



## CPAF, HSP60 and MOMP antigens elicit pro-inflammatory cytokines production in the peripheral blood mononuclear cells from genital *Chlamydia trachomatis*-infected patients

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### ABSTRACT

**Background:** Persistent inflammation caused by *Chlamydia trachomatis* in the female genital compartment represents one of the major causes of pelvic inflammatory disease (PID), ectopic pregnancy and infertility in females. Here, we examined the pro-inflammatory cytokine response following stimulation with three different types of *C. trachomatis* antigens, viz. chlamydial protease-like factor (CPAF), heat shock protein 60 (HSP60) and major outer membrane protein (MOMP).

**Methods:** A total of 19 patients with genital *C. trachomatis* infection and 10 age-matched healthy controls were recruited for the study. Peripheral blood mononuclear cells (PBMCs) isolated from genital *C. trachomatis*-infected females were cultured in the presence of CPAF, HSP60 and MOMP antigens, and cytokines were measured by ELISA assay.

**Results:** We reported that pro-inflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$  and IL-6) were robustly secreted following antigenic exposure. Notably, CPAP and MOMP were more potent in triggering IL-1 $\beta$ , as compared to HSP60. Elevated levels of the proinflammatory cytokines were also noted in the samples infected with plasmid-bearing *C. trachomatis* as compared to those infected with plasmid-free strains.

**Conclusions:** Our study highlights distinct ability of chlamydial antigens in triggering pro-inflammatory response in the host immune cells.

### 1. Introduction

*Chlamydia trachomatis* is a Gram-negative, obligate intracellular bacterium that primarily infects the epithelial cells lining the reproductive mucosa and causing sexually transmitted disease in humans. *C. trachomatis* genital infection leads to substantial worldwide morbidity with a global prevalence of ~131 million infections annually according to recent estimates (Newman et al., 2015). In females, *C. trachomatis* infection causes a myriad of urogenital tract pathologies, which manifests as cervicitis and urethritis (Bebear and de Barbeyrac,

2009; Paavonen and Eggert-Kruse, 1999). A vast majority of asymptomatic individuals (50–70%) display recalcitrant infections leading to significant challenges to the successful management and control of genital chlamydial infection (Spiliopoulou et al., 2005). *C. trachomatis* also has the ability to subvert the functions of the host cell for its own advantage during infection by remodeling host proteome regulation and host transcription initiation (Olive et al., 2014; Tan et al., 2016), which underscores the need for an effective chlamydial vaccine. To achieve this goal requires a thorough understanding of the interaction between host immune responses with the chlamydial antigens following

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intracellular infection with *C. trachomatis*.

Chlamydial protease-like factor (CPAF), heat shock protein 60 (HSP60) and major outer membrane protein (MOMP) are three chlamydial antigens with potent immunopathogenic responses. Notably, the MOMP of *C. trachomatis* comprises ~60% of the total protein content of the bacterial cell wall (Caldwell et al., 1981), and is known to be an immunodominant antigen in humans. MOMP plays a vital role in chlamydial biology acting primarily as an adhesion agent, which promotes attachment to the host cell during infection and also as a porin that facilitates the selective passage of nutrients across the chlamydial cell membrane (Su et al., 1990; Jones et al., 2000). CPAF is a serine protease secreted by *C. trachomatis* midway during its development cycle into the cytosol of the infected host cell (Zhong et al., 2001). Although the function of CPAF remains largely unclear, recent experimental data points to its likely role in chlamydial pathogenesis by suppressing host immune responses via degradation of host antimicrobial peptides and complement components, as well as inhibition of pro-inflammatory transcription factors (Tang et al., 2015; Yang et al., 2016; Patton et al., 2016). Upon encountering growth stress, *C. trachomatis* enters into a persistent phase characterized by an arrest in cell division (Wyrick, 2010), that is accompanied by production of copious amounts of a 60-kDa heat shock protein (HSP60). HSP60 has been shown to contribute to prolonged local inflammatory response (LaRue et al., 2007), and poses greater risks for development of tubal factor infertility in females (Tiitinen et al., 2006; Witkin et al., 1994).

Here, we examined the host inflammatory response to three different chlamydial antigens (MOMP, CPAF, and HSP60) using peripheral blood mononuclear cells (PBMCs) derived from the *C. trachomatis*-infected females. Specifically, we sought to determine the profile of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ) and IL-6 cytokines secreted against chlamydial antigens in our patient cohort with a view to understand the host immune responses following infection with *C. trachomatis*.

## 2. Materials and methods

### 2.1. Study population

A total of 49 female individuals attending the obstetrics and gynecology outpatient clinic at the University of Malaya Medical Centre between 2013 and 2014 were recruited into the cohort. Exclusion criteria were positive urine pregnancy test, recent antibiotic therapy, vaginal candidiasis and genital tuberculosis. The control group was composed of a cohort of voluntarily participating females aged between 21 and 42 years without previous history of sexually transmitted infections. Only 19 samples that were diagnosed to be *C. trachomatis*-positive were used in this study. A total number of 10 age-matched healthy controls were also included. All participants were briefed about the objectives of the study and written informed consents were obtained prior to collecting the blood and vaginal swab specimens. The information pertaining to visits and gynecology including reasons for referral, menstruation, symptoms of genital and urinary tract infections, obstetric and medical histories were recorded. The study has been approved by the institutional Medical Ethics Committee (reference 908.109).

### 2.2. Detection of *C. trachomatis* infection

*C. trachomatis* was diagnosed using a real-time polymerase chain reaction (RT-PCR) and a serological method, as described previously (Yeow et al., 2016). Endocervical swabs were collected from the patients' vulva and cervix into prescribed UTM-RT universal transport media tubes (Copan, Brescia, Italy), vortex mixed and were centrifuged at 10,000  $\times$ g. The resultant pellet was lysed and DNA was extracted using a commercial QIAquick Gel extraction kit (Qiagen, Hilden, Germany) and amplified using primers targeting the *Momp* and a cryptic

plasmid as previously described (Jalal et al., 2006). To determine the serovars, *OmpA* fragments were sequenced by using a BigDye<sup>®</sup> Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) in a 3730 DNA Analyzer. The primers used for sequencing were P3, P4, 191S, C214, NL-F and NL-R (Lysen et al., 2004; Stevens et al., 2004; Yang et al., 1993), to ensure sufficient sequence overlap and fidelity spanning the four *OmpA* variable domains. The genotype was determined by comprising to the BLAST result at the National Center for Biotechnology Information.

For serological diagnosis, anti-*C. trachomatis* MOMP IgG or IgM in the patient sera were detected using a commercial RIDASCREEN *C. trachomatis* IgG/IgM kit (R-Biopharm, Darmstadt, Germany) as indicated in the manufacturer's guidelines. In brief, 96-well microtiter plates that were precoated with chlamydial LPS were used. All positive, negative and cut-off controls as well as samples were run in duplicates. For the IgM determination wells, a total of 25  $\mu$ L RIDA RF Absorbents was added to block IgG binding. Two-fold diluted sera samples were applied in duplicate and incubated at 37  $^{\circ}$ C for 45 min followed by addition of avidin HRP-conjugated anti-human IgG or IgM for an additional 30 min at 37  $^{\circ}$ C. Later, the wells were washed and the color developed following the addition of 3,3',5,5'-tetramethylbenzidine (TMB) solution at 37  $^{\circ}$ C for 20 min was quenched using 0.5 M H<sub>2</sub>SO<sub>4</sub>. Optical density (OD) was read at 450 nm with a reference wavelength of 620 nm on a microplate reader (Tecan, Männedorf, Switzerland). Results were expressed as sample index, with the formula (sample index = average OD for sample / average OD for cut-off controls); and values of > 1.1 were considered positive. Samples that showed positive results in diagnostic PCR and ELISA were labelled as *C. trachomatis*-positive and were used in the study.

### 2.3. PBMC culture and stimulation

PBMCs were purified using Ficoll-Hypaque density gradient centrifugation (Sigma-Aldrich, MO, USA). A total of 9 ml of heparinized blood was diluted two-fold with phosphate buffered saline (PBS) and layered on top of 6 ml Ficoll-Hypaque at a 3:1 ratio and centrifuged at 400  $\times$  g for 25 min at room temperature. The buffy coat at the interface layer was harvested, transferred to a sterile 15 ml tube containing PBS, and centrifuged at 225  $\times$  g for 10 min at 4  $^{\circ}$ C. Later, the cells were suspended in 10 ml RPMI1640 medium and viable cell count was obtained using 0.4% trypan blue in a hemocytometer. For cryopreservation, cells were resuspended in freezing media containing 90% fetal bovine serum (FBS) and 10% dimethyl sulfoxide (DMSO) and cooled overnight in a Mr. Frosty Freezing Container (Thermo Fisher Scientific, MA, USA) at -80  $^{\circ}$ C before transferring into liquid nitrogen until further analyses. Before antigen stimulation, frozen PBMCs were thawed, washed three times with RPMI 1640, and suspended in RPMI 1640 supplemented with 10% heat inactivated human AB serum. Cells (6.25  $\times$  10<sup>4</sup> cells/well) from each sample were seeded in separate wells (non-pooled) in a flat-bottomed 96-well plates and were subsequently stimulated with MOMP, CPAF, and HSP60 antigens at a final concentration of 1  $\mu$ g/mL and incubated for 72 h at 37  $^{\circ}$ C at a 5% CO<sub>2</sub> atmosphere. PBMCs were also stimulated with 1  $\mu$ g/mL phytohemagglutinin (PHA) (Sigma-Aldrich, MO, USA) as positive control. Chlamydial antigens were prepared as previously described (Li et al., 2007). Briefly, genes encoding for MOMP, CPAF and HSP60 with an N-terminal glutathione S-transferase were cloned into pGEX vectors and expressed in *Escherichia coli* system (Sharma et al., 2004). Proteins were purified using Centrplus YM-10 tubes (Millipore, MA) with endotoxin levels at < 1 endotoxin unit (EU)/mg.

### 2.4. Quantification of cytokines

Concentrations of cytokines in the culture supernatant were analyzed using a commercial Ready-SET-Go! ELISA kit (eBiosciences, CA, USA) in accordance with the manufacturer's protocol. Nunc MaxiSorp

ELISA plates (Biolegend, CA, USA) were coated with 100  $\mu$ L/well of capture antibody in 1  $\times$  coating buffer overnight at 4 °C. Plates were then washed and non-specific binding was blocked with 200  $\mu$ L/well of assay diluent at room temperature for 1 h. Wells were washed and PBMC supernatants were added and incubated for 2 h. After washing, detection antibody was loaded into each well and incubated for another 1 h. TMB substrate solution was added 30 min after the addition of Avidin-HRP and the color was allowed to develop for 15 min. The color reaction was subsequently stopped with 1 M H<sub>2</sub>SO<sub>4</sub>. Absorbance was measured in a microplate reader (Tecan, Männedorf, Switzerland) at 450 nm using a reference wavelength of 570 nm. The cell culture supernatants of each subject after stimulated with different antigens were collected and run separately in duplicates.

### 2.5. Statistical analysis

Statistical analyses were conducted using GraphPad Prism Version 5. Data were presented as mean  $\pm$  SEM. Comparison between the groups were carried out using two-tailed Mann-Whitney U test. Statistical significance was determined when  $P < 0.05^*$ , or  $P < 0.01^{**}$ . Correlation study was performed using Microsoft excel software.

## 3. Results

### 3.1. Patients demography

The study comprised a total of 49 female individuals of child-bearing age who visited the obstetrics and gynecology clinic at the Universiti Malaya Medical Center, Kuala Lumpur. Out of 49 subjects examined, only 19 (38.7%) who were positively infected with genital *C. trachomatis* infection were used in the following cytokine study (Table 1). The study subjects aged between 21 to 42 years old (median: 31 years old). Reasons for clinic visit included issues of infertility or spontaneous abortion ( $n = 9$ ), abdominal pain ( $n = 4$ ), and irregular menstrual cycle ( $n = 3$ ). Clinical examination revealed endometriosis in 4 individuals, mucopurulent cervicitis was noted in 2 individuals and 1 individual had polycystic ovarian syndrome (PCOS). This cohort consisted of different ethnicities including Malays ( $n = 12$ ), Chinese ( $n = 3$ ) and Indians ( $n = 3$ ) and Indonesian ( $n = 1$ ). *C. trachomatis* infection were diagnosed through real time PCR amplification of the DNA extracted from cervical cytobrush samples and serum anti-*C. trachomatis* IgG/IgM assays. As mentioned above, 19 out of 49 samples were diagnosed to be positive for the *C. trachomatis* infection. Sequencing

data revealed that all samples were infected by *C. trachomatis* serovar D. Common practice for diagnosis relies on amplification of *C. trachomatis* DNA from vaginal swabs, however the data provides information for current infection, but not past, present asymptomatic or persistent infections. Therefore, we further detected presence of infection by examining chlamydial specific IgG and IgM in patients' sera using ELISA assay. Overall, 13 of the subjects were IgM + IgG +, 5 were IgM + IgG-, and one IgM-IgG+. A total of 10 healthy control female volunteers who were seronegative (IgM-IgG-) were included as negative control in this study. Given that presence of plasmid in the *C. trachomatis* has been shown to evoke immune response and is associated with a more severe disease progress in human (Donati et al., 2009; Comanducci et al., 1994; Li et al., 2008), we further investigated if the samples were infected with plasmid-bearing or plasmid-free strains. A total of 12 out of 19 samples (63%) showed positive amplification with a separate set of primers targeting chlamydial plasmid, indicating infection with plasmid-bearing chlamydial strain.

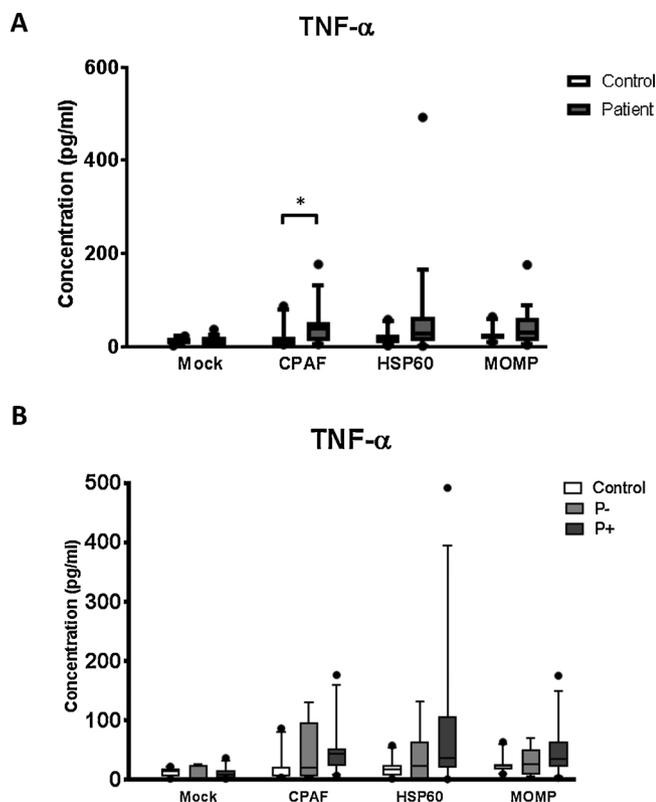
### 3.2. Chlamydial CPAF, HSP60 and MOMP antigens trigger TNF- $\alpha$ secretion from patients' PBMCs

The production of pro-inflammatory cytokine TNF- $\alpha$  was examined in chlamydial antigens (CPAF, HSP60 and MOMP)-stimulated PBMCs (Fig. 1A and Table 2). Our data showed that CPAF stimulation induced a 2.7-fold higher ( $*P = 0.0105$ ) TNF- $\alpha$  levels in the genital *C. trachomatis*-infected subjects ( $49.9 \pm 11.0$ ; median: 38.9 pg/ml), compared to healthy control ( $18.5 \pm 7.9$ ; median: 6.9 pg/ml). MOMP stimulation induced a 1.7-fold increase of TNF- $\alpha$  in *C. trachomatis*-infected patients ( $42.3 \pm 9.2$ ; median: 30.4 pg/ml), compared to healthy control ( $24.8 \pm 4.7$ ; median: 22.9 pg/ml); whereas HSP60 induced a 3.6-fold increase of TNF- $\alpha$  in the PBMCs from chlamydial subjects ( $69.6 \pm 25.8$ ; median: 28.4 pg/ml), compared to healthy controls ( $19.1 \pm 5.1$ ; median: 18.3 pg/ml). Hence, all three types of antigens were able to induce comparable levels of TNF- $\alpha$  production from PBMCs of *C. trachomatis*-infected patients. Among them, HSP60 demonstrated highest potency to trigger TNF- $\alpha$  secretion.

Furthermore, we examined if the production of TNF- $\alpha$  were varied between the PBMCs from the subjects infected with plasmid-bearing versus plasmid-free *C. trachomatis* strains (Fig. 1B). CPAF antigen stimulation caused a small increase of 1.16-fold higher level of TNF- $\alpha$  in the PBMCs from plasmid-bearing ( $52.7 \pm 14.2$ ; median: 43.3 pg/ml) versus plasmid-free ( $45.2 \pm 18.8$ ; median: 19.9 pg/ml) subjects. MOMP induced a 1.69-fold higher level of TNF- $\alpha$  in plasmid-bearing ( $49.8 \pm 13.4$ ; median: 34.8 pg/ml) versus plasmid-free ( $29.4 \pm 9.1$ ;

**Table 1**  
Demographic data and clinical symptoms of study population.

Patient	Age	Ethnic	Marital Status	Fertility	Inflammation	Menses	Other remarks
#1	25	Malay	married	miscarriage	mucopurulent cervicitis	regular	menorrhagia
#2	34	Chinese	married	Infertile	endometriosis	irregular	laparotomy cystectomy
#3	36	Malay	married	fertile	endometriosis	regular	multiple uterine fibriod
#4	28	Malay	married	Infertile	normal	irregular	PCOS
#5	35	Malay	married	Infertile	endometriosis	regular	-
#6	31	Indian	married	fertile	normal	regular	abnormal PAP smear
#7	42	Malay	married	fertile	normal	regular	HPV infection
#8	31	Malay	?	miscarriage	normal	regular	-
#9	27	Malay	married	fertile	normal	regular	parity 1
#10	21	Indonesian	?	Infertile	normal	irregular	-
#11	33	Malay	married	fertile	normal	regular	adenomyosis
#12	27	Malay	married	Infertile	normal	regular	-
#13	26	Malay	married	fertile	normal	regular	postcoital bleeding
#14	30	Malay	married	Infertile	normal	regular	-
#15	24	Chinese	?	fertile	mucopurulent cervicitis	regular	-
#16	34	Indian	?	fertile	endometriosis	regular	recurrent ovarian cyst
#17	32	Chinese	married	fertile	normal	regular	-
#18	31	Malay	married	miscarriage	normal	regular	-
#19	34	Malay	married	fertile	normal	regular	parity 2



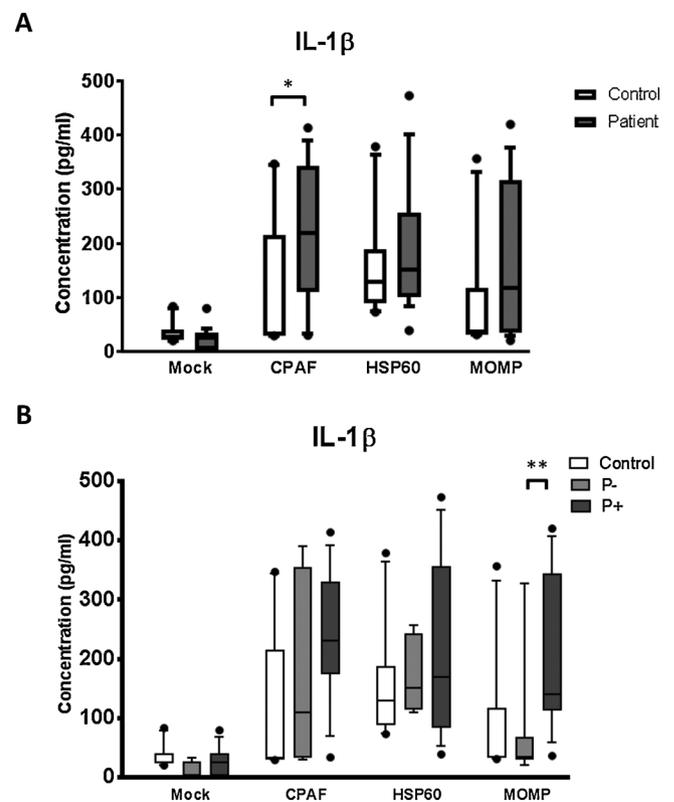
**Fig. 1.** Production of TNF- $\alpha$  from the chlamydial antigen-stimulated PBMCs. PBMCs from the healthy controls (n = 10) and *C. trachomatis*-infected individuals (n = 19) were unstimulated (mock) or stimulated with different chlamydial antigens (CPAF, HSP60 and MOMP) at a final concentration of 1  $\mu$ g/mL for 72 h. Cytokine concentration (pg/ml) secreted in the culture supernatants was measured by ELISA assay. Tukey whisker box plot shows (A) comparison between healthy control and *C. trachomatis*-infected groups; and (B) comparison of the control, plasmid-free (P-) and plasmid-bearing (P+) groups. Dots represent outliers. \*P < 0.05 by a Mann-Whitney U test.

median: 26.8 pg/ml) subjects. Whereas HSP60 induced a 2.11-fold increased TNF- $\alpha$  in plasmid-bearing (86.4  $\pm$  17.8; median: 36.8 pg/ml) versus plasmid-free (40.8  $\pm$  17.8; median: 22.8 pg/ml) subjects. Overall, higher concentrations of TNF- $\alpha$  were observed in plasmid-bearing than those of plasmid-free groups upon stimulation with chlamydial antigens, in accordance with previous studies which showed a more severe immunopathology associated with plasmid-bearing strains

**Table 2**

Cytokines secretion from chlamydial antigens-stimulated PBMCs. The concentrations (pg/ml) of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 cytokines secreted in the culture supernatant of PBMCs. PBMCs were unstimulated (mock), stimulated with control (PHA) or three different types of chlamydial antigens (CPAF, HSP60 and MOMP). HC: healthy control PBMCs from age-matched female volunteers (n = 10); and Ctr: PBMCs from chlamydial-infected female patients (n = 19). P-: PBMCs from plasmid-free chlamydia-infected patients (n = 7); and P+: PBMCs from plasmid-bearing chlamydial-infected female patients (n = 12).

Samples	Mock				CPAF				HSP60				MOMP				PHA			
	HC	Ctr	P-	P+	HC	Ctr	P-	P+	HC	Ctr	P-	P+	HC	Ctr	P-	P+	HC	Ctr	P-	P+
<b>TNF-<math>\alpha</math></b>																				
Mean	13.9	11.6	11.5	11.6	24.8	42.3	29.4	49.8	18.5	49.9	45.2	52.7	19.1	69.6	40.8	86.4	33.9	75.5	76.7	74.7
Std. Error	2.3	2.4	4.4	3.01	4.7	9.2	9.1	13.4	7.9	11.0	18.8	14.2	5.1	25.8	17.8	39.5	15.2	19.6	29.3	26.8
Median	15.1	7.0	2.5	8.8	22.9	30.4	26.8	34.8	6.9	38.8	19.9	43.4	18.3	28.4	22.8	36.8	23.2	29.1	29.1	27.7
<b>IL-1<math>\beta</math></b>																				
Mean	33.9	21.7	16.7	24.6	85.4	159.9	78.3	207.5	119.5	220.3	187.3	239.5	156.2	196.4	169.9	211.8	98.2	57.8	50.1	62.2
Std. Error	6.0	4.8	5.2	6.9	32.0	31.0	41.9	37.0	40.3	29.5	64.1	29.4	29.4	28.3	22.3	43.1	13.9	13.1	20.2	17.7
Median	25.6	25.3	25.3	25.3	34.6	117.6	34.5	140.3	32.4	219.5	109.1	231.2	129.1	150.7	150.7	170.0	106.6	34.5	28.8	57.3
<b>IL-6</b>																				
Mean	20.3	17.4	14.2	19.2	356.5	370.6	352.8	381.0	370.4	391.7	374.9	401.4	406.3	422.3	377.9	448.2	40.3	30.2	21.7	35.2
Std. Error	2.2	4.2	4.2	6.3	46.1	43.5	69.1	58.0	49.6	39.4	74.04	47.5	52.4	39.0	74.7	44.7	11.7	4.5	5.1	6.1
Median	22.3	18.9	18.9	15.9	275.4	261.8	261.8	399.8	281.9	305.2	305.2	392.1	326.1	491.0	352.5	526.2	24.7	25.4	22.1	27.4



**Fig. 2.** Production of IL-1 $\beta$  from the chlamydial antigen-stimulated PBMCs. PBMCs from the healthy controls (n = 10) and *C. trachomatis*-infected individuals (n = 19) were unstimulated (mock) or stimulated with different chlamydial antigens (CPAF, HSP60 and MOMP) at a final concentration of 1  $\mu$ g/mL for 72 h. Cytokine concentration (pg/ml) secreted in the culture supernatants was measured by ELISA assay. Tukey whisker box plot shows (A) comparison between healthy control and *C. trachomatis*-infected groups; and (B) comparison of the control, plasmid-free (P-) and plasmid-bearing (P+) groups. Dots represent outliers. \*P < 0.05, \*\*P < 0.01 by a two-tailed Mann-Whitney U test.

(Donati et al., 2009; Comanducci et al., 1994; Li et al., 2008).

### 3.3. Chlamydial CPAF and MOMP antigens induce high levels of IL-1 $\beta$

In addition to TNF- $\alpha$ , our results showed that exposure to chlamydial antigens resulted in markedly elevated concentrations of another

pro-inflammatory cytokine, IL-1 $\beta$ , in the PBMCs derived from genital *C. trachomatis*-infected subjects (Fig. 2A). When stimulated with CPAF, IL-1 $\beta$  levels were elevated at 1.84-fold ( $*P = 0.0162$ ) in the PBMCs from chlamydial-infected subjects ( $220.3 \pm 29.6$ ; median: 219.5 pg/ml), compared to healthy controls ( $119.5 \pm 40.3$ ; median: 32.4 pg/ml). Likewise, when stimulated with MOMP, a 1.87-fold increase of IL-1 $\beta$  level was observed in the PBMCs from chlamydial-infected subjects ( $159.9 \pm 31.0$ ; median: 117.6 pg/ml), compared to healthy controls ( $85.5 \pm 32.0$ ; median: 34.7 pg/ml). Only a marginal 1.25-fold induction of IL-1 $\beta$  was also observed upon HSP60 stimulation in the PBMCs from chlamydial-infected subjects ( $196.4 \pm 28.3$ ; median: 150.7 pg/ml), compared to the healthy controls ( $156.2 \pm 9.4$ ; median: 129.1 pg/ml). Hence, our data suggest that both CPAF and MOMP elicited high IL-1 $\beta$  response; whereas the HSP60 stimulation elicited a greater expression of TNF- $\alpha$  instead of IL-1 $\beta$ .

Interestingly, a noticeable difference of IL-1 $\beta$  secretion upon chlamydial antigens stimulation was observed between the PBMCs derived from subjects infected with plasmid-free and plasmid-bearing strains (Fig. 2B). When stimulated with CPAF, plasmid-bearing samples showed a 1.5-fold higher secretion of IL-1 $\beta$  ( $239.5 \pm 29.5$ ; median: 231.2 pg/ml), versus the plasmid-free samples ( $187.3 \pm 64.1$ ; median: 109.1 pg/ml). MOMP stimulation induced a 2.64-fold ( $**P = 0.0048$ ) higher IL-1 $\beta$  in the plasmid-bearing samples ( $207.5 \pm 37.1$ ; median 140.3 pg/ml) versus ( $78.4 \pm 41.9$ ; median: 34.5 pg/ml); whereas HSP60 induced a marginal level of 1.24-fold higher IL-1 $\beta$  level in plasmid-bearing ( $211.8 \pm 43.1$ ; median: 170.0 pg/ml) versus plasmid-free ( $169.9 \pm 22.3$ ; median: 150.7 pg/ml) samples. Our findings suggest that CPAF and MOMP were more robust than HSP60 in their ability to trigger IL-1 $\beta$  response, particularly in the PBMCs from the patients infected with plasmid-bearing bacteria.

### 3.4. Marginal increased IL-6 secretion in the chlamydial antigens-stimulated PBMCs from chlamydial-infected patients and healthy controls

In contrast to TNF- $\alpha$  and IL-1 $\beta$ , no obvious difference ( $< 1.1$ -fold) was observed in the IL-6 production upon antigenic exposure in the PBMCs derived from chlamydial-infected subjects or healthy control individuals (Fig. 3A). When stimulated with CPAF, the concentration of IL-6 in the chlamydial-infected patients were merely 1.05-fold higher ( $391.7 \pm 39.4$ ; median: 305.2 pg/ml), compared to those of healthy control individuals ( $370.4 \pm 49.6$ ; median: 281.9 pg/ml). The levels of IL-6 produced after MOMP stimulation were also comparable (1.02-fold) between samples from chlamydial-infected subjects ( $370.6 \pm 43.6$ ; median: 261.8 pg/ml) versus healthy controls ( $356.5 \pm 46.2$ ; median: 275.4 pg/ml); and after HSP60 stimulation (1.03-fold) in chlamydial-infected patients ( $422.3 \pm 39.0$ ; median: 491.0 pg/ml) versus healthy controls ( $406.3 \pm 52.4$ ; median: 326.1 pg/ml).

When compared between the plasmid-free and plasmid-bearing samples, there was no significant difference observed between two groups, but plasmid-bearing samples demonstrated a slightly higher IL-6 levels upon stimulation with chlamydial antigens (Fig. 3B). When stimulated with CPAF, plasmid-bearing samples showed a 1.07-fold higher IL-6 level ( $401.4 \pm 47.5$ ; median: 392.1 pg/ml) compared to plasmid-free samples ( $374.9 \pm 74.0$ ; median: 305.2 pg/ml). MOMP stimulation induced a 1.07-fold increase in the plasmid-bearing ( $381.0 \pm 58.0$ ; median: 399.8 pg/ml) versus plasmid-free ( $352.8 \pm 69.2$ ; median: 261.8 pg/ml) samples; whereas HSP60 stimulation induced a 1.18-fold higher IL-6 in plasmid-bearing ( $448.2 \pm 44.7$ ; median: 526.2 pg/ml) versus plasmid-free ( $377.9 \pm 74.8$ ; median: 352.5 pg/ml) samples. Overall, IL-6 production was triggered by chlamydial antigens stimulation, however limited differences were observed between chlamydia-infected subjects versus healthy controls. Overall, the standard errors for the ELISA data set were relatively high likely due to variations in the history of infection, inflammatory status or clinical symptoms among the study subjects at

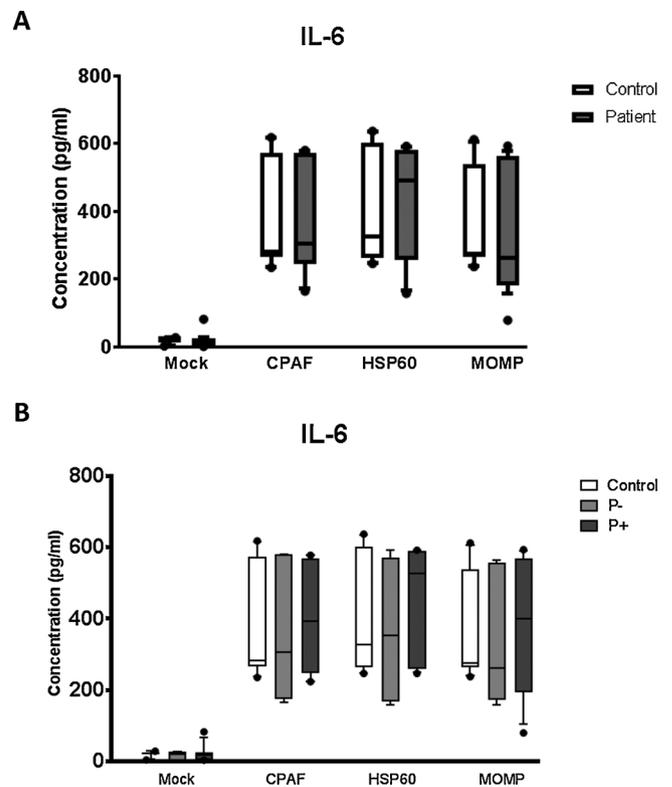


Fig. 3. Production of IL-6 from the chlamydial antigen-stimulated PBMCs. PBMCs from the healthy controls ( $n = 10$ ) and *C. trachomatis*-infected individuals ( $n = 19$ ) were unstimulated (mock) or stimulated with different chlamydial antigens (CPAF, HSP60 and MOMP) at a final concentration of 1  $\mu$ g/mL for 72 h. Cytokine concentration (pg/ml) secreted in the culture supernatants was measured by ELISA assay. Tukey whisker box plot shows (A) comparison between healthy control and *C. trachomatis*-infected groups; and (B) comparison of the control, plasmid-free (P-) and plasmid-bearing (P+) groups. Dots represent outliers.

the time of sample collection, as listed in Table 1.

### 3.5. Correlation analysis between the levels of cytokines

A significant correlation analysis has been noted between the cytokine levels (Fig. 4). After stimulation with CPAF, positive correlations were observed in the concentrations of IL-1 $\beta$  and IL-6 ( $R^2 = 0.626$ ), IL-1 $\beta$  and TNF- $\alpha$  ( $R^2 = 0.475$ ), or TNF- $\alpha$  and IL-6 ( $R^2 = 0.571$ ). Stimulation with MOMP also generated positive correlations between the concentrations of IL-1 $\beta$  and IL-6 ( $R^2 = 0.430$ ), IL-1 $\beta$  and TNF- $\alpha$  ( $R^2 = 0.548$ ), or TNF- $\alpha$  and IL-6 ( $R^2 = 0.458$ ). Whereas stimulation with HSP60 antigen generated a positive correlation between IL-1 $\beta$  and TNF- $\alpha$  ( $R^2 = 0.501$ ); however, lower degrees of correlation were noted in the concentrations of IL-1 $\beta$  and IL-6 ( $R^2 = 0.226$ ), or TNF- $\alpha$  and IL-6 ( $R^2 = 0.251$ ). The positive correlations suggest the existence of interaction network among three pro-inflammatory cytokines. This result is as predicted as these three cytokines work collaboratively to promote persistence inflammation during infection.

## 4. Discussion

The main objective of this study was to assess the host immune profile in the patient cohort upon antigenic challenge with chlamydial CPAF, HSP60, and MOMP antigens. In general, chlamydial antigens-stimulated PBMCs presented consistent increase of pro-inflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$  and IL-6). The present study is limited by the fact that only one concentration of each antigen was used during stimulation; a better comparison of the antigens' ability to trigger immune

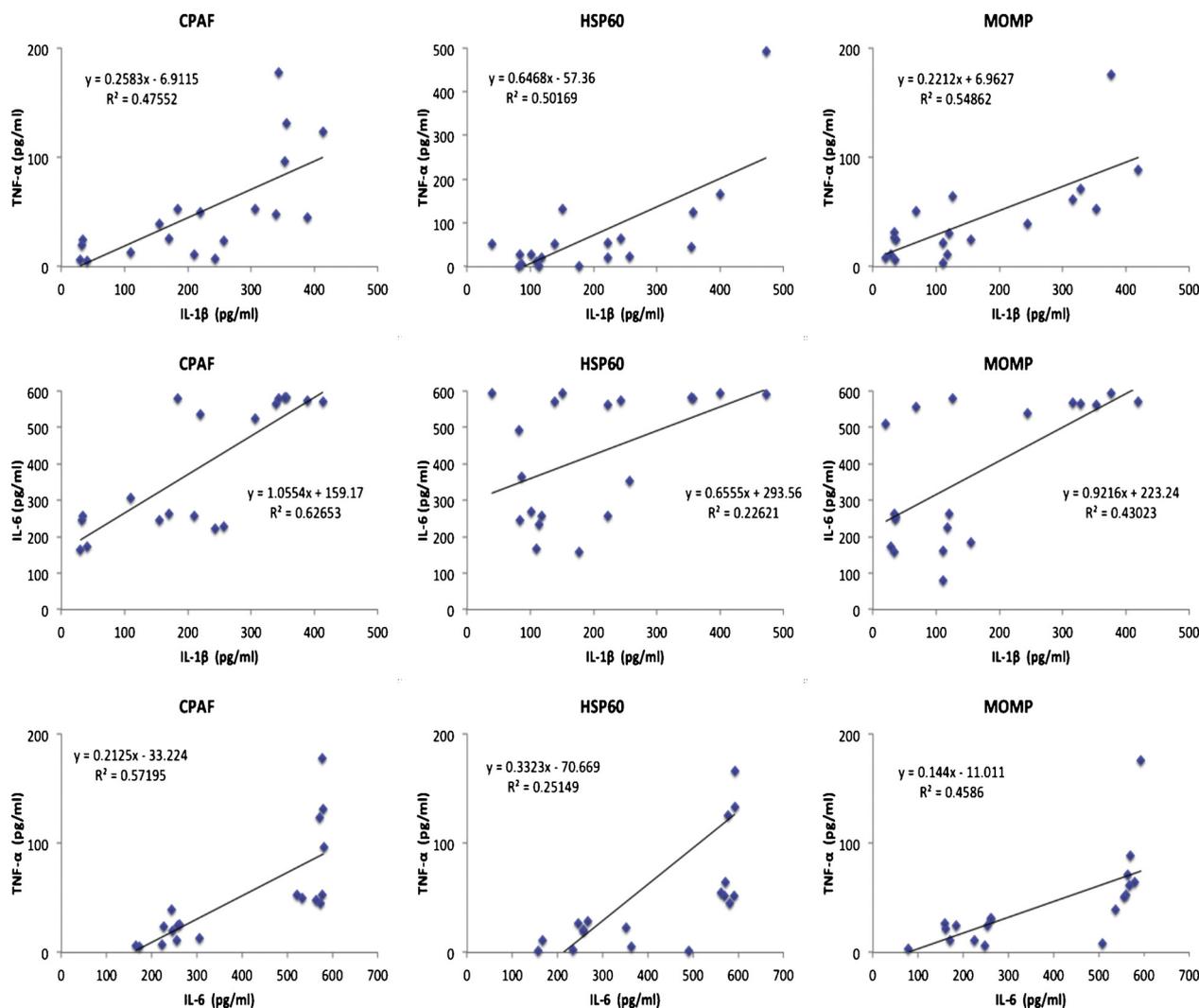


Fig. 4. Correlation of the proinflammatory cytokines secreted by chlamydial antigen-stimulated PBMCs. Correlation between each pair of cytokine concentration in the PBMCs from *C. trachomatis*-infected individuals ( $n = 19$ ) after stimulation with different chlamydial antigens (CPAF, HSP60 and MOMP) at a final concentration of 1  $\mu\text{g}/\text{mL}$  for 72 h. X- and y-axis represent the cytokine concentration (pg/ml).

response could be obtained if a series of different concentrations were included in the experimental design. TNF- $\alpha$  has been implicated in many types of inflammatory diseases, and its inhibitor has been widely used as a therapeutic agent for autoimmune diseases such as rheumatoid arthritis. It is primarily produced by activated macrophages to regulate immune response through inducing cytokine secretion and E-selectin on endothelium. As endogenous pyrogen, its release causes inflammation, fever and apoptotic cell death. In the *in vivo* study of *C. trachomatis* infection in mouse model, depletion of TNF- $\alpha$  through intravenous antibody injection leads to a higher bacteria burden and accelerated mortality (Williams et al., 1990), suggesting a protective role of TNF- $\alpha$  in host defense against *C. trachomatis* infection. In human experiment, HSP60 antigen stimulation has been shown to induce higher expression of TNF- $\alpha$ , along with IL-10 and IFN- $\gamma$  in the cells derived from infertile females with *C. trachomatis* infection (Srivastava et al., 2008). Here, we reported a similar response of TNF- $\alpha$  release not only to HSP60, but also CPAF and MOMP stimulations. Multivariate analysis study in human also suggests an association of TNF-308 A allele with increased risk of sequelae of chlamydial infection (Natividad et al., 2007), indicating the vital role of TNF- $\alpha$  in limiting the *C. trachomatis* infection-mediated pathogenesis.

Our study showed that CPAF and MOMP, but not HSP60 antigens were most potent in inducing IL-1 $\beta$ . In general, IL-1 $\beta$  shares certain

common biological properties with TNF- $\alpha$  and IL-6, for example their ability to enhance T or B lymphocyte proliferation and maturation. IL-1 $\beta$  is able to induce chemokine on endothelium, and production of prostaglandins that lead to reduced pain threshold. Previous studies have shown that mice deficient in IL-1 $\beta$  or IL-1 receptor (IL-1R) display a higher bacteria burden and delayed bacteria clearance when infected intravaginally with *C. muridarum* (Nagarajan et al., 2012; Prantner et al., 2009). In contrast, mice deficient in IL-1R antagonist (IL-1Ra) display a faster bacterial clearance rate (Nagarajan et al., 2012). Despite multiple evidences from animal studies, genotypic study in human showed no direct correlation as IL-1 $\beta$  and IL-1R antagonist gene polymorphism are not associated with higher risk for chlamydial infection-mediated clinical sequelae such as tubal pathology in a group of Caucasian females (Murillo et al., 2003). Our study using a cohort of *C. trachomatis*-infected patients from multiethnic background demonstrated IL-1 $\beta$  as one of the most prominently induced cytokines from the patients' PBMCs during exposure to chlamydial CPAF and MOMP antigens. Expression and maturation of IL-1 $\beta$  depends on NOD-like receptor (NLR) signaling which activates caspase 1 and apoptosis associated speck-like protein containing CARD domain (ASC) inflammasome, which are switched on upon *C. trachomatis* infection in human cervical epithelial cells as well as THP1 monocytes (Abdul-Sater et al., 2009, 2010). Hence, we anticipate that CPAF and MOMP antigens

likely recognized by NLR, which activates IL-1 $\beta$  expression and maturation. On the other hand, HSP60 antigen, which did not induce IL-1 $\beta$  response may activate the cells through a distinct receptor and signaling pathway.

Although stimulation by CPAF, HSP60 or MOMP antigens induced IL-6 secretion from PBMCs, no remarkable difference was observed between *C. trachomatis*-infected patients and healthy controls. It is not surprised to see a difference in IL-1 $\beta$  and IL-6 as their productions utilized different signaling pathways, although both were universally grouped as pro-inflammatory cytokines. Primary human endometrial and endocervical cells were shown to produce IL-6 in response to chlamydial antigens stimulation, which might act as a determinant of disease outcome (Cunningham et al., 2013). IL-6 can be induced by chlamydial stress response proteases (CtHtrA and CtTsp) and UV-inactivated, but not by live *C. trachomatis*, suggesting a potential pathway modulation by live bacteria. Besides, significant amounts of IL-6 were induced in the lung of mice infected with *C. trachomatis* especially during optimal early phase of infection as IL-6 knockout mice demonstrated elevated bacteria burden in lung tissue and increased mortality rate (Williams et al., 1998). Interestingly, IL-6 acts as a double-edged sword to limit the bacteria burden and exacerbate bacterial pathogenicity, as shown in the *C. muridarum*-mediated infection in IL-6 knockout mouse model.

Noteworthy, we observed an elevation of cytokines in the group infected with *C. trachomatis* plasmid-bearing strain, in comparison with the plasmid-free group. Despite its unknown biological significance, the conserved 7.5 kb *C. trachomatis* plasmid encodes 8 proteins (Pgp1-8) that were shown to evoke immune response in humans and animals (Donati et al., 2009; Comanducci et al., 1994; Li et al., 2008). A previous study showed that plasmid Pgp3 causes secretion of a number of proinflammatory cytokines, including TNF- $\alpha$ , IL-8, as well as IL-1 $\beta$  from human cell line THP-1 monocytes, presumably exerting its effect via activation of MAPK and (ERK)/MAPK cascades (Zhou et al., 2013). Importantly, plasmid-deficient *C. trachomatis* has been demonstrated to cause reduced pathology than its plasmid-bearing counterparts in animal studies, indicating that the plasmid is a virulence factor from *C. trachomatis* (Lei et al., 2014; O'Connell et al., 2007; Liu et al., 2014; Huang et al., 2015). Our result provides further evidence for the role of the plasmid in *C. trachomatis* pathogenesis. It is noteworthy that the percentages of plasmid-free samples are higher among the current cohort (7/19, 36%) compared to most reported studies that suggest a lower occurrence of the natural plasmid-free strains (Peterson et al., 1990). This could be due to the fact that our cohort was not limited to patients who showed severe gynecological symptoms and that we have also included those who visited the clinic for a check-up. Inclusion of females with no overt symptoms may increase the detection rate of the plasmidless strain as it is known to be clinically non- or less-virulent (Last et al., 2014). Besides, another possible explanation might be related to the lack of sensitivity of the PCR plasmid primers. The *C. trachomatis* plasmid has been shown to be a low-copy plasmid with an average copy number of  $4 \pm 0.8$  plasmids per chromosome (Pickett et al., 2005). Some strains of *C. trachomatis* with low copy numbers of plasmid may escape detection during RT-PCR amplification cycles, hence development and usage of a more sensitive detection method is required for the determination of plasmid-bearing strains in the clinics.

## 5. Conclusion

In summary, our study suggests the importance of inflammatory cytokines in the host immune response to chlamydial antigens. Our study also highlights the plasmid-bearing *C. trachomatis* in inducing higher level pro-inflammatory cytokines, in line with previous studies that indicate plasmid as a key virulence factor for *C. trachomatis* infection. Nonetheless, more research is warranted to obtain a more thorough understanding of the host immune responses following intracellular infection with *C. trachomatis*, which is key to induction of

optimal protective immune responses against genital chlamydial infections. As the current understanding of chlamydial plasmid is somehow limited in murine infection model, there is a pressing need for further study to characterize the role of plasmid positive serovars and its association with the pathogenesis of human infections.

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## Conflict of interest

The authors declared no financial interest or potential conflict of interest.

## Ethical approval

All procedures performed in our study with human participants were in accordance to regulation by the institutional medical ethics board.

## Informed consent

Informed consent was obtained from all individuals participated in this study.

## Declarations of interest

None

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