50
40
30
20
10
0
*
*
Naïve
LIC
MOMP
CT/CpG
MOMP+CT/CpG

Figure 3


A)
Pathology score
3
2
1
0
No VL
Naive VL
LIC
MOMP
CT/CpG
LHS
RHS
B)
PMNs per HPF
6
4
2
0
No VL
Naive VL
LIC
IN-MOMP
IN-CT/CpG
IN-MOMP+CT/CpG
C)
lymphocytes per HPF
30
20
10
0
*
No VL
Naive VL
LIC
IN-MOMP
IN-CT/CpG
IN-MOMP+CT/CpG