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# *Plac8*-dependent and *iNOS*-dependent mechanisms clear *Chlamydia muridarum* infections from the genital tract[1]

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## Abstract

*Chlamydia trachomatis* urogenital serovars replicate predominately in genital tract epithelium. This tissue tropism poses a unique challenge for host defense and vaccine development. Studies utilizing the *Chlamydia muridarum* mouse model have shown that CD4 T cells are critical for clearing genital tract infections. *In vitro* studies have shown that CD4 T cells terminate infection by up regulating epithelial *iNOS* transcription and nitric oxide production. However, this mechanism is not critical as *iNOS*-deficient mice clear infections normally. We recently showed that a subset of *Chlamydia*-specific CD4 T cell clones could terminate replication in epithelial cells using an *iNOS*-independent mechanism requiring T cell degranulation. We advance that work using microarrays to compare *iNOS*-dependent and *iNOS*-independent CD4 T cell clones. *Plac8* was differentially expressed by clones having the *iNOS*-independent mechanism. *Plac8*-deficient mice had delayed clearance of infection, and *Plac8*-deficient mice treated with the *iNOS*-inhibitor N-monomethyl-L-arginine were largely unable to resolve genital tract infections over 8 weeks. These results demonstrate that there are two independent and redundant T cell mechanisms for clearing *C. muridarum* genital tract infections; one dependent on *iNOS*, the other dependent on *Plac8*. While T cells subsets are routinely defined by cytokine profiles, there may be important subdivisions by effector function, in this case $CD4_{Plac8}$.

## Introduction

*Chlamydia trachomatis* serovars D-K are sexually transmitted bacterial pathogens that cause urethritis, endometritis, salpingitis, epididymitis, and pelvic inflammatory disease. In women, infections ascend into the upper reproductive tract causing scarring responsible for ectopic pregnancies and infertility. Aggressive public health measures based on antibiotic treatment of source cases and their sexual partners are partially counterproductive due to deleterious effects on herd immunity (1–3). There is a consensus among researchers and public health experts that a *Chlamydia* vaccine is likely necessary to reduce the incidence of *Chlamydia trachomatis* infections, and the medical resources committed to treating them. A critical component of rational vaccine development is defining the immune parameters that mediate/correlate with protective immunity.

For viral vaccines, neutralizing antibodies are the immune parameter that correlates with protective immunity against primary infections. Existing data from the *C. muridarum* mouse model suggests that immunity to urogenital serovars of *Chlamydia* is complex. Passive

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transfer of antibodies from immune mice has little effect on primary infections of the genital tract (4), and B cell-deficient mice clear primary genital tract infections similar to wild type controls (5). Clearance of primary genital tract infections is dependent on T cell immunity, and T cell-immunity is sufficient to clear the genital tract (6). MHC class I antigen presentation is dispensable for protective host immunity while MHC class II is absolutely required (7). Recent data in experimental mouse models argues for a supportive role for antibodies in vaccine-generated immunity in the genital tract (8, 9). A large body of research has identified CD4 T cells of the Th1 subset as the critical parameter for protective host immunity (6). The protective Th1 subset generated by vaccination appears to be multifunctional CD4 T cells that secrete IFN-γ & TNF-α, with or without IL-2 production (10).

*In vitro* studies using a *Chlamydia*-specific CD4 T cell clone capable of terminating *C. muridarum* replication in epithelial cells identified a probable mechanism for sterilizing CD4 T cell-mediated immunity that was dependent on *iNOS*, IFN-γ, and T cell-epithelial cell contact via LFA-1 - ICAM-1 (11–14). However, follow up studies showed *iNOS*-knockout mice were not compromised in clearance of *C. muridarum* genital tract infections (15, 16), and that IFN-γ-knockout mice cleared 99.9% of *C. muridarum* from the genital tract with near normal kinetics (17, 18). Furthermore, mice deficient in the known T cell killing mechanisms perforin & *FasL-Fas* were also able to clear *C. muridarum* genital tract infections with normal kinetics (19). Additional knockout mice and antibody depletion studies have shown that mice deficient in TNF-α receptors (20), IL-12 (18, 20), or IL-1β (21) have varying degrees of compromised *C. muridarum* genital tract infection clearance. However, cytokine deficiencies have pleiotropic effects on host innate and adaptive immunity, and do not identify specific effector mechanisms responsible for terminating *Chlamydia* replication in reproductive tract epithelium.

Our lab recently identified an *iNOS*-independent mechanism for terminating *C. muridarum* replication in epithelial cells (22). Using a panel of *Chlamydia*-specific CD4 T cell clones we showed that a subset of clones could terminate *C. muridarum* replication in the presence of the *iNOS* inhibitor N-monomethyl-L-arginine (MLA), while a separate clone was completely inhibited by MLA as previously reported in the literature. The *iNOS*-independent CD4 T cell clones were also relatively independent of IFN-γ, and could be partially blocked using phenyl arsenic oxide (PAO), an inhibitor of T cell degranulation. Potent blocking of the *iNOS*-independent CD4 clones required both an inhibitor of degranulation and an iNOS inhibitor, suggesting two independent mechanisms capable of terminating *C. muridarum* replication in infected epithelial cells. All the *Chlamydia*-specific CD4 T cell clones were Th1, made IFN-γ when activated, and were capable of recognizing infected epithelial cells (23).

To further investigate the findings above, we compared two *iNOS*-independent CD4 T cell clones to two *iNOS*-dependent CD4 T cell clones using gene expression micro arrays. Micro array analysis identified a candidate second mechanism for terminating *C. muridarum* replication in epithelial cells that was then investigated *in vivo* utilizing an existing knockout mouse. Interesting results of those studies are reported here.

## Materials and Methods

### Mice

4–5 week old female C57BL/6J and B6.129S6-Plac8 (009598) mice were purchased from The Jackson Laboratories (Bar Harbor, MA). All mice were housed in Indiana University Purdue University-Indianapolis (IUPUI) specific-pathogen-free facilities (SPF). The IUPUI Institutional Animal Care and Utilization Committee approved all experimental protocols.

### Cells, T cell clones and bacteria

C57epi.1 epithelial cells and *Chlamydia*-specific CD4 T cell clones uvmo-1, uvmo-2, uvmo-4, and spl4-10, were derived from C57BL/6 mice and cultured as previously described (23). Mycoplasma-free *Chlamydia muridarum (Nigg)*, previously known as *C. trachomatis* strain mouse pneumonitis (MoPn) (Nigg) was grown in McCoy cells as previously described (23).

### Cytokine ELISAs

$5\times10^4$ T cell clones were activated in 96 well tissue culture plates by immobilized anti-CD3 monoclonal antibody 145-2c11 (BD Biosciences, San Jose, CA), 0.5 μg/ml in PBS overnight at 4°C (washed once), in RPMI media containing 1 ηg/ml recombinant murine IL-7 (R&D Systems, Minneapolis, MN). Relative levels of interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), IL-2, and IL-10 in culture supernatants were determined by ELISA using capture and biotinylated monoclonal antibody pairs with recombinant murine standards according to the manufacturer's protocols. IFN-γ ELISA: XMG1.2; IL-2 ELISA 5H4/1A12 (Pierce-Endogen; Rockford, IL). TNF-α ELISA: TN3-19.12/C1150-14; IL-10 ELISA: JES5-2A5/SXC-1 (BD Biosciences). Detection was accomplished with Streptavidin-HRP (BD Biosciences) and TMB substrate (Sigma Chemical Co., St. Louis, MO).

### Gene expression micro array analysis

*Chlamydia*-specific CD4 T cell clones uvmo-2, uvmo-3, uvmo-4, and spl4-10 were purified by ficoll-hypaque (histopaque 1083; Sigma Chemical Co.) at the end of their culture cycle and then grown for 3 days in their usual culture media including growth factors, without antigen stimulation. On day three, total RNA was isolated from each T cell clone using a protocol that included an RNAse-free DNAse I treatment step (RNAeasy; Qiagen, Valencia, CA). With assistance from The Indiana University Center for Medical Genomics, gene expression patterns were analyzed using the Affymetrix Mouse ST 1.0 Array that analyzes 28,853 murine genes. Samples were labeled using the standard Affymetrix protocol for the WT Target Labeling and Control Reagents kit according to the Affymetrix user manual: GeneChip® Whole Transcript (WT) Sense Target Labeling Assay GeneChip. Individual labeled samples were hybridized to the Mouse Gene 1.0 ST GeneChips® for 17 hours then washed, stained and scanned with the standard protocol using Affymetrix GCOS (GeneChip® Operating System). GCOS was used to generate data (CEL files). Arrays were visually scanned for abnormalities or defects. CEL files were imported into Partek Genomics Suite (Partek, Inc., St. Louis, Mo). RMA signals were generated for the core probe sets using the RMA background correction, quantile normalization and summarization by Median Polish. Summarized signals for each probe set were $\log_2$ transformed. These log transformed signals were used for Principal Components Analysis, hierarchical clustering and signal histograms to determine if there were any outlier arrays. Untransformed RMA signals were used for fold change calculations. Data was analyzed using a 1-way Anova (analysis of variance) using $\log_2$ transformed signals for all four CD4 T cell clones, and contrasts were made comparing iNOS-dependent uvmo-4 and spl14-10 individually to the combined expression of the iNOS-independent T cell clones uvmo-2 and uvmo-3. Fold changes were calculated using the untransformed RMA signals. Genes up or down regulated 5-fold with *p values* <0.001 for either uvmo-4 or spl4-10 compared to uvmo-2/uvmo-3 were considered in the final analysis (supplemental table 1). The microarray data presented here is available in the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo) under accession number GSE32128.

### RT-PCR analysis

*Chlamydia*-specific CD4 T cell clones uvmo-2, uvmo-3, uvmo-4, and spl4-10 were purified by ficoll-hypaque at the end of their culture cycle and then grown for 3 days in their usual culture media including growth factors, without antigen stimulation. On day three, total RNA was isolated from each T cell clone using a protocol that included an RNAse-free DNAse I treatment step. Specific mRNA gene reverse transcription and applification was performed using AMV reverse transcriptase/*Tfl* DNA polymerase in a one step system (AccessQuick RT-PCR; Promega, Madison, WI). Amplification conditions were (1) 48°C for 45 min; (2) 95°C 2 min; (3) 95°C 30 sec; (4) 56°C 20 sec; (5) 72°C 30 sec; (6) goto step three 37 times; (7) 72°C 7 min; (8) hold 4°C, using an MJ Research J200 PCR machine. All gene analyses were done on the same set of RNA samples. PCR primers, amount of total RNA in amplification, and expected product sizes shown in table 1. RT-PCR reactions were run on 2% agarose gels with ethidium bromide; images inverted for presentation. The “no RT” controls generated no RT-PCR products (*data not shown*). PCR primers used are shown in table 1.

### Epithelial cell infections/*Chlamydia* replication/titration

C57epi.1 cell monolayers in 48 well plates were untreated or treated with IFN-γ (10 ηg/ml) for 10 h prior to infection. Wells were infected with 3 IFU per cell. After addition of *C. muridarum* the plates were spun at 300 × g for 30 minutes. 4 h post infection the inoculums were removed and CD4 T cell clones were added in RPMI 1640 T cell medium. 32 h post infection, wells were scraped, harvested with an equal volume of sucrose-phosphate-glutamate acid buffer (SPG buffer) and stored at −80° C until *C. muridarum* titers determined on McCoy cell monolayers using an anti-*Chlamydia* LPS antibody and FITC-labeled goat anti-mouse IgG (Rockland Immunochemicals, Gilbertsville, PA) as previously described (24).

### Genital tract infections

One week prior to infection mice were treated with 2.5 μg of medroxyprogesterone delivered subcutaneously (depo-Provera, Pfizer Pharmaceuticals, New York, NY). Lightly anesthetized mice were infected vaginally with $5\times10^4$ inclusion forming units (IFU) of *C. muridarum biovar Nigg* in 10 μl of SPG buffer. Mice were swabbed M-W-F to monitor the clearance of *C. muridarum* from the genital tract as previously described (25).

### Pathology Scoring

A simple scoring system was utilized to assess macroscopic genital tract pathology. Hydrosalpinx was scored as 0, 1, or 2 reflecting no, unilateral, or bilateral hydrosalpinx respectively. Mice have a bi-fed uterus (2 uterine horns) that was similarly scored 0, 1, or 2 for hydro-uterus. The maximum pathology score for an individual mouse was 4.

### Degranulation experiments

$5\times10^4$ CD4 T cell clone cells were incubated in wells with media, phorbol myristate acetate (PMA; Sigma) (10 ηg/ml) & calcium ionophore A23187 (500 ng/ml; Sigma), PMA & A23187 + immobilized anti-CD3 (0.5 μg/ml; 50 μl PBS overnight 4° C) (145-2C11; BD Biosciences), or media containing 0.1% tritonX-100 for 4 h at 37° C. Culture supernatants were collected and levels of granzyme B were determined using a murine granzyme B ELISA (mouse granzyme B duoset; R&D systems, Minneapolis, MN).

$$\%\text{Degranulation}=\frac{\text{measured value in experimental well}-\text{spontaneous release (media)}\times 100}{\text{tritonX}-100\text{ value (maximal release)}-\text{spontaneous release}}$$

### Statistical Analysis

Summary figures for each experimental investigation are presented means and standard deviations (SD) or as `pooled' means and standard error of the mean (SEM). Figure legends indicate the number of independent experiments pooled to generate each figure. Student's two-tailed *t*-tests were used to assess significance of experimental data. Homogeneity of variances was assessed using a folded F-test. Statistical analysis of *Chlamydia* shedding was performed using repeated measures analysis of variance to test for differences between groups over time. All data were verified to meet analytic assumptions. Nonparametric tests were also performed and showed similar results. Analyses were performed using SAS 9.2 (SAS Institute, Cary, NC).

## Results

### Characteristics of Chlamydia-specific CD4 T cell clones used in gene expression micro array

We previously characterized a panel of ten *Chlamydia*-specific CD4 T cell clones including uvmo-2, uvmo-3, uvmo-4, and spl4-10 (22, 23). The uvmo-2 and uvmo-3 T cell clones are potent terminators of *C. muridarum* replication, with or without IFN-γ pretreatment of epithelial monolayers, and make significant amounts of IFN-γ when activated by infected epithelial cells (23). They have two potent mechanisms to terminate replication; one dependent on *iNOS*, the other dependent on T cell degranulation. Significant reversal of the ability of uvmo-2 and uvmo-3 to terminate infection required simultaneous use of two inhibitors; an *iNOS*-inhibitor (MLA) and an inhibitor of T cell degranulation (PAO) (22). Availability of the degranulation-dependent mechanism allowed uvmo-2 & uvmo-3 to terminate *C. muridarum* replication in the presence of *iNOS* inhibitors (*iNOS*-independent). The uvmo-4 and spl4-10 CD4 T cell clones make modest amounts of IFN-γ when activated by infected epithelial cells, and they require IFN-γ pretreatment of the epithelial monolayer to control *C. muridarum* replication (23). The ability of Spl4-10 to terminate *C. muridarum* replication in epithelial cells is *iNOS*-dependent, and was completely reversed by *iNOS* inhibition with MLA (22). The mechanism utilized by uvmo-4 to terminate *C. muridarum* replication in epithelial cells was not previously determined. Because uvmo-4 required IFN-γ pretreatment of epithelial cells to control *C. muridarum* replication, we hypothesized that it was dependent on *iNOS* similar to spl4-10. C57epi.1 epithelial monolayers pretreated 10 h with 10 ηg/ml IFN-γ in the absence or presence of 1 mM MLA were infected with *C. muridarum* for 4 h, then co-cultured without and with uvmo-4 at a T cell:epithelial cell ratio of ~0.75:1. Thirty-two hours post infection the wells were harvested by addition of SPG buffer plus scraping. Replication of *C. muridarum* under each condition was quantified by plating recovered IFU on McCoy monolayers (Figure 1). The ability of uvmo-4 to block >90% of *C. muridarum* recovery required IFN-γ pretreatment of the epithelial monolayer. *iNOS* inhibition with MLA restored *C. muridarum* recovery from untreated epithelial cells, and restored *C. muridarum* recovery to >50% of control values in IFN-γ pretreated epithelial cells. Termination of *C. muridarum* replication by uvmo-4 was dependent on *iNOS* production of nitric oxide. Identification of uvmo-4 as an *iNOS*-dependent CD4 T cell clone gave us the desired CD4 T cell panel for gene expression micro array analyses; *i.e.* two *iNOS*-dependent CD4 T cell clones (uvmo-4 and spl4-10) and two *iNOS*-independent CD4 T cells clones (uvmo-2 and uvmo-3).

Uvmo-2, -3, -4, and spl4-10 would be classified Th1 based on IFN-γ production, but there are significant differences in their cytokine profiles (Figure 2). When activated via the T cell receptor complex uvmo-2, uvmo-3, and uvmo-4 produce TNF-α (50–100 ηg/ml) and IL-2 (3–10 ηg/ml) in addition to IFN-γ; spl4-10 produces significant amounts of IL-10 (14 ηg/ml), lesser amounts of IFN-γ & TNF-γ, and a negligible amount of IL-2.

To assess the role of cytokines in the anti-*Chlamydia* effector mechanism of these four CD4 T cell clones, we activated each T cell clone with UV-inactivated *C. muridarum* pulsed irradiated splenocytes in media containing IL-2 (25 units/ml) and IL-7 (2 ηg/ml), harvesting the conditioned media 48 h later. Control RPMI media and fresh cell-free conditioned media from each clone (40% vol/vol in RPMI media) were exchanged for the epithelial media covering monolayers of C57epi.1 epithelial cells infected 4 h earlier with 3 IFU of *C. muridarum* per cell. 32 h post infection the wells were harvested by adding SPG buffer and scraping. Replication of *C. muridarum* under each condition was quantified using McCoy monolayers (Figure 3). The final IFN-γ concentration under each experimental condition was comparable for all four clones, as was the relative inhibition of *C. muridarum* replication. Fresh T cell conditioned media was 2–3 logs more potent than recombinant murine IFN-γ at 10 ηg/ml, which causes only a 2–3-fold reduction in recovered IFU/well in C57epi.1 cells (22). The cytokine-mediated inhibition of replication was almost completely reversed in the presence of MLA, arguing that the mechanism of replication inhibition by cytokines in the absence of T cells was potent induction of epithelial *iNOS* and nitric oxide production.

## Gene expression micro arrays comparing iNOS-independent T cell clones to iNOS-dependent T cell clones

Our previous study showed that the most potent CD4 T cell clone terminators of *C. muridarum* replication, uvmo-2 and uvmo-3, utilized induction of epithelial *iNOS*, and a degranulation-dependent effector mechanism that was not functional in spl4-10. We also showed that uvmo-2 and uvmo-3 recognized and lysed infected epithelial cells late in replication cycle; too late to catch *C. muridarum* in the non-infectious reticulate body stage (eclipse phase). Late recognition of infected epithelial cells by uvmo-2 & uvmo-3 argued against their termination mechanism being a function of earlier presentation of their nominal antigens, *i.e.* not early recognition. Because the uvmo-2 & uvmo-3 could terminate replication in the presence of *iNOS* inhibitor MLA, we hypothesized that they had an unidentified T cell effector mechanism. To gain insight into this unidentified degranulation-dependent effector mechanism we performed gene expression micro arrays comparing *iNOS*-independent (uvmo-2 & uvmo-3) to *iNOS*-dependent (uvmo-4 & spl4-10) T cell clones. We recognized that micro array analyses of four T cell clones would not permit statistically significant conclusions about T cell mechanisms, but postulated that the resulting data would suggest testable hypotheses. T cell clones at the end of their culture cycle were ficoll-hypaqued to remove cellular debris, washed, then cultured for 72 h in media with usual growth cytokines. Total RNA was harvested and used for Affymetrix mouse ST1.0 gene expression micro arrays; four independent RNA isolations for four independent micro array experiments were done to minimize background noise. Figure 4 shows principal component analysis from the micro arrays comparing the four T cell clones. Interestingly the two *iNOS*-independent CD4 T cell clones derived from different mice (uvmo-2 & 3) clustered tightly together and away from the *iNOS*-dependent clones (uvmo-4 & spl4-10), indicating that they had a similar expression pattern for the genes that accounted for the greatest variability in the data set. In supplemental table 1 we list the 193 genes that differed + or – 5-fold comparing the individual expression patterns of spl4-10 and uvmo-4 to the combined expression pattern of uvmo-2 & uvmo-3. The genes that were the most intriguing to us and the primary focus of this paper were those potentially useful as biomarkers or possibly involved in an effector mechanism (Table 2). Uvmo-2 & uvmo-3, the *iNOS*-independent terminators of *C. muridarum* replication, had high mRNA levels of *Casd1* (CAS1 domain containing 1) and *Plac8* (placenta-specific 8), and low levels of perforin (*Prf1*). The *iNOS*-dependent T cell clones uvmo-4 & spl4-10 had very low *Casd1* and *Plac8* levels and high levels of perforin. RT-PCR analysis confirmed the micro array

data for *Casd1*, *Plac8*, and perforin in *iNOS*-independent (uvmo-2,-3) and *iNOS*-dependent (uvmo-4 & spl4-10) CD4 T cell clones (Figure 5).

## Plac8 knockout mice are compromised in their ability to clear C. muridarum genital tract infections

High level expression of *Plac8* by the *iNOS*-independent CD4 T cell clones was particularly interesting because *Plac8* knockout mice were previously shown to have a defect in clearing *Klebsiella pneumoniae* peritoneal infections; a phenotype ascribed to a killing defect in *Plac8*-deficient neutrophils (26). In the original *Plac8* knockout mouse study, investigation of tissue distribution showed expression of *Plac8* in bulk T cell populations by western blotting. To investigate whether *Plac8* was important in host defense against *C. muridarum* genital tract infections we challenged *Plac8* knockout mice, comparing them to C57BL/6J control mice.

C57BL/6J and *Plac8* knockout mice were treated with medroxyprogesterone to synchronize estrous, then infected vaginally with $5\times10^4$ IFU of *C. muridarum*. The *iNOS* inhibitor MLA was added to the drinking water on day 52 and continued thereafter. Mice were swabbed thrice weekly through 80 days post-infection then assessed for genital tract pathology. Recovered IFUs from genital swabs were titered on McCoy monolayers (Figure 6). *Plac8* mice had a clear defect in clearance of *C. muridarum* from the genital tract. C57BL/6J control mice cleared the infection by day 26 while *Plac8* knockout mice cleared at day 47. Clearance kinetics were identical through the first three weeks of infection before diverging during clearance of the final ~1000 IFU/swab from the genital tract. Addition of *iNOS* inhibitor MLA to the drinking water post-clearance on day 52 led to transient recovery of viable *C. muridarum* from *Plac8* knockout mice on days 56 & 59, but not from C57BL/6J mice at any time point. With MLA treatment all the *Plac8* knockout mice shed viable *Chlamydia* on at least one day; two mice on a single day (either day 56 or day 59), and four mice on both days. These results mirror previously published experiments done by Ramsey *et al* with *iNOS* knockout mice where cyclophosphamide treatment (lymphocyte/neutrophil depletion) post-clearance led to transient recovery of viable *C. muridarum* from *iNOS* knockout mice but not C57BL/6J control mice (27). Our data combined with those of Ramsey *et al* argue that sterilizing immunity in the genital tract is dependent on two separate mechanisms; one mechanism dependent on *iNOS*, the other mechanism dependent on *Plac8*. Clearance of transient *C. muridarum* shedding in *iNOS* and *Plac8* knockout mice in post-clearance experiments with cyclophosphamide and MLA respectively argues for a third mechanism, possibly the antibody-dependent mechanism described by Morrison *et al* (4, 28).

To further investigate the role of *Plac8* in clearance of genital tract infections, C57BL/6J and *Plac8* knockout mice were pretreated with medroxyprogesterone on day T-7, continuous MLA was added to the drinking water at day T-1, and then the mice were infected vaginally on day 0 with $5\times10^4$ IFU of *C. muridarum*. Mice were swabbed thrice weekly through day 56 post-infection; recovered IFU were titered on McCoy monolayers (Figure 7). *Plac8* knockout mice treated with MLA were markedly compromised in their ability to clear *C. muridarum* compared to C57BL/6J mice under the same experimental conditions. Clearance kinetics diverged after day 14, with all C57BL/6J mice clearing the infection by day 40 compared to only 1 of 8 *Plac8* knockout mice clearing the infection by the day 56. In addition to the absolute difference in final clearance, there is a difference in the slope of the clearance curve with the *Plac8* clearance curve being nearly flat from day 21 through the end of the experiment. Clearance of *C. muridarum* from one *Plac8* knockout mouse on the last day of the experiment raises the possibility that *Plac8* knockout mice treated with MLA would clear genital tract infections over a longer time course. Two *Plac8* knockout mice infected with continuous MLA drinking water experienced an inflammatory response that

sealed the vaginal vault, making it impossible to do sequential monitoring of *Chlamydia* shedding. This outcome is unique in the authors' experience with the *C. muridarum* mouse model. These mice were killed at day 28 due to increasing abdominal girth. Both mice appeared to be well by activity level but were judged to be at risk for acute decompensation. Gross reproductive tract pathology in these mice is shown in Figure 8. Both *Plac8* knockout mice had gross distortion of the genital tract anatomy with pan dilatation, hydrouterus or hemouterus, and severe hydrosalpinx. The lumenal fluid and spleen were harvested from the second mouse and *C. muridarum* quantified on McCoy monolayers. Significant IFUs were recovered from the hydrosalpinx fluid (25,000 IFU/ml), and from the spleen (8,400 IFU) suggesting dissemination and extra-genital tract replication when both the *iNOS*-dependent and the *Plac8*-dependent mechanisms are compromised.

Assessment of macroscopic genital tract pathology for the experiments shown in figures 6 and 7 showed no difference between C57BL/6J mice and *Plac8* knockout mice when MLA was administered on post-infection day 52 (Delayed MLA), but a significant difference when MLA was administered concurrently with infection (Initial MLA) (see Figure 9).

### CD4 T cell clones that do not express Plac8 have no apparent defect in degranulation

Our previous study showed that the *iNOS*-independent mechanism utilized by uvmo-2 & uvmo-3 was dependent on T cell degranulation. In neutrophils, *Plac8* localizes to the granules. It is unclear from existing data whether *Plac8* is in granules or regulates trafficking of granules (*see discussion*). With respect to degranulation-dependent mechanism utilized by uvmo-2 & uvmo-3, the two simplest explanations for *Plac8* function would be either directly as an antimicrobial effector molecule analogous to human granulysin, or indirectly as a regulator of events in degranulation, *i.e.* participation in delivery of an unidentified effector molecule. To test whether T cells that do not express *Plac8* are deficient in degranulation, we compared the degranulation capacity of our C57BL/6 derived $Plac8^{+}$ CD4 T cell clones uvmo-2 & uvmo-3 to our $Plac8^{-}$ CD4 T cell clones spl4-10 & uvmo-4. The T cell clones were degranulated with PMA/A23187 and PMA/A23187 plus anti-CD3. Degranulation was measured as % granzyme B release as measured by ELISA (Figure 10). The four CD4 T cell clones had a wide range of intracellular granzyme B levels (26–390 pg/ml). There was no obvious defect in degranulation in any of the CD4 T cell clones, or any correlation of degranulation efficiency with presence or absence of *Plac8* expression. *Plac8* does not appear to have a role in T cell degranulation.

## Discussion

Data presented in this study establish that there are two redundant mechanisms for clearance of *C. muridarum* from the genital tract. The first mechanism is dependent on T cell & IFN-γ–mediated up regulation of epithelial *iNOS* transcription and nitric oxide production as previously described in the literature by Igietseme *et al* (29). The second mechanism is dependent on *Plac8*, and likely on T cell degranulation based on our *in vitro* studies (22). The existence of an *iNOS*-independent mechanism capable of clearing *C. muridarum* from the genital tract explains longstanding confusion about the role of *iNOS* in protective immunity. Mice deficient in *iNOS* clear *C. muridarum* because they still have the *Plac8*-dependent mechanism; conversely, *Plac8*-deficient mice clear infection because they still have the *iNOS*-dependent mechanism. In this study we showed that dual deficient mice, genetically deficient in *Plac8* and pharmacologically deficient in *iNOS* activity (nitric oxide), were severely compromised in their ability to resolve a *C. muridarum* genital tract infection over 8 weeks. In addition, we showed that *Plac8* contributes to sterilizing immunity as live *C. muridarum* could be recovered from *Plac8* knockout mice that had cleared genital tract infections by administration of an *iNOS* inhibitor (MLA). That result combined with a previous study showing that cyclophosphamide treatment led to recovery

of viable *C. muridarum* from *iNOS* knockout mice that had cleared a genital tract infection argues that sterilizing immunity requires both *iNOS*-dependent and *Plac8*-dependent mechanisms.

Data from our *in vitro* studies and others *in vivo* studies strongly suggest that clearance of *Chlamydia* from the genital tract is not dependent on physical killing of infected epithelial cells. We have previously shown that three *Chlamydia*-specific CD4 T cell clones that were potent terminators of *C. muridarum* replication in epithelial cells recognized infected cells late in the replication cycle (22). Those clones did not lyse 18 h infected epithelial cells (optimal recognition time point) in 4 h killing assays, but eventually lysed the epithelial monolayer after the reticulate body (RB) to elementary body (EB) transition was complete. On that basis we concluded that cytolysis was not likely critical for these clones to terminate infection, and that termination of infection required a direct antimicrobial attack on EB. The *iNOS*-independent CD4 T cell-mediated termination of infection was inhibited by phenyl arsenine oxide (PAO), an inhibitor of T cell degranulation, suggesting delivery of an anti-*Chlamydia* effector molecule. That data combined with *in vivo* studies showing that perforin knockout mice, *FasL* knockout mice, and perforin-*Fas* dual knockout mice clear infections with normal kinetics argues in favor of clearance mechanisms that are independent of cytolysis.

In the current study we have additional data that support a non-cytolytic mechanism for T cell-mediated clearance of genital tract infections. CD4 T cell clones that are potent terminators of *Chlamydia* replication in epithelial cells turn out to have low levels of perforin, while less potent *Chlamydia*-specific CD4 T cell clones have high levels of perforin. On that basis it does not appear that high perforin expression correlates with terminating infection in epithelial cells. The small amounts of perforin present in perforin low CD4 T cell clones must be sufficient to deliver an anti-*Chlamydia* effector molecule to the epithelial cytosol, or there may be a perforin independent pathway. Our previous report showed that PAO and concanamycin A prevented potent CD4 T cell clone-mediated termination of *Chlamydia* replication in epithelial cells. Concanamycin A is an H+/ATPase inhibitor that causes perforin degradation by raising the pH of T cell granules. We equivocated about interpreting the concanamycin A (CMA) results because CMA was also a potent inhibitor of *C. muridarum* replication necessary for T cell recognition of infected cells. It is possible that concanamycin A elevation of pH in T cell granules may also cause degradation of molecules other than perforin, including the anti-*Chlamydia* effector molecule.

The biological function of *Plac8* is unclear from existing data. *Plac8* is a small 112 aa cysteine-rich protein originally described as being enhanced in the reproductive tract (30). *Plac8* is expressed by macrophages, neutrophils, B cells, T cells and epithelial cells (lung & small bowel specifically), and has a highly conserved human homolog (26). Some studies have linked *Plac8* to hematopoetic cell survival (31–33), though that biology did not appear to be relevant for neutrophils from wild type and *Plac8* knockout mice (26). *Plac8* knockout mice were more susceptible to *Klebsiella pneumoniae* peritonitis, and their neutrophils were compromised in their ability to kill ingested bacteria *in vitro.* In neutrophil fractionation experiments *Plac8* localized to granules and was not found in the cytosol. SDS-PAGE eletrophoretic mobility suggested that *Plac8* was not processed (26). *Plac8* does not have a conventional signal sequence. There is a potential signal peptide cleavage site between amino acids 17 and 18, but only 7% of known mammalian cleavage sites share these amino acid sequence characteristics (SignalP; http://www.cbs.dtu.dk/services/SignalP/). Our data and the existing *Klebsiella* data are most easily explained by one of two scenarios. *Plac8* is either an antimicrobial effector molecule analogous to human granulysin, or it is a critical component of the pathway that delivers or activates an anti-*Chlamydia* effector molecule in

the cytosol of infected epithelial cells. In this report we showed that *Plac8* does not appear to have a role in T cell degranulation. It is attractive to speculate that reactive sulfhydryl groups on cysteine-rich *Plac8* react with cysteine-rich OmcA & OmcB in the *Chlamydia* EB outer membrane leading to neutralization. Our data show that *Plac8* is not ubiquitously expressed by CD4 T cells. Limited by an n=4, it appears that CD4 T cells that express *Plac8*, $CD4_{Plac8}$, utilize both iNOS-dependent and degranulation-dependent mechanisms for terminating *C. muridarum* replication in epithelial cells, while $Plac8^{-}$ CD4 T cells appear to be dependent on nitric oxide. It is plausible that dual effector *Chlamydia*-specific $CD4_{Plac8}$ T cells are the optimal protective subset in the genital tract.

The susceptibility of *Plac8* knockout mice to *C. muridarum* genital tract infections is likely due to a defect in T cell immunity because nude mice sufficient in neutrophils, *Plac8*, and *iNOS* are incapable of clearing primary *C. muridarum* primary genital tract infections (34), time to clearance in wild type mice is not affected by neutrophil depletion (35, 36), and clearance is entirely dependent on MHC class II- restricted T lymphocytes (7). The details of how *Plac8* is critical for cellular immunity to *Chlamydia* remain to be determined. It is interesting to note that both *iNOS*- and *Plac8*-dependent mechanisms for terminating *Chlamydia* replication in epithelial cells are not likely to be cytolytic mechanisms that expose intracellular bacteria to extracellular effector mechanisms such as neutralizing antibodies or neutrophils. Rather both mechanisms appear to kill *Chlamydia* within intact epithelial cells. In this “toxic bag” model, *Chlamydia* is trapped and neutralized within the infected cells by a combination of nitric oxide and *Plac8* or a *Plac8*-dependent effector. This type of mechanism is intellectually satisfying as the majority of CD4 T cells appear to recognize infected epithelial cells after the noninfectious RB to infectious EB transition has occurred (23). Because *Plac8* is also expressed by epithelial cells that are susceptible to infection, we speculate that it exists in an inactive state in those cells. It is possible that CD4 T cells not only deliver active *Plac8* to infected cell cytosol, but that active *Plac8* or other injected T cell molecule triggers activation of epithelial *Plac8* in an amplified final pathway.

In this study CD4 T cell conditioned media was a very potent inhibitor of *C. muridarum* replication in murine epithelial cells through an *iNOS*-dependent mechanism; greater than two logs more potent than recombinant IFN-γ in our previous studies and those of others. This finding suggests that *in vitro* studies using recombinant IFN-γ (22, 37–39) may have significantly underestimated the contribution of cytokines to controlling *C. muridarum* replication in murine cells due either to lesser biologic activity of recombinant IFN-γ or more likely, contributions of additional cytokines such as TNF-γ to a cytokine-mediated anti-*Chlamydia* epithelial response. The *iNOS* promoter once activated by IFN-γ can be further up regulated by TNF-α (40). TNF-α has been shown to be an important cytokine during genital tract clearance (20) and in protective immunity induced by *Chlamydia* T cell vaccines (10, 41). Scurlock *et al* in a recent paper focused on possible IL-17 contributions to *Chlamydia* immunity showed that IFN-γ and TNF-γ in genital tract secretions of *C. muridarum* infected mice peaked on day 4 and were essentially undetectable by day 14 (42). Doxycycline experiments suggest that adaptive immunity takes hold about day 10 post-infection (43). We propose a working model in which early innate immunity in a genital tract awash with cytokines including IFN-γ & TNF-α clears 2–3 logs of *C. muridarum* during the first 10 days of infection, and that clearance of the last 3–4 logs of *C. muridarum* by the adaptive cellular immune response can be accomplished by either cytokine-dependent (*iNOS*) or *Plac8*-dependent mechanisms; sterilizing cellular immunity requires both *iNOS*- & *Plac8*-dependent mechanisms.

Identifying the *Plac8*-dependent mechanism for clearing *Chlamydia* from the reproductive tract resolves longstanding confusion about the role of *iNOS* in protective immunity to *Chlamydia* genital tract infections by identifying a redundant second clearance mechanism.

Investigating the $CD4_{Plac8}$ T cell subset will likely contribute to our understanding of protective immunity, and hopefully identify surrogate biomarkers for protective immunity that prove useful during rational vaccine development. *Casd1* is an uncharacterized open reading frame predicted to be a transmembrane glycoprotein with an extracellular domain. We are investigating *Casd1* as a possible T cell accessory molecule, and potentially a convenient biomarker for the $CD4_{Plac8}$ T cell subset.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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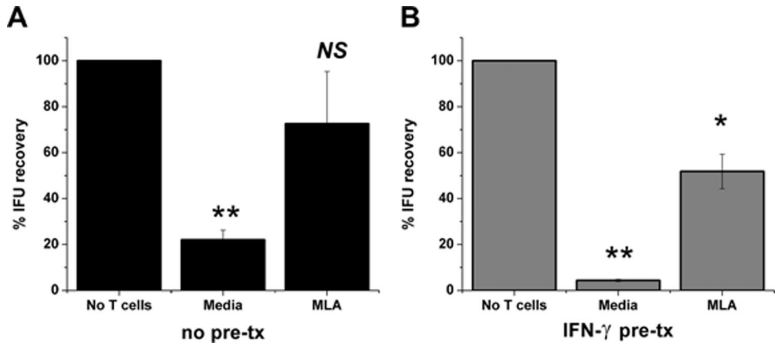


**Figure 1.**

Uvmo-4 is an iNOS-dependent CD4 T cell clone. C57epi.1 epithelial cells untreated (panel A)or pretreated for 10 h with IFN-β (panel B), in the absence or presence of iNOS inhibitor MLA, were infected with *C. muridarum* at 3 IFU per cell. Inocula were removed 4 h later and infected epithelial cells co-cultured without and with $1.5\times10^5$ uvmo-4 T cells in the absence of MLA (Media), in the presence of MLA (MLA). 32 h post infection wells were harvested and *C. muridarum* quantified on McCoy monolayers. Means and SD from one experiment done in triplicate; statistical comparisons were made to the no T cell control wells set at 100%. * = *p value* < 0.05; ** = *p value* < 0.005; *NS* = not statistically significant.

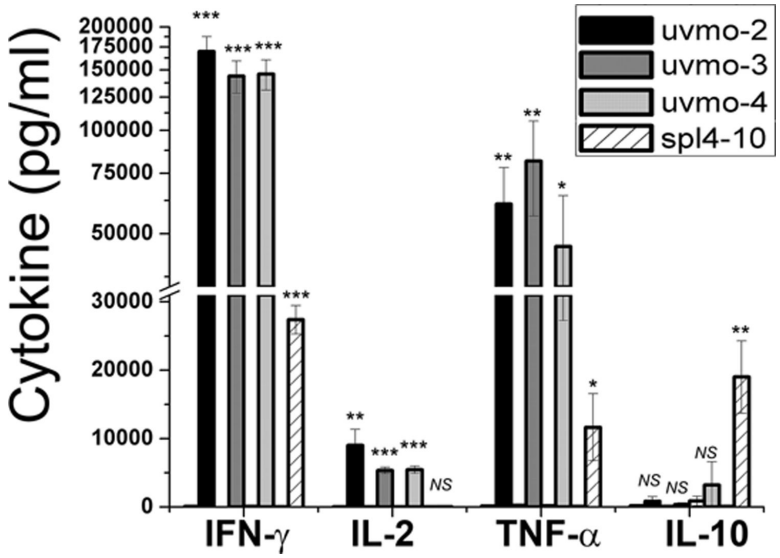


**Figure 2.**

Multifunctional *Chlamydia*-specific CD4 T cell clones with differing cytokine profiles. $5\times10^4$ T cells were cultured, without activation or activated by immobilized anti-CD3 monoclonal antibody, in RPMI media containing 1 ηg/ml IL-7. 20 h later supernatants were collected and analyzed for individual cytokines by ELISA. Cytokine production in media alone was negligible for all clones (graphed but not easily visualized to left of each colored or hatched bar). Aggregate means and SEM from two independent experiments; statistical comparisons were made to the media control. * = *p value* < 0.05; ** = *p value* < 0.005; *** = *p value* < 0.0005. (Note that scale is linear to 30,000; logarithmic thereafter).

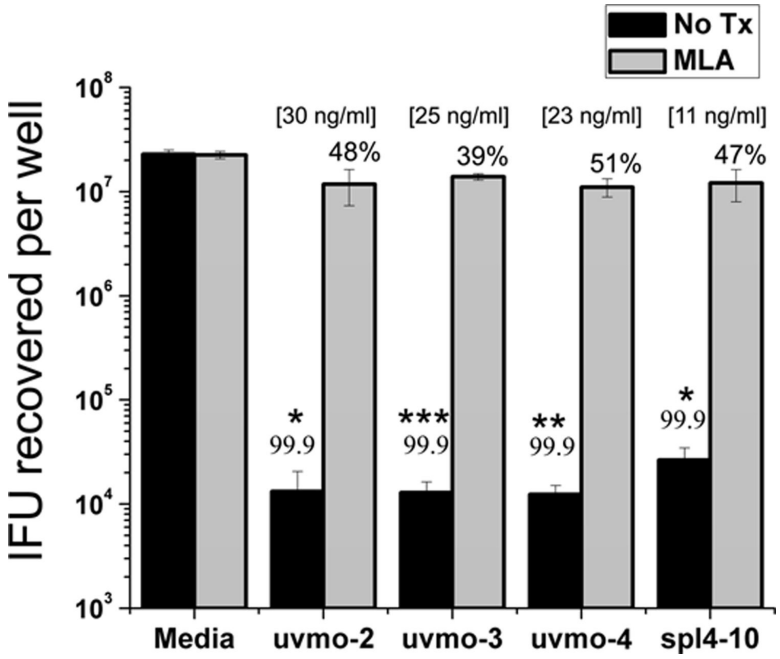


**Figure 3.**

The role of T cell cytokines and iNOS in controlling *C. muridarum* replication in epithelial cells. Fresh T cell clone conditioned culture supernatant (40% vol/vol), without and with 1mM MLA, was exchanged for the infecting inocula 4 h post-infection of C57epi.1 epithelial monolayers with *C. muridarum* (3 IFU per cell). 32 h later the wells were harvested and *C. muridarum* quantified on McCoy monolayers. Concentration of IFN-γ in 40% conditioned media for each clone is shown in brackets. Percent inhibition of *C. muridarum* replication is shown immediately above each bar. Black bars = no treatment; gray bars = 1mM MLA. Mean and standard deviations from one experiment done as triplicates; statistical comparisons were made between untreated and MLA-treated wells for each clone. * = *p value* < 0.05; ** = *p value* < 0.005; *** = *p value* < 0.0005.

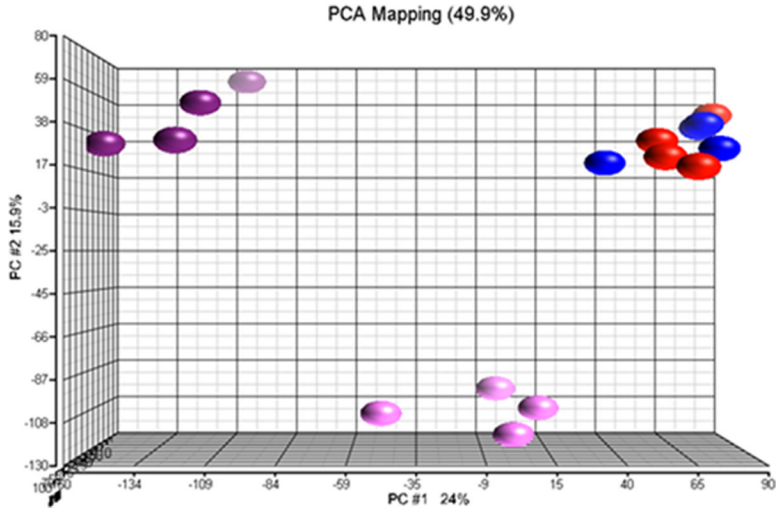


**Figure 4.**

Principal Component Analysis of the gene expression micro array data for the four CD4 T cell clones. **Red** = uvmo-2; **blue** = uvmo-3; **light purple** = uvmo-1; **dark purple** = spl4-10.

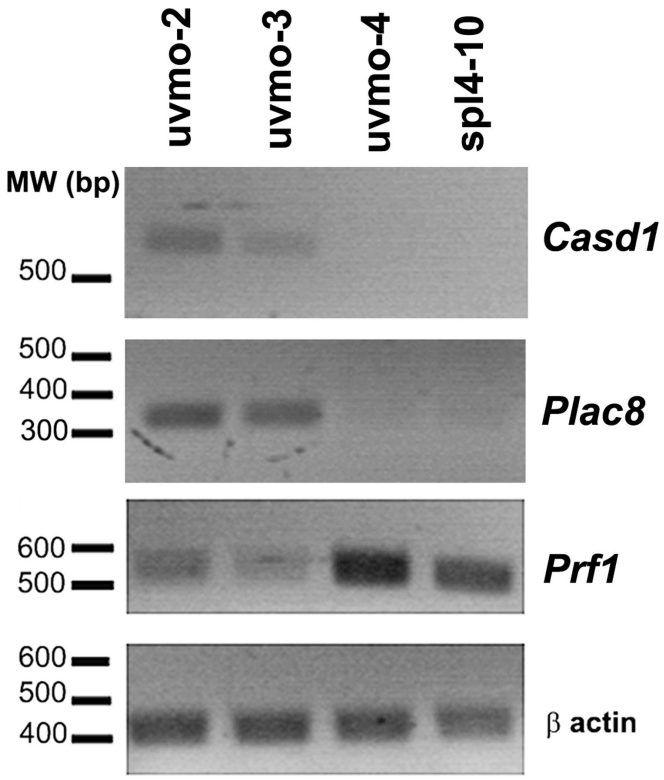


**Figure 5.**
RT-PCR confirmation of micro array data. mRNA levels for *Casd1*, *Plac8*, perforin (*Prf1*), and β actin were analyzed using an independent total RNA isolation for each CD4 T cell clone. RT-PCR products were run on a 2% ethidium bromide agarose gel; molecular weight markers on the left margin. Image shown is inverted for presentation purposes.

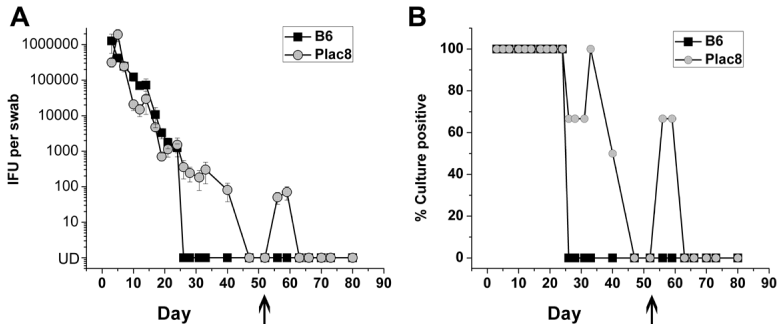


**Figure 6.**

*Plac8* is required for sterilizing immunity. Wild type C57BL/6J and *Plac8* female knockout mice in experimental groups of 6 mice each were treated with medroxyprogesterone, then infected vaginally one week later with $5\times10^4$ IFU of *C. muridarum*. On day 52 (arrow), regular drinking water was replaced with water containing 50 μM MLA (iNOS inhibitor). Genital tract shedding was monitored through day 80 post-infection. Data presented are from one experiment; differences in the clearance kinetics for C57BL/6J *versus Plac8* knockout mice were statistically significant with an overall *p value* of 0.003.

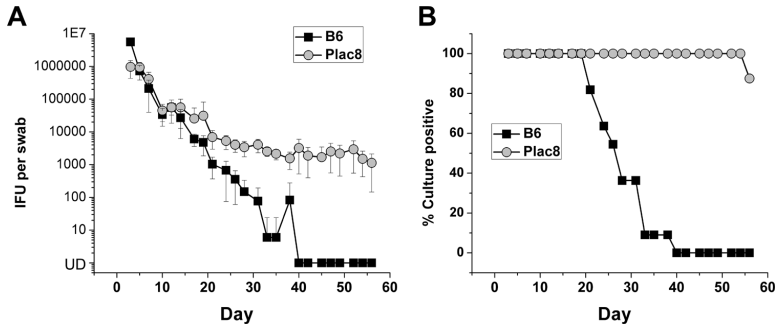


**Figure 7.**

Inhibition of iNOS at initiation of infection compromises clearance of *C. muridarum* from the genital tract of *Plac8* knockout mice. Wild type C57BL/6J (11 mice) and *Plac8* knockout (10 mice) female mice were treated with medroxyprogesterone, then infected vaginally one week later with $5\times10^4$ IFU of *C. muridarum*. The drinking water contained 50 μM MLA (iNOS inhibitor) on the day before infection through day 56 post-infection. Genital tract shedding was monitored through day 56 post-infection. Two of the ten *Plac8* knockout mice could not be monitored due to inflammatory obstruction of the vaginal vault; eight *Plac8* knockout mice were included in the final data set. Aggregate data from two experiments; differences in the clearance kinetics for C57BL/6J *versus Plac8* knockout mice were statistically significant with an overall *p value* of <0.0001.

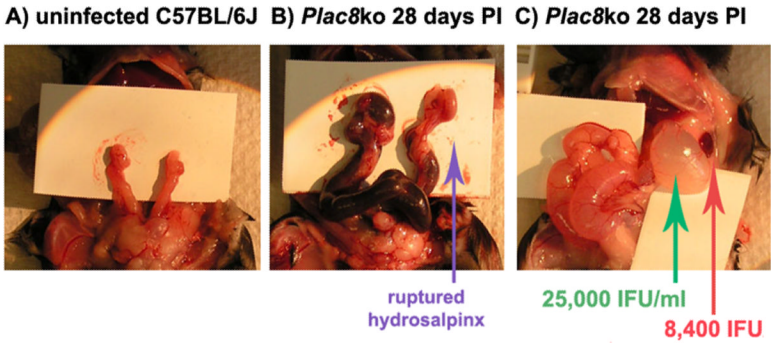


**Figure 8.**

Severe pathology in *Plac8* knockout mice treated with MLA at time of vaginal infection with *C. muridarum*. The two *Plac8* knockout mice that could not be monitored in the experiment shown in figure 7 were killed on day 28 post-infection and compared to an uninfected C57BL/6J mouse. Uterine horns, oviducts, and ovaries were exposed by dissection. Recoverable *C. muridarum* in hydrosalpinx fluid (*green*) and spleen (*red*) was determined for the mouse in panel C.

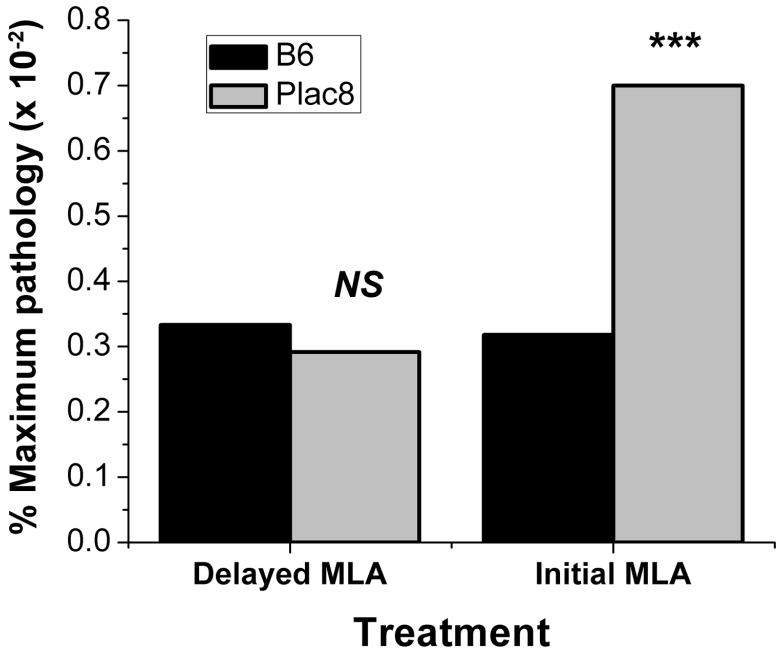


**Figure 9.**

Mice in delayed-MLA (figure 6) and initial-MLA (figure 7) infection experiments were scored for macroscopic pathology at the end of those experiments. Statistical comparisons were made between the C57BL/6J and *Plac8* knockout mice for each experiment. *** = *p value* <0.0005; *NS* = not statistically significant.

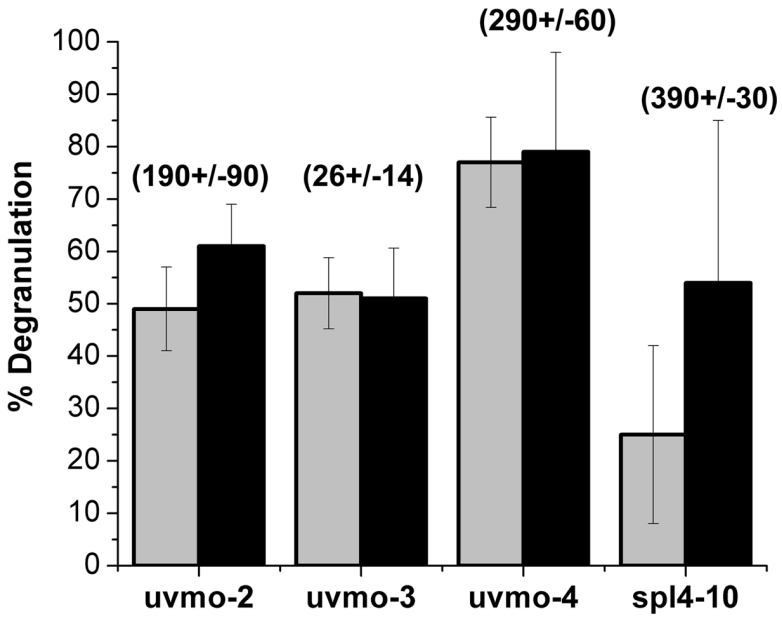


**Figure 10.**

*Plac8* expression is not required for degranulation. The CD4 T cell clones were degranulated with either PMA & A23187 (*gray bars*) or PMA & A23187 + immobilized anti-CD3 (*black bars*). There was no correlation between *Plac8* expression (uvmo-2,-3) or lack of expression (uvmo-4, spl4-10) and the ability to degranulate. Maximal release of granzyme B in ρg/ml for each T cell clone is shown in parentheses. Aggregate data from two independent experiments with the SEM; comparisons made between the uvmo-2 & uvmo-3 *versus* uvmo-4 & spl4-10 were not statistically significant.

## Table 1

RT-PCR primers

| gene | Sense primer | Antisense primer | Product size (bp) | Total RNA in amplification (vg) |
|---|---|---|---|---|
| *Casd1* | GGGAGATCAACCACTACTTCAG | GACCCAATAAACATCACTAGTC | 590 | 250 |
| *Plac8* | ATGGCTCAGGCACCAACAGTTA | GAAAGCGTTCATGGCTCTCCTC | 336 | 250 |
| *Prfl* | TGGATGTGAACCCTAGGCCAGAG | AAGTACTTCGACGTGACGCTCAC | 515 | 100 |
| β actin | ATGGATGACGATATCGCTGCGC | CGTACATGGCTGGGGTGTTGAA | 400 | 100 |

**Table 2**

Biomarker and Mechanism Genes

| Gene Symbol | max average signal | *p value uvmo-2/3 vs. uvmo-4* | Fold-change*uvmo-2/3 vs. uvmo-4* | *p value uvmo-2/3 vs. spl4-10* | Fold-change*uvmo-2/3 vs. spl4-10* |
|---|---|---|---|---|---|
| ***Casd1*** | 9.15 | 1.58E-16 | **31.68** | 1.11E-16 | **35.15** |
| ***Plc8*** | 11.30 | 2.33E-16 | **138.39** | 1.40E-16 | **171.60** |
| ***Prf1*** | 11.17 | 1.30E-11 | − **22.62** | 1.80E-12 | − **39.88** |