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Chlamydia-Specific CD4 T Cell Clones Control Chlamydia muridarum Replication in Epithelial Cells by Nitric Oxide-Dependent and -Independent Mechanisms

Krupakar Jayaraju,* Micah Kerr,* Susan Ofner,† and Raymond M. Johnson*

Chlamydia trachomatis serovars D–K are sexually transmitted intracellular bacterial pathogens that replicate in epithelial cells lining the human reproductive tract. It is clear from knockout mice and T cell depletion studies using Chlamydia muridarum that MHC class II and CD4 T cells are critical for clearing bacteria from the murine genital tract. It is not clear how CD4 T cells interact with infected epithelial cells to mediate bacterial clearance in vivo. Previous work using an epithelial tumor cell line showed that a Chlamydia-specific CD4 T cell clone was able to inhibit C. muridarum replication in vitro via induction of epithelial NO production. We have previously shown that Chlamydia-specific CD4 T cell clones can recognize and be activated by infected reproductive tract epithelial cells and block Chlamydia replication in them. We extend those observations by investigating the mechanism used by a panel of CD4 T cell clones to control Chlamydia replication in epithelial cells. We found that Chlamydia-specific CD4 T cell clones were cytolytic, but that cytolysis was not likely critical for controlling C. muridarum replication. For one, CD4 T cell clone-induced epithelial NO production was critical for controlling replication; however, the most potent CD4 T cell clones were dependent on T cell degranulation for replication control with only a minor additional contribution from NO production. We discuss our data as they relate to existing knockout mouse studies addressing mechanisms of T cell-mediated control of Chlamydia replication and their implications for intracellular epithelial pathogens in mouse models. The Journal of Immunology, 2010, 185: 000–000.

Chlamydia trachomatis serovars D–K are intracellular bacterial pathogens that cause a common sexually transmitted urethritis/cervicitis throughout the world. In the United States, 1,210,523 cases were reported to the Centers for Disease Control in 2008, the largest number of cases ever reported and an increase of 9.2% from the previous year (1). A similar upward trend in C. trachomatis infections has been previously documented in Canada and thought to reflect an unintended negative consequence of treatment and control efforts on herd immunity (2). In women, untreated Chlamydia infections ascend into the Fallopian tubes, causing tubal scarring that leads to chronic pelvic pain, tubal pregnancies, and infertility (3).

T cell depletion and knockout mouse studies have clearly shown that MHC class II and CD4 T cells are necessary for primary clearance of Chlamydia muridarum from the murine genital tract (4, 5). C. muridarum is closely related to C. trachomatis serovar D, including gene-for-gene synteny excepting a small region known as the plasticity zone that is associated with species-specific evasion of IFN-γ-induced innate immunity (6, 7). Because rodent and human Chlamydia strains have evolved to evade IFN-γ-induced innate immune defenses in their natural host (8, 9), it is likely that analogous to mice, human clearance of Chlamydia infections requires CD4 T cell-mediated immunity. Because the components of adaptive cellular immunity are highly conserved between mice and humans, it is also likely that mice and humans use similar T cell effector mechanisms to clear Chlamydia from the genital tract. Although the broad identity of the relevant effector T cell population, CD4 T cells, has been determined using the murine model, the mechanism used by CD4 T cells to clear Chlamydia from the reproductive tract is unknown (10).

CD4 T cell lines protective in adoptive transfer studies have also been shown to control C. muridarum replication in polarized epithelial monolayers (11). The mechanism of control was dependent on IFN-γ and physical interaction between T cells and infected epithelial cells via LFA-1–ICAM-1. In the presence of IFN-γ, T cell engagement of epithelial cells via LFA-1 was shown to augment epithelial NO production above that induced by IFN-γ alone, and NO was shown to be the effector molecule responsible for controlling Chlamydia replication (12). These data identified T cell–induced NO production as the probable mechanism for clearing Chlamydia from the genital tract. However, subsequent studies showed no difference in Chlamydia genital tract clearance kinetics between inducible NO synthase (iNOS)-deficient mice and wild-type mice (13, 14), though there are important differences in immunopathology (15). In addition, mice deficient in LFA-1, perforin, Fas, Fas ligand (FasL), perforin and FasL, p47phox, and TNFRs all resolve C. muridarum genital tract infections with normal or near normal kinetics, though there are important differences in intensity of shedding (TNFR knockout) (reviewed in Ref. 4). Furthermore, mice deficient in IFN-γ clear 99.9% of C. muridarum from the genital tract with near normal kinetics (16, 17). These knockout mouse data argue against direct T cell-mediated killing via perforin and Fas–FasL making a major contribution to bacterial clearance and argue against indirect mechanisms for CD4 T cell-mediated clearance via IFN-γ/LFA-1 induction of epithelial iNOS.
(NO) and IFN-γ/TNF-α induction of epithelial NAPDH oxidase (reactive oxygen species) defense mechanisms, begging the question of how mice clear 

Chlamydia
genital tract infections.

C. trachomatisreplicates predominantly in the reproductive tract epithelium during natural human infections (18, 19) and experimental murine
c. muridaruminfections in wild-type mice (4).

Because we have previously shown that 

Chlamydia-specific CD4 T cell clones can recognize C. muridarumAggs in the context of epithelial MHC class II molecules and block Chlamydia replication in epithelial cells, it is reasonable to propose that bacterial clearance from the genital tract involves the physical interaction of 

Chlamydia-specific CD4 T cells with infected epithelial cells. For this report, we used our 

Chlamydia-specific CD4 T cell clones and an upper reproductive tract epithelial cell line to investigate how CD4 T cells control 

Chlamydia replication in infected epithelial cells. We present the interesting results of those studies in this paper.

Materials and Methods

Mice

Four- to 5-wk-old female C57BL/6 mice were purchased from Harlan Laboratories (Indianapolis, IN). 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

Bml.11 epithelial cells (H-2Kk) have been previously described (20); C57epi.1 epithelial cells and 

Chlamydia-specific CD4 T cell clones splk-10, uvmo-1, uvmo-2, and uvmo-3 were derived from C57BL/6 mice (H-2Kk) and cultured as previously described (21). Mycoplasma-free C. muridarum (Nigg), previously known as C. trachomatis strain mouse pneumonitis (MoPn) (Nigg), was grown in McCoy cells as previously described (20).

Alloreactive CD8 T cell clone specific for H-2Kmp was derived from a C7BL/6 (H-2b) mouse primed with full-thickness dorsal tail skin graft from MHC-CH2-H−/H− mice (30 U/ml) overnight. This cytokine milieu reflects that produced by C. muridarum-infected oviduct epithelial cells (20) and bone marrow-dendritic cell pulsed with heat-inactivated C. muridarum (23). Final cytokine concentrations in primary wells were 0.3333 that of the overnight pretreatment values; final well volume was 3 cc. The resulting polyclonal T cell populations were serially passed and limiting dilutions and that earliest recognition/activation occurred

Ags in the context of epithelial MHC class II mole-

ules and that additional Ag recognition/activation in epithelial cells in vitro (26). We have previously shown that 

C. muridarumreplication in epithelial cells in vitro (26). We have previously shown that 

mice without centrifugation. Eighteen hours post-

fection, 1,000 purified (histopaque 1083, Sigma-Aldrich) CD4 T cells were added to the C57epi.1 monolayers in 10 μg/ml tetracycline in the presence of inhibitors: 200 nM PAO, 50 nM of CMA, or 10 μg/ml of Ab specific for CD4 (GK1.5, eBioscience), Fas (Jo2, NA/LE, BD Biosciences), and FasL (MLF3, functional grade, eBioscience). T cell clones were incubated with PAO and CMA for 1 h preaddition to epithelial cells. After 2 h of coculture, wells were fixed with 1% paraformaldehyde for 20 min and stained with Cyto-Quik (Fisher Scientific, Pittsburgh, PA). Photos of representative fields were taken with a Nikon diaphot 200 inverted microscope (Nikon, Melville, NY) equipped with a Diagnostic Instruments spot color camera (Diagnostic Instruments, Sterling Heights, MI) at ×20 original magnification.

NO measurement

C57epi.1 epithelial monolayers in 48-well plates were untreated or pre-

treated with 10 ng/ml IFN-γ for 14 h in the absence or presence of 1 nM MLA or 100 nM NIL, then infected with 3 IFU/cell C. muridarum. Four hours later, the inocula were removed and monolayers cocultured with media or 1,500 T cell clone cells in the presence of iNOS inhibitors where indicated. The T cell to epithelial cell ratio under these conditions was ~1:1.

Thirty-two hours later, 36 h postinfection, culture supernatants were col-

lected and NO quantified (measured as nitrite) using a commercial Greiss reagent system (Promega) according to the manufacturer’s protocol (25).

Statistical analysis

Summary figures for each experimental investigation are presented as mean and SD or pooled means with their associated SEM. Figure legends indicate the number of independent experiments pooled to generate each figure. Student two-tailed t test was used to assess significance of experimental data. The p values <0.05 were considered statistically significant. For Fig. 3, Sidak methodology was used to adjust p values for multiple compar-

Results

IFN-γ-independent CD4 T cell clones control C. muridarum replication without NO production

Igetseme et al. (12) previously showed that a 

Chlamydia-specific CD4 T cell clone designated 2-14.0 controlled C. muridarum replication in an LFA-1- and IFN-γ-dependent fashion through in-

duction of epithelial NO. Blocking NO production with MLA completely negated 2-14.0’s ability to control C. muridarum replication in epithelial cells in vitro (26). We have previously shown that the CD4 T cell clones designated uvmo-1, -2, and -3 recognized C. muridarum Ags in the context of epithelial MHC class II mole-

cules and that earliest recognition/activation occurred ~12 h post-

fection, with maximal T cell activation at 18–21 h postinfection. These three CD4 T cell clones were able to control replication without IFN-γ pretreatment of syngeneic epithelial monolayers. We investigated whether NO production was critical for CD4 T cell clones uvmo-1, -2, and -3 to control C. muridarum replication in epithelial cells in vitro.
C57epi.1 oviduct epithelial cell monolayers, untreated or pretreated with IFN-γ in the absence or presence of NO synthetase inhibitors MLA or NIL for 14 h, were infected with C. muridarum and then cocultured with media or CD4 T cell clones uvmo-1,-2, and -3. Supernatants were collected at 36 h postinfection, 32 h post T cell coculture, and analyzed for NO production measured as nitrite (Fig. 1A). IFN-γ significantly induced NO production in C57.1 epithelial cells, roughly 6-fold with p < 0.0001. MLA and NIL were effective inhibitors of NO production, as MLA blocked >90% of IFN-γ-induced NO; NIL blocked ~60%. Levels of NO production in the presence of MLA, without or with CD4 T cell coculture, were not statistically different from NO produced by infected epithelial cells in media without IFN-γ pretreatment. Uninfected C57epi.1 cells grown in regular culture media for 36 h had basal NO production of <1.5 μM. CD4 T cell clones uvmo-1 and uvmo-2 were able to boost NO produced by infected C57epi.1 epithelial cells that had not been pretreated with IFN-γ. Coculture of the CD4 T cell clones with IFN-γ-pretreated infected epithelial cells did not boost NO production above levels seen with IFN-γ pretreatment alone. As seen previously, IFN-γ pretreatment had a modest negative effect on C. muridarum replication in C57epi.1 epithelial cells (~1.5–2-fold). The modest inhibitory effect of IFN-γ was reversed by blocking NO production with either MLA or NIL (Fig. 1B).

Having demonstrated that MLA and NIL were effective inhibitors of NO production by C57epi.1 epithelial cells in absence or presence of CD4 T cell clones, we tested whether NO was critical for CD4 T cell clones uvmo-1,-2, and -3’s ability to control C. muridarum replication in epithelial cells. Darville et al. (27) have previously shown detectable IFN-γ levels in genital secretions as soon as 2 d postinfection that increase to ~4000 pg/ml by day 3 and remain elevated at ≥1000 pg/ml through day 10, the last day tested. Based on that data, it is reasonable to postulate that CD4 T cell interactions with infected epithelial cells occur in a microenvironment that includes IFN-γ. C57epi.1 oviduct epithelial cell monolayers, pretreated with IFN-γ in the absence or presence of NO synthetase inhibitors MLA and NIL, were infected with C. muridarum. Four hours later, the inocula were removed and T cells added at a T cell to epithelial cell ratio of 0.75:1 in medium (control) or medium containing NO synthetase inhibitors MLA or NIL. Thirty-two hours later, the wells were harvested by scraping and the C. muridarum yield quantified by titration on McCoy monolayers (Fig. 2). Blocking NO production with MLA (and to a lesser extent NIL) had very modest negative effects on the abilities of uvmo-1 (98% inhibition dropped to 95% → raw IFU data: no T cell control = 3.4 × 10^6 IFU recovered per well; + uvmo-1 = 6.4 × 10^4 IFU/well; MLA + uvmo-1 = 1.7 × 10^5 IFU/well) and uvmo-3 (92% inhibition dropped to 89%) and no effect on the ability of uvmo-2 to control C. muridarum replication in vitro.

Role of cytolysis in controlling replication

Replicating Chlamydia species are theoretically vulnerable to T cell-mediated cytolysis during the early stages of infection after infectious elementary bodies (EBs) transition to noninfectious replicating reticulate bodies (RBs), analogous to the eclipse phase in viral replication exploited by CTL to mediate viral clearance (28). Lysis of infected cells during the Chlamydia eclipse phase of replication would release noninfectious RBs and terminate replication. In Fig. 3, we show the replication kinetics of C. muridarum in C57epi.1 cells without centrifugation, the protocol used in an upcoming section for investigating the role of CD4 T cell cytolysis in blocking Chlamydia replication. C57epi.1 monolayers, untreated or pretreated with IFN-γ, were infected, then sequentially harvested at 0, 3, 6, 9, 12, 15, 18, 21, 24, 32, and 36 h and IFU quantified on McCoy cell monolayers. In C57epi.1 cells, C. muridarum enters a noninfectious RB replication phase spanning ~3–18 h postinfection. From ~18 h forward, infectious EBs accumulate with maximal yield at ~30 h postinfection. Pretreatment with IFN-γ for 14 h caused a modest reduction (~2-fold) of C. muridarum replication in C57epi.1 cells.

The kinetics of C. muridarum replication in C57epi.1 epithelial cells defines a window, roughly 3–18 h postinfection, during which Chlamydia-specific T cells could terminate replication by lysing

![FIGURE 1](http://www.jimmunol.org/figures/figure1.png)

**FIGURE 1.** Infected epithelial cell production of NO in the absence and presence of CD4 T cell clones: C. muridarum replication in C57epi.1 epithelial cells in the absence and presence of IFN-γ. A, C57epi.1 epithelial cells, untreated (hatched bars) or pretreated with IFN-γ (10 ng/ml × 14 h) in the absence (black bars) or presence of IFN-γ inhibitors MLA (gray bars) or NIL (light gray bars), were infected with C. muridarum (3 IFU/cell). Four hours later, the inocula were removed and monolayers cocultured without and with CD4 T cell clones uvmo-1, -2, and -3 for an additional 32 h. Culture supernatants were collected 36 h postinfection, 32 h postcoculture, and NO quantified as nitrite; aggregate means and SEM from two independent experiments. Asterisks indicate comparisons with IFN-γ treatment (black bars) for each of the four conditions (No T cells, uvmo-1, uvmo-2, uvmo-3). Daggers indicate comparisons with the No T cells infected control (hatched bar). B, Effects of IFN-γ pretreatment (black bars) on C. muridarum replication in infected C57epi.1 epithelial cells. C57epi.1 cells were untreated (hatched bar) or pretreated with IFN-γ (10 ng/ml × 14 h) in absence (black bar) or presence of MLA (gray bar) or NIL (light gray bar), and then infected with C. muridarum (3 IFU/cell). Wells were harvested 36 h postinfection and C. muridarum quantified on McCoy monolayers. Comparisons were made to the infected control (hatched bar). Aggregate means and SEM from two independent experiments. **,*****p < 0.001; ****p < 0.0005.
infected epithelial cells. Our previous work showed that under optimal conditions (pretreatment of the epithelial monolayer with IFN-γ preinfection), the earliest time point for recognition by our CD4 clones was ∼12 h postinfection and that the maximal recognition/activation time point was 18–21 h postinfection. To investigate the role of cytolysis in controlling C. muridarum replication, we first investigated the cytolytic potential of our three Chlamydia-specific CD4 T cell clones, comparing them to a cytolytic alloreactive CD8 T cell clone specific for H-2Kbm1 (designated CD8bm1; see Materials and Methods).

Redirected lysis assays were performed using an FcR-bearing target cell (P815) and an activating anti-CD3 mAb 145-2C11. T cell–target conjugate formation occurs via the anti-CD3 Abs simultaneously binding to P815 FcRs and the TCR–CD3 complex. Binding of the CD3–TCR complex triggers T cell-mediated cytolysis. This assay has the advantage of providing a uniform activation signal to the T cells that is independent of their Ag specificity or coreceptor (CD4 or CD8). We also used the redirected lysis assays to determine the potency and stability of the perforin inhibitor (CMA) and the degranulation inhibitor (PAO), used in upcoming experiments.

The perforin-mediated short-term cytolytic potential of our Chlamydia-specific CD4 T cell clones was similar to that of an alloreactive CD8 T cell clone CD8bm1. The percent specific cytolyis at an E:T ratio of 3:1 in a 4-h assay is shown in parentheses for each clone (Fig. 4). Because investigating T cell mechanisms for controlling Chlamydia replication required a 24-h in vitro assay (see CD4 T cell clones lysis of infected epithelial cells in delayed fashion), we did standard 4-h assays with each inhibitor and also tested the stability of CMA and PAO in culture media overnight (labeled as 24 h). In standard 4-h killing assays, the perforin inhibitor CMA significantly blocked cytolysis by Chlamydia-specific CD4 T cell clones uvmo-2, uvmo-3, and alloreactive clone CD8bm1, with minimal effects on uvmo-1 (Fig. 4, light gray bars); in 4-h assays, the degranulation inhibitor PAO significantly blocked cytolysis by all clones (Fig. 4, black bars).

To assess stability of the inhibitors in 24-h assays, working concentrations of the inhibitors were incubated in medium at 37°C for 18 h then used in 4-h redirected lysis assays (18 h incubation plus 2 h assay setup plus 4 h assay = 24 h) (Fig. 4). CMA was stable in medium, retaining its ability to partially inhibit the T cell clones after 18 h in medium at 37°C relative to fresh CMA in the 4-h assays. Conversely, PAO lost nearly all its inhibitory activity after 18 h in culture media at 37°C. The data in Fig. 4 show that CMA is a partial inhibitor based on intrinsic activity; PAO is a potent inhibitor in short-term assays but was unstable in culture medium over the course of longer assays. Based on these results, CMA and PAO are useful experimental tools for investigating cytolyis, but should be viewed as partial inhibitors in the upcoming 24-h assays used to study epithelial cytolysis and Chlamydia replication.

**CD4 T cell clones lysis of infected epithelial cells in delayed fashion**

We tested whether the Chlamydia-specific CD4 T cell clones could lyse infected epithelial cells in 4-h killing assays. We have previously shown that maximal recognition/activation for all three CD4 T cell clones occurs 18–21 h postinfection using IFN-γ–pretreated C57epi.1 epithelial cells. C57epi.1 cells were pretreated with IFN-γ for 14 h, then infected with C. muridarum. At 18 h postinfection, the cells were harvested and used as targets in 4-h killing assays in the presence of tetracycline to halt Chlamydia replication. Despite using an optimal time point postinfection, and earlier demonstration that the CD4 T cells were cytolytic via perforin pathways (Fig. 4), none of the CD4 T cell clones showed killing of 18-h infected targets in 4-h [51Cr]release or non-radioactive killing assays (data not shown). Although there was no short-term killing, we knew that the CD4 T cell clones were able to lyse 18-h infected C57epi.1 cells dramatically within 24 h of coculture based on direct visualization under phase-contrast microscopy.

To investigate this delayed killing phenomenon, we set up C57epi.1 cells in chamber slides and used direct visualization/photodocumentation as the readout for delayed cytolysis. C57epi.1 monolayers in eight-well chamber slides were mock-infected or infected with C. muridarum for 18 h, then tetracycline and T cell clones were added in the absence or presence of inhibitors of cytolysis/apoptosis. In the first set of experiments, we used inhibitors of the two classic pathways for T cell–mediated killing: CMA, which blocks perforin-mediated cytolysis by raising the pH in T cell exocytotic granules causing perforin degradation (29), and Abs to Fas and FasL to block activated cell death receptor-ligand interactions (apoptosis) (30, 31) (Fig. 5). Photos were taken 24 h later to semiquantitatively score cytolysis. Addition of tetracycline at 18 h
postinfection preserved the viability of the infected C57epi.1 monolayer for the duration of the assays (Fig. 5, top panels). CMA and mAbs had little or no toxicity for C57epi.1 monolayers at the concentrations used (Fig. 5, top panels). None of the clones exhibited visible cytotoxicity toward mock-infected C57epi.1 cells (Fig. 5 A). All of the clones lysed 18-h infected epithelial cells within 24 h (Fig. 5 B). CMA partially protected infected C57epi.1 monolayers from uvmo-2 and uvmo-3, but not uvmo-1, consistent with uvmo-1’s relative indifference to CMA documented in the redirected lysis assays (Fig. 4). mAbs to Fas/FasL had no visible effect on delayed cytolysis.

Next, we investigated the role of T cell degranulation and the CD4 coreceptor in delayed killing of infected epithelial monolayers (Fig. 6). PAO is a tyrosine kinase inhibitor that blocks T cell degranulation (32); GK1.5 is a mAb specific for CD4 that blocks the interaction between the CD4 coreceptor and MHC class II molecules (33). Again, the CD4 T cell clones did not lyse mock-infected cells (Fig. 6 A), and the inhibitors were not toxic for the C57epi.1 monolayers at the concentrations used (Fig. 6, top panels). Blocking T cell degranulation with PAO partially protected infected C57epi.1 monolayers from uvmo-2 and uvmo-3, but not uvmo-1, consistent with uvmo-1’s relative indifference to CMA documented in the redirected lysis assays (Fig. 4). mAbs to Fas/FasL had no visible effect on delayed cytolysis.

Having shown that CMA (perforin) and PAO (degranulation) partially blocked delayed cytolysis by the CD4 T cell clones uvmo-2 and uvmo-3, we investigated whether blocking cytolysis would interfere with their control of C. muridarum replication in C57epi.1 cells. We first tested whether CMA and PAO affected C. muridarum replication in C57epi.1 cells. Untreated C57epi.1 monolayers were infected with C. muridarum in the presence of anti-CD3 Ab 145-2c11 in 4-h assays. T cells were untreated (hatched bars) or preincubated with CMA (light gray bars) or PAO (black bars) for 1 h preassay. Culture supernatants were assayed for release of lactate dehydrogenase activity using a commercial nonradioactive CTL assay to determine percent specific lysis. Absolute percent specific lysis values for each clone absent inhibitors are shown in parentheses. Aggregate means and SEM from three independent experiments. Comparisons are made to the untreated T cell clone (hatched bars) for each of the two conditions (4 h and 24 h). *p < 0.05; **p < 0.005; ***p < 0.0005.

![Figure 4](http://www.jimmunol.org/)
on *C. muridarum* replication. Although *C. muridarum* replication inhibition by CD4 T cells in the presence of CMA would be normalized to the absolute replication of *C. muridarum* in the presence of CMA, interpretation of CMA experimental results are less straightforward, as *Chlamydia* replication is needed to generate *Chlamydia* epitopes required for T cell recognition.

Cognizant of the above, we tested whether blocking perforin (CMA) or T cell degranulation (PAO), with and without MLA inhibition of epithelial inducible NO synthetase, would negatively affect the ability of CD4 T cell clones uvm-2 and uvm-3 to control *C. muridarum* replication in C57epi.1 epithelial cells. C57epi.1 monolayers pretreated with IFN-γ, in the presence of MLA where indicated, were infected with *C. muridarum*. Four hours later, the inocula were removed, and T cells, pretreated with CMA or PAO where indicated, were added at a T cell to epithelial cell ratio of 0.75:1 in medium (no inhibitor), medium containing MLA (inhibits iNOS), medium containing CMA (inhibits perforin), containing PAO (inhibits degranulation), medium containing MLA and CMA (blocking iNOS and perforin), and medium containing MLA and PAO (blocking iNOS and degranulation). Thirty-two hours later, 36 h postinfection, supernatants were collected from each well to measure IFN-γ production; then, the wells were harvested by scraping and the *C. muridarum* yield quantified by titration on McCoy monolayers. T cell inhibition-of-replication results were normalized to *C. muridarum* replication in identical parallel C57epi.1 wells exposed to the respected inhibitor(s) without the T cell clones. IFN-γ results, reflecting recognition/activation of each T cell clone by infected epithelial cells, are shown in the top panels of Fig. 8. For uvm-2, the presence of CMA caused a 3-fold reduction in IFN-γ production; for uvm-3, the presence of CMA caused roughly a 10-fold reduction in IFN-γ production, whereas MLA modestly enhanced (2-fold) and PAO modestly decreased (40% reduction) IFN-γ production for uvm-3. These results are consistent with the previously documented need for *C. muridarum* replication for TCR recognition of infected cells by uvm-2 and uvm-3 (21), the former apparently requiring less replication than the latter to have its epitope visible in the context of epithelial MHC class II molecules. Consistent with that hypothesis, MLA signi-

***FIGURE 5.*** *Chlamydia*-specific CD4 T cell clones lyse infected C57epi.1 epithelial cells in a delayed fashion: roles of perforin and Fas/FasL. C57epi.1 epithelial monolayers in eight-well chamber slides were mock-infected and cocultured without and with 1 × 10⁵ T cells (A), infected 18 h with 5 IFU/cell *C. muridarum* and cocultured without and with T cells (B), infected and cocultured without and with T cells pretreated and exposed to CMA (C), and infected cocultured without and with T cells in the presence of MLA (D). All wells, mock-infected or infected for 18 h, included addition of 10 μg/ml tetracycline in the coculture media after 18 h to halt progression of the *Chlamydia* infection and preserve epithelial cell viability for duration of the assay. Twenty-four hours postaddition of the T cells, epithelial monolayers were stained with Cyto-Quik (Fisher Scientific) and photographed at ×20 original magnification. Representative data from two independent experiments.

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***FIGURE 6.*** *Chlamydia*-specific CD4 T cell clones lyse infected C57epi.1 epithelial cells in a delayed fashion: roles of T cell degranulation and the CD4 coreceptor. C57epi.1 epithelial monolayers in eight-well chamber slides were mock-infected and cocultured without and with 1 × 10⁵ T cells (A), infected 18 h with 5 IFU/cell *C. muridarum* and cocultured without and with T cells (B), infected and cocultured without and with T cells pretreated and exposed to PAO (C), and infected cocultured without and with T cells in the presence of mAb GK1.5 specific for CD4 (10 μg/ml) (D). All wells, mock-infected or infected for 18 h, included addition of 10 μg/ml tetracycline in the coculture media after 18 h to halt progression of the *Chlamydia* infection and preserve epithelial cell viability for duration of the assay. Twenty-four hours postaddition of the T cells, epithelial monolayers were stained with Cyto-Quik (Fisher Scientific) and photographed using an inverted microscope at ×20 original magnification. Representative data from two independent experiments.
significantly increases replication of C. muridarum under these conditions (Fig. 1B), and MLA significantly increased the amount of IFN-γ released by uvmo-3 during coculture with infected epithelial cells (Fig. 8, top right panel). CD4 T cell clones uvmo-2 and uvmo-3’s inhibition of Chlamydia replication was relatively indifferent to inhibition of epithelial NO production (as previously shown in Fig. 2), but both were significantly affected by partial blocking of T cell degranulation with PAO or partial inhibition of perforin activity with CMA (bottom panels of Fig. 8). For both clones, PAO was the most potent single inhibitor, but major reductions, complete reversal for uvmo-3 and 50% reversal for uvmo-2, of T cell-mediated inhibition of Chlamydia replication required inhibition of both NO production and perforin/degranulation. These results are consistent with dual effector mechanisms, with one pathway dependent on epithelial NO production and the other on T cell degranulation and likely perforin. Keeping in mind that MLA inhibition of NO production is >90% and that CMA/PAO inhibition was only partial, complete reversal of uvmo-2 inhibition would be theoretically possible with MLA plus a more stable/potent inhibitor of degranulation or perforin. The alternative explanation for residual inhibition of Chlamydia replication by uvmo-2 in the presence of MLA plus PAO/CMA would be presence of a third anti-Chlamydia mechanism.

Other Chlamydia-specific CD4 T cell clones

In this study, we initially chose to focus on the most potent Chlamydia-specific CD4 T cell clones identified during an earlier investigation. However, it is reasonable to ask whether all Chlamydia-specific CD4 T cells use this dual, apparently redundant, mechanism for controlling replication in epithelial cells. Therefore, we investigated the mechanism used by a previously described Chlamydia-specific CD4 T cell clone designated spl4-10 that was dependent on IFN-γ pretreatment of C57epi.1 epithelial cells to control C. muridarum replication (21) and compared it to uvmo-2.
in the same experiments. C57epi.1 monolayers pretreated with IFN-γ, in the presence of MLA when indicated, were infected with Chlamydia muridarum. Four hours later, the inocula were removed, and T cells, pretreated with PAO where indicated, were added at an E:T ratio of 0.75:1 in medium (control), medium containing MLA, or medium containing PAO. Thirty-two hours later, the wells were harvested by scraping and the C. muridarum yield quantified by titration on McCoy monolayers. T cell inhibition–of–replication results were normalized to C. muridarum replication in identical parallel C57epi.1 wells exposed to the respected inhibitor without the T cell clones (no T cell; 0% inhibition). As seen in previous experiments, uvmo-2 inhibition of C. muridarum replication was minimally reversed by MLA, and partially reversed by PAO (Fig. 9). In contrast, spl4-10s ability to inhibit Chlamydia replication was completely reversed by either MLA or PAO. These results are consistent with generation of two different Chlamydia–specific CD4 T cell subsets during the adaptive immune response to experimental C. muridarum genital tract infections. One subset, represented by spl4-10 and previously published clone 2-14.0, is absolutely dependent on NO production for controlling Chlamydia replication; the second subset, represented by uvmo-1, -2, and -3, can effectively control Chlamydia replication in epithelial cells in the absence of epithelial production of NO.

Discussion

The relevance of the C. muridarum mouse genital tract model to human C. trachomatis genital tract infections is a critical issue for pathogenesis research and vaccine development. Existing data from pathogenesis studies using knockout mice are difficult to reconcile with basic immunologic paradigms. Mice deficient in perforin, Fas, FasL, or both perforin and FasL have normal clearance kinetics (35), raising doubts about the role of cytolysis in controlling Chlamydia infections of the genital tract. A strong body of existing research has identified an IFN-γ–dependent mechanism based on induction of epithelial NO, as a mechanism for clearing Chlamydia from the reproductive tract; yet IFN-γ–deficient mice clear 99.9% of C. muridarum from the genital tract with near normal kinetics (16, 17), and iNOS–deficient mice clear C. muridarum genital tract infections with normal kinetics (13, 14). These perplexing results could be reconciled if there were redundant mechanisms for clearing Chlamydia from the reproductive tract, one dependent on IFN-γ and iNOS and the other independent of IFN-γ and iNOS. Knocking out either mechanism in isolation theoretically would not adversely affect C. muridarum clearance in the mouse model.

Existing knockout mouse data clearly show that MHC class II is critical for clearing C. muridarum from the genital tract (5). We have shown the Chlamydia–specific CD4 T cell clones control C. muridarum replication in oviduct epithelial cells by recognizing Chlamydia Ags in the context of MHC class II molecules and that IFN-γ was important for upregulating epithelial MHC class II molecules, which correlated with improved T cell recognition, measured as IFN-γ production, and control of C. muridarum replication in epithelial cells (21). Having a panel of Chlamydia–specific CD4 T cell clones allowed us to further investigate mechanisms for controlling C. muridarum replication in epithelial cells in vitro.

One potential mechanism for clearance would be recognition and lysis of infected epithelial cells during the noninfectious RB stage of Chlamydia development. We had previously shown that the earliest CD4 T cell recognition of infected epithelial cells by our clones occurred roughly 12 h postinfection, with maximal recognition 18–21 h postinfection. In this study, we looked at short-term perforin–mediated lysis with 18-h infected targets and found no cytolysis in 4-h assays. However, there was a delayed lysis phenomenon readily visible via microscopy that occurred 18–24 h post T cell coculture with 18-h infected epithelial cells. This visually dramatic lytic event was dependent on T cell degranulation and perforin, but did not occur soon enough in the Chlamydia developmental cycle to lyse infected cells in the RB stage of replication (i.e., optimal epithelial targets at 18 h postinfection were not lysed in 4 h assays [18–22 h postinfection] and were only visually lysed after 18–24 h of coculture [36–42 h postinfection]). C. muridarum replication in oviduct epithelial cells is nearly complete by 24 h postinfection and is maximal by ~30 h post-infection. Based on the in vitro data presented in this study and data from perforin/FasL knockout mice (35), the mechanism(s) used by CD4 T cells to clear genital tract infections are not likely to be critically dependent on the physical lysis of infected epithelial cells at the RB stage of the Chlamydia life cycle, though we cannot rule out the possibility that replication kinetics in vivo differ from those observed in vitro.

![FIGURE 9. Role of iNOS and T cell degranulation in IFN-γ–dependent (spl4-10) and –independent (uvmo-2) Chlamydia–specific CD4 T cell clones. C57epi.1 epithelial cells were pretreated for 14 h with IFN-γ in the absence or presence of iNOS inhibitor MLA, and then infected with C. muridarum at 3 IFU/cell. Inocula were removed 4 h later, and infected epithelial cells were cocultured without (white bars; set as 0% inhibition) and with 1.5 x 10^5 uvmo-2 T cells (left panel) or spl4-10 T cells (right panel) in the absence of inhibitors (control black bars), in the presence of MLA (gray bars), and with T cells pretreated and exposed to PAO (light gray bars). Thirty-six hours postinfection, the wells were harvested and C. muridarum quantified on McCoy monolayers. Percent T cell inhibition of MoPn replication for each treatment condition (inhibitor) was normalized to C. muridarum replication in identical parallel wells that were not cocultured with T cells. Aggregate means and SEM from two independent experiments. Comparisons were made to the no inhibitor control (black bars) for each of the T cell clones. *p < 0.05; ***p < 0.0005.](http://www.jimmunol.org/)
The existing data for controlling Chlamydia replication in epithelial cells support an IFN-γ- and NO-dependent mechanism, with *Chlamydia*-specific CD4 T cells upregulating epithelial NO production to control infection. This iNOS-dependent mechanism did not require direct TCR recognition of *Chlamydia* Ags presented by epithelial MHC class II molecules; it required recent T cell activation (<48 h) and physical contact between T cells and epithelial cells, with LFA–1–ICAM-1 interactions being critical for boosting epithelial NO production to *Chlamydia*-cidal levels (12). This iNOS-dependent mechanism was demonstrable in vivo using systemic MLA treatment and adoptive transfer of 2-14.0 T cells into heterozygous +/nu BALB/c mice (36). However, the iNOS-dependent mechanism is not critical in T cell-sufficient mice based on normal *C. muridarum* genital tract clearance kinetics in iNOS knockout mice (13, 14) and wild-type mice treated with the iNOS inhibitor MLA (14). In this study, we investigated the role of NO production in controlling *C. muridarum* replication in oviduct epithelial cells using a small panel of *Chlamydia*-specific CD4 T cell clones and found that one clone, spl4-10, was dependent on epithelial NO production for controlling *C. muridarum* replication similar to the previously reported *Chlamydia*-specific CD4 T cell clone 2-14.0 (26). However, other potent *Chlamydia*-specific CD4 T cell clones, uvmo-1, -2, and -3, were not dependent on epithelial NO production to control *C. muridarum* replication. This finding confirms that there are two independent/redundant CD4 T cell-mediated mechanisms for controlling *C. muridarum* replication in epithelial cells that are mediated by different CD4 T cell subsets and, by extrapolation, two independent/redundant T cell-mediated mechanisms for clearing *C. muridarum* from the genital tract in the mouse model. iNOS-dependent CD4 T cells apparently have only the IFN-γ- and iNOS-dependent pathway, as they are completely inhibited by MLA (e.g., spl4-10 [this paper]) and 2-14.0 (26), whereas iNOS-independent T cells, uvmo-1, -2, and -3, have both pathways, with PAO having a greater effect than MLA but maximal reversal of T cell-mediated inhibition of *C. muridarum* replication requiring inhibition of both iNOS (MLA) and T cell degranulation (PAO).

The iNOS-independent mechanism represented by CD4 T cell clones uvmo-1, -2, and -3 is dependent on T cell degranulation and likely perforin, though our perforin database on CMA needs to be interpreted cautiously because of the significant inhibition of *C. muridarum* replication by this H⁺/ATPase inhibitor. Though tangential to the focus of the investigations reported in this paper, the finding that two H⁺/ATPase inhibitors, CMA (this paper) and bafilomycin (34), inhibit replication of *C. muridarum* in murine epithelial cells identifies a potentially interesting host–pathogen interaction. The iNOS-independent CD4 T cell mechanism used by CD4 clones uvmo-1, -2, and -3 for limiting *C. muridarum* replication in vitro is not dependent on the physical lysis of infected cells based on the relative kinetics of *C. muridarum* replication and timing of CD4 T cell recognition/lysis of infected target cells. The fact that recognition and lysis of infected epithelial cells in vitro occurs after the transition from noninfectious RBs to infectious EBs suggests that the in vitro iNOS-independent mechanism for controlling *C. muridarum* replication is a direct attack on EBs, presumably by molecules resident within T cell granules based on a requirement for degranulation, but potentially including T cell–induced epithelial effector molecules other than NO. Delineation of the specific T cell and possibly epithelial cell anti-*Chlamydia* effector molecules for the iNOS-independent T cell–mediated mechanism is the focus of ongoing investigations.

In vitro confirmation of two redundant CD4 T cell mechanisms for interrupting *Chlamydia* replication provides an explanation for otherwise perplexing knockout mouse data. Perforin/degranulation is not critical if the iNOS-dependent mechanism is available; iNOS is not critical if the degranulation-dependent pathway (iNOS-independent) pathway is available. This conclusion is supported by the data showing that heterozygous +/nu BALB/c mice adoptively transferred with a protective iNOS-dependent T cell clone 2-14.0 had reduced clearance of the *C. muridarum* from the genital tract when mice were treated systemically with MLA (36), but that wild-type mice treated with MLA show no delay in resolution of *C. muridarum* genital tract infections (14).

The presence of two IFN-γ–regulated innate defense mechanisms in mice, p47 GTPases and iNOS, that inhibit *Chlamydia* replication in mouse epithelial cells but are not operative in human reproductive tract epithelium, compromises the mouse model for studying human-relevant *Chlamydia* immunobiology. Similar concerns can be raised about mice as experimental models for other human intracellular pathogens that replicate in epithelial cells. Human reproductive tract epithelial cells do not express iNOS (7, 9), and iNOS has been shown to inhibit replication of both *C. muridarum* and human *Chlamydia* serovars in mouse epithelial cells in vitro (this paper, 12, 37) and in vivo (36). Second, human epithelial cells do not express IFN-γ–regulated murine p47 GTPases that limit replication of human *Chlamydia* strains, not *C. muridarum*, in mouse epithelial cells when IFN-γ is present (7, 8, 38, 39). Consistent with susceptibility to these dual IFN-γ–regulated defense mechanisms, IFN-γ knockout (8, 40) and IFN-γ receptor knockout mice (41) are much more permissive for genital tract infections with human *C. trachomatis* serovars than are wild-type mice. Further complicating the mouse model, human reproductive tract epithelial cells have IFN-γ regulated IDO, an innate defense mechanism that is not present in mouse reproductive epithelial cells (7, 9).

One proposed approach to optimizing the mouse model is to knockout murine p47 GTPases and knockin IDO to create a mouse that would mirror the IFN-γ–-induced innate defenses present in human reproductive tract epithelium (7). Such a mouse would presumably be dependent on human-relevant innate and adaptive immunity to clear infections and make the mouse model permissive for replication of human *Chlamydia* serovars. However, the finding of two redundant CD4 T cell-mediated mechanisms for controlling *C. muridarum* replication makes this approach problematic because a p47 GTPase knockout/IDO knockin mouse would still have an iNOS-dependent T cell–mediated mechanism for controlling replication of rodent or human *Chlamydia* species that is not operative in epithelium of the human reproductive tract. In light of the findings presented in this report, and the fact that human and rodent *Chlamydia* serovars are likely relatively indifferent to IFN-γ–induced innate defenses when replicating in their natural host species [human-IDO (6); mouse p47 GTPases (7)], we propose that an optimized mouse model would be a tissue-specific knockout of iNOS in the epithelial cell lineage. Such a mouse, at least when infected with rodent *C. muridarum*, would be dependent on the iNOS-independent CD4 T cell–mediated mechanism for controlling and clearing *Chlamydia*. Based on the high degree of conservation of components of T cell–adaptive immunity between mice and humans, the iNOS-independent mechanism identified in mouse CD4 T cells is likely to be the critical pathway during resolution of *C. trachomatis* genital tract infections in humans. A tissue-specific knockout of epithelial iNOS mouse would still be problematic for use with human *Chlamydia* serovars due to presence of murine p47 GTPases. The existing global iNOS knockout mouse is likely a suboptimal model based on significant dissemination of *C. muridarum* to the...
lunge and spleen during genital tract infections (13), potentially altering the nature of the adaptive immune response compared with adaptive immune responses seen during typical genital tract infections in wild-type mice and humans.

Disclosures
The authors have no financial conflicts of interest.

References