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SHORT COMMUNICATION

OT-1 mice display minimal upper genital tract pathology following primary intravaginal *Chlamydia muridarum* infection

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In this straightforward study, Murthy *et al.* show that mice whose CD8 T cells that are 'locked-in' for the recognition of one antigen (OVA), do not show any deficiencies in the ability to clear chlamydia from infected oviducts. However, unlike wild type mice, these OVA-specific mice do not develop pathology, suggesting that *Chlamydia*-specific CD8 T cells contribute significantly to immune pathology despite a lack of a role for *in vivo* clearance of the pathogen.

Keywords
Chlamydia trachomatis; *Chlamydia muridarum*; antigen-specific CD8 T cells; upper genital tract; pathogenesis.

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Abstract

Chlamydia trachomatis is the most common bacterial sexually transmitted disease worldwide and leads to serious pathological sequelae in the upper genital tract (UGT) including pelvic inflammatory disease, ectopic pregnancy, and infertility. Several components of the host immune responses have been shown to contribute to the UGT pathology following genital chlamydial infection. We have shown recently that $CD8^+$ T cells induce the chlamydial UGT pathology via the production of TNF-α. However, those studies did not determine whether the pathology is mediated by bystander or antigen-specific $CD8^+$ T cells. In this study, we compared chlamydial clearance and UGT pathology in OT-1 transgenic mice and the corresponding C57BL/6J wild-type mice following primary intravaginal *Chlamydia muridarum* infection. All $CD8^+$ T cells in the OT-1 mice respond only to the Ova 257-264 peptide and are incapable of responding to other antigenic epitopes including those of *Chlamydia*. OT-1 mice displayed vaginal chlamydial clearance comparable to the wild-type animals. However, both oviduct and uterine horn pathology were minimal in the OT-1 mice compared with the high degree of pathology observed in the wild-type animals. These results strongly suggest that *Chlamydia*-specific, not bystander, $CD8^+$ T cells mediate the UGT pathological sequelae following genital chlamydial infection.

Sexually transmitted infections caused by *Chlamydia trachomatis* affect approximately 90 million people annually worldwide (Brunham & Rey-Ladino, 2005). A significant proportion of infected females develop severe pathological sequelae in the upper genital tract (UGT) including pelvic inflammatory disease, ectopic pregnancy, and infertility (Brunham & Rey-Ladino, 2005). A mouse model using a closely related pathogen *Chlamydia muridarum* has been used extensively to study genital chlamydial infections, because the pathological phenotype in this model is closely comparable to those found in human *C. trachomatis* infections (Morrison & Caldwell, 2002). Using the mouse model, several components of the host immune response including Toll-like receptor 2 signaling (Darville *et al.*, 2003), IL-1 β (Prantner *et al.*, 2009), and neutrophils and matrix metalloproteases (Imtiaz *et al.*, 2007; Lee *et al.*, 2010), have been shown to contribute to the immunopathogenesis of the disease sequelae following genital chlamydial infection. In this model, we also have shown recently that $CD8^+$ T cells, via TNF-α production, mediate the UGT pathology following genital chlamydial infections (Murthy *et al.*, 2011). Specifically, mice deficient in CD8 (CD8 KO mice) display similar kinetics of chlamydial clearance, but significantly reduced pathology following primary *C. muridarum* infection, when compared with corresponding wild-type animals. Moreover, repletion of CD8 KO mice with $TNF^{+/+}$ $CD8^+$ T cells, but not with $TNF^{-/-}$ $CD8^+$ T cells, at the time of bacterial challenge, restores pathology to the levels found in wild-type animals. In these experiments, a likely but not confirmed possibility was that a very small frequency of *Chlamydia*-specific $CD8^+$ T cells among the adoptively transferred naïve cells proliferated clonally in the CD8 KO mice response to the

infection and induced the pathology. However, it also is possible that the large numbers of bystander, non-*Chlamydia*-specific, $CD8^+$ T cells mediated the pathology. Bystander $CD8^+$ T cells have been shown to get activated and possibly play a role in other diseases with infectious and noninfectious etiologies (Chen *et al.*, 2005; Sobottka *et al.*, 2009).

We sought to evaluate whether the *Chlamydia*-induced UGT pathology is mediated by antigen-specific or bystander $CD8^+$ T cells. The adoptive transfer of $CD8^+$ T cells derived from *Chlamydia*-infected wild-type mice into recipient CD8 KO mice may seem as a straightforward method to evaluate this possibility. However, the frequency of antigen-specific $CD8^+$ T cells in *Chlamydia*-infected mouse spleens would be very low, and a large fraction of transferred cells would be non-*Chlamydia*-specific. Therefore, the development of pathology in recipient CD8 KO mice could still be due to the effects of non-*Chlamydia*-specific $CD8^+$ T cells. To overcome this hurdle, we made an unconventional use of the OT-1 transgenic mice (catalog number 003831, Jackson Laboratories, Bar Harbor, ME). OT-1 mice have a full $CD8^+$ T-cell compartment; however, all CD8 T cells respond only to the Ova 257-264 peptide, and therefore, these mice are used typically to study antigen-specific responses against the Ova 257-264 peptide. However, as the $CD8^+$ T cells in these mice cannot respond to chlamydial antigens in an antigen-specific fashion, we reasoned that a reduction in UGT pathology following chlamydial genital infection in these mice would suggest a role for *Chlamydia*-specific, not bystander, $CD8^+$ T cells in chlamydial pathogenesis. On the contrary, if pathology in OT-1 mice was comparable to that in the wild types, it could be interpreted as an effect of the bystander $CD8^+$ T cells.

We compared the course of chlamydial clearance from the lower genital tract and the development of UGT pathology in OT-1 mice and the corresponding C57BL/6J wild-type mice (Jackson Labs). The mice were housed in the animal facility at Midwestern University (MWU). Food and water were provided ad libitum, and all procedures were performed in accordance with an approved protocol (number 2088) from the Institutional Animal Care and Use Committee at MWU. *Chlamydia muridarum* Nigg was grown on confluent HeLa cell monolayers as described previously (Murthy *et al.*, 2011). The infected cells were lysed by sonication and elementary bodies (EBs) purified on Renografin gradients. Aliquots of bacteria were stored at −70 °C in sucrose–phosphate–glutamine (SPG) buffer. Groups ($n = 5$ in experiment 1 and $n = 6$ in experiment 2) of 4- to 6-week-old female OT-1 and C57BL/6J mice were infected intravaginally with 5×10^4 inclusion forming units (IFU) of *C. muridarum* on day 0. Mice were given subcutaneous Depo-provera® (2.5 mg per mouse, Upjohn, Kalamazoo, MI) 10 and 3 days before challenge to render them anestrous and more receptive to the infection (Murthy *et al.*, 2011). Vaginal swab material was collected on the indicated days after challenge and chlamydial enumeration conducted by plating swab material on HeLa cell monolayers followed by immunofluorescent staining (Murthy *et al.*, 2011). As shown in Fig. 1a, C57BL/6J mice displayed a high level of chlamydial shedding on day 3 followed by progressive clearance of infection and complete clearance by day 32 following the primary inoculation. The group of OT-1 mice displayed kinetics of chlamydial shedding and clearance similar to the wild-type animals. These results suggest that neither *Chlamydia*-specific nor bystander $CD8^+$ T cells are required for clearance of the bacterium from the lower genital tract.

The mice were rested until day 80, euthanized, and genital tracts collected to evaluate the UGT pathology. The collected tissues were immersed in 2% paraformaldehyde for 24 h and then soaked in ethanol for 48 h. The processed tissues were photographed using a 10 megapixel Panasonic Lumix TZ3 digital camera. A representative set of three genital tracts each from C57BL/6J mice (left column) and OT-1 mice (right column) is shown in Fig. 1b. Individual mouse genital tracts were placed next to a standard metric ruler and photographed, the pictures printed out on 8×11.25 inch sheets of paper, the greatest cross-sectional diameter measured for each oviduct, and reported individually and as mean ± SEM in a group in Fig. 1c. As described by us previously (Murthy *et al.*, 2011), we have found that a threshold of 0.5 mm can be used to distinguish between normal and dilated oviducts. Additionally, the greatest cross-sectional diameter for every 5-mm longitudinal section of the uterine horn was measured, and the average diameter per uterine horn was calculated. The individual averages per uterine horn and the mean ± SEM of the average uterine horn diameter in a group are reported in Fig. 1d. We also have found that a threshold of 1 mm can be used to distinguish between normal and dilated uterine horns. The enumeration of chlamydial counts, oviduct, and uterine horn measurements were conducted in a blinded fashion. In Fig. 1c and d, the ratio of oviducts or uterine horns that were normal to the total number evaluated is shown on the *x*-axis. The horizontal line at 0.5 mm in Fig. 1c and at 1 mm in Fig. 1d distinguishes the normal from dilated tissues. As shown in Fig. 1c, all oviducts in C57BL/6J mice displayed oviduct dilatation reflecting a significantly ($P < 0.01$, Fisher's exact test) higher incidence of oviduct dilatation compared with one dilated oviduct in OT-1 mice. The degree of oviduct dilatation in C57BL/6 mice also was obviously and significantly ($P < 0.01$, two-tailed Student's *t*-test) higher than that in the OT-1 mice. Similarly, the incidence ($P < 0.01$, Fisher's exact test) and degree of uterine horn dilatation in C57BL/6J mice were significantly ($P < 0.01$, Fisher's exact test) higher than that in OT-1 mice. These results suggest that non-*Chlamydia*-specific $CD8^+$ T cells in the OT-1 mice were incapable of mediating UGT pathology following genital *C. muridarum* infection.

Collectively, these results suggest that neither *Chlamydia*-specific nor bystander $CD8^+$ T cells are required for clearance of primary genital chlamydial infection, whereas *Chlamydia*-specific, but not bystander, $CD8^+$ T cells mediate UGT pathology. The inability of OT-1 $CD8^+$ T cells to induce pathology following chlamydial infections may theoretically be due to these cells not trafficking to the infected genital tracts and/or due to the lack of clonal selection and

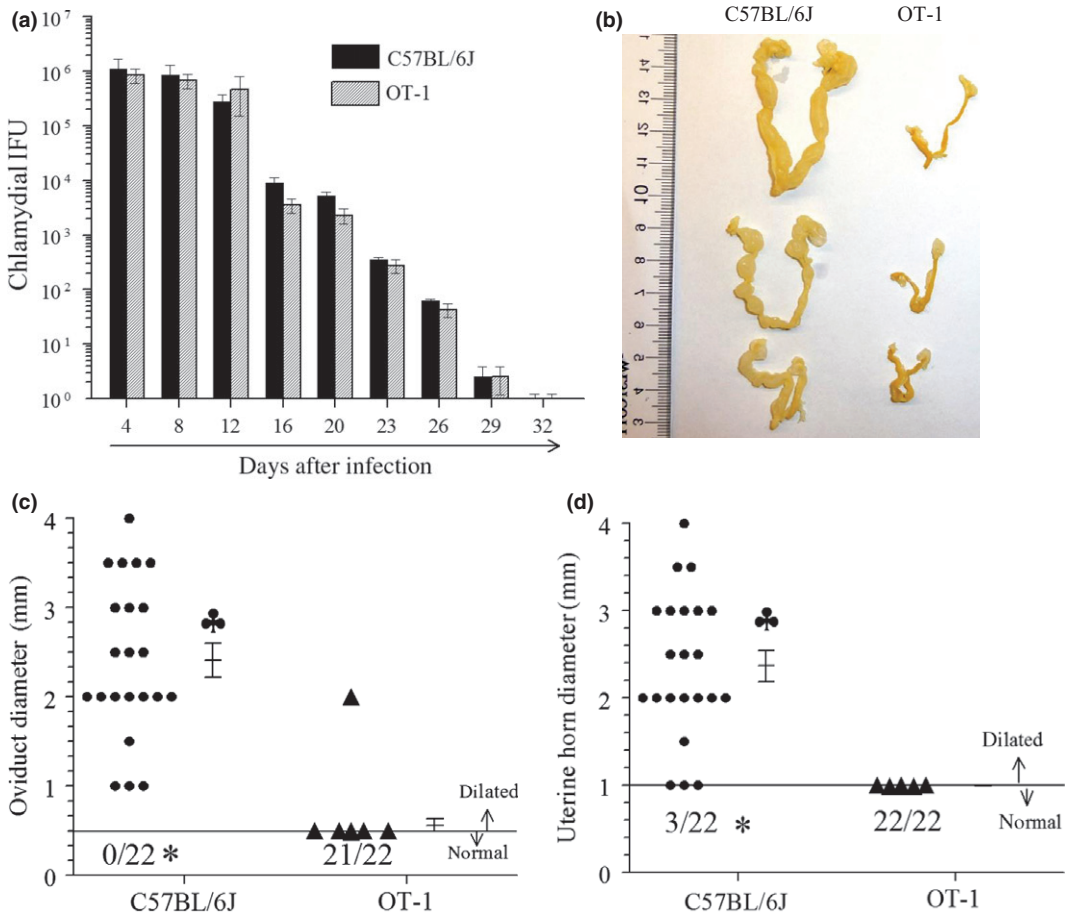

Fig. 1 Chlamydial clearance and UGT pathology in OT-1 mice. Groups of OT-1 and C57BL/6J mice were infected intravaginally with 5×10^4 IFU of *Chlamydia muridarum*. (a) Chlamydial shedding was measured at the indicated time-points after inoculation. On day 80 after inoculation, mice were euthanized and genital tract tissues analyzed for oviduct and uterine horn pathology. (b) Representative oviduct and uterine horn dilatation in C57BL/6J mice compared with OT-1 animals. (c) Oviduct and (d) uterine horn dilatation. Each individual marker represents one oviduct or uterine horn, and the mean ± SEM of greatest cross-sectional oviduct diameter or mean ± SEM of the average uterine horn diameters per group of mice also is shown. The number of normal oviducts or uterine horns (numerator) and the total number of oviducts or uterine horns evaluated (denominator) have been indicated in parentheses on the *x*-axis. *Significant difference in incidence ($P < 0.01$, Fisher's exact test) and ♣degree ($P < 0.01$, Student's *t*-test) of oviduct or uterine horn dilatation following primary genital *C. muridarum* infection in C57BL/6J mice vs. OT-1 mice. Results are composite of two independent experiments. The statistical significance holds true when the experiments are analyzed independently.

expansion following exposure to the infection in the genital tract. Although not evaluated specifically in this study or in the *Chlamydia* infection model in general, there is ample evidence in literature from other infection models to suggest the likelihood of the latter possibility. First, intravenously OT-1 $CD8^+$ T cells have been shown to accelerate the clearance of Ova-expressing Herpes Simplex Virus-2 infection in the mouse vaginal epithelium, suggesting that these cells retain the ability to traffic to the mouse genital tract (Dobbs *et al.*, 2005). Second, it has been shown that both antigen-specific and bystander $CD8^+$ T cells are recruited in comparable numbers into the sites of infection within the first week of primary intracerebral infection with neurotropic corona virus in a mouse model of virus-induced encephalitis (Chen *et al.*, 2005). Furthermore, that study demonstrated that the frequencies of bystander $CD8^+$ T cells reduce beyond the first week, whereas the virus-specific $CD8^+$ T cells are maintained at high frequencies in the infected tissues due to clonal selection and expansion (Chen *et al.*, 2005). Similarly, it is likely that OT-1 $CD8^+$ T cells traffic into the genital tract of *C. muridarum*-infected mice, but do not undergo clonal selection or expansion in response to the chlamydial (non-Ova 257-264) antigens presented during the infection, and therefore do not contribute to oviduct pathology.

The findings of this study have important implications. (1) Previous reports suggest that certain $CD8^+$ T cell clones that recognize specific chlamydial epitopes may assist in bacterial clearance (Wizel *et al.*, 2008) and thus may be suitable as vaccine candidates. Our findings do not exclude this possibility; however, they suggest the need to exercise due diligence in applying such immune responses for the

induction of protective immunity, without inducing clinically relevant pathology. (2) These findings suggest that *Chlamydia*-specific $CD8^+$ T cells do not assist significantly in bacterial clearance and therefore may not recognize infected cells effectively. However, they mediate pathology suggesting the possibility that neighboring uninfected cells are targeted by the *Chlamydia*-specific $CD8^+$ T cells. In essence, that begs the question as to how chlamydial antigens are acquired by uninfected cells. One straight forward possibility is that uninfected cells acquire chlamydial antigens from the extracellular matrix following lysis of *Chlamydia*-infected cells. Alternatively, there is ample evidence that gap junction-mediated antigen transport between neighboring cells via connexon channels may contribute to this phenomenon (Handel *et al.*, 2007). Thus, our findings are relevant and provide new perspectives to further evaluate the mechanism of pathogenesis following genital chlamydial infections and apply them to antichlamydial vaccine development.

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