Chlamydial-Secreted Protease Chlamydia High Temperature Requirement Protein A (cHtrA) Degrades Human Cathelicidin LL-37 and Supresses Its Anti-Chlamydial Activity

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Background: Chlamydia trachomatis is an obligate intracellular pathogen that can cause severe reproductive tract complications while ascending infection occurs. When spreading from cell to cell in a host, C. trachomatis utilizes various survival strategies to offset host defense mechanisms. One such strategy is to degrade host antimicrobial defense proteins before they can attack the invading C. trachomatis cells.

Material/Methods: We expressed and purified recombinant chlamydia high temperature requirement protein A (cHtrA) including 2 cHtrA mutants (MT-H143A and MT-S247A), and also extracted endogenous cHtrA. Proteins were identified and their purity evaluated by SDS-PAGE and Western blot. The anti-chlamydial activity and degradation of 5 antimicrobial peptides (cathelicidin LL-37, α-defensin-1 and -3, and β-defensin-2 and -4) by cHtrA and 2 cHtrA mutants (MT-H143A and MT-S247A) were tested by immunoassay and Western blot.

Results: Of the 5 antimicrobial peptides (cathelicidin LL-37, α-defensin-1 and -3, and β-defensin-2 and -4) tested, cathelicidin LL-37 showed the strongest anti-chlamydial activity. Interestingly, cHtrA effectively and specifically degraded LL-37, suppressing its anti-chlamydial activity. The 2 cHtrA mutants (MT-H143A and MT-S247A) were unable to degrade LL-37. Comparison of cHtrA activity from C. trachomatis D, L2, and MoPn strains on LL-37 showed similar responses.

Conclusions: cHtrA may contribute to C. trachomatis pathogenicity by clearing the passage of invasion by specific LL-37 degradation.

MeSH Keywords: Cathelicidins • Chlamydia trachomatis • Serine Proteases

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**Background**

*Chlamydia trachomatis* (*C. trachomatis*), a gram-negative, obligate, intracellular pathogen, causes the most globally common sexually transmitted bacterial infection [1,2]. Most patients report no or mild symptoms [3], but some females experience severe reproductive tract complications, including chronic pelvic pain, ectopic pregnancy, and infertility [4]. Venereal lymphogranuloma caused by *C. trachomatis* serovars L1-L3 is a less common but severe disease characterized by genital ulcers and inguinal lymphadenopathy [5].

*C. trachomatis* develops efficient strategies to avoid host immune response mechanisms [6], displaying different forms inside or outside of host cells as part of its development cycle. The noninfectious but metabolically active reticulate bodies (RBs) form inside cells. They specialize in nutrient acquisition and replication, and highly express effector proteins, such as chlamydia high temperature requirement protein A (cHtrA), to assist in all periods of the infection cycle [7,8]. To invade greater numbers of host cells, RBs need to change into elementary bodies (EBs) to survive exposure to the extracellular environment. To resist osmotic and physical stress, EBs create a cell wall stabilized by a network of proteins cross-linked by disulfide bonds [9]. However, the extracellular period still poses the greatest risk of exposure to attack the host immune system.

CHtrA is a serine protease expressed by *C. trachomatis* and transported to the host cytosol. Research has proved that CHtrA is vital to the replication phase of the chlamydial developmental cycle [10], and plays multifunctional roles in virulence and outer-membrane protein assembly [11]. CHtrA can also degrade extracellular matrix components such as aggrecan, fibronectin, and numerous proteoglycans to promote invasiveness while exposed [12]. CHtrA performs protease and chaperone activities with a broad range of substrate specificity; therefore, it is assumed that CHtrA could also degrade some antimicrobial peptides (AMPs), just as the chlamydial protease, CPAF, does [13] to block strong AMP anti-chlamydial activity before EBs are released from host cells and exposed to the harsh extracellular environment.

AMPs, also called host defense peptides, are involved in the first-line of defense in the human innate immune response to pathogens [14]. Their broad-spectrum strong antimicrobial activity ranges from gram-positive and gram-negative bacteria to prokaryotes, fungi, viruses, and even cancer cells [15,16]. There are 2 distinct groups of AMPs in mammals, named defensins and cathelicidins. α-defensins and β-defensins are the most researched AMPs at present [17,18], while LL-37, the only member of cathelicidins in humans, remains a little-understood peptide. LL-37 is widely distributed in the urogenital mucosa or glands of the urinary tract, vagina, and cervix, and plays an important role against bacteria, viruses, spirochetes, and chlamydia [19]. Proteolysis of cathelicidin LL-37 peptide was previously reported and biologically active smaller peptides generating from that was suggested remaining antimicrobial and/or immunomodulatory activities as LL-37 did [20,21].

CHtrA is actively transported into, and stored in, the host cytosol. There is a strong possibility that CHtrA may contact the extracellular environment before EBs. The proteolytic ability of CHtrA may help to change the microenvironment by degrading AMPs, blocking their anti-chlamydial activity, and facilitating EB diffusion and invasion. We hypothesize that CHtrA has the ability to degrade AMPs, and can block AMP anti-chlamydial activity.

**Material and Methods**

**Cell culture and chlamydial infection**

*C. trachomatis* serovar D (UW-3/Cx strain), along with L2 (434/Bu strain) and mouse pneumonitis (MoPn) were all separately propagated in human cervical carcinoma epithelial cells (HeLa 229 cells, ATCC cat#CCL2). Host cells were grown in tissue culture dishes or 24-well plates with coverslips, which contained Dulbecco’s modified Eagle’s medium (DMEM; Gibco BRL, Rockville, MD, USA) and 10% fetal calf serum (FCS; Gibco BRL). We cultivated them at 37°C with 5% CO₂ and then inoculated them with chlamydial organisms.

**IC50 titration**

To obtain human AMPs 50% inhibition concentrations (IC50, i.e. minimal concentrations required for inhibiting 50% chlamydial infection), we serially diluted and incubated them, respectively, with chlamydial organisms [13]. After that, we inoculated the incubation mixtures onto monolayers of HeLa cells. Twenty-four hours after inoculation, we visualized chlamydial inclusions through immunofluorescence assay. The following antimicrobial peptides were used: HNP1 (human neutrophil peptide 1 or human alpha-defensin 1, cat# 60743 from AnaSpec, Fremont, CA), HNP3 (cat# PDF-4146-s), HBD2 (human beta-defensin 2, cat# PDF-4338-s), HBD4 (cat# PDF-4406-s all 3 are from Peptides International, Louisville, KY, USA). We also purchased LL-37 (with a sequence of LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES; Cat#61302) from AnaSpec, Fremont, CA.

**Immunofluorescence assay**

HeLa cells, already infected by *C. trachomatis*, were fixed with cold methyl alcohol (Concord Technology Co., Ltd., Tianjin, China) for 15 min, then permeabilized with 0.1% (vol/vol) Triton X-100 (Solarbio, Beijing, China) dissolved in phosphate-buffered...
saline (PBS) for 8 min. We washed and blocked those samples. DAPI solution (blue; Solarbio) was used for DNA visualization. Rabbit anti-chlamydiaal organism antibodies (cat# OTB0797), along with goat anti-rabbit IgG secondary antibodies conjugated with FITC (green; Zhongshan Golden Bridge Bio-technology Co., Ltd., Beijing, China) were used for visualizing chlamydiaal inclusion bodies. Fluorescence microscope was needed.

**Expression and purification of recombinant cHtrA**

The genes encoding cHtrA were amplified from *C. trachomatis* serovar D (purified DNA from UW-3/Cx strain was used as a template). Primers to amplify cHtrA included BamHI and NotI recognition sequences (F: CGCGGATCCATGAGGAAGATTATTTATG; R: AAATATGCGGCCGCATTACGCTGATCTAAGAC). The cHtrA mutant was constructed using 2 round single point mutation. Sequences of Primers used were:

- F (H143A): GGAAGCGGCGATTCTCCCGAGGGACAA; R (H143A): TTGTTTCCTGGAGAAGCAATAGCCTGGTTCC;
- F (S247A): TTAATCCCTGGGAATGCAGGCGGTCCATTG; R (S247A): CAATGGACCGCTGATCTCCAGGATTAA.

In virtue of clone technology and pGEX-6P2 vectors (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA), we obtained fusion proteins as the cHtrA genes expression products, which fused glutathione-s-transferase (GST) to the N terminus. Vectors were transformed into E. coli BL21 strains. We cultured the strains in super optimal broth with catabolite repression medium at 37°C for 45 min, and then in LB solid medium overnight. Monoclonal colonies were collected and added to the liquid medium for incremental culture. Recombinant protein expression was induced for 3 h with isopropyl β-D-thiogalactopyranoside (Yinglang Chemical Co., Ltd., Shandong, China) at 30°C. Strains were harvested by centrifugation at 3000 g for 5 min, then suspended in PBS, lysed by lysozyme and Triton X-100, and sonicated on ice until the lysate cleared. The liquid supernatant was also collected following centrifugation and analyzed by SDS-PAGE.

Expressed proteins were purified from the cleared lysate by GST beads. A PreScission protease (Pharmacia) was used. We collected the eluents with the cleaved proteins and used Centricon units to concentrate purified proteins, while the fusion proteins were still left onto the glutathione beads. Proteins were identified and their purity evaluated by SDS-PAGE and Western blot (Figure 1). (µg/ml)10

**Extraction and purification of endogenous cHtrA**

Infected HeLa cells (5-10×10⁶) were suspended in growth medium, then centrifuged at low-speed and washed twice. Cold cytosol protein extract (200 µL) and protease inhibitor (2 µL) (Cytosol Protein Extraction Kit, BestBio Science Co., Ltd., Shanghai, China) were added per 20 µL of sediment cells (packed cell volume), then the mixture was shaken on a high-speed vortex for 15 seconds followed by 10 min of cooling on ice. The mixture was then shaken for another 15 seconds on a high-speed vortex, and centrifuged (16 000×g, 4°C, 5 min). The obtained liquid supernatant was the cytosol protein solution from HeLa cells infected by *C. trachomatis* serovar L2 strain, called L2 S100. Thirty-two milliliters of mouse anti-cHtrA monoclonal antibodies (MAB; clone 6A2) was mixed with 65 µL of GST beads and kept at 4°C overnight. This mixture was incubated with 1 mL of L2 S100 at room temperature for 2 h. As the endogenous cHtrA in L2 S100 would bind to the protein-G beads because of their attached antibodies (UTHSCSA, Texas, USA), the beads were softly washed once with PBS and the liquid supernatant was discarded. We incubated 2.5 µg of LL-37 with 5 µL, 15 µL, and 45 µL of treated beads, respectively, on a shaker at 37°C for 2 h. After all control groups were disposed of well, SDS-PAGE and Western blot were performed.

**SDS-PAGE and Western blot**

Polyacrylamide gels were prepared following the manufacturer’s instructions (Bio-Rad, Hercules, CA, USA). The running buffer (pH 8.3) used for the denaturing gels contained 15.15 g Tris base, 94 g glycine and 50 ml 10% sodium dodecyl sulfate (SDS), and was diluted with distilled water to volume of 5000 ml. Protein samples that were run on denaturing gels were boiled for 5 min in a 4×sodium dodecyl sulfate (SDS) sample buffer (0.25 mol/L Tris-HCl, pH 6.8, 40% glycerol, 0.004% bromophenol blue, 8% (wt/vol) SDS, and 20% (vol/vol) 2-mercaptoethanol). Incubating the AMPs with different concentrations/capacities of recombinant/endogenous cHtrA and cHtrA mutants (MT-H143A and MT-S247A) were identified by SDS-PAGE. Molecular weight of cHtrA (no matter wild-type or mutants) is 56KD. One µg/mL cHtrA can hardly be detected, but the bands of 10 µg/mL and 100 µg/mL cHtrA were clear on SDS gel.

**Figure 1.** Recombinant cHtrA and cHtrA mutants (MT-H143A and MT-S247A) were identified by SDS-PAGE. Molecular weight of cHtrA (no matter wild-type or mutants) is 56KD. One µg/mL cHtrA can hardly be detected, but the bands of 10 µg/mL and 100 µg/mL cHtrA were clear on SDS gel.
2 mutants (MT-H143A, recombinant wild-type cHtrA carrying substitution of the histidine at the position of 143 with alanine, and MT-S247A, recombinant wild-type cHtrA carrying substitution of the serine at the position of 247 with alanine) which lost the function of enzyme digestion at 37°C for 30 min and boiling for 5 min, then loaded 20 µL of them respectively into each lane.

Proteins we obtained through electrophoresis could be visualized directly by staining with Coomassie blue (Sigma), or used for further steps, such as Western blot. The latter required certain primary antibodies: mouse monoclonal antibodies (MAb; clone 6A2) for detecting cHtrA, mouse MAbs (Yacolt Biotech, Plymouth Meeting, PA, USA) for detecting LL-37, mouse MAbs (clone 9G9) for detecting CT795. We probed these primary antibodies with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.) and then used an Enhanced Chemiluminescence Kit (Santa Cruz Biotech, Santa Cruz, CA, USA) to see the results.

Measurements and statistical analysis

Chlamydial infection was quantitated immediately after immunofluorescence assay by counting 5 random views for every coverslip. The numbers of inclusion forming units (IFUs) would be calculated as the results. Based on the concentrations of AMPs that we used and IFUs we counted, the values of IC50 was calculated for each AMP using IBM SPSS Statistics 24 software. The titration was duplicated 3 times for each experiment. The ultimate value of IC50 for each AMP was presented as the mean±standard deviation (SD).

As for chlamydial infection rates, we counted 5 random views for each coverslip, with rates calculating as the value of IFUs divided by the cell quantities. Three duplicates were performed for each group, and the final infection rate for each group was expressed as the mean±SD. Quantitative data was assessed by an independent-sample T test.

Results

Cathelicidin LL-37 has the strongest anti-chlamydial activity in 5 human AMPs

Evaluation of the anti-chlamydial activity of cathelicidin LL-37, α-defensin-1 & 3, and β-defensin-2 & 4 against C. trachomatis serovar L2 showed that the α-defensins displayed the weakest anti-chlamydial activity with an IC50 of 60 µg/mL (Table 1). The IC50 of the 2 β-defensins were 47 µg/mL for β-defensin-4 and 28 µg/mL for β-defensin-2, indicating that β-defensins have slightly stronger anti-chlamydial activity than α-defensins.

The IC50 of cathelicidin LL-37 was 4.62±0.94 µg/mL. These results demonstrate that LL-37 has the most stable and strongest anti-chlamydial activity of the 5 AMPs tested, and moreover, that AMPs can effectively suppress C. trachomatis infection.

Table 1. Anti-chlamydial activities of 5 human antimicrobial peptides (AMPs). AMPs were titrated for their ability to inhibit C. trachomatis serovar L2 growth. The minimal concentrations required for inhibiting 50% chlamydial infection (IC50) were determined for each AMP. The data are expressed as µg/mL (mean±SD) and were derived from triplicate testing with duplicates in independent experiments.

| AMPs      | IC50/µg/mL (±S) |
|-----------|-----------------|
| Cathelicidin LL-37 | 4.82±0.94       |
| α-defensins | 60.67±5.13      |
| HNP1      | 69.87±7.45      |
| β-defensins | 28.33±9.45     |
| HBD2      | 47.70±6.15      |
| HBD4      | 47.70±6.15      |

Recombinant cHtrA can cleave LL-37 in vitro

Evaluation of 0, 1, 10, and 100 µg/mL cHtrA co-incubated with 100 µg/mL each of cathelicidin LL-37, α-defensin-1 and -3, and β-defensin-2 and -4 by Coomassie blue staining showed no evident changes in groups incubated with α-defensins or β-defensins (Figure 2). However, co-incubation of LL-37 with 100 µg/mL cHtrA degraded all LL-37. Evaluation of the mutant cHtrAs, MT-H143A and MT-S247A, showed that neither mutant was able to degrade any AMPs. Therefore, we speculate that cHtrA has the ability to degrade LL-37, and the degradation of antimicrobial peptide required cHtrA proteolysis ability.

Antibody purified endogenous cHtrA can cleave LL-37 in vitro

After adding 2.5 µg cathelicidin LL-37 to 5 µL, 15 µL, and 45 µL of endogenous cHtrA and control groups, those without a cHtrA ingredient (HeLa S100, untreated protein-G beads, and CT795 adhered or free beads) all revealed high LL-37 concentration bands (Figure 3). The sample of unpurified L2 HeLa S100 revealed an evident cHtrA peptide band, meanwhile, the LL-37 band was thin, weak, and comparable to that of the 15 µL sample of purified endogenous cHtrA. When LL-37 was incubated with cHtrA purified by protein-G beads, the more chHtrA was added, the thicker the revealed band, and the thinner the LL-37 band appeared until it faded away. These findings support the conclusion that cHtrA can effectively degrade LL-37, no matter the source it is obtained from.
Recombinant cHtrA can specifically cleave LL37 in vitro. Each of the AMPs (100 µg/mL) was co-incubated with variable concentrations (0, 1, 10, and 100 µg/mL) of cHtrA and 100 µg/mL of the cHtrA mutants (MT-H143A and MT-S247A) for 30 min and we analyzed the results by Western blot. cHtrA concentrations above 10 µg/mL are able to degrade LL-37, and the degradation of antimicrobial peptide required cHtrA proteolytic ability (mutants are unable to degrade LL-37).

cHtrA blocks the anti-chlamydial effect of LL-37

Based on previous reports [13,22–24], we adopted 30 µg/mL LL-37 in the current study. The result showed that administration of 30 µg/mL of LL-37 decreased the chlamydial infection rate (7.67±2.08%; Figure 4) compared to the control group (54±6.24%; P<0.01). However, pre-incubation of LL-37 with cHtrA restored the chlamydial infectivity in a cHtrA dose-dependent manner. Adding 1 µg/mL cHtrA with 30 µg/mL LL-37 caused a slight infection rate recovery (11.3±3.51%). Incubation of 10 µg/mL of cHtrA with 30 µg/mL of LL-37 further increased the infection rate (28.7±7.51%), and significantly differed from the LL-37 alone treatment (P<0.01). Neither cHtrA alone (47±6.24%), nor 100 µg/mL cHtrA with 30 µg/mL LL-37 affected chlamydial infectivity (44.7±5.13%). These 2 groups showed no statistical difference from the blank control group, but both differed significantly from the LL-37 only group (P<0.01). MT-H143A and MT-S247A mutants (100 µg/mL) incubated with 30 µg/mL of LL-37 were unable to affect LL-37 activity.

Repeating the infectivity experiment on C. trachomatis L2, D, and MoPn strains showed strikingly consistent results (Figure 5). LL-37 significantly lowered the chlamydial infection rate in all groups (P<0.01); pre-incubation of LL-37 with cHtrA restored the chlamydial infectivity in a cHtrA dose-dependent manner, and when the cHtrA concentration reached 10 µg/mL, all the infection rates significantly differed from the LL-37 alone group (P<0.05); The presence or absence of LL-37 made no difference at 100 µg/mL cHtrA, and neither mutant was able to degrade LL-37.

These results further proved that cathelicidin LL-37 exhibits strong anti-chlamydial activity, and chlamydial-secreted protease cHtrA inhibits C. trachomatis death by degrading LL-37. Furthermore, C. trachomatis L2, D, and MoPn strains all displayed similar results.

Discussion

C. trachomatis secreted proteins (CtSPs) are synthesized by C. trachomatis and transported into the host cell cytosol [11]. They are crucial to the C. trachomatis intracellular developmental cycle and interfere with the host immune system in multiple ways, such as preventing apoptosis, and blocking host identification and attack [25–28]. Valdivia hypothesized that CtSPs synthesized late in the C. trachomatis infectious cycle prepare inclusions for exit from the host cells, and pack EBs with CtSPs required for the infection of new host cells [8]. cHtrA is one such CtSP synthesized late in the infectious cycle. The cHtrA protein can be detected inside inclusions as early as 12 h after infection, and cHtrA secretion into the cytosol becomes apparent by 24 h post infection [29].

As cHtrA is actively secreted into the host cell cytosol in the late infectious cycle [29], we assume it plays an important role in the invasion of neighboring cells. However, the mechanism is still unknown. cHtrA is essential to the replication phase of the chlamydial developmental cycle [30]. Researchers designed an experiment lethal to C. trachomatis by adding JO146 (which can inhibit the proteinase activity of cHtrA) to the replication phase of developmental cycle [10,31]. Moreover, as cHtrA has proteolytic activity over a broad range of substrate
specificity [32], it can cleave cell-to-cell junction factors such as E-cadherin, and claudin-8, as well as extracellular matrix components including aggrecan, fibronectin, and numerous proteoglycans while exposed [12,33]. We assume that cleavage of cellular factors using cHtrA is a strategy used by C. trachomatis to disrupt the epithelial barrier, thereby producing host cell damage, changing the microenvironment outside host cells, and promoting invasion of neighboring cells.

Making epithelial cells more invadable is one aspect of increasing the EB survival rate when they exit host cells and are exposed to the extracellular matrix, but is not the entire survival strategy. Host epithelial cells can recruit inflammatory cells, such as neutrophils, to the infection site by releasing cytokines [34]. Although neutrophils cannot directly attack RBs hiding inside the host cells, the neutrophil-secreted AMPs outside the host cells are able to attack EBs any time EBs exit the host cell. When chlamydial infected host cells activate adjacent

Figure 4. cHtrA blocks the anti-chlamydial effect of LL-37 on C. trachomatis serovar L2. (A) C. trachomatis L2 infection (a) was compared with the effect of LL-37 (30 µg/mL) pre-incubated alone (b), or with 1 (c), 10 (d), or 100 µg/mL (e) wild-type (WT) cHtrA, or 100 µg/mL of MT-H143A (g) or MT-S247A (h) prior to LL-37 treatment of chlamydial organisms. 100 µg/mL of WT cHtrA incubated alone (f) was used as a control to investigate its effect on chlamydial infectivity. As shown in the figure, the C. trachomatis L2 infection (a) was strongly inhibited by 30 µg/mL of LL-37 (b), which was then successfully reversed by pre-incubation of LL-37 with WT cHtrA (c–e) but not mutants (g, h). Five random views per coverslip were counted, and the infection rates are expressed as the mean±SD. Three duplicates were performed for each test. cHtrA concentrations above 30 µg/mL apparently block the anti-chlamydial activity of LL-37. (B) Quantitative data obtained from this experiment. The colors of the tubes represent different experiment operates, and the details are contained in the legends. * P<0.05, ** P<0.01.
cells to secrete additional cytokines, and the cytokines recruit additional neutrophils which can increase the expression of LL-37 [35], the likelihood of EB survival is reduced. For *C. trachomatis*, spreading from cell to cell is about survival and reproduction, so survival strategies include the ability to defuse the threat posed by AMPs. Chlamydial proteins such as chlamydial-secreted protease, CPAF, and chlamydial plasmid-encoded virulence factor, pgp3, have been discussed in previous reports about their ability of interacting with LL-37 [13,24]. It suggested that *C. trachomatis* might have developed various evolutionary strategies evading AMPs for survival.

In the current study, we confirmed that the cathelicidin LL-37 possess a potent anti-chlamydial activity. We repeated part of the experiments previously reported [13] and found that there was difference between our data (about HNP1, HNP3, HBD4) and theirs, but the anti-chlamydial activity of LL-37 was undoubtedly the strongest of all tested. cHtrA might be a part of the *C. trachomatis* survival strategy, as it had the ability to degrade LL-37 and suppress its anti-chlamydial activity. In addition, the degradation required cHtrA proteolysis ability, as the mutant cHtrAs (MT-H143A and MT-S247A) failed to degrade a significant amount of LL-37. We hypothesize that the pre-stored cHtrA in the host cell cytoplasm may be rapidly released upon cell lysis to degrade the extracellular LL-37 before the intra-inclusion organisms are exposed to the extracellular environments, which may facilitate chlamydial survival and cell-to-cell spreading. Although we have presented *in vitro* evidence for cHtrA suppressing the anti-chlamydial activity of human cathelicidin LL-37 by degradation, these observations must be validated in animal models.

**Figure 5.** cHtrA cleavage of LL-37 reverses anti-chlamydial activity on D and MoPn strains. *C. trachomatis* serovar D and MoPn strains were used to evaluate the cHtrA-mediated blockade of LL-37 anti-chlamydial activity. The colors of the tubes represent different experiment operates, and the details are contained in the legends. * P<0.05, ** P<0.01.
Conclusions

ChTrA effectively and specifically degraded LL-37 and suppressed its anti-chlamydial activity, suggesting a potential contribution of ChTrA to chlamydial pathogenesis in vivo by invading LL-37.

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Conflict of interest

None.

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