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Chlamydial Protease-Like Activity Factor- Insights into Immunity and Vaccine Development

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Abstract

Chlamydia trachomatis is a Gram-negative obligate intracellular pathogen that remains the leading cause of bacterial sexually transmitted disease worldwide, despite the availability of efficacious antimicrobial therapy. Given that chlamydial infections cause severe pathological sequelae in the upper genital tract, a licensed vaccine to prevent infection and disease would be an ideal solution. Chlamydial protease-like activity factor (CPAF) is a protein secreted in considerable amounts into the cytosol of infected cells and released into the extracellular milieu upon cellular lysis, which therefore is accessible to the host immune system. This is further substantiated by the observation that CPAF is immunodominant among other antigens in *Chlamydia* sero-positive humans. The efficacy of vaccination with CPAF against genital chlamydial challenge has been evaluated extensively in the murine model. This review will discuss important insights into the potential of CPAF as a component of an anti-chlamydial vaccine.

Introduction

Chlamydia trachomatis is a Gram negative obligate intracellular bacterial pathogen that has a tropism for mucosal surfaces, and is the leading cause of bacterial sexually transmitted disease worldwide (Morrison & Caldwell, 2002; Brunham & Rey-Ladino, 2005). Efficacious antimicrobial regimens are available to treat these infections. However, the majority of genital chlamydial infections are asymptomatic and go untreated leading to ascending infection and pathological sequelae in the upper genital tract, such as pelvic inflammatory disease, ectopic pregnancy, and infertility (Morrison & Caldwell, 2002; Brunham & Rey-Ladino, 2005). Given the serious nature of these sequelae, and the continual rise in incidence of genital chlamydial infections over the last decade (Rekart & Brunham, 2008), there is an urgent need for a preventive vaccine (Morrison & Caldwell, 2002; Brunham & Rey-Ladino, 2005; Rekart & Brunham, 2008). Several studies have been carried out to identify potential vaccine candidates, but the complex biphasic developmental cycle, strict host tropism, and serovar variations

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remain important constraints that need to be overcome before attaining an effective anti-chlamydial vaccine for human use. This review focuses on the promising potential of the chlamydial protease-like activity factor (CPAF) as a component of a vaccine to confer optimal protective immunity against genital chlamydial infection and subsequent sequelae.

Biological characteristics of CPAF

It was first reported in 1999 that *Chlamydia* inhibits IFN γ inducible major histocompatibility (MHC) class II expression by degradation of the host upstream stimulatory factor-1 (USF-1, Zhong *et al.*, 1999), and later that degradation of the transcription factor RFX-5 results in the inhibition of both constitutive and IFN γ inducible MHC I expression in *Chlamydia* infected cells (Zhong *et al.*, 2000). Both processes were found to involve a chlamydial protease or proteasome-like activity (CPA). The CPA was present in the cytosol of infected cells, but not uninfected cells, was distinct from the host 20S proteasome activity, and could be inhibited only by an irreversible proteasomal inhibitor lactacystin. Subsequently, two distinct cytosolic protein bands were purified from *Chlamydia trachomatis* serovar L2-infected HeLa cell lysates that correlated with the CPA and corresponded to the -NH₂ and -COOH terminal fragments of a chlamydial protein encoded by open reading frame CT858 (Zhong *et al.*, 2001), leading to the designation of this protein as CPA factor (CPAF: 70 kDa), and the bands as CPAFn (29 kDa) and CPAFc (35 kDa). It was shown that CPAF was necessary for the observed enzymatic activity, and also was sufficient since the protein (encoded by the full length CPAF gene and expressed as a glutathione S-transferase-fusion in a bacterial system) exhibited similar enzymatic activity within a cell-free system (Zhong *et al.*, 2001). Moreover, the cleavage of full-length CPAF into CPAFn and CPAFc, and intramolecular dimerization of the fragments were both found to be essential for the enzymatic activity (Dong *et al.*, 2004a). The presence of cytosolic CPAF during *C. pneumoniae* infection was reported initially (Shaw *et al.*, 2002; Fan *et al.*, 2002), and this observation was extended when proteolytically active CPAF was found to be expressed by five known chlamydial species (Dong *et al.*, 2005) and to exhibit a high degree of amino acid identity. Recently, it was shown that mature CPAF, a serine protease, is a homodimer of the catalytic domains, each comprising two distinct subunits (Huang *et al.*, 2008). The dormancy of the zymogen was shown to be maintained by an internal inhibitory segment, with trans-autocatalytic cleavage initiating activation.

Biological functions of CPAF

CPAF is encoded on the chlamydial genome, produced by the chlamydial reticulate body (RB), secreted out of the inclusion, and localizes predominantly to the cytosol of the infected cell (Zhong *et al.*, 2001). CPAF production is initiated during the middle of the chlamydial developmental cycle around 12 h post infection (p.i.), and progressively accumulates within the cytosol of the infected cell. To this end, CPAF has been found to be highly stable within the infected cells with an approximate half-life of 48 h (Shaw *et al.*, 2002). The kinetics of cytosolic localization of CPAF correlates well with the onset of degradation of multiple target host proteins that have been identified using cells infected with either *C. trachomatis* or *C. pneumoniae* (Shaw *et al.*, 2002). The original discovery of the degradation of host MHC transcription factors, USF-1 and RFX-5, suggested a specific immune evasion mechanism of *Chlamydia* mediated by CPAF (Zhong *et al.*, 1999; Zhong *et al.*, 2000). More recently, CPAF also has been shown to degrade the pro-apoptotic BH3-only Bcl2 family proteins (Pirbhai *et al.*, 2006), which may contribute to the strong anti-apoptotic effect exerted by *Chlamydia* upon the infected host cell (Fan *et al.*, 1998), thereby prolonging survival and allowing successful completion of the developmental cycle. Additionally, CPAF has been shown to degrade cytoskeletal elements, including keratin-8 (Dong *et al.*, 2004b), keratin-18, vimentin and β -tubulin (Savijoki *et al.*, 2008), within infected cells and is thought to allow the expansion of the inclusion and subsequent release of infectious chlamydial elementary bodies (EBs).

Intermediate filament proteins also were found to be processed by CPAF to form filamentous structures, at the inclusion surface, with altered structural properties (Kumar *et al.*, 2008), leading to the proposition that such function would stabilize the inclusion by providing dynamic scaffolding and also minimize exposure of the intra-inclusion contents to the cytoplasmic innate immune surveillance pathways. Collectively, it appears that the protease-like activity of CPAF interacts with key host proteins to manipulate the host immune response in multiple ways towards rendering the developing inclusion less detectable by immune surveillance.

CPAF as a potential vaccine candidate

Chlamydia sero-positive humans make anti-CPAF antibodies (Sharma *et al.*, 2004), including some with the ability to neutralize the enzymatic activity of this molecule (Sharma *et al.*, 2005). CPAF was found to be one of the seven highly immunodominant proteins in an array of 156 fusion proteins cloned from ORFs in the chlamydial genome (Sharma *et al.*, 2006). These findings suggest that CPAF is presented substantially to the host immune system during an infection, presumably due to abundance and cytosolic and/or extracellular localization (Zhong *et al.*, 2001). Thus, for a protein that effects the immune evasion of chlamydial antigens contained within or on the membrane of the chlamydial inclusion, CPAF itself may be the “Achilles' heel” of *Chlamydia* in the context of vaccination-induced immune recognition. When considered with the fact that CPAF is highly conserved among different chlamydial serovars and species (Dong *et al.*, 2005), CPAF appears to be a promising candidate for a pan-serovar anti-chlamydial vaccine.

Protective immunity induced by CPAF vaccination

Intranasal vaccination of mice with three doses of recombinant CPAF (rCPAF), cloned from *C. trachomatis* L2 genome, expressed and purified from an *Escherichia coli* expression vector system, induces a robust anti-CPAF splenic Th1 type cellular IFN γ response, and serum and vaginal antibody responses (Murthy *et al.*, 2007). When administered with murine recombinant interleukin-12 (IL-12) as an adjuvant, the responses are significantly elevated and also induce mucosal anti-CPAF immunoglobulin A (IgA) responses (Murthy *et al.*, 2007). As shown in Fig.1, mice vaccinated intranasally with rCPAF+IL-12 demonstrate significantly reduced bacterial shedding as early as day 6–8 and resolve the infection by day 15 after vaginal *C. muridarum* challenge, or in approximately half the time taken to resolve the infection in unimmunized mice. Vaccination with rCPAF+IL-12 also substantially protects mice from the upper genital tract pathological sequelae of the infection including prolonged inflammatory cellular infiltration, fibrosis, hydrosalpinx, oviduct and uterine horn dilatation (Murthy *et al.*, 2007), suggesting the preservation of reproductive health. The administration of rCPAF with an alternative Th1 adjuvant, CpG deoxynucleotides, also induces comparable immunity to that of rCPAF plus IL-12 (Cong *et al.*, 2007). Moreover, mucosal (intranasal) or systemic (intraperitoneal) immunization with rCPAF plus CpG induces comparable protective immunity against primary chlamydial challenge. Transgenic mice expressing human leukocyte antigen (HLA)-DR4 molecules (in place of mouse MHC II) exhibit robust protective immunity against genital chlamydial challenge following rCPAF plus IL-12 vaccination, indicating the presence of protective epitopes within CPAF that are processed and presented by human HLA molecules (Murthy *et al.*, 2006) and suggesting the translational value of this antigen.

CPAF vaccination-induced protective immunity brings about comparable reductions in upper genital tract pathologies to that induced by live replicating chlamydial organisms. However, while CPAF-vaccinated mice shed high numbers of bacteria at early time-points, a primary chlamydial infection induces a high degree of resistance to re-infection in approximately 60% of animals, and the mice that get infected shed much lower numbers of bacteria with complete resolution of the infection within a week after challenge (Morrison & Caldwell, 2002). The

high degree of protective immunity against secondary challenge has been shown to be mediated by both *Chlamydia*-specific CD4⁺ T cells and antibodies, but not CD8⁺ T cells (Morrison *et al.*, 2000). The mechanisms of chlamydial clearance induced by CPAF vaccination may differ from those induced by primary chlamydial infection, as described below, which may explain the differences in kinetics of shedding.

Mechanisms of protective immunity induced by CPAF vaccination

The protective immunity induced by rCPAF vaccination is dependent upon the induction of the MHC II pathway (Murthy *et al.*, 2006), Ag-specific CD4⁺ T cells (Murphey *et al.*, 2006), and endogenous IFN γ production (Murthy *et al.*, 2007). Protective immunity comparable to that induced by vaccination in mice can be transferred by CPAF-specific CD4⁺ T cells into naïve recipient mice (Murphey *et al.*, 2006). Considering that natural immunity conferred by a primary chlamydial challenge against rechallenge is the “gold standard” for vaccine-mediated protection, CPAF-specific CD4⁺ T cells induce comparable protective immunity to that of *Chlamydia*-specific CD4⁺ T cells primed by a live replicating infection (Murphey *et al.*, 2006). The enhanced local infiltration of CPAF-specific CD4⁺ T cells and IFN γ production in the genital tract on day 6 after challenge correlates with the significantly reduced bacterial shedding in vaccinated mice at this time-point (Li *et al.*, 2008). IFN γ production from CPAF-specific CD4⁺ T cells is required and sufficient, despite the absence of all other sources of IFN γ production, to mediate the protective immunity (Li *et al.*, 2008). rCPAF-mediated immune protection, in terms of either chlamydial clearance or reduction of upper genital tract pathology, is not dependent upon the MHC I pathway (CD8⁺ T cells) (Li *et al.*, 2008), or B cells and antibody (Murthy *et al.*, 2008). Thus, the protective immunity induced by rCPAF vaccination is mediated predominantly by antigen-specific CD4⁺ T cells.

Proposed model for the dependence of CPAF-mediated anti-chlamydial immunity on CD4⁺ T cells

CPAF vaccination-induced chlamydial resolution is very similar, albeit significantly accelerated, to the immune process that has been shown previously to occur during a primary genital chlamydial infection with an important role for CD4⁺ T cells (Su *et al.*, 1995) and IFN γ (Rank *et al.*, 1992), and a less dominant role for antibody (Ramsey *et al.*, 1988; Su *et al.*, 1997) and CD8⁺ T cells (Igietseme *et al.*, 1994; Magee *et al.*, 1995). Since CPAF is not expressed on the surface of the infective chlamydial EB, it may not induce antibodies that neutralize chlamydial infectivity and relies instead upon CD4⁺ T cell/IFN γ mediated clearance of infection. It is counter-intuitive to a certain degree that CPAF, a predominantly cytosolic antigen, does not induce protective immunity via the MHC I pathway and CD8⁺ T cells (Li *et al.*, 2008). Thus, we propose a working model as described in Fig. 2 to explain the dependence of CPAF-mediated protective immunity, and anti-chlamydial immunity in general within the mouse model, on CD4⁺ T cell responses.

A targeted combinatorial approach for optimal anti-chlamydial immunity

Vaccination with CPAF as the sole chlamydial antigen may significantly reduce the duration of chlamydial shedding and prevent the development of pathological sequelae (Murthy *et al.*, 2007) which are highly desirable consequences. However, when compared to mock-immunized animals, vaccinated mice display comparable bacterial shedding at day 3, but only exhibit significantly reduced numbers as early as day 6 after chlamydial challenge (Murthy *et al.*, 2007), resulting from an approximate 5 day delay in the considerable infiltration of effector CD4⁺ T cells into the genital tract after chlamydial challenge of vaccinated mice (Li *et al.*, 2008). Given that CPAF-mediated immunity occurs predominantly via CD4⁺ T cells (Murphey *et al.*, 2006), there is an inherent limitation to reducing bacterial shedding at very early time-

points when rCPAF is used as the only immunogen. In contrast, the induction of long-lasting absolute resistance to infection would be ideal; and optimal immunity that results in reduced load and duration of bacterial shedding, and prevention of pathological consequences is a realistic goal for an efficacious anti-chlamydial vaccine. Neutralizing antibody has been shown to confer resistance to (Su *et al.*, 1997; Pal *et al.*, 1997) and play a predominant role in resolution (Morrison *et al.*, 2005) following chlamydial rechallenge, and thus can be used to supplement the CD4⁺ T cell driven immunity towards achieving optimal protection. In theory (as shown in Fig. 2), the induction of sufficient antibodies against EB surface antigens could neutralize infectivity and confer complete resistance to infection. The chlamydial major outer membrane protein (MOMP), which is abundantly expressed on the surface of the EB has been considered a suitable candidate for such an approach (Morrison & Caldwell, 2002; Brunham & Rey-Ladino, 2005). However, the requirement of appropriate tertiary conformation and therefore tedious refolding (Pal *et al.*, 2005), the presence of surface-structure based sero-variability (Morrison & Caldwell, 2002; Brunham & Rey-Ladino, 2005), and the expected need for repeated booster immunizations to maintain high levels of pre-formed neutralizing antibody over long periods of time, may limit the use of MOMP as the only vaccine antigen. Apart from antibody, Th1 type CD4 T cell responses also are induced by MOMP vaccination (Berry *et al.*, 2004; Pal *et al.*, 2005; Li *et al.*, 2007), but may not be highly protective since vaccination with non-refolded MOMP only induces partial immune protection (Pal *et al.*, 2006; Li *et al.*, 2007), for reasons raised in the model (Fig. 2).

Summary

A single ideal chlamydial vaccine antigen would be one that is abundantly expressed on the surface of the EB, contains conserved neutralizing epitopes, and also is secreted abundantly into the host cytosol and mediates T cell responses. Such an antigen has yet to be discovered, although the polymorphic membrane protein D (pmpD) of *Chlamydia* fits this description to some extent, since it is expressed predominantly on the surface of the chlamydial reticulate body (RB), to a lesser extent on the EB, and secreted outside the bacterium but confined within the inclusion membrane (Crane *et al.*, 2006; Kiselev *et al.*, 2007). Therefore, it is generally agreed upon that multiple chlamydial antigens and the induction of a robust Th1 type CD4⁺ T cell response, as well as antibody, will be required for a successful anti-chlamydial vaccine (Morrison & Caldwell, 2002; Brunham & Rey-Ladino, 2005). The results from immunological characterization of CPAF as a vaccine candidate, as described in this review, support and extend this view. This leads us to propose that a targeted induction of a combination of neutralizing antibodies against EB surface-exposed conserved epitopes, and CD4⁺ T cell mediated immunity against an abundant, highly conserved antigen that is secreted into the host cytosol (such as CPAF) may be the key towards achieving long-lasting optimal protection against multiple serovars responsible for genital chlamydial infection.

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Fig. 1. rCPAF+IL-12 vaccination enhances the resolution of genital chlamydial infection. Animals (10 mice/group) were treated with three doses of rCPAF+IL-12, rCPAF, IL-12, or PBS (Mock). One month following the final vaccination, mice were challenged intravaginally with 5×10^4 IFU of *C. muridarum*. On the days indicated following challenge, chlamydial shedding was measured; the numbers of chlamydial IFU recovered from vaginal swabs were determined. Each symbol represents an individual animal. The number(s) of animals in each group that had resolved the infection are indicated on the X-axis. An asterisk indicates that there is a significant difference between the groups indicated ($P < 0.05$, as determined by the Kruskal-Wallis test). The results are representative of three independent experiments. (Reproduced with permission from Infection and Immunity; 2007, 75: 666–676).

Fig. 2.

Proposed model for the importance of CD4⁺ T cells in rCPAF-mediated anti-chlamydial immunity. **(A)** The infectious EBs enter the host cell and are contained within the endosome. At this stage, the infection can be prevented by neutralizing antibodies against surface structures on the EB. **(B)** The infection is at an advanced stage with chlamydial reticulate bodies (RBs) that are actively multiplying and producing chlamydial proteins, including CPAF. At this juncture, most chlamydial proteins are walled-off from the host by the sturdy inclusion membrane, whereas abundant amounts of CPAF localize to the cytosol of the infected cell. The activation of either CD4⁺ or CD8⁺ T cells is minimal since CPAF actively downregulates of MHC I and II expression. **(C)** The rupture of the infected cell releases the intracellular contents into the extracellular matrix. At this stage, antibodies that bind to the surface of EB can neutralize infectivity, whereas others (i.e. anti-CPAF antibodies) cannot. **(D)** The extruded antigens are taken up by neighboring host cells in endosomes. Apart from the infectious EB, various chlamydial proteins (such as CPAF), RBs, and inclusion membrane proteins also are endocytosed. Antibody-bound antigens may be taken up more efficiently via Fc-receptor mediated processes at this stage. Due to the abundant amounts of CPAF, compared to other chlamydial antigens, relatively more molecules are taken up into the endosomes. **(E)** While the infecting EB progresses through the developmental cycle, the endocytosed proteins are processed and presented via the exogenous MHC II pathway to CD4⁺ T cells; due to the greater uptake, more CPAF epitopes are processed and presented. Although MOMP is expressed abundantly on surface of chlamydial EBs and RBs, only MOMP on the RBs or dead EBs, but not on live EBs, have the opportunity to be processed and presented by MHC II pathways. The surface presentation of MHC II molecules loaded with these chlamydial antigens occurs before

CPAF-mediated MHC down-regulation begins within this cell. The activated CD4⁺ T cells secrete IFN γ which aborts the chlamydial developmental cycle, leading to (F) elimination of infection. Antigen presentation to CD4⁺ T cells may happen more efficiently in macrophages and dendritic cells, but the secreted IFN γ can act in a paracrine fashion to eliminate infection from epithelial cells. This model explains the predominant role of CD4⁺ T cells, followed by antibody and then CD8⁺ T cells, in rCPAF vaccination-induced immunity against genital chlamydial infection.