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HOST RESPONSE AND INFLAMMATION

T Cell-Independent Gamma Interferon and B Cells Cooperate To Prevent Mortality Associated with Disseminated *Chlamydia muridarum* Genital Tract Infection

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ABSTRACT CD4 T cells and antibody are required for optimal acquired immunity to *Chlamydia muridarum* genital tract infection, and T cell-mediated gamma interferon (IFN-γ) production is necessary to clear infection in the absence of humoral immunity. However, the role of T cell-independent immune responses during primary infection remains unclear. We investigated this question by inoculating wild-type and immune-deficient mice with *C. muridarum* CM001, a clonal isolate capable of enhanced extragenital replication. Genital inoculation of wild-type mice resulted in transient dissemination to the lungs and spleen that then was rapidly cleared from these organs. However, CM001 genital infection proved lethal for *STAT1*$^{-/-}$ and *IFNG*$^{-/-}$ mice, in which IFN-γ signaling was absent, and for *Rag1*$^{-/-}$ mice, which lacked T and B cells and in which innate IFN-γ signaling was retained. In contrast, B cell-deficient muMT mice, which can generate a Th1 response, and T cell-deficient mice with intact B cell and innate IFN-γ signaling survived. These data collectively indicate that IFN-γ prevents lethal CM001 dissemination in the absence of T cells and suggests a B cell corequirement. Adoptive transfer of convalescent-phase immune serum but not naive IgM to *Rag1*$^{-/-}$ mice infected with CM001 significantly increased the survival time, while transfer of naive B cells completely rescued *Rag1*$^{-/-}$ mice from CM001 lethality. Protection was associated with a significant reduction in the lung chlamydial burden of genitally infected mice. These data reveal an important cooperation between T cell-independent B cell responses and innate IFN-γ in chlamydial host defense and suggest that interactions between T cell-independent antibody and IFN-γ are essential for limiting extragenital dissemination.

KEYWORDS B cell responses, *Chlamydia*, interferons

Chlamydia trachomatis is the most prevalent sexually transmitted bacterial infection and a significant cause of female reproductive tract morbidity. The development of a vaccine remains a top global health priority (1). Preclinical *C. trachomatis* vaccine development includes utilization of the murine genital infection model for determination of protective immune responses against *Chlamydia muridarum*. Genital infection of mice with specific immune deficiencies has provided a method for determining the protective contribution afforded by humoral and cell-mediated immunity.

Previous studies revealed a central role for CD4 T cells in protection against primary intravaginal *C. muridarum* infection, and wild-type mice demonstrate clearance comparable to that in B cell-deficient mice (2). Multiple experiments have demonstrated a protective role for Th1 gamma interferon (IFN-γ) production in primary and acquired

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immunity against chlamydial infection (3–7). Furthermore, recent evidence suggests that antibody and CD4 T cell-derived IFN-γ optimally cooperate to protect against infection through neutrophil activation and subsequent chlamydial killing (8, 9). Thus, the requirement for Th1 cells and T cell-dependent antibody during protective adaptive responses is well accepted (10).

Earlier studies demonstrated that T cell-deficient athymic nude mice and severe combined immune-deficient (SCID) mice, which lack functional T and B lymphocytes because of impaired VDJ rearrangement, uniformly fail to resolve genital infection with the *C. muridarum* Weiss and Nigg strains, respectively (11, 12). SCID mice demonstrate high levels of dissemination, while IFN-γ-deficient mice exhibit enhanced chlamydial dissemination and a portion fail to resolve the genital tract infection (11). Infection of B cell-deficient mice results in a transient disseminated infection that is likely cleared through enhanced systemic CD4 T cell responses (13). Furthermore, T and B cell-deficient *Rag1*$^{-/-}$ mice that fail to express functional Rag1 proteins, required for somatic recombination, develop a lethal systemic infection after intravaginal infection with *C. muridarum* Weiss (14). Together, these data suggest an important, less characterized T cell-independent IFN-γ and B cell corequirement for protection against a primary disseminated infection.

We recently identified a clonal isolate (CM001) from a *C. muridarum* Nigg stock (6, 15) that was capable of enhanced extragenital replication compared to the parental stock. CM001 allowed us to explore the mechanisms of protection against dissemination during primary intravaginal infection of immune-deficient mice. Wild-type, B cell-deficient, and T cell-deficient mice survived the CM001 infection. However, mice lacking IFN-γ signaling and *Rag1*$^{-/-}$ mice died. Adoptive transfer of convalescent-phase immune serum or naive B cells protected *Rag1*$^{-/-}$ mice from CM001 lethality. B cell adoptive transfer to *Rag1*$^{-/-}$ hosts reduced the disseminated lung chlamydial burden to the levels found in T cell-deficient mice. These are the first studies to demonstrate a T cell-independent corequirement for B cells and innate IFN-γ for control of extragenital chlamydial infection.

RESULTS

Genetic characterization of *C. muridarum* clonal isolates. We performed whole-genome sequencing of *C. muridarum* strains CM001, CM002, CM003, CM004, CM005, CM006, CM007, CM008, CM009, CM010, CM012, and CM021 with the goal of identifying unique genetic differences that might account for the dissemination properties of the variants. Single molecule real-time (SMRT) sequencing via a PacBio platform yielded high-quality draft genomes, with a mean coverage of between 27- and 114-fold per strain (see Table S5 in the supplemental material). This coverage was not always sufficient to achieve single contigs and to close genomes *de novo* (16), so we used the published sequence of CM972 (17), a strain derived from CM001 via plasmid curing (18), as a scaffold to facilitate comparisons of all isolates. A draft assembly of the conserved virulence plasmid was generated for each of the isolates, confirming its presence in all sequenced strains. Likely sequence errors ($n = 5$; Table S5) were identified in the CM972 sequence as single nucleotide calls that diverged from those detected in the parental CM001 strain and all other sequenced isolates, so these were excluded from further evaluation. Overall, we identified variations at a total of 119 loci among the 12 isolates.

Nine single nucleotide polymorphisms (SNPs) were mapped to seven coding sequences and one intragenic region (Table S5). Of these, five were unique to the isolates screened. These included a C-for-A substitution in *ompA* of CM001 (Fig. S2), predicted to exchange cysteine for glycine in the translated protein. We identified two genetic differences unique to CM001 in the *ompA* gene (TC_052, Y015_RS00285), which encodes the major outer membrane protein (MOMP), the C-for-A SNP described above, and a 6-bp insertion (AGCTTA). All the remaining isolates were predicted to express an MOMP with a double amino acid deletion and a glycine-to-cysteine substitution adjacent to the conserved portion of the VD4 region (Fig. S3) of the protein (19), changes that have been predicted to alter the MOMP conformation (20). Broadening

the comparison of the MOMP sequence of CM001 and the other clonal isolates to *C. muridarum* sequences available in GenBank confirmed that it is an outlier with respect to the AR-Nigg population because its sequence is identical to the sequences of *C. muridarum* Weiss (21), *C. muridarum* Nigg (22), and *C. muridarum* Nigg3 derivatives (23), while all other strains sequenced expressed an MOMP protein identical to that of *C. muridarum* Nigg2 (21) and CmVar004 (24) (Fig. S3), clonal isolates recovered independently from the AR-Nigg population (21, 24).

***C. muridarum* clonal isolates reveal a variant with enhanced dissemination.** Previous studies revealed that *C. muridarum* Weiss and Nigg stocks contain variants with genotypic and phenotypic differences (21). We hypothesized that plaque-purified isolates (Fig. S1) derived from such stocks would yield clonal isolates that exhibited differential cervicovaginal burdens during infection. We explored this possibility by inoculating wild-type mice with a panel of plaque-purified isolates derived from a polyclonal population of *C. muridarum* Nigg (AR-Nigg). Groups of mice were intravaginally inoculated with Nigg or one of five different clones. Clone CM001 demonstrated a significantly increased cervicovaginal burden over the course of infection compared to that for Nigg, while infection with clone CM012 resulted in a reduced burden (Fig. 1A). However, plaque assay, a highly sensitive means to measure infectivity in cell culture (18, 25), did not reveal any differences in the efficiency of plaquing (EOP) (18) between the strains (for CM001, $3.77 \times 10^{-1} \pm 1.01 \times 10^{-1}$; for Nigg, $1.77 \pm 1.04 \times 10^{-1}$; for CM012, $3.46 \times 10^{-1} \pm 3.76 \times 10^{-2}$). We also examined the ability of these clones to ascend to the oviduct and to disseminate to the lungs and spleen. Mice intravaginally infected with CM001 demonstrated a significantly higher chlamydial burden in their lungs on day 8 and in their spleens on days 8 and 10 postinfection than mice intravaginally infected with Nigg (Fig. 1B). Both strains were undetectable in the lungs and spleen on day 14. In contrast, no significant difference in the oviduct burden between mice infected with CM001 and mice infected with Nigg was detected on any day examined. These data indicate that although both Nigg and CM001 ascend to the oviducts of wild-type mice, CM001 exhibits enhanced dissemination to the lungs and spleen during early infection.

CM001-mediated lethality and dissemination are plasmid independent. We previously observed that *Rag1*$^{-/-}$ mice intravaginally infected with plaque-purified *C. muridarum* (now designated CM001) succumbed to infection (15). Since *C. muridarum* virulence is linked to the presence of its plasmid (17, 26), we investigated if lethality and dissemination to the lungs and spleen were plasmid dependent. *Rag1*$^{-/-}$ mice infected with CM001 or plasmid-cured CM3.1 displayed a significantly increased bacterial burden compared to the Nigg-infected controls (Fig. 2A), but the infection course between the strains was not significantly different. Mice infected with CM3.1 succumbed, as did the mice infected with CM001 (Fig. 2B). Mortality was associated with high chlamydial burdens in the oviducts, lungs, and spleens of CM001- and CM3.1-infected mice (Fig. 2C). Mice infected with CM012 and Nigg demonstrated a low burden in the lungs and spleens at the time of sacrifice on day 40. Mice infected with CM018, a clonal isolate that had spontaneously lost the chlamydial virulence plasmid, also survived their infection (data not shown). These data collectively show that CM001 lethality in *Rag1*$^{-/-}$ mice is plasmid independent and is associated with a high chlamydial burden in distal organs at the time of death.

IFN-γ signaling is required for protection against CM001 lethality. B cell-deficient mice clear disseminated infection with *C. muridarum* Nigg (13), but *IFNG*$^{-/-}$ mice demonstrate extragenital dissemination, and a portion of the mice succumb to infection with strain Weiss (11). We next investigated if CM001 infection of these immune-deficient mice would yield similar results. B cell-deficient mice survived infection with CM001 and demonstrated a course of infection clearance similar to that demonstrated by wild-type mice (Fig. 3A). However, CM001 infection of *IFNG*$^{-/-}$ and *STAT1*$^{-/-}$ mice, deficient in IFN-γ signaling, proved lethal, while Nigg-infected mice survived (Fig. 3B). Based on these results, we extended our analysis to determine if Th1

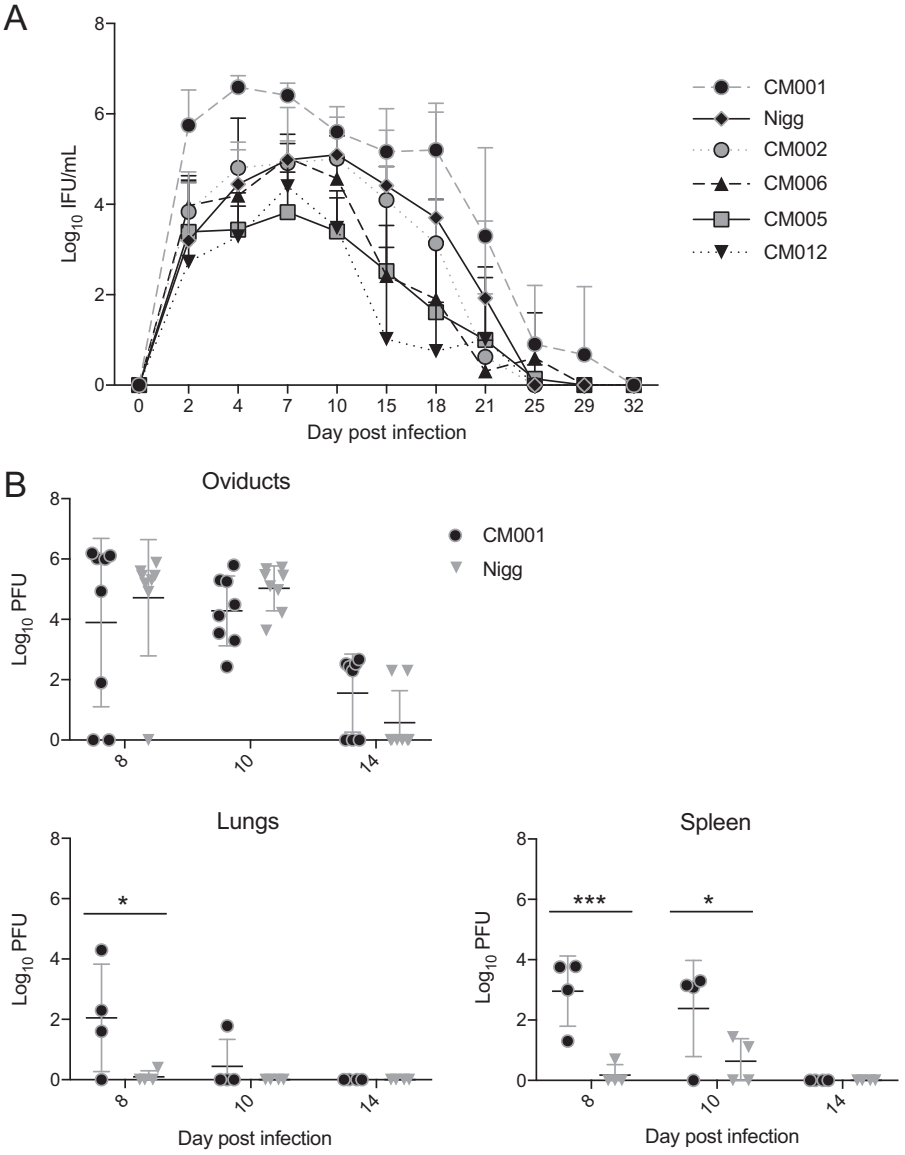

FIG 1 *C. muridarum* CM001 intravaginal infection disseminates to distal organs in wild-type mice and is rapidly cleared. (A) C57BL/6J mice were intravaginally infected with Nigg or *C. muridarum* clones, and the course of primary infection was monitored with lower genital tract swab specimens. Significance was determined by two-way RM ANOVA. Data represent the mean + SD for swab specimens from infected mice or swab specimens from infected and uninfected mice ($n = 4$ mice per group). *P* values for comparison of the groups over the primary infection course were as follows: $P = 0.02$ for Nigg versus CM001 and $P = 0.05$ for Nigg versus CM012. (B) CM001 and Nigg lung, spleen, and oviduct burdens were compared by determination of the numbers of PFU on the indicated days postinfection. Statistical significance, determined by two-way ANOVA, is indicated by asterisks, as follows: *, $P < 0.05$; ***, $P < 0.001$.

cells were dispensable for protection against CM001. We had previously noted that *Tcra*$^{-/-}$ mice do not succumb to CM001 infection (6) and nude mice develop chronic chlamydial genital infection with strain Nigg (12), so we were curious to discover if they would succumb to CM001 intravaginal infection like *Rag*1$^{-/-}$ mice. Nude mice survived the intravaginal CM001 infection (Fig. 3C), indicating that IFN-γ prevents lethal CM001 infection in the absence of T cells and suggests a B cell corequirement for the control of dissemination.

B cells cooperate with IFN-γ to protect against CM001 lethality in the absence of T cells. A recent study revealed that convalescent-phase immunoglobulin and IFN-γ are required for neutrophil activation and chlamydial clearance (9). Based on this

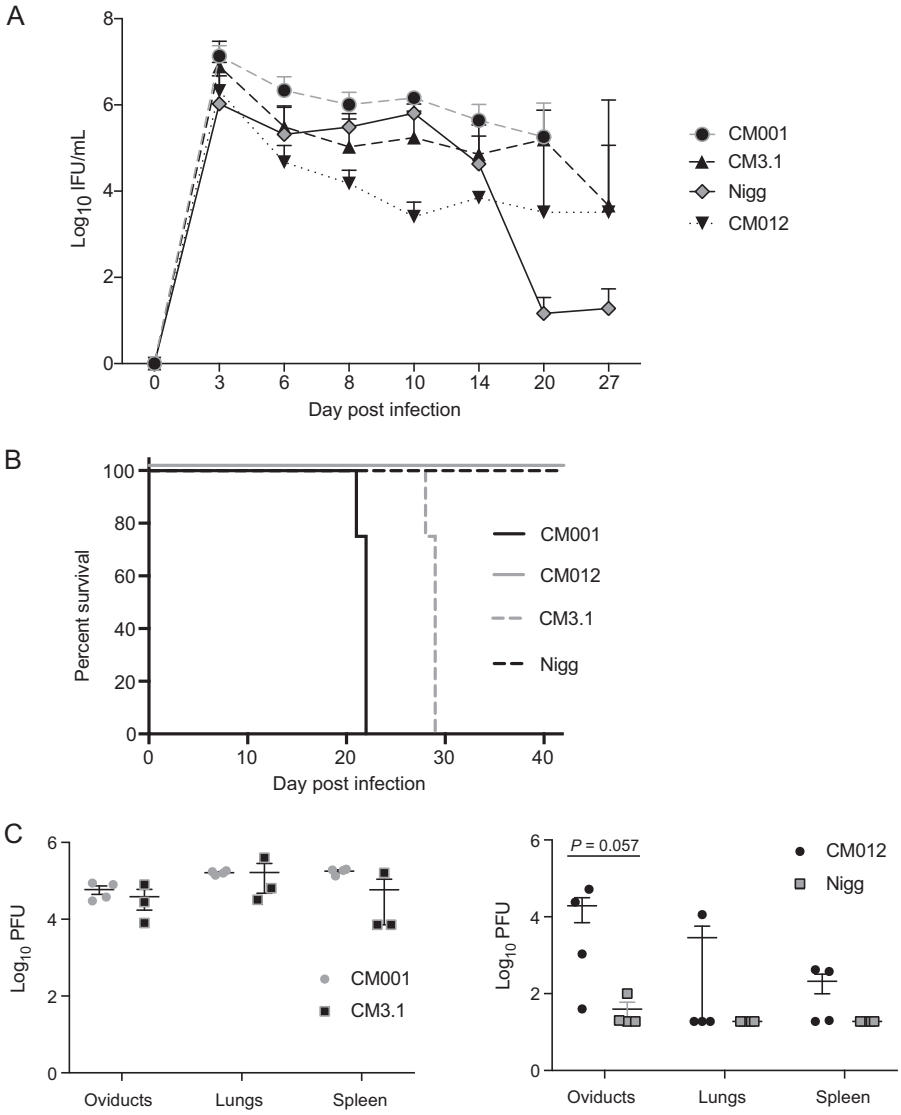

FIG 2 *C. muridarum* CM001 intravaginal infection in *Rag1*$^{-/-}$ mice is associated with fatal dissemination to distal organs. (A) *Rag1*$^{-/-}$ mice were intravaginally infected with Nigg, CM001, CM012, or plasmid-deficient CM3.1, and the course of primary infection was monitored with lower genital tract swab specimens. Significance was determined by two-way RM ANOVA. Data represent the mean + SD for 4 mice per group. *P* values for comparison of the groups over the primary infection course were as follows: $P = 0.01$ for Nigg versus CM001, $P < 0.001$ for Nigg versus CM3.1, $P = 0.01$ for CM012 versus CM001, $P = 0.001$ for CM012 versus CM3.1, and $P =$ not significant for the remaining group comparisons. (B) Mice were euthanized after reaching a body condition score of 2−, and an exact log-rank test was used to analyze survival differences between the Nigg- and CM001- or CM3.1-infected groups ($P < 0.01$). (C) Chlamydial loads in the lungs, spleen, and both oviducts were determined by plaque assay (by determination of the number of PFU) at the time of euthanasia (left) or on day 40 (right). Statistical significance was determined by the Mann-Whitney U test.

finding, we hypothesized that adoptive transfer of wild-type mouse convalescent-phase immune serum, consisting of T cell-dependent antibody, to *Rag1*$^{-/-}$ mice would delay the lethal response. Adoptive transfer of immune serum significantly increased the survival time compared to that for mice receiving naive IgM or the untreated controls (Fig. 4A). We next investigated if *Rag1*$^{-/-}$ mice could be completely rescued in the absence of T cells or T cell-dependent antibody. Adoptive transfer of B cells derived from the spleens of naive T cell-deficient mice rescued *Rag1*$^{-/-}$ mice from an otherwise lethal CM001 infection (Fig. 4B). T cell-deficient mice and B cell-reconstituted *Rag1*$^{-/-}$ mice demonstrated a respective 1.2- and 1.5-log reduction in the lung

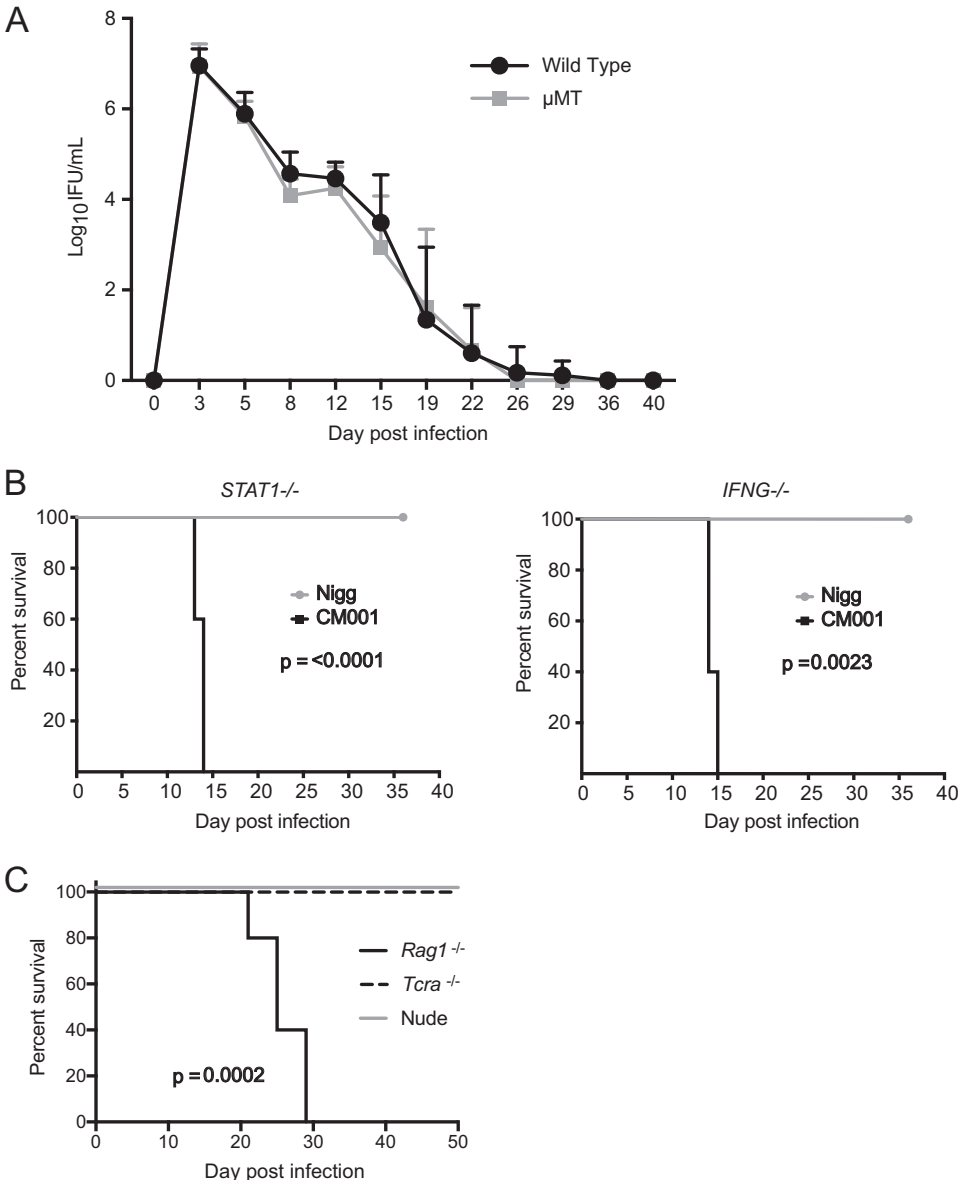

FIG 3 Genetic ablation of IFN-γ signaling results in CM001-mediated lethality. (A) C57BL/6J ($n = 24$) and muMt ($n = 14$) deficient mice were intravaginally infected with CM001, and the course of primary infection was monitored with lower genital tract swab specimens. Data represent the mean + SD. Significance was determined by two-way RM ANOVA (P was not significant). (B) *STAT1*$^{-/-}$ (Nigg group, $n = 10$; CM001 group, $n = 5$) and *IFNG*$^{-/-}$ (5 mice per group) mice were intravaginally infected with Nigg or CM001. Animal welfare was monitored daily, and an exact log-rank test was used to analyze survival differences between Nigg- and CM001-infected *STAT1*$^{-/-}$ ($P < 0.0001$) and *IFNG*$^{-/-}$ ($P = 0.0023$) mice. (C) Nude, *Rag1*$^{-/-}$, and *Tcra*$^{-/-}$ mice were infected with CM001 and monitored daily ($P = 0.0002$ for nude mice versus both *Rag1*$^{-/-}$ and *Tcra*$^{-/-}$ mice).

chlamydial burden on day 19 postinfection compared to that in the *Rag1*$^{-/-}$ controls (Fig. 4C). The chlamydial burden in the genital tract was not significantly different between groups over the course of primary infection (Fig. 4D). These data collectively demonstrate the ability of B cells to mediate protection against extragenital chlamydiae in the absence of T cell helper and effector functions.

DISCUSSION

This study highlights the previously underappreciated contribution of IFN-γ and B cells to the control of extragenital chlamydial dissemination, particularly during early infection, after murine intravaginal inoculation. We did not detect differences in the

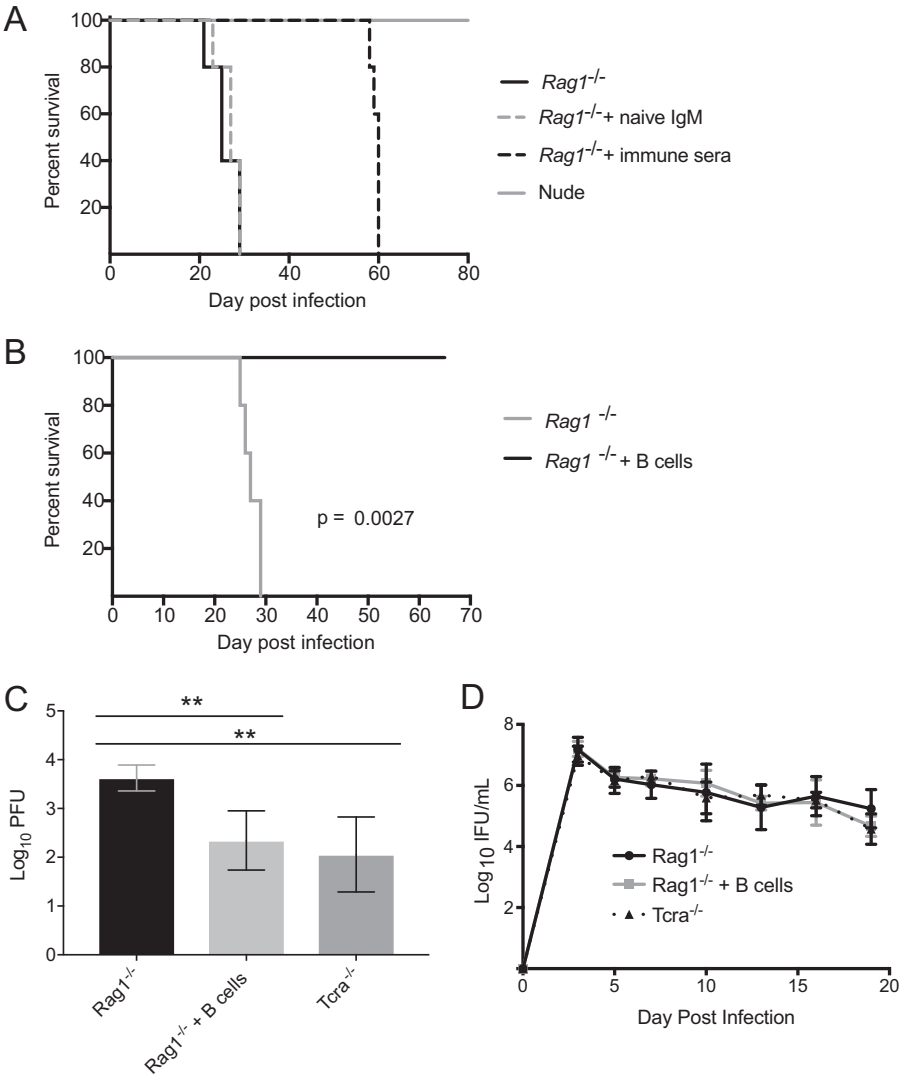

FIG 4 B cells and IFN-γ cooperate to prevent CM001 lethality independently of T cells. (A) Nude mice and *Rag1*$^{-/-}$ mice receiving an intravenous transfer of naive mouse IgM, convalescent-phase immune serum, or PBS ($n = 5$ per group) were infected with CM001 and monitored daily. An exact log-rank test was used to analyze the survival differences between nude mice ($P < 0.0001$) and *Rag1*$^{-/-}$ mice ($P = 0.0026$) receiving immune serum. (B) *Rag1*$^{-/-}$ mice were mock treated or intravenously injected with 3×10^5 B cells ($n = 5$ per group) from *Tcra*$^{-/-}$ mice prior to CM001 infection and were monitored daily ($P = 0.0027$). (C) *Tcra*$^{-/-}$ ($n = 3$), *Rag1*$^{-/-}$ ($n = 5$), and B cell-reconstituted *Rag1*$^{-/-}$ ($n = 5$) mice were infected with CM001 and sacrificed on day 19 postinfection. The chlamydial load in the lungs was determined by plaque assay (determination of the number of PFU). Statistical significance, determined by one-way ANOVA, is indicated by asterisks, as follows: **, $P < 0.01$. (D) The course of primary infection was monitored with lower genital tract swab specimens (*P* was not significant). Significance was determined by two-way RM ANOVA.

extent of extragenital infection between wild-type mice infected with CM001 and wild-type mice infected with the other isolates when studied after day 10, coincident with the development of a *Chlamydia*-specific T cell response (6, 13). Cell culture assays of replication or infectivity failed to reveal differences between CM001 and its siblings that might account for these findings. However, when we compared the consequences of infection with CM001 in immune-deficient mice to those of infection with the other isolates, we observed high chlamydial burdens in the lungs and spleens of moribund *Rag1*$^{-/-}$ mice that were euthanized after intravaginal infection. These findings reveal that immunologic control of extragenital chlamydiae after intravaginal inoculation is an important component of host defense by preventing pneumonia and weight loss, which are ultimately fatal (11, 14).

TABLE 1 Outcome of CM001 infection and immune status of mouse strains[a]

Mouse genotype	Mortality	Clearance	IFN-γ signaling	Antibody	$\alpha\beta$ T cells
Wild type	No	Yes	Yes	Yes	Yes
Rag1$^{-/-}$	Yes	No	Yes	No	No
STAT1$^{-/-}$	Yes	No	No	Yes	Yes
IFNG$^{-/-}$	Yes	No	No	Yes	Yes
muMT	No	Yes	Yes	No	Yes
Tcra$^{-/-}$	No	No	Yes	Yes	No
Rag1$^{-/-}$ + B cells	No	No	Yes	Yes	No

[a]The survivability of infection, clearance of infection, and immune profiles were compared in wild-type and gene-deficient C57BL/6J mice.

This lethal phenotype allowed us to identify determinants necessary to prevent fatal chlamydial dissemination. CM001 intravaginal infection of mice with defective IFN-γ signaling resulted in universal lethality at between 10 and 14 days postinfection. These observations are consistent with the findings of previous studies demonstrating a protective role for IFN-γ in the early control of dissemination (11, 27, 28). However, *Rag1*$^{-/-}$ mice, lacking both T and B cells, succumbed to CM001 infection after 20 days postinfection, despite the presence of IFN-γ (Table 1). This result suggests a potential cooperation between B cells and IFN-γ in prevention of chlamydial dissemination. Indeed, *Rag1*$^{-/-}$ mice were transiently rescued by the adoptive transfer of convalescent-phase immune serum from wild-type mice. This result demonstrates that antibody generated in the presence of T cell help can afford protection against disseminated chlamydial infection, in the absence of an effector Th1 response. Furthermore, transfer of naive B cells taken from T cell-deficient mice completely rescued *Rag1*$^{-/-}$ mice from lethality. This protection was associated with a decreased chlamydial burden in the lungs of genitally infected mice and suggests a protective role for T cell-independent antibody. These experiments reveal a previously undescribed T cell-independent role for B cells in concert with IFN-γ for protection against *C. muridarum* disseminated disease.

Genomic sequencing of the clonal isolates derived from the parental Nigg population revealed very little diversity among the strains. Nevertheless, we noted variations in the lengths of homopolymeric G and C tracts situated close to the start codon of three genes, TC_0436, TC_0440, and TC_0447, indicating the potential for slip-strand mispairing (29) during DNA replication to throw expression in or out of phase (see Fig. S2 in the supplemental material). Interestingly, these proteins comprise part of a conserved, eight-member, paralogous family of phosphatidylcholine-hydrolyzing phospholipase D (PLD) proteins (30, 31) containing NucT motifs (32), indicative of a PLD subfamily with nuclease activity. The extracellular, outer membrane-associated NucT nuclease of *Helicobacter pylori* enables it to use extracellular DNA as a purine source (33). Although the role of these proteins in *C. muridarum* is unknown, their high sequence homology suggests that they have a similar function and may be subject to antigenic variation, with expression altering in response to selective pressures. All of the strains investigated were predicted to express at least one of the three proteins, and we did not detect genetic variation in the remaining members of the family.

We were particularly interested in identifying genetic variation unique to CM001 because it appeared to be more virulent than the other isolates in both wild-type and immune-deficient mice. We have demonstrated the importance of the chlamydial virulence plasmid to infectivity (18, 25, 34) and in eliciting damaging innate inflammation (26), but the presence or absence of the plasmid did not influence the ability of the *C. muridarum* isolates to kill the immune-deficient mice examined in this study. CM3.1, a plasmid-deficient derivative of CM001, still caused lethal infection of *Rag1*$^{-/-}$ mice, while CM018, a spontaneous plasmid-deficient isolate from the Nigg population, did not. CM3.1 resembled its parent by also killing *IFNG*$^{-/-}$ and *STAT1*$^{-/-}$ mice. Our analysis revealed two sequence differences in *ompA*, a gene encoding MOMP, the

trimeric beta-barrel porin that comprises ~60% of the outer membrane protein component (20). The *ompA* sequence present in CM001 was conserved in CM972 and CM3.1 (data not shown) but not in any other isolates investigated. These changes mapped to the conserved region of VD4 (Fig. S4), a portion of MOMP that has proven difficult to resolve structurally and that differentiates it from other beta-barrel bacterial porins (20). It has been proposed that this stretch of the protein forms a loop that is important for eukaryotic cell attachment (35). Our cell culture-based infectivity assays did not reveal any differences in infectivity between CM001 and other plasmid-containing *C. muridarum* isolates. However, differences in infectious burden were observed, potentially illustrating the impact of the VD4 conformation on chlamydial attachment, replication, and dissemination *in vivo*. Our comparison of the *C. muridarum* sequences available in GenBank indicated that many independent laboratory stocks express MOMPs identical to the MOMP expressed by CM001, suggesting that this represents the wild-type conformation. In the case of the parental Nigg stock, prolonged cell culture (21, 36) in the absence of selective immune pressure appears to have supported the outgrowth of variants, predominantly those represented by CM002 and the remaining isolates that we characterized, but also isolates with other *ompA* mutations (24). Yeruva et al. recently reported their isolation of a virulent clone from the Nigg stock that they determined was virulent based on its ability to kill *Rag1*$^{-/-}$ mice (36). It would be interesting to determine if the *ompA* in this clone is identical to that in CM001. These observations may also help reconcile the irregular chlamydial lethality reported in immune-deficient mouse models, as these differences are likely due to genetic variation between laboratory stocks.

Our analyses were limited to delineating general requisites for protection against disseminated extragenital infection. While IFN-γ and B cells were shown to play a pivotal role in protection, the cellular source of IFN-γ and the mechanism of B cell protection were not investigated. However, previous experiments have demonstrated that NK cells are a critical source of IFN-γ in the absence of T cells (37), and B cell protection is likely dependent on their production of antibody, based on the observation that antibody-deficient (AID$^{-/-}$ μS$^{-/-}$) B cells are unable to control dissemination from the genital tract (38). A role for antibody is further suggested by the transient protection of *Rag1*$^{-/-}$ mice from CM001 lethality by administration of convalescent-phase serum. However, we cannot rule out the possibility of antibody-independent mechanisms of protection, such as cytokine production (39). Recent investigation suggests that IgG and IFN-γ are necessary for neutrophil activation prior to chlamydial killing (9), but only T cell-dependent antibody production was investigated. Lastly, we did not determine the mechanism for dissemination; however, previous data suggest that *C. muridarum* may directly ascend from the genital tract into the peritoneal cavity (11). However, the possibility of dissemination through the lymphatics and blood cannot be excluded, and future investigation of this process may help further define the role of B cells and IFN-γ in host defense.

Significant T cell-independent immunity has been described for *Ehrlichia muris* (40), *Salmonella* (41), and *Streptococcus pneumoniae* (42). Our analyses indicate that T cell-independent B cell responses contribute to protection against lethal extragenital chlamydial infection. Interestingly, spleen-derived B cells adopt a predominately marginal zone phenotype *in vivo* after adoptive transfer to lymphopenic hosts (43), and neutrophil-derived cytokines can directly stimulate marginal zone B cells for T cell-independent IgG (44). T cell-independent IgG could activate neutrophils to phagocytose bacteria (42, 45), which would be enhanced in the presence of IFN-γ (9, 46). However, we cannot rule out an alternative model where antibodies limit dissemination and, independently, IFN-γ provides cell-autonomous immunity in lung epithelial cells and/or alveolar macrophages (47). Detectable, low levels of IgG have been observed in nude mice intravaginally infected with *C. muridarum* (12), and T cell-independent IgG can provide robust protection against viral (48) and bacterial infections (42). It is tempting to speculate that MOMP-reactive IgG is responsible for controlling chlamydial dissemination, since bacterial porins have been shown to constitute a rare group of

protein antigens capable of inducing protective T cell-independent antibodies (41). If the murine model of extragenital dissemination and immunological control reflects human *C. trachomatis* infection, then this T cell-independent protection may help prevent the development of reactive arthritis and Fitz-Hugh-Curtis syndrome.

In conclusion, we have demonstrated that IFN-γ and B cells cooperate to provide protection against a lethal disseminated chlamydial infection independently of T cells. Examination of T cell-independent responses may further reveal detailed mechanisms of extragenital host defense against *Chlamydia*. Increased understanding of T cell-independent mechanisms of antichlamydial host defense may also inform methods to augment the T cell responses generated with targeted vaccines.

MATERIALS AND METHODS

Strains, cell lines, and culture conditions. The *Chlamydia muridarum* Nigg stock (AR-Nigg) was obtained from Roger Rank at the University of Arkansas for Medical Sciences (21). A group of clonal variants was derived from this population via plaque assay (18, 49). Individual plaques were harvested, diluted, and repurified via plaques before being amplified to high-titer stocks for subsequent experiments. A total of 24 isolates were recovered, and a subset (strains CM002, CM005, CM006, CM007, CM008, CM009, CM010, CM012, CM018, and CM021) was selected for further analysis. Also included in the study were strains CM001, the parent of the avirulent, plasmid-cured strain CM972 (18, 26), and CM3.1, a derivative of CM972 with normal infectivity (26). Plaque-purified *C. muridarum* strains were propagated in *Mycoplasma*-free L929 cells (50) and titrated as inclusion-forming units (IFU) (51), using a fluorescently tagged antichlamydial lipopolysaccharide monoclonal antibody (Bio-Rad). Intracellular growth kinetics and infectivity in cell culture were evaluated for the isolates as previously described (18, 25, 26).

Sequencing. Gradient-purified bacterial preparations (52) were treated with RNase/DNase to degrade the nucleic acid carried over from before isolation of genomic DNA using a MasterPure complete DNA and RNA extraction kit (Epicentre, Madison, WI). Genomic DNA was sheared and processed for single molecule real-time (SMRT) sequencing on a PacBio RSII sequencer (Pacific Biosciences, Menlo Park, CA), as previously described (53). Data were analyzed using the hierarchical genome assembly process (HGAP) method (54), and the assembled contigs were polished using the Quiver program (PacBio). Whole-genome alignments of the clonal isolates and reference sequence were performed using the progressive Mauve algorithm (55), supported in the Geneious program (version 11.0; Biomatters, Auckland, New Zealand). Where indicated, Sanger sequencing of the PCR-amplified products was performed to resolve ambiguities or to confirm predicted sequence polymorphisms.

Animals. Six- to 8-week-old female C57BL/6J (stock no. 000664), B6.129S2-*Ighm*^{tm1Cgn}/J (muMT negative; stock no. 002288), B6.129S(Cg)-*Stat1*^{tm1Div}/J (*STAT1*$^{-/-}$, stock no. 012606), B6.129S7-*Ifng*^{tm1Ts}/J (*IFNG*$^{-/-}$; stock no. 002287), B6.129S7-*Rag1*^{tm1Mom}/J (Rag1 knockout; stock no. 002216), B6.129S2-*Tcra*^{tm1Mom}/J (*Tcra*$^{-/-}$; stock no. 002116), and B6.Cg-*Foxn1*^{nu}/J (C57BL/6 nude; stock no. 000819) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were given food and water *ad libitum* in an environmentally controlled pathogen-free room with a cycle of 12 h of light and 12 h of darkness. All animal experiments were approved by the Institutional Animal Care and Use Committees at the University of Pittsburgh and University of North Carolina.

Murine *Chlamydia* infection and monitoring. Female mice at least 8 weeks old were subcutaneously injected with 2.5 mg medroxyprogesterone (Depo-Provera; Upjohn) 5 to 7 days prior to infection to induce a state of anestrous (56). Mice were intravaginally inoculated with 5×10^5 inclusion-forming units (IFU) of Nigg or the *C. muridarum* clones diluted in 30 μl sucrose-sodium phosphate-glutamic acid buffer. The mice were monitored for cervicovaginal shedding via the collection of endocervical swab specimens (51) over the course of their infection (57). Ascending genital infection and extragenital dissemination were confirmed via quantitation of the bacterial load in homogenized oviduct tissues, spleens, and lungs from sacrificed mice (26). Animal welfare was monitored daily, and immune-deficient mice were euthanized if they achieved a body conditioning score of 2− (58).

B cell isolation and adoptive transfer. B cells were isolated from the spleens of naive *Tcra*$^{-/-}$ mice by negative magnetic selection (Miltenyi Biotech), according to the manufacturer's protocol. A sample of isolated cells was analyzed by flow cytometry to confirm 98% CD45$^+$ CD5$^-$ CD19$^+$ resting B cell purity using LIVE/DEAD Fixable Yellow (Life Technologies) in combination with the following fluorochrome-labeled antibodies from BD Biosciences: anti-CD45 (clone 30-F11), anti-CD5 (clone 53-7.3), and anti-CD19 (clone 1D3). Stained cells were analyzed on a CyAN ADP analyzer (Beckman Coulter), and the resultant data were analyzed using FlowJo software. At least 3×10^6 spleen-derived B cells were injected intravenously into medroxyprogesterone-treated *Rag1*$^{-/-}$ mice 5 days prior to intravaginal infection.

C57BL/6J mice were bled at sacrifice following resolution of intravaginal *C. muridarum* Nigg infection to recover convalescent-phase immune serum (10). Serum was pooled and complement inactivated at 56°C for 30 min. Naive mouse-derived IgM (Rockland Immunochemicals, Inc.) was dialyzed overnight prior to intraperitoneal (i.p.) injection. Naive medroxyprogesterone-treated *Rag1*$^{-/-}$ mice received i.p. injections of phosphate-buffered saline (PBS), 0.5 ml of immune serum, or 200 μg IgM on days −1, 0, 1, 3, 7, 10, and 13 of infection.

Statistical analysis. Differences between the means for experimental groups after infection were calculated using the Mann-Whitney U test or a two-way repeated-measures (RM) analysis of variance

(ANOVA). Comparisons of animal survival were performed by an exact log rank test. Prism software (GraphPad Software) was utilized for statistical analyses, and values of P of ≤ 0.05 were considered significant.

Accession number(s). The genome sequencing data for the strains sequenced in this study are deposited under NCBI BioProject accession number PRJNA435732.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00143-18.

SUPPLEMENTAL FILE 1, PDF file, 1.3 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

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