Evidence of the Anti-inflammatory Effect of Silver-Polyvinyl Pyrrolidone Nanoparticles (Ag-PVP) in *Chlamydia trachomatis* Infected Macrophages and HeLa Cells

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ABSTRACT

Chlamydia trachomatis infects macrophages and epithelial cells, which evoke TNF, IL-6, and IL-8 that are key protagonists of acute inflammation. These cytokines put the patient at risk for major health issues such as pelvic inflammation disease (PID) and infertility, if not controlled. Surface coated pure metal such as silver-polyvinyl pyrrolidone nanoparticle (Ag-PVP) has been studied extensively for its anti-inflammatory role. Here we explored the hypothesis that Ag-PVP will inhibit inflammatory cytokines that are produced during the early phase of a *C. trachomatis* infection. We used human epithelial cells and mouse J774 macrophages as target cells, and live *C. trachomatis* serovar L2 and its major outer membrane protein (MOMP) as stimulants. Ag-PVP added to 2 day *C. trachomatis*-infected cells down-regulated TNF, IL-6 and IL-8. When MOMP was used as the stimulant, Ag-PVP similarly down-regulated these cytokines. Results from the MTT cytotoxicity assay clearly show that the anti-inflammatory effect of Ag-PVP was not due to cell death. Our data imply that Ag-PVP maybe an important therapeutic agent to regulate inflammation during the early stage of a *C. trachomatis* infection.

Keywords: Bacteria, Silver nanoparticles, MOMP, cytokines, inflammation

1. INTRODUCTION

Chlamydia trachomatis is an obligate intracellular bacterial pathogen responsible for sexually transmitted infections worldwide [1]. During *Chlamydia* infection of epithelial cells and macrophages, IL-6, IL-8, and TNF [2] are secreted as a part of the innate immune response to mitigate the infection [3]. However, if produced excessively, these cytokines contribute to the disease manifestation by damaging nearby cells [2].

Surface coated silver nanoparticles have been found to exhibit different biological properties [4]. One of the main advantages of using nanoscale materials in life science research is that it can easily cross physiological barriers such as the blood-brain barrier and the tight epithelial junctions of mucosal surfaces [5]. However, one possible disadvantage of nanoscale materials is their potential for indiscriminate entry into various organs [4, 5]. In our previous study we have demonstrated that Ag-PVP of 4-8 nm could inhibit respiratory syncytial virus replication [4]. This prompted us to test the anti-inflammatory role of Ag-PVP in a *C. trachomatis* infection model. In this study, for the first time, we investigated the ability of Ag-PVP to down-regulate the expression of IL-6, IL-8 and TNF as induced by live *C. trachomatis* serovar L2 and its recombinant major outer membrane protein (MOMP) in human epithelial cells and mouse macrophages.

2. MATERIALS AND METHODS

2.1 Cell lines and culture

HeLa cells and mouse J774 macrophages were obtained from the American type culture collection (ATCC, Rockville, MD). HeLa cells were cultured in minimal essential medium (MEM/H) (Sigma, St Louis, MO, USA) supplemented with 10 % heat-inactivated fetal bovine serum (FBS) (Gibco Invitrogen, Carlsbad, CA), 2 mM L-glutamine (Invitrogen) and 1 µg/mL antibiotic and antimycotic (Invitrogen). J774 mouse macrophage cell line was cultured in Dulbecco Modified Eagle Medium (DMEM) (ATCC) supplemented with 10% heat-inactivated FBS, 1 µg/mL antibiotic and antimycotic (Invitrogen). All cells were maintained at 37°C in a humidified incubator containing 5% CO_2.

2.2 Infection of HeLa and J774 cells

Ag-PVP was synthesized following the method of Elechiguerra et. Al. [6] and found to be a monodispersed molecule with a size of 5-10 nm. HeLa or J774 (10^5 cells/well) cells were infected with

infectious particle of *C. trachomatis* serovar L2 (10^4 IFU/well) for 2 days in the presence of 1 µg/mL cycloheximide (Sigma). After day 2 infection of cells, infection media were replaced with media containing various concentrations of Ag-PVP. Cell-free-supernatants were collected after 48 hr by centrifugation at 450 × g/10 min at 4°C and stored at -80°C until used. In separate experiments, J774 and HeLa cells were stimulated for 24 hr with *C. trachomatis* rMOMP (5 µg/mL) in the presence or absence of various concentrations of Ag-PVP.

2.3 Cytokines

All reagents and antibodies for IL-6, IL-8 and TNF cytokine ELISAs were purchased from BD Biosciences (San Jose, CA). ELISAs were performed according to the manufacturer's suggested protocol.

2.4 Confocal

To support our ELISA result, the expression of IL-6 and TNF was detected by confocal microscopy. Mouse J774 cells (2.5×10^4 cells/well) were left uninfected or infected with *C. trachomatis* (2.5×10^3 IFU/well) and after a 2 day infection, the media were replaced with fresh media supplemented with or without Ag-PVP (2.5 g/mL). After 24 hr post-Ag-PVP incubation, the cells were fixed with 2% PFA and immunostained with anti-mouse TNF (FITC) and IL-6 (PE) antibodies (BD Biosciences). The slides were next stained with DAPI combined with anti-fade mounting solution (Invitrogen), and examined using a confocal microscope (Leica True Confocal Laser Scanning Microscope, Nikon Instrument, Melville, NY).

2.5 MTT cytotoxicity assay

HeLa and J774 cells (10^4 cells/well) were seeded in 96-well plates and after 24 hr of incubation; various concentrations of Ag-PVP were added to cells. Cells were incubated for an additional 24 hr after which they were treated with the MTT dye and then incubated for 4 hr at 37°C. The plates were removed from 37°C, stop solution added, covered and left in the dark for 3 hr at room temperature. Absorbance was measured at 570 nm using a microplate reader. Percent viability was calculated based on cells cultured in the absence of Ag-PVP.

2.6 Statistics analysis

All data are expressed as mean ± SD, and were analyzed using the two-tailed unpaired Student's *t*-test. $P < 0.05$ was considered significant.

3. RESULTS

3.1 Ag-PVP inhibits cytokines induced by *C. trachomatis* and rMOMP in HeLa cells

During *Chlamydia* invasion of epithelial cells IL-6 and IL-8 are expected to be secreted. Therefore, we measured their levels *in vitro* in *C. trachomatis* infected HeLa cells. Compared with uninfected cells infected HeLa cells produced significant levels of IL-6 and IL-8 (Fig. 1A). However, when Ag-PVP was added to infected cells the levels of cytokines were reduced in a dose-dependent manner. Significant ($P < 0.005$) reduction was shown at concentrations of 1.25 and 2.5 µg/mL (Fig. 1A), suggesting Ag-PVP inhibitory role on cytokines levels in infected cells. Similar results were obtained when rMOMP was used as the stimulant (Fig. 1B). Collectively our results clearly show an anti-inflammatory role for Ag-PVP in reducing IL-6 and IL-8 in *C. trachomatis*-infected epithelial cells.

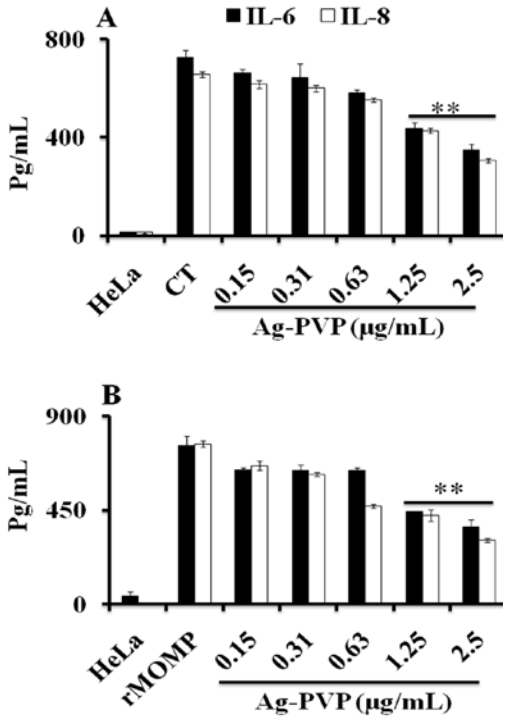

Fig. 1. Ag-PVP inhibits IL-6 and IL-8 production in a dose-dependent manner in HeLa cells

3.2 Ag-PVP inhibits cytokines induced by *C. trachomatis* and rMOMP in J774 cells

During invasion of *Chlamydia* there is a complex interaction between macrophages and the pathogen resulting in cytokines production [3]. Therefore, we measured the effect of Ag-PVP on accumulation of IL-6 and TNF in *C. trachomatis*-infected macrophage cultures. Our result shows significant ($P < 0.005$) accumulation of IL-6 and TNF (Fig. 2A) in infected J774 cell cultures as compared to that of uninfected cultures. When Ag-PVP was added, the production levels of IL-6 and TNF were diminished in a dose-dependent fashion with the highest reduction observed at concentrations of 1.25 and 2.5 µg/mL of Ag-PVP (Fig. 2A). Like *C. trachomatis*-infected cells, rMOMP-stimulated J774 cells (Fig. 2B) showed similar reduction profiles. Our result suggests that Ag-PVP greatly perturbed the interaction between macrophages and *C. trachomatis* by diminishing the production levels of IL-6 and TNF by J774 cells.

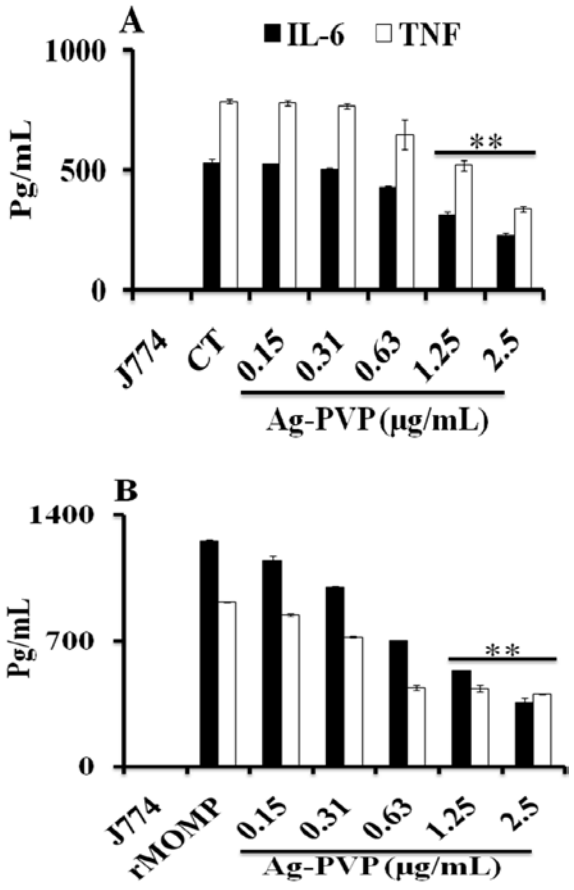

Fig. 2. Ag-PVP inhibits TNF and IL-6 production in a dose-dependent manner in J774 cells

3.3 Confocal microscopy analyses confirm the anti-inflammatory effect of Ag-PVP

To confirm our ELISA results, confocal microscopy was employed to assess the expression of TNF and IL-6 induced by *C. trachomatis* in J774 cells. TNF (green) and IL-6 (red) were expressed in infected cells as shown by florescence aggregating around the nucleus (Fig. 3A and 3B). However, infected cells exposed to Ag-PVP had lower patterns of IL-6 and TNF expression (Fig. 3A and B). Our confocal microscopy analyses are consistent with our ELISA observation. Together with the above results, we have clearly demonstrated that Ag-PVP is capable of reducing the levels of IL-6, IL-8 and TNF in response to *C. trachomatis* and its major outer membrane protein in HeLa and J774 cells.

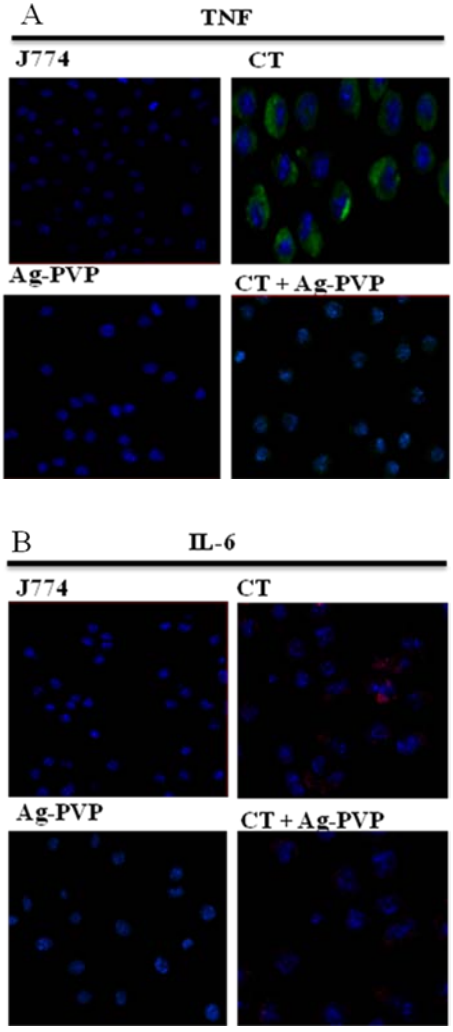

Fig. 3. Ag-PVP inhibits expression of TNF (blue) and IL-6 (red) in *C. trachomatis*-infected J774 cells (blue, nuclei stain)

3.4 The decreased level of TNF, IL-6 and IL-8 by Ag-PVP is not due to cell death

One of the disadvantages of using nanoparticles in life science research is their cytotoxicity effect. Therefore, we investigated whether or not the observed decreased inflammatory response rendered by Ag-PVP in infected HeLa and J774 cells is due to its cytotoxcicity effect. Here, we evaluated cell viability under the influence of Ag-PVP in the concentration range of 0.15 to 5.0 μg/mL for 24 hr. With the exception of 5 μg/mL, more than 90% of cells survived all Ag-PVP concentrations tested (Fig. 4), suggesting that concentrations ≤ 2.5 μg/mL are not toxic to cells. More specifically, it implies that the anti-inflammatory effect of Ag-PVP observed at 1.25 and 2.5 μg/mL is not due to increased cell death.

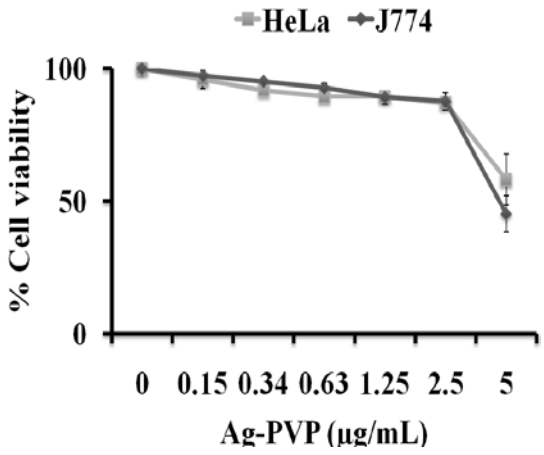

Fig. 4. Cytotoxicity effect of Ag-PVP on HeLa and J774 cells

4. DISCUSSION

Pro-inflammatory cytokines such as IL-8, IL-6 and TNF play important role in *Chlamydial* immunopathology [1-3]. However, overproduction of them promotes infiltration of immune cells that can release protease that damage other cells [2]. Furthermore, induction of these cytokines promotes other genital viral infections such as human immunodeficiency virus and human papilloma virus [3]. Thus, it is important to control excess production of these cytokines during a *C. trachomatis* infection.

Silver nanoparticles have been investigated as anti-inflammatory molecule in various infectious models [5]. For the first time, in this paper, we show by cytokine ELISA and confocal microscopy the ability of Ag-PVP to diminish the production of IL-6, IL-8, and TNF in an *in vitro C. trachomatis*-infection model. We clearly demonstrated by the MTT assay that the effect of Ag-PVP is not a result of cell death. Although we have not demonstrated it here, one plausible hypothesis for Ag-PVP mode of action is that it may interrupt the signaling pathway used by *C. trachomatis* to evoke the production of cytokines. Current studies are ongoing in our laboratory to investigate this hypothesis. Our data imply that Ag-PVP maybe an important therapeutic agent to regulate inflammation during an early *C. trachomatis* infection.

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