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Oral *Chlamydia* vaccination induces transmucosal protection in the airway

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ABSTRACT

Although *Chlamydia* has been frequently detected in the gastrointestinal tracts of both humans and animals, it is not associated with any gastrointestinal pathology. We have recently shown that gastrointestinal *Chlamydia muridarum* is not only non-pathogenic but also induces protective immunity in the genital tract. We now report that the transmucosal immunity induced by a single oral immunization with *C. muridarum* protected the mouse airway from a subsequent challenge infection. The oral immunization significantly reduced chlamydial burden in the airway as early as day 3 after intranasal challenge. As a result, the airway chlamydial spreading to extra-airway tissues was completely prevented on day 3 and significantly reduced on day 9. The immunized mice were protected from any significant systemic toxicity caused by the intranasal challenge since there was no significant bodyweight drop in the immunized mice. This robust protection correlated well with *Chlamydia*-specific antibodies that recognize chlamydial organism surface antigens and T cell responses that are dominated with a Th1 phenotype. The immunized mice developed high ratios of IgG2b/c over IgG1 levels and IFNγ-producing over IL-5- or IL-13-producing lymphocytes. Thus, we have demonstrated that oral vaccination with *C. muridarum* can induce Th1-dominant transmucosal immunity in the airway. Together with previous studies, we propose that non-pathogenic colonization of *Chlamydia* in the gastrointestinal tract be explored as an oral delivery system for inducing protection against infections and pathologies in extra-gastrointestinal tissues.

1. Introduction

The genus *Chlamydia* contains various species that can affect human health. *C. trachomatis* serovars D to K that are transmitted sexually between individuals may ascend to the upper genital tract and cause ectopic pregnancy and infertility [1] while serovars A to C are transmitted via contact causing ocular infection and trachoma [2,3]. The genital *C. trachomatis* serovars can also be vertically transmitted to newborns and cause conjunctivitis [4]. The *C. pneumoniae* species that is transmitted via the airway may cause pneumonia in immune-compromised individuals [5] and also associate with asthma [6] and atherosclerosis [7]. *C. psittaci* that is transmitted to humans via inhalation or contact of feces from infected animals can cause life-threatening pneumonia and systemic toxicity [8,9].

The *C. muridarum* species, although not a cause of any known human diseases, has been extensively used to study human chlamydial pathogenesis and immunity. *C. muridarum* induction of long-lasting tubal fibrosis and hydrosalpinx in mice [10–12], which mimicks the tubal adhesion/infertility observed in women urogenitally infected with *C. trachomatis* [13–15], has been used to investigate chlamydial pathogenic mechanisms and immune responses in the reproductive tract [16–23]. Intranasal inoculation with *C. muridarum* for inducing mouse pneumonia and systemic toxicity, leading to significant bodyweight drop and even fatality within a few weeks, has been used as a robust model for both analyzing chlamydial pathogenic mechanisms [24,25] and evaluating vaccine efficacy [26–29]. Studies based on the *C. muridarum* genital and airway infection mouse models have consistently revealed that both IFNγ-dominant Th1-like and B cell responses are essential for preventing and controlling chlamydial infection [25,30–34].

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Chlamydial organisms have also been detected in the gastrointestinal (GI) tracts of both animals [35] and humans [36–39]. However, the medical significance of the GI tract *Chlamydia* remains unclear. Careful clinical investigations under various transmission settings may be necessary for addressing whether GI tract *Chlamydia* can affect the extra-GI tissues' susceptibility to chlamydial infection and pathogenicity. Using the *C. muridarum* murine model, we have recently shown that *C. muridarum* colonizes the mouse GI tract for long periods of time [40], confirming various previous reports [41–44]. Furthermore, the genital *C. muridarum* can spread to the GI tract [42,45]. This spreading is dependent on a hematogenous route [46]. Once arriving at the GI tract, *C. muridarum* organisms establish long-lasting colonization there [45]. Interestingly, the genital *C. muridarum* spreading to the GI tract is associated with *C. muridarum* pathogenicity in the upper genital tract [47,48]. On the contrary, we have recently found that when mice were pre-colonized with *C. muridarum* in the GI tract prior to their exposure to *C. muridarum* in the genital tracts, the mice became immunized against a subsequent infection in the genital tract [49], suggesting that GI tract *C. muridarum* can act as an oral vaccine. However, it remains unknown whether oral vaccination with *C. muridarum* can also induce protective immunity in the airway, which is addressed in the current study.

We now report that a single oral immunization with *C. muridarum* is sufficient for inducing protective immunity in the mouse airway. The oral immunization significantly reduced bacterial burden in the lung and protected mice from any significant bodyweight loss. The robust protection correlated well with *Chlamydia*-specific antibody and T cell responses dominated with a Th1 phenotype, which is consistent with our recent finding that the oral immunization-induced Th1-dominant responses protected mice in the genital tract [49]. Thus, the non-pathogenic colonization of *Chlamydia* in the GI tract may be explored as an oral delivery system for inducing transmucosal protection in the extra-GI tissues.

2. Materials and methods

2.1. Chlamydial organism growth

Both *Chlamydia muridarum* (CM) clones G13.32.1 [50] and CMmCherryG5 [51,52] used in the current study were derived from strain Nigg3 (Genbank accession# CP009760.1). CMmCherryG5 was created by transforming a plasmid-free clone CMUT3G5 (Genbank accession# CP006974.1) with pmCherry:CM as described previously [51,52]. The genome sequences of the *C. muridarum* G13.32.1 and CMmCherryG5 are nearly identical with the exception in the gene coding for TC0412. Although mutations in this gene in *C. trachomatis* have been shown to alter the infectivity of *C. trachomatis* in the mouse genital tract [53], similar mutations in *C. muridarum* had no significant effect on either the infectivity or the pathogenicity in the genital tract infection model [50,54]. Both G13.32.1 and CMmCherryG5 were propagated in HeLa cells and purified as elementary bodies (EBs) as reported previously [45,55]. Aliquots of the purified EBs were stored at −80 °C until use. It is worth noting that for maintenance of HeLa cells and growing up *Chlamydia* for purification, DMEM medium with 10% fetal calf serum (both from ThermoFisher Scientific, Grand Island, NY 14072) with or without cycoheximide (C104450, Sigma-Aldrich, 2 μg/ml for suppressing HeLa cells and growing up *Chlamydia*) but without any antibiotics was used. However, for titrating chlamydial organism titers from animal samples, we included vancomycin (V1130) and gentamycin (G1272, both from Sigma-Aldrich) in the medium for preventing bacterial growth.

2.2. Mouse immunization and challenge infection

Mouse experiments were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Laboratory Animal Experiments of the University of Texas Health Science Center at San Antonio.

Purified *C. muridarum* EBs were used to inoculate six to seven week-old female C57BL/6J mice (stock number 000664, Jackson Laboratories, Inc., Bar Harbor, Maine) intragastrically with 2×10^5 inclusion-forming units (IFUs) of CMmCherryG5 as oral immunization. The intragastric inoculation was performed only once. There was no need to anesthetize mice for the intragastric inoculation since animals only experienced minor and transient distress. To carry out the intragastric inoculation, an experimentalist used one hand to hold the mouse upright with the abdomen facing the experimentalist, then slowly inserted a feeding needle with ball-head into the mouse esophagus and let mouse swallow down the needle. Finally, the content was gently injected and the needle was pulled out. Special attention was paid to avoid contamination of the airway by lowering the feeding needle only after the needle touched the back wall of the esophagus. In our pilot experiments, we monitored chlamydial organisms and genomes in both the GI tract and airway tissues on days 1 and 3 after the intragastric inoculation and found chlamydial organisms and genomes only in the GI tract but not airway tissues, indicating lack of contamination. After the intragastric inoculation, rectal swabs were taken weekly for monitoring CMmCherryG5 colonization in the gastrointestinal tract. Four weeks after the oral immunization, some mice were sacrificed for monitoring immune responses while others were challenged intranasally with 2500 IFUs of G13.32.1. Pilot experiments with 500, 2500 and 12,500 IFUs of this particular stock showed that an inoculation dose of 2500 IFUs induced severe lung infection and systemic toxicity but without fatality and oral immunization induced protection against infections with all 3 doses. On days 3 and 9 after intranasal challenge, mice were sacrificed for quantitating chlamydial burdens in the lung and extra-airway tissues as described below.

2.3. Enumerating viable chlamydiae recovered from rectal swabs and tissue homogenates

To quantitate viable chlamydiae in rectal swabs, each swab was soaked in 0.5 ml of SPG (sucrose phosphate glutamate), vortexed with glass beads, and the chlamydial organisms released into the supernatants were titrated on HeLa cell monolayers in duplicate. The infected cultures were processed for immunofluorescence assay as described previously [56,57] and below. Inclusions were counted in five random views per coverslip using a fluorescence microscope. For coverslips with less than one IFU per field, entire coverslips were counted. Coverslips showing obvious cytotoxicity of HeLa cells due to insufficient dilutions were excluded and instead, coverslips with higher dilutions of the same samples were used for counting inclusions. The total number of IFUs per swab was calculated based on the mean IFUs per view, the ratio of the view area to that of the entire well, dilution factor, and inoculation volumes. Where possible, a mean IFUs/swab was derived from the serially diluted and duplicate samples for any given swab. The total number of IFUs/swab was converted into $\log_{10}$, which was used to calculate the mean and standard deviation across mice in the same group at each time point.

For quantitating viable chlamydiae from mouse organs and tissue segments, each organ or tissue segment was transferred to a tube containing 0.5–5 ml SPG depending on the sizes of the organs. Each GI tract was cut into 7 segments/portions including stomach,