

Original Article

CPAF selectively degrades chlamydial T cell antigens for inhibiting antigen presentation

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Abstract

Introduction: *Chlamydia trachomatis* is the leading cause of sexually transmitted bacterial disease, which may cause significant threats, such as pelvic inflammatory disease and tubal factor infertility, to women if untreated. The pathological mechanisms of chlamydia-induced disease remain largely unknown, but it has been proposed that CPAF, a chlamydia-secreted serine protease, may play major roles in aiding chlamydial infection and contribute to chlamydia pathogenesis during *in vivo* infection. According to previous results, CPAF targets host immunity by degrading antimicrobial peptides and neutralizing complement activity; however, whether CPAF is involved in chlamydial antigen presentation has never been reported.

Methodology: Antigen presentation assay was used to monitor the effects of CPAF on OT1-, OT2-, and chlamydia T cell antigen-mediated antigen presentation. *In vitro* cell-free degradation assay was used to detect CPAF processing of chlamydia T cell antigens.

Results: We found that CPAF preferably inhibits OT2- but not OT1-mediated antigen presentation. CPAF inhibits OT2 antigen presentation by direct proteolytic cleavage in the wild type CPAF, but not enzymatic mutants. Importantly, several previously identified chlamydial T cell antigens were selectively degraded by CPAF when co-incubated *in vitro*. In addition, specific inhibition T cell antigen presentation by CPAF was correlated with T cell antigen cleavage by CPAF *in vitro* assay.

Conclusions: Our experiments demonstrated that CPAF selectively and specifically degrades chlamydial T cell antigens, which chlamydia may utilize as a novel mechanism for evading host immune responses to promote chlamydia survival.

Key words: *Chlamydia*; CPAF; antigen presentation; host immunity.

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Introduction

Chlamydia trachomatis (*C. trachomatis*) infection in the lower genital tract (LGT) of women can lead to inflammatory pathologies such as hydrosalpinx in the upper genital tract (UGT), resulting in severe complications, including ectopic pregnancy and infertility [1-3]. More than 1.4 million cases of chlamydial infection are reported to the Centers for Disease Control and Prevention (CDC) in the United States annually [4], but the actual number of *C. trachomatis* infections is likely to be significantly higher since many infections are asymptomatic and remain undiagnosed. Recently, cases of chlamydial infection keep increasing steadily [5].

Although extensive research has been done, the mechanisms by which *C. trachomatis* induces disease

in human fallopian tubes remain unknown, especially how chlamydial organisms ascend and induce hydrosalpinges in the fallopian tube. Recently, it was hypothesized that both adequate ascension to and induction of the appropriate inflammatory response in the upper genital tract is necessary for hydrosalpinx development [6-9]. To overcome the host immunity barriers, and establish the successful infection, chlamydia may utilize virulence factors as previously proposed [10]. Many virulence factor candidates have been identified, such as the polymorphic outer membrane autotransporter family of proteins [11,12], the putative large cytotoxin [13], stress response proteins [14], and chlamydial secretion effectors [15-17]. It is known that *C. trachomatis* organisms can secrete numerous proteins into host cell cytoplasm

[18,19]. Among them, Chlamydial Protease-Like Activity Factor (CPAF), a chlamydial type II secretion protein, may serve as a promising candidate of virulence factor as proposed [16,20-22].

CPAF was initially recognized as a serine protease which is abundantly secreted in the chlamydial infection cell cytosol at a very early stage of infection. Numerous substrates have been identified during the past decade [23-26]. However, these CPAF substrates appear to be generated during cell processing and not following chlamydia *in vivo* [27], which leads to further discussions of authentic substrates to CPAF [28-33]. However, recent studies show that CPAF can process chlamydia outer membrane complex protein B (OmcB) [34] and cellular nuclear envelope protein LAP1 [35] during chlamydia infection, and CPAF exhibits anti-chlamydial activity by targeting antimicrobial peptides *in vitro* [36]. More importantly, CPAF deficient organisms (L2-17) are unable to establish a sufficient infection in mouse lower genital infection [37], which highlights that CPAF plays crucial roles during chlamydia infection *in vivo*. We report that CPAF was involved in inhibiting chlamydia T cell antigen-mediated antigen presentation *in vitro* and that inhibition was dependent on CPAF proteolytic activity by directly targeting T cell antigens, which may provide a new mechanism for chlamydia to evade host immune response during *in vivo* infection.

Methodology

Isolation of naive T cells from spleen

For the OT1 and OT2 antigen presentation experiment, CD8⁺ and CD4⁺ T lymphocytes were isolated from OT1 and OT2 transgenic mouse spleens by negative selection using commercially available kits, (BD IMag™ Mouse CD8/CD4 T Lymphocyte Enrichment Set (BD Biosciences, San Jose, USA). The enrichment cocktail contain non CD8 and CD4 T lymphocyte monoclonal antibodies which recognize antigens expressed on peripheral erythrocytes and leukocytes. This enrichment technique thus avoid the inadvertent activation of the enriched CD8 or CD4 T lymphocytes making it sufficiently sensitive for our antigen presentation experiment. Specifically, for T cell isolation, the transgenic OT1 and OT2 mice were sacrificed, and the spleens were isolated, and transferred to PBS resuspension buffer. The excised spleen was then sliced into small pieces, and a syringe plunger was used to generate a single cell suspension. All the cells were collected and passed through a cell strainer (BD Biosciences, San Jose, USA) to isolate the whole splenocytes for purification using the IMag™

Mouse CD8 or CD4 T Lymphocyte Enrichment Set according to the manufacturer's instructions.

For the chlamydia purified protein antigen presentation experiment, the T lymphocytes were enriched by using the same procedures, but the spleen was harvested from chlamydia EB vaccinated mice. In this study, BALB/c were selected, and purified chlamydia EBs were used for vaccination [38]. After confirmation of the presence of positive chlamydia antibodies, the mice were sacrificed and the spleens subjected to CD4⁺ T cells isolation. Freshly isolated CD4⁺ T cells were used for the antigen presentation experiments performed in this study.

Generation of dendritic cells from bone marrow-derived dendritic cells

The dendritic cells (DC) used for the antigen presentation experiment were generated from bone marrow-derived dendritic cells (BMDCs) according to a method previously described [39]. Briefly, mice were sacrificed and bone marrow cells were isolated from mouse femurs and tibiae by flushing with RPMI 1640 medium. The cells were harvested and centrifuged at 400g for 10 min to spin down the cell pellet, and RBC lysis buffer (Gey's solution, BD Biosciences, Biosciences, San Jose, USA) was applied to remove the red blood cells. The cells were resuspended in BMDC medium (RPMI 1604 medium supplement with 10% FCS, 1% L-glutamine, 1% non-essential amino acid, 1% sodium pyruvate, 1% penicillin-streptomycin, GM-CSF of 10 ng/ mL, and IL-4 at 5 ng/ mL) and 2×10⁶ cells/dish were seeded with BMDC media in a petri dish for culturing. Three days later, additional BMDC medium was refreshed once. Seven days later, the non-adherent cells along with the media were transferred to a new cell culture plate, and the next day (day 8 after culture), all non-adherent matured dendritic cells were harvested, counted, and subjected to antigen presentation experiments.

Antigen presentation experiments

For the OT1 and OT2 antigen presentation experiment, the peptides of OT1 (OVA 257-264, InvivoGen, San Diego, USA) and OT2 (OVA 323-339, InvivoGen, San Diego, USA) were dissolved in phosphate-buffered saline (PBS) and aliquoted; fresh aliquots were used for each experiment. When performing the antigen presentation experiments, the peptides were incubated with dendritic cells for 6 hours before adding the purified CD8⁺ or CD4⁺ T cells for further stimulation for 24 hours to monitor the T cell proliferation. At the same time, dendritic cells (DC)

alone, T cells (TC) alone, dendritic cells plus OT1/OT2 peptides, and T cells plus peptide OT1/OT2 peptides were set up and incubated respectively to serve as experimental controls. For the chlamydial purified proteins antigen presentation experiments, all the procedures were performed the same way, but D694 and D111 purified proteins were used instead of OT1/OT2 peptides. After 24 hours, the T cell clusters were imaged under a light microscope, and the supernatant was harvested for measuring IL-2 production by ELISA as described below.

Detection of IL-2 in the supernatant by ELISA

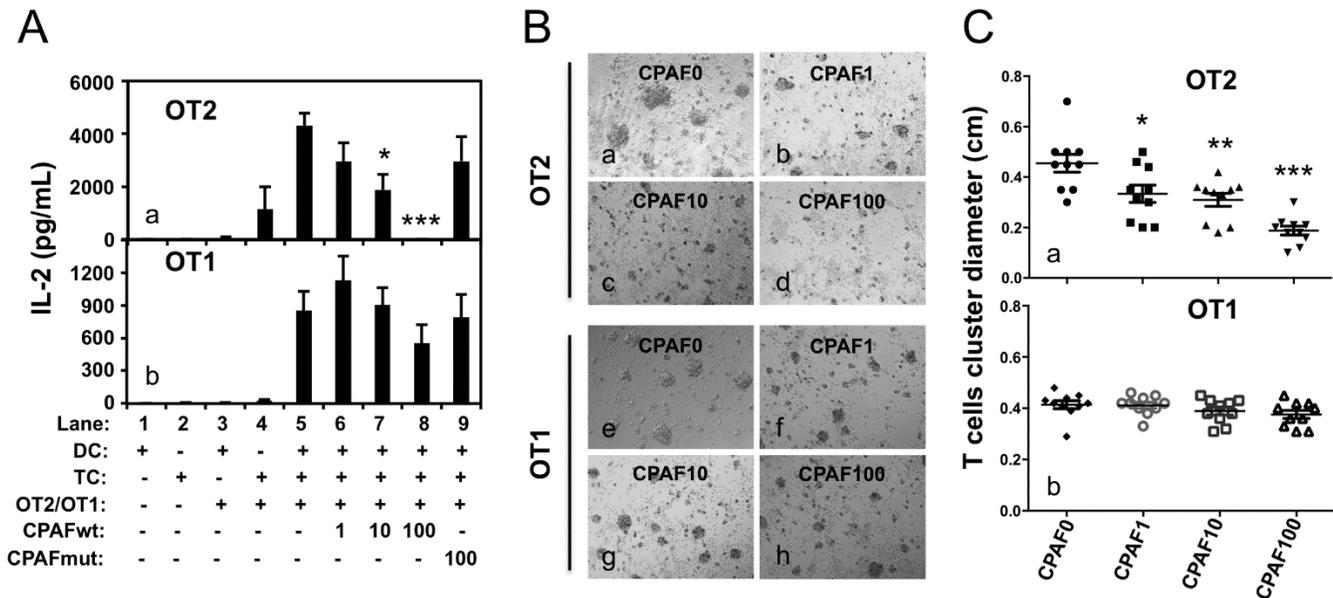
The secretion of IL-2 in the supernatant was analyzed by enzyme-linked immunosorbent assay (ELISA) [40], which was used to monitor the antigen presentation activity in the current study. To test this, a 96-well ELISA microplate (Nalge Nunc International, Rochester, USA) was coated with a capture Ab (R&D Systems, Minneapolis, USA) and after blocking, the samples (supernatant harvested from the antigen presentation assays) or standards were added to the coated plates followed by a biotin conjugated detection Ab (R&D Systems, Minneapolis, USA). The Ab

binding signals were detected with an HRP-conjugated Avidin plus a soluble colorimetric substrate (ABTS). The absorbance was taken at 405 nm using a microplate reader (Molecular Devices, Sunnyvale, USA). The cytokine concentrations were calculated based on absorbance values, IL-2 standard, and sample dilution factors, and expressed as picograms per milliliter.

Cell-free degradation assay and Coomassie blue staining

GST-fusion recombinant proteins of D111, D628, D622, D702, D509, and D694 were expressed in a pGEX expression system (GST was fused to the N terminus of the chlamydial proteins) and purified by glutathione-conjugated agarose beads (Amersham Biosciences, Pittsburgh, USA) as described previously [41-43]. To monitor the processing of chlamydia T cell antigens by CPAF, a cell-free pre-incubation assay was used as described elsewhere [36]. Briefly, GST-fusion purified proteins were preincubated with or without wild type (WT) CPAF in digestion buffer for 2 hours at 37°C. After the reaction, the mixture was loaded onto a 12% SDS polyacrylamide gel and electrophoresis separation was carried out, and the gel was stained with

Figure 1. CPAF inhibits OT2-but not OT1-mediated antigen presentation.



A) Different doses of CPAFwt at 1 µg/mL (lane #6), 10 µg/mL (lane #7), and 100µg/mL (lane #8), and high dose of mutant CPAF (CPAFmut 100 µg/mL, lane#9) preincubated with dendritic cells for 2 hours. Antigen OT2 peptide (top panel) or OT1 peptide (bottom panel) were then added for further incubation of 6 hours, and at a later time T cells were added for stimulation for 24 hours. After incubation the supernatant was harvested for measuring the production of the cytokine IL-2 by ELISA assay. Dendritic cells alone (lane #1), T cells alone (lane #2), dendritic cells plus OT2 peptide (lane #3), and T cells plus peptide OT2 (lane #4) with corresponding times served as experimental controls by measuring the IL-2 production as well. Our results show that wt CPAF but not mutant CPAF can significantly inhibit OT2-mediated antigen presentation, as significantly reduced IL-2 production was observed. T cell clusters were imaged (B), and the diameter of clusters calculated (C) in the same experimental setting as panel A, to further confirm that CPAF significantly inhibits OT2 but not OT1 antigen presentation, as shown by small T cell cluster formations after CPAF inhibition, in a dose-dependent manner. *, p < 0.05, **, p < 0.01, analyzed by Mann-Whitney test.

a Coomassie blue dye (Sigma, St.Louis, USA) for visualizing protein bands.

Statistical analysis

Mann-Whitney test was used for analyzing both IL-2 differences among different treatments of antigen presentation groups and the diameters of T cell clusters. $P < 0.05$ was considered as statistically significant.

Results

CPAF inhibits OT2- but not OT1-mediated antigen presentation

To test whether CPAF can inhibit antigen presentation, we monitored IL-2 production from the supernatant that was harvested from stimulated T cells, which indicates the activity of antigen presentation. We found that when OT2 peptide was incubated with dendritic cells, followed by T cells, the IL-2 production was significantly high (Figure 1A, panel a, column 5), but the controls of DC alone, TC alone, DC plus peptide, and TC plus peptide (Figure 1A, panel a, columns 1-4) showed negative or minimum levels of IL-2. However, when wild type (wt) CPAF was preincubated with OT2 peptides, the IL-2 production was significantly reduced, and it presented in a dose-dependent (CPAF from 1 $\mu\text{g}/\text{mL}$ to 100 $\mu\text{g}/\text{mL}$) manner as shown in columns 6-7 of Figure 1A. Mutant CPAF, however, failed to inhibit antigen presentation, showing similar levels of IL-2 production even with high doses of mutant CPAF (100 $\mu\text{g}/\text{mL}$) (Figure 1A column 9). To our surprise, when OT1 was replaced with OT2 to perform the same experiment, we found that even wt CPAF failed to inhibit any antigen presentation activity, showing that IL-2 production was similar between groups from OT1 with pre-incubation of wt CPAF or without preincubation of wt CPAF (Figure 1A, panel b, columns 5-8). This experiment demonstrates that CPAF can inhibit OT2- but not OT1-mediated antigen presentation and that inhibition is dependent upon CPAF enzymatic activity.

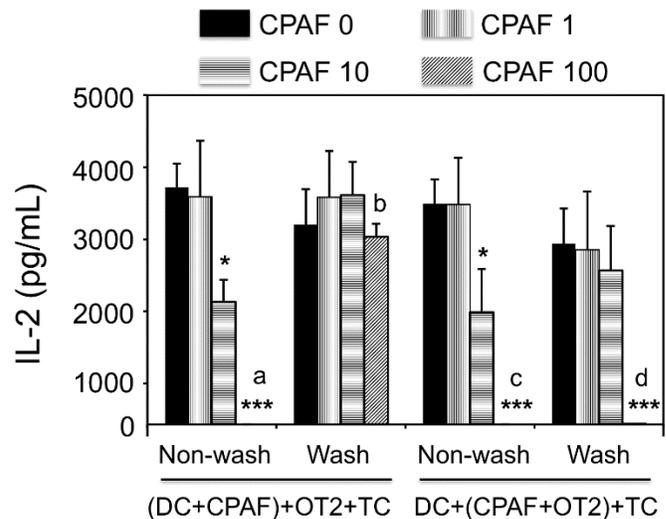
To further validate our observations, we tested the effects of CPAF impact on T cell proliferation by directly measuring the T cell clusters, as shown in Fig1B. Different doses of CPAF were preincubated with OT2 or OT1; then DCs and purified T cells were added to test T cell proliferation. We found that wt CPAF preincubated with OT2 but not OT1 significantly inhibited T cell cluster formation, as shown by the CPAF concentration increases. T cell clusters were decreased for OT2 (Fig1B, panel a-d), but not OT1 (Figure 1B, panels e-h), indicating that the inhibition operated in a dose-dependent manner. Additionally, we

used a semi-quantitative method to monitor the diameters of T cell clusters by testing the T cell cluster sizes, which revealed similar results (Figure 1C). Taken together, these experiments demonstrated that CPAF can inhibit OT2- but not OT1-mediated antigen presentation activity.

CPAF inhibit antigen presentation via CPAF directly targeting OT2

To further dissect the mechanisms that CPAF uses to inhibit antigen presentation, we designed a test where CPAF was either preincubated with DC or preincubated with OT2 peptides, then the remaining component for T cell stimulation was added. In this way we attempted to find the target of CPAF impacted by antigen presentation. To stimulate the T cell we used different doses of CPAF (1 $\mu\text{g}/\text{mL}$ to 100 $\mu\text{g}/\text{mL}$) or without CPAF (0 $\mu\text{g}/\text{mL}$) were pre-incubated with DCs. After incubation, CPAF was either washed from the resulting supernatant or not (non-wash), then OT2 and T cells

Figure 2. CPAF inhibits antigen presentation via direct cleavage of OT2.



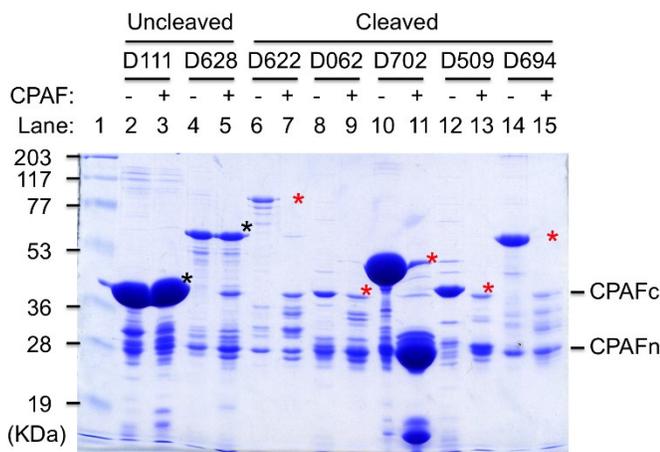
To dissect the mechanisms of how CPAF inhibits antigen presentation, different doses of CPAF were preincubated with dendritic cells (DC), with or without washing away the CPAF after two hours. OT2 was then added before finally adding T cells (TC) for testing antigen presentation. 24 hours later supernatant was collected for measuring the IL-2 production to monitor the activity of OT2 mediated antigen presentation. It is shown that when prewashed before adding OT2, CPAF fails to inhibit OT2-mediated antigen presentation indicated by normal IL-2 production in the supernatant (column b), while without the wash procedure, CPAF inhibits antigen presentation as demonstrated before (column a). On the contrary, with same experiment setting, if CPAF were preincubated with OT2, then CPAF will eventually inhibit OT2 antigen presentation despite the washing procedures or not, as shown in both (column c and d) by the lack of IL-2 production. This experiment indicates that CPAF inhibition of antigen presentation directly targets the OT2 peptide antigen. *, $p < 0.05$, ***, $p < 0.001$, analyzed by Mann-Whitney test.

were added as described in the materials and methods. To our surprise, we found that when CPAF was not washed away, IL-2 secretion from T cells was significantly reduced with increasing preincubation doses of DCs into the CPAF (Figure 2, column a). However, when CPAF was washed away in the supernatant, CPAF failed to further inhibit OT2-mediated antigen presentation (Figure 2, column b), suggesting that CPAF must remain in the system for inhibition of antigen presentation, but dose not play a direct effect on DCs. In contrast, when CPAF was preincubated with OT2, the IL-2 secretion from T cells was significantly reduced whether CPAF was washed away or not (Figure 2, columns c and d), which indicates that a direct targeting of OT2 by CPAF occurred during incubation.

CPAF selectively digests T cell antigens

The experiments described above demonstrate that CPAF can inhibit OT2-mediated antigen presentation, and CPAF directly targets OT2 for inhibition. Because CPAF was identified as a serine protease, we hypothesized that CPAF may also be able to process chlamydial T cell antigens for evading host immunity response. To test this hypothesis, we took several previously identified chlamydia T cell antigens (data was not shown), including D111, D628, D622, D062, D702, D509, and D694 to test processing by CPAF.

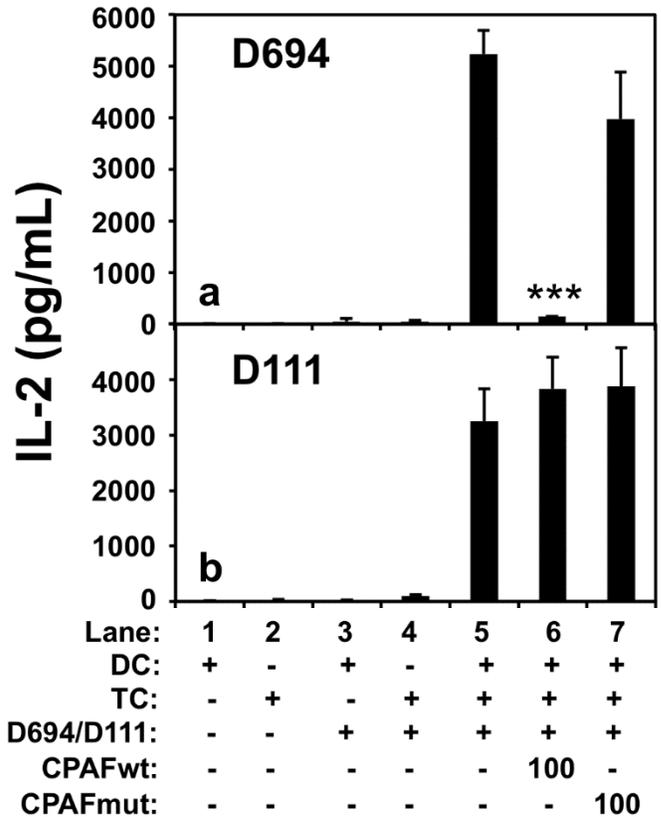
Figure 3. CPAF selectively degrades T cells antigens.



Several previously identified T cell antigens were preincubated with or without CPAF to test CPAF processing of T cell antigens. As shown in the top panel, T cell antigens with CPAF (+) or without CPAF (-) were preincubated with chlamydial T cell antigens, including D111, D628, D622, D026, D752, D509 and D694 for 2 hours for processing. After digestion, all the samples were loaded into SDS-PAGE gels for measuring protein degradation by direct Coomassie Blue Staining. The results show that CPAF selectively degrades chlamydial T cell antigens D622, D026, D752, D509 and D694 (red asterisks), but not D111 and D628 (black asterisks).

Indeed, we found that five (D622, D062, D702, D509, and D694) out seven purified proteins can be processed by CPAF under *in vitro* degradation assay (Figure 3, red markers), while the other two (D111 and D628) failed to be processed by CPAF (Figure 3, black markers), which suggests that CPAF selectively processes chlamydial T cell antigens. Furthermore, we performed the antigen presentation experiment again to further validate that CPAF also functions to inhibit T cell antigen presentation during *in vitro* assay. CPAF cleavable antigen D694 and CPAF non-cleavable antigen D111 were preincubated with DCs and T cells from chlamydia EB-vaccinated mice. After monitoring IL-2 production again for testing the antigen presentation activity, it was interesting to find that

Figure 4. CPAF inhibits chlamydial T cell antigen presentation *in vitro*.



T cell antigens D694 (top panel) and D111 (bottom panel) were selected to test whether CPAF can directly inhibit T cell antigen-mediated antigen presentation *in vitro*. To test this, CPAFwt 100 µg/mL (lane 6) or CPAFmut 100 µg/mL (lane 7) preincubated with dendritic cells for 2 hours. D694/D111 antigen was then added for further incubation of 6 hours and T cells added for stimulation of 24 hours. After 24 hours, the supernatant was harvested to measure the production of the cytokine IL-2 by ELISA assay. Various controls (lanes 1-4) were also included in the experiment. The results showed that CPAF could significantly block D694-mediated antigen presentation, while failing to block D111-mediated antigen presentation. ***, p < 0.001, analyzed by Mann-Whitney test.

CPAF specifically inhibits CPAF-cleavable antigen presentation only (Figure 4, panel a), but not non-cleavable antigen (Figure 4, panel b), indicating that inhibition of antigen presentation by CPAF was accordingly correlated with CPAF cleavage. In addition, this inhibition was dependent on CPAF proteolytic activity, since mutant CPAF failed to inhibit any T cell antigen presentation.

Discussion

Previous studies demonstrated that chlamydia utilizes its virulence factor of CPAF to degrade host antimicrobial peptides [36] and targets the complement system for neutralizing anti-chlamydial activity [44], suggesting that CPAF plays a critical role in promoting chlamydia survival during genital infection. In the current study, we unexpectedly identified that CPAF can block antigen presentation by using a classical antigen presentation system, which depended on CPAF enzymatic activity. Furthermore, we identified that CPAF can process T cell antigens *in vitro*, and CPAF selectively blocks T cell antigen presentation since this inhibition was correlated with CPAF cleavage. These data were consistent with the previous findings that CPAF plays critical roles of evading host immunity and promoting chlamydia infection [35-37,44].

Chlamydia infection begins in the lower genital tracts; however, the diseases induced by chlamydia such as pelvic inflammatory disease (PID), infertility, and ectopic pregnancy take place in the fallopian tubes in the upper genital tracts [2,45,46]. Therefore, chlamydia must overcome host immunity, particularly in the genital mucosa that controls the spread and ascension of chlamydia. Although the pathological consequences of infection are well known, the mechanisms of how chlamydia overcomes these host immunities are not fully understood. Notably, many host factors were proposed and identified *in vitro* and *in vivo* to play crucial roles for controlling chlamydia infection in the genital tract environment [36,44,47,48], including antimicrobial peptides, complement, and cytokines and chemokines that are stimulated following chlamydia infection. Besides those factors, it is important to note that T cell accumulation at the site of chlamydial infection also demonstrates a critical step for restricting chlamydial infection, as reported previously [49-51]. Particularly, T cell proliferation and the profound proinflammation responses induced by T cells generate a quick host response for clearing the chlamydia infection and resolving the inflammation induced by chlamydia. In addition, a chlamydial inclusion body forms during *in vivo* infection in

epithelial cells, the inclusion lyses, and a multitude of chlamydia proteins and organisms are released to the mucosa system. These antigens are predominantly detected by the host's immune system, especially the T cell antigens encoded by chlamydia, which would, upon T cell detection, induce a robust host immune response and cause the host to clear the infection [52]. As such, cleavage of these specific T cell antigens would be essential for chlamydial survival. It is also possible that CPAF cleaves chlamydial T cell antigens and limits their detection by microbial pattern recognition receptors or their processing and displaying on MHC class II molecules as discussed before [52].

CPAF secreted into cell cytosol following chlamydial infection starts at a very early stage of infection, and accumulates in abundance during the late stage of chlamydia inclusion, which provides a chance to cleave the substrates once released from the inclusion body. Initially identified as a serine protease, multiple substrates for CPAF have been identified by, including the cellular proteins RFX5 [25], USF-1 [53], Keratin-8 [54], Keratin-18 [55], and the chlamydial proteins CPAF [56], CT005 [57], IncD [57], and TARP [57]. However, caution should be taken when analyzing these results since many substrates were proven to be artificial during cell processing after lysis of infected cells, which did not actually appear during chlamydial infection *in vivo*. Therefore, the T cell antigens processed by CPAF and identified in the current study should be further validated during *in vivo* infection in the future. In addition, identifying specific T cell antigens through the dendritic cell-based immunoprecipitation approach is considered a promising way to research a chlamydia vaccine [39,58], and several promising T cell antigens were identified to be likely candidates for vaccine but require further validation. In this study, we took advantage of this system to identify several T cell antigens and we further investigated whether CPAF blocks the presentation of these T cell antigens. However, whether the T cell antigens we identified really have immunological effects during chlamydia infection and the efficacy of vaccines targeted to these antigens need further investigation. As suggested, epitopes of these T cell antigens should continue to be identified, and further monitoring of *in vivo* antigen processing and presentation by immunofluorescent labeling assay or other related techniques is critically needed.

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