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Plac8-Dependent and Inducible NO Synthase-Dependent Mechanisms Clear Chlamydia muridarum Infections from the Genital Tract

Raymond M. Johnson,* Micah S. Kerr,* and James E. Slaven†

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 C. 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 Abs are the immune parameter that correlates with protective immunity against primary infections. Existing data from the Chlamydia muridarum mouse model suggest that immunity to urogenital serovars of Chlamydia is complex. Passive transfer of Abs 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 Ag presentation is dispensable for protective host immunity whereas MHC class II is absolutely required (7). Recent data in experimental mouse models argue for a supportive role for Abs 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 and 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 inducible NO synthase (iNOS), IFN-γ, and T cell/epithelial cell contact via LFA-1/ICAM-1 (11–14). However, follow-up studies showed that 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 and Fas ligand/Fas were also able to clear C. muridarum genital tract infections with normal kinetics (19). Additional knockout mice and Ab 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.

*Department of Medicine, Indiana University School of Medicine, Indianapolis, IN 46202; and †Department of Biostatistics, Indiana University School of Medicine, Indianapolis, IN 46202

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Address correspondence and reprint requests to Dr. Raymond M. Johnson, Division of Infectious Diseases, Department of Medicine, Indiana University School of Medicine, 635 Barnhill Drive, MS 224, Indianapolis, IN 46202. E-mail address: raymjohn@iupui.edu

The online version of this article contains supplemental material.

Abbreviations used in this article: EB, elementary body; IFU, inclusion-forming unit; iNOS, inducible NO synthase; MLA, N-monomethyl-L-arginine; PMO, phenyl arsenic oxide; RMA, robust multiarray analysis; SPG, sucrose-phosphate-glutamic acid.

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Our laboratory 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), whereas 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 phenylarsenic 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 of the Chlamydia-specific CD4 T cell clones were Th1, and they made IFN-γ when activated and were capable of recognizing infected epithelial cells (23).

To further investigate these findings, we compared two iNOS-independent CD4 T cell clones to two iNOS-dependent CD4 T cell clones using gene expression microarrays. Microarray analysis identified a candidate second mechanism dependent on Plac8 for terminating C. muridarum replication in epithelial cells that was then investigated in vivo utilizing an existing Plac8 knockout mouse. Plac8 knockout mice were significantly compromised in their ability to clear C. muridarum genital tract infections.

Materials and Methods

Mice

Four- to five-week-old female C57BL/6 and B6.129S6-Plac8tm1Bhk/J (stock no. 009598) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were housed in Indiana University–Purdue University Indianapolis specific pathogen-free facilities. The Indiana University–Purdue University Indianapolis 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 C. muridarum (Nigg), previously known as C. trachomatis strain mouse pneumonitis, was grown in McCoy cells as previously described (23).

Cytokine ELISAs

T cell clones (5 × 10^5) were activated in 96-well tissue culture plates by immobilized anti-CD3 mAb 145-2C11 (BD Biosciences, San Jose, CA), 0.5 µg/ml in PBS overnight at 4 °C (washed once), in RPMI 1640 media containing 1 ng/ml recombinant murine IL-7 (R&D Systems, Minneapolis, MN). Relative levels of IFN-γ, TNF-α, IL-2, and IL-10 in culture supernatants were determined by ELISA using capture and biotinylated mAb pairs with recombinant murine standards according to the manufacturers’ protocols (IFN-γ ELISA, XMG1.2; IL-2 ELISA, 5H4/1A12 [Pierce–Endogen; Rockford, IL]; TNF-α ELISA, TN3-19.12/C11.150-14; IL-10 ELISA, JESS-2AS/SXC-1 [BD Biosciences]). Detection was accomplished with streptavidin-HRP (BD Biosciences) and tetramethylbenzidine substrate (Sigma-Aldrich, St. Louis, MO).

Gene expression microarray analysis

Chlamydia-specific CD4 T cell clones uvmo-2, uvmo-3, uvmo-4, and spl4-10 were purified by Ficoll-Hypaque ( Histopaque 1083; Sigma-Aldrich) at the end of their culture cycle and then grown for 3 days in their usual culture media including growth factors, without Ag stimulation. On day 3, total RNA was isolated from each T cell clone using a protocol that included an RNase-free DNase I treatment step (RNasey, 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 Whole Transcript Sense Target Labeling and Control Reagents kit according to the Affymetrix user manual, that is, the GeneChip Whole Transcript Sense Target Labeling Assay Manual. Individual labeled samples were hybridized to the Mouse Gene 1.0 ST GeneChips for 17 h and then washed, stained and scanned with the standard protocol using Affymetrix GeneChip Operating System. The GeneChip Operating System was used to generate data (CEL files). Arrays were visually scanned for abnormalities or defects. CEL files were imported into Partek Genomic Suite (Partek, St. Louis, MO). Robust multiarray analysis (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 transformed. These log-transformed signals were used for principal components analysis, hierarchical clustering, and signal histograms to determine whether there were any outlier arrays. Untransformed RMA signals were used for fold change and untransformed one-way ANOVA using log-transformed signals for all four CD4 T cell clones, and contrasts were made comparing iNOS-dependent uvmo-4 and spl4-10 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 downregulated 5-fold with p values of <0.001 for either uvmo-4 or spl4-10 compared with uvmo-2/uvmo-3 were considered in the final analysis (Supplemental Table I).

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 d in their usual culture media including growth factors, without Ag stimulation. On day 3, 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 amplification were performed using AMV reverse transcriptase/Taq 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 for 2 min; 3) 95 °C for 30 s; 4) 56 °C for 20 s; 5) 72 °C for 30 s; 6) go to step 3 for 37 times; 7) 72 °C for 7 min; and 8) hold at 4 °C using an MJ Research J2000 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 are shown in Table I. RT-PCR reactions were run on 2% agarose gels with ethidium bromide; images are inverted for presentation. The "no RT" controls generated no RT-PCR products (data not shown). PCR primers used are shown in Table I.

Epithelial cell infections, Chlamydia replication, and titration

C57epi.1 cell monolayers in 48-well plates were untreated or treated with IFN-γ (10 ng/ml) for 10 h prior to infection. Wells were infected with 3 inclusion-forming units (IFU) per cell. After addition of C. muridarum the plates were spun at 300 × g for 30 min. Four hours postinfection, the inoculums were removed and CD4 T cell clones were added in RPMI 1640 T cell medium. Thirty-two hours postinfection, wells were scraped, harvested with an equal volume of sucrose-phosphate-glutamic acid (SPG) buffer, and stored at −80 °C until C. muridarum titers were determined on McCoy cell monolayers using an anti-Chlamydia LPS Ab 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 medroxyprogesterone delivered s.c. (Depo-Provera; Pfizer Pharmaceuticals, New York, NY) and were inoculated intravaginally with 10^6 C. muridarum CFU. Vaginal lavage was collected daily from zero to 10 days postinfection, and the numbers of C. muridarum were determined by quantitative culture (23).

Table I. RT-PCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense Primer (3′ → 5′)</th>
<th>Antisense Primer (3′ → 5′)</th>
<th>Product Size (bp)</th>
<th>Total RNA in Amplification (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casd1</td>
<td>GGGGATCTACACCTACTCTCAG</td>
<td>GACCCATAATACCTCTACTGA</td>
<td>590</td>
<td>250</td>
</tr>
<tr>
<td>Plac8</td>
<td>ATGGCTCTAGGACAAACGTTTA</td>
<td>AAAAAAGCTCCATAGTCTCTTCT</td>
<td>356</td>
<td>250</td>
</tr>
<tr>
<td>Prf1</td>
<td>TGAGTGACAGCCTCTAGGCGAG</td>
<td>AAGGACTTCGACGAGCTGGCTT</td>
<td>515</td>
<td>100</td>
</tr>
<tr>
<td>β-actin</td>
<td>AATGGATGAGCTGAACAGTCTG</td>
<td>CTACAGTGGGTTGTTGGAAG</td>
<td>400</td>
<td>100</td>
</tr>
</tbody>
</table>
of C. muridarum 23). The uvmo-2 and uvmo-3 T cell clones are potent terminators of T cell clones, including uvmo-2, uvmo-3, uvmo-4, and spl4-10 (22, 23). We previously characterized a panel of 10 Chlamydia C. muridarum biovar Nigg in 10 μl SPG buffer. Mice were swabbed Monday, Wednesday, and Friday to monitor the clearance of C. muridarum from the genital tract as previously described (25).

Pathology scoring
A simple scoring system was used to assess macroscopic genital tract pathology. Hydrodistalpinx was scored as 0, 1, or 2 reflecting no, unilateral, or bilateral hydrodistalpinx, respectively. Mice have a bifed uterus (two 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
CD4 T cell clones (5 × 10^4) were incubated in wells with media, 10 μg/ml PMA (Sigma-Aldrich), and 500 ng/ml calcium ionophore A23187 (Sigma-Aldrich), PMA and A23187 plus immobilized anti-CD3 (0.5 μg/ml; 50 μl PBS overnight at 4˚C) (145-2C11; BD Biosciences), or media containing 0.1% Triton X-100 for 4 h at 37˚C. Culture supernatants were collected and levels of granzyme B were determined using a mouse granzyme B ELISA (mouse granzyme B DuoSet; R&D Systems). To determine percentage degranulation, the following equation was used: % degranulation = [(measured value in experimental well – spontaneous release (media)]/ [Triton X-100 value (maximal release) – spontaneous release)] × 100.

Statistical analyses
Summary figures for each experimental investigation are presented as means and SD or as “pooled” means and SEM. Figure legends indicate the number of independent experiments pooled to generate each figure. A Student two-tailed t test was 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 ANOVA 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 microarray analyses
We previously characterized a panel of 10 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 they 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, and 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 and 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 used 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 ng/ml IFN-γ in the absence or presence of 1 mM MLA were infected with C. muridarum for 4 h, then cocultured without and with uvmo-4 at a T cell/epithelial cell ratio of 0.75:1. Thirty-two hours after 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 (Fig. 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 NO. Identification of uvmo-4 as an iNOS-dependent CD4 T cell

FIGURE 1. Uvmo-4 is an iNOS-dependent CD4 T cell clone. C57epi.1 epithelial cells untreated (A) or pretreated for 10 h with IFN-γ (B), in the absence or presence of iNOS inhibitor MLA, were infected with C. muridarum at 3 IFU/cell. Inocula were removed 4 h later and infected epithelial cells were cocultured without and with 1.5 × 10^3 uvmo-4 T cells in the absence of MLA (Media) or in the presence of MLA. Thirty-two hours after infection wells were harvested and C. muridarum was quantified on McCoy monolayers. Means and SD are from one experiment done in triplicate; statistical comparisons were made to the no T cell control wells set at 100%. *p < 0.05, **p < 0.005.

Pathology scoring
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FIGURE 2. Multifunctional Chlamydia-specific CD4 T cell clones with differing cytokine profiles. T cells (5 × 10^4) were cultured, without activation or activated by immobilized anti-CD3 mAb, in RPMI 1640 media containing 1 ng/ml IL-7. Twenty hours 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 are from two independent experiments; statistical comparisons were made to the media control. *p < 0.05, **p < 0.005, ***p < 0.0005. (Note that scale is linear to 30,000 and is logarithmic thereafter.)
monolayers. Concentration of IFN-γ was quantified on McCoy monolayers with C. muridarum (3 IFU/cell). Thirty-two hours later the wells were harvested and C. muridarum was quantified on McCoy monolayers. Concentration of IFN-γ in 40% conditioned media for each clone is shown in brackets. Percentage inhibition of C. muridarum replication is shown immediately above each bar. Black bars indicate no treatment; gray bars indicate 1 mM MLA. Mean and standard deviations are from one experiment done in triplicate; statistical comparisons were made between untreated and MLA-treated wells for each clone. *p < 0.05, **p < 0.005, ***p < 0.0005.

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% v/v), without and with 1 mM MLA, was exchanged for the infecting inocula 4 h postinfection of C57epi.1 epithelial monolayers with C. muridarum (3 IFU/cell). Thirty-two hours later the wells were harvested and C. muridarum was quantified on McCoy monolayers. Concentration of IFN-γ in 40% conditioned media for each clone is shown in brackets. Percentage inhibition of C. muridarum replication is shown immediately above each bar. Black bars indicate no treatment; gray bars indicate 1 mM MLA. Mean and standard deviations are from one experiment done in triplicate; statistical comparisons were made between untreated and MLA-treated wells for each clone. *p < 0.05, **p < 0.005, ***p < 0.0005.

1640 media and fresh cell-free–conditioned media from each clone (40% v/v in RPMI 1640 media) were exchanged for the epithelial media covering monolayers of C57epi.1 epithelial cells infected 4 h earlier with 3 IFU C. muridarum per cell. Thirty-two hours after infection the wells were harvested by adding SPG buffer and scraping. Replication of C. muridarum under each condition was quantified using McCoy monolayers (Fig. 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 ng/ml, which causes only a 2- to 3-fold reduction in recovered IFU per 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 NO production.

Gene expression microarrays 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, used 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, which was too late to catch C. muridarum in the noninfectious reticulate body stage (eclipse phase). Late recognition of infected epithelial cells by uvmo-2 and uvmo-3 argued against their termination mechanism being a function of earlier presentation of their nominal Ags (i.e., not early recognition). Because the uvmo-2 and 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 microarrays comparing iNOS-independent (uvmo-2 and uvmo-3) to iNOS-dependent (uvmo-4 and spl4-10) T cell clones. We recognized that microarray analyses of four T cell clones would not permit statistically significant conclusions about T cell mechanisms, but we postulated that the resulting data would suggest testable hypotheses. T cell clones at the end of their culture cycle were purified by Ficoll-Hypaque to remove cellular debris, washed, and then cultured for 72 h in media with usual

FIGURE 4. Principal components analysis of the gene expression microarray data for the four CD4 T cell clones: uvmo-2, red; uvmo-3, blue; uvmo-1, light purple; spl4-10, dark purple.

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growth cytokines. Total RNA was harvested and used for Affymetrix mouse ST1.0 gene expression microarrays. Four independent RNA isolations for four independent microarray experiments were done to minimize background noise. Fig. 4 shows principal components analysis from the microarrays comparing the four T cell clones. Interestingly, the two iNOS-independent CD4 T cell clones derived from different mice (uvmo-2 and uvmo-3) clustered tightly together and away from the iNOS-dependent clones (uvmo-4 and 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 I we list the 193 genes that differed ±5-fold comparing the individual expression patterns of spl4-10 and uvmo-4 to the combined expression pattern of uvmo-2 and uvmo-3. The genes that were the most intriguing to us and the primary focus of this study were those potentially useful as biomarkers or possibly involved in an effector mechanism (Table II). Uvmo-2 and 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 and spl4-10 had very low Casd1 and Plac8 levels and high levels of perforin. RT-PCR analysis confirmed the microarray data for Casd1, Plac8, and perforin in iNOS-independent (uvmo-2 and uvmo-3) and iNOS-dependent (uvmo-4 and spl4-10) CD4 T cell clones (Fig. 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 × 10^4 IFU 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 d postinfection and then assessed for genital tract pathology. Recovered IFU from genital swabs were titered on McCoy monolayers (Fig. 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 whereas Plac8 knockout mice cleared at day 47. Clearance kinetics were identical through the first 3 wk infection before diverging during clearance of the final ~1000 IFU/swab from the genital tract. Addition of iNOS inhibitor MLA to the drinking water postclearance on day 52 led to transient recovery of viable C. muridarum from Plac8 knockout mice on days 56 and 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 1 d, 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. (27) with iNOS knockout mice where cyclophosphamide treatment (lymphocyte/neutrophil depletion) postclearance led to transient recovery of viable C. muridarum from iNOS knockout mice but not C57BL/6J control mice. 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 postclearance experiments with cyclophosphamide and MLA, respectively, argues for a third mechanism, possibly the Ab-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 treated with medroxyprogesterone 1 wk prior to infection, continuous MLA was added to the drinking water 1 d prior to infection, and then the mice were infected vaginally with 5 × 10^4 IFU C. muridarum. Mice were swabbed thrice weekly through day 56 postinfection; recovered IFU were titered on McCoy monolayers (Fig. 7). Plac8 knockout mice treated with MLA were markedly compromised in their ability to clear C. muridarum compared with 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 with only one of eight 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

Table II. Biomarker and mechanism genes

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Maximum Average Signal</th>
<th>p Value of uvmo-2/3 Versus uvmo-4</th>
<th>Fold Change uvmo-2/3 Versus uvmo-4</th>
<th>p Value uvmo-2/3 Versus spl4-10</th>
<th>Fold Change uvmo-2/3 Versus spl4-10</th>
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<tbody>
<tr>
<td>Casd1</td>
<td>9.15</td>
<td>1.58 × 10^-16</td>
<td>31.68</td>
<td>1.11 × 10^-16</td>
<td>35.15</td>
</tr>
<tr>
<td>Plac8</td>
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<td>2.33 × 10^-16</td>
<td>138.39</td>
<td>1.40 × 10^-16</td>
<td>171.60</td>
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<tr>
<td>Prf1</td>
<td>11.17</td>
<td>1.30 × 10^-11</td>
<td>-22.62</td>
<td>1.80 × 10^-12</td>
<td>-39.88</td>
</tr>
</tbody>
</table>

FIGURE 5. RT-PCR confirmation of microarray 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 mass markers are on the left margin. Image shown is inverted for presentation purposes.
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 our 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 Fig. 8. Both Plac8 knockout mice had gross distortion of the genital tract anatomy with pan dilatation, hydrouterus or hemouterus, and severe hydrosalpinx. The luminal fluid and spleen were harvested from the second mouse and C. muridarum was quantified on McCoy monolayers. Significant IFU were recovered from the hydrosalpinx fluid (25,000 IFU/ml) and from the spleen (8,400 IFU), suggesting dissemination and extragenital tract replication when both the iNOS-dependent and the Plac8-dependent mechanisms are compromised.

Assessment of macroscopic genital tract pathology for the experiments in Figs. 6 and 7 showed no difference between C57BL/6J mice and Plac8 knockout mice when MLA was administered on postinfection day 52 (delayed MLA), but a significant difference was shown when MLA was administered concurrently with infection (initial MLA) (see Fig. 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 used by uvmo-2 and 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 used by uvmo-2 and 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, that is, 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 and uvmo-3 to our Plac8+/+ CD4 T cell clones spi4-10 and uvmo-4. The T cell clones were degranulated with PMA/A23187 and PMA/A23187 plus anti-CD3. Degranulation was measured as percentage of granzyme B release as measured by ELISA (Fig. 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 NO 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

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**FIGURE 6.** Plac8 is required for sterilizing immunity. Wild-type C57BL/6J and Plac8 female knockout mice in experimental groups of six mice each were treated with medroxyprogesterone and then infected vaginally 1 wk later with $5 \times 10^3$ IFU $C.~muri-\textit{daram}$. 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 postinfection. 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 $C.~muri-\textit{daram}$ 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 and then infected vaginally 1 wk later with $5 \times 10^3$ IFU $C.~muri-\textit{daram}$. The drinking water contained 50 μM MLA (iNOS inhibitor) on the day before infection through day 56 postinfection. Genital tract shedding was monitored through day 56 postinfection. Two of the 10 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 are 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.
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 (NO), were severely compromised in their ability to resolve a C. muridarum genital tract infection over 8 wk. Additionally, 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 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 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-Fasl dual knockout mice clear infections with normal kinetics argues in favor of clearance mechanisms that are independent of cytolysis.

In the present study we have additional data that support a noncytolytic 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, whereas 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 CD4 T cell clone-mediated termination of Chlamydia replication in epithelial cells. Concancamycin 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 results because concanamycin A 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 and small bowel specifically) and has a highly conserved human homolog (26). Some studies have linked Plac8 to hematopoetic cell survival (31–33), although 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 K. pneumoniae peritonitis, and their neutrophils were compromised in their ability to kill ingested bacteria in vitro. In neutrophil fractionation experiments Plac8 localized to granules.

**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 Fig. 7 were killed on day 28 postinfection and compared with an uninfected C57BL/6J mouse. Uterine horns, oviducts, and ovaries were exposed by dissection. Recoverable C. muridarum was isolated from the mouse. Uterine horns, oviducts, and ovaries were exposed by dissection. Recoverable C. muridarum was isolated from the mouse.

**FIGURE 9.** Mice in delayed MLA (Fig. 6) and initial MLA (Fig. 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 < 0.0005.

**FIGURE 10.** Plac8 expression is not required for degranulation. The CD4 T cell clones were degranulated with either PMA and A23187 (gray bars) or PMA and A23187 plus immobilized anti-CD3 (black bars). There was no correlation between Plac8 expression (uvmo-2, uvmo-3) or lack of expression (uvmo-4, spl4-10) and the ability to degranulate. Maximal release of granzyme B (in pg/ml) for each T cell clone is shown in parentheses. Aggregate data are from two independent experiments with the SEM; comparisons made between the uvmo-2 and uvmo-3 versus uvmo-4 and spl4-10 were not statistically significant.
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 aa 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 study 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 and 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 a sample size of 4, it appears that CD4 T cells that express Plac8, CD4Plac8, use both iNOS-dependent and degranulation-dependent mechanisms for terminating C. muridarum replication in epithelial cells, whereas Plac8−/− CD4 T cells appear to be dependent on NO. It is plausible that dual effector Chlamydia-specific CD4Plac8 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 1) nude mice sufficient in neutrophils, Plac8, and iNOS are incapable of clearing primary C. muridarum primary genital tract infections (34), 2) time to clearance in wild-type mice is not optimal protective subset in the genital tract. Chlamydia that expose intracellular bacteria to extracellular effector mechanisms such as neutralizing Abs or neutrophils. Instead, 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 NO and Plac8 or a Plac8-dependent effector. This type of mechanism is intellectually satisfying, as most CD4 T cells appear to recognize infected epithelial cells after the noninfectious reticulate body 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 cells cytosol, but that active Plac8 or other injected T cell molecules trigger activation of epithelial Plac8 in an amplified final pathway.

In this study CD4 T cell-conditioned media were a very potent inhibitor of C. muridarum replication in murine epithelial cells through an iNOS-dependent mechanism, being >2 logs more potent than rIFN-γ in our previous studies and those of others. This finding suggests that in vitro studies using rIFN-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 rIFN-γ or, more likely, to contributions of additional cytokines such as TNF-α to a cytokine-mediated anti-Chlamydia epithelial response. The iNOS promoter once activated by IFN-γ can be further upregulated 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). Scurock et al. (42), in a recent paper focused on possible IL-17 contributions to Chlamydia immunity, showed that IFN-γ and TNF-α in genital tract secretion of C. muridarum-infected mice peaked on day 4 and were essentially undetectable by day 14. Doxycycline experiments suggest that adaptive immunity takes hold around day 10 postinfection (43). We propose a working model in which early innate immunity in a genital tract awash with cytokines including IFN-γ and TNF-α clears 2–3 logs of C. muridarum during the first 10 d infection, and that clearance of the last 3–4 logs C. muridarum by the adaptive cellular immune response can be accomplished by either cytokine-dependent (iNOS) or Plac8-dependent mechanisms, whereas sterilizing cellular immunity requires both iNOS- and 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 CD4Plac8 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 CD4Plac8 T cell subset.

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Disclosures
The authors have no financial conflicts of interest.

References


