Poly-Vinyl Pyrrolidone (PVP)-coated Silver (Ag) Nanoparticles (Ag-PVP): a Potential to Regulate *Chlamydia trachomatis* Inflammation

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ABSTRACT

Chlamydia trachomatis is an important cause of sexually transmitted infection worldwide. Excessive inflammatory responses play a key role in its disease progression. Early intervention strategies that can reduce inflammatory responses could benefit control efforts in reducing the disease severity. Previously we reported that Ag-PVP reduced pro-inflammatory cytokines produced by *C. trachomatis*-infected mouse J774 macrophages. Here we reveal that Ag-PVP also exerts its anti-inflammatory effect on primary mouse bone marrow-derived macrophages (BMDM). The MTT assay showed that Ag-PVP was relatively non-toxic to BMDM (up to 6.25 µg/mL) over 72 hr. By ELISA we document that Ag-PVP (2.5 µg/mL) inhibited IL-6 (cytokine) and CXCL10 (chemokine) as produced by *C. trachomatis*-infected BMDM. Employing qRT-PCR we demonstrate that Ag-PVP down-regulated the expression of the surface receptors, TLR2 and NOD2, as triggered by *C. trachomatis* on J774 macrophages, suggesting that Ag-PVP may exert its anti-inflammatory effect by interfering with their expression. Our results show the anti-inflammatory effect of Ag-PVP on BMDM is not associated with cell death. Of importance our data demonstrates that Ag-PVP may mediate its anti-inflammatory effect in infected macrophages by reducing macrophage surface receptors expression.

Key words: *Chlamydia trachomatis*, silver nanoparticles, Toll like receptor, NOD like receptor

1. INTRODUCTION

Sexually transmitted *C. trachomatis* infection is of widespread public health concern because of its prevalence and potentially devastating reproduction consequences, including pelvic inflammatory disease (PID), infertility, and ectopic pregnancy [1]. Like other infectious organisms, *Chlamydia* infection of macrophages allows the secretion of various small proteins such as cytokines and chemkines [1]. Toll like receptor (TLR) and NOD like receptors play a key role for the secretion of these proteins [2]. Both receptors are expressed on the extracellular compartment of host cells to recognize microbial products. Microbial recognition by TLR or NOD permits activation of several adaptor molecules and inflammatory signaling pathways. The activation of one or more of these signaling pathways leads to the activation of the transcription factor for the secretion of cytokines and chemokines. The secretion of cytokines and chemokines enhances the clearance of infection. However, for some diseases cytokines and chemokines are produced massively and contribute to the disease manifestations. *C. trachomatis* is one of these diseases, where it up-regulates the production of cytokines and chemokines and avoids clearance. Various studies have shown that *C. trachomatis* persists for a longer period of time by inducing excessive production of cytokines and chemokines [3]. Therefore, it is important to search for potential anti-inflammatory molecules that may limit the production levels of cytokine and chemokines that contribute to the pathogenesis of disease.

Poly-vinyl pyrrolidone coated silver (Ag-PVP) nanoparticles have been found to exhibit various biological properties [4]. Previously Ag-PVP was shown to reduce the production levels of pro-inflammatory cytokines during viral and bacterial infections [4, 5]. Our laboratory has recently focused on understanding the anti-inflammatory effects and mode of action of different sizes of Ag-PCP in controlling host innate immune inflammatory responses during a *C. trachomatis* infection using mouse J774 macrophages [6, 7]. Here we further demonstrated the anti-inflammatory effect of Ag-PVP (20 nm) in primary cells by employing mouse bone marrow-derived macrophages (BMDM) as target cells and live infectious particles of *C. trachomatis* as stimulant. We tested the toxic effects of Ag-PVP against BMDM over 72 hr. We also determined Ag-PVP ability to reduce the production level of major cytokines and chemokines that are induced by *C. trachomatis*-infected BMDM. Finally we demonstrated the role of Ag-PVP in the mRNA gene transcripts of TLR2 and NOD2. Here we

present our data and discuss the role of Ag-PVP as regulators of the inflammatory response during a *C. trachomatis* infection.

2. MATERIALS AND METHODS

2.1 Cell lines and culture

Mouse J774 macrophages were obtained from the American type culture collection (ATCC, Rockville, MD) and 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% CO2. Bone marrow derived macrophages (BMDM) were isolated from bone marrow cells of female BAL/c mice (Charles River Laboratories). We used macrophage-colony stimulating factor (M-CSF) to differentiate the cells into macrophages following a method of Gautam et al [8]. The efficiency of the differentiation was assessed using fluorescence-activated cells sorting (FACS) analysis of mouse F4/80 macrophage surface antigen expression.

2.2 Preparation of Ag-PVP nanoparticles

Ag-PVP at 20 nm was purchased from Nanostructured & Amorphous Materials, Inc. (Houston, TX, USA) and stock solution of 1 mg/mL was prepared by dissolving 1 mg of Ag-PVP with 1 mL of PBS, and stored at 4°C until used. From the stock solution desired concentrations of Ag-PVP were prepared for all assay applications using culture media. Ag-PVP solutions were subjected to water bath sonication, before being used, to prevent particle aggregations.

2.3 Cell cytotoxicity assay

After seven days of differentiation, BMDM (10^5 cells/well) were plated in 96-well plates and after 24 hr incubations, various concentrations of Ag-PVP were added to the cells. The cells were incubated for 24, 48 and 72 hr and after each time-point 3-(4,5-dmethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromine (MTT) (15 µL) reagent was added to each well. The plates were then covered and left at 37ºC in a humidified incubator containing 5% CO_2 for 3 hr. Solubilization stop/mix solution (100 µL) was added to each well and incubated for an additional 1 h in the dark prior to the absorbance measurements. The absorbance was measured at 570 nm using a TECAN Sunrise ELISA plate reader (TECAN US Inc., Durham, North Carolina). Percent cell viability was calculated by subtracting the optical density reading compared to normal cells (control).

2.4 Infection of BMDM

C. trachomatis MoPn Nigg II was purchased from ATCC (ATCC #VR-123) and propagated in HeLa cells monolayers in MEM/H supplemented with 10% FBS. To determine the effect of Ag-PVP on the production levels of IL-6 and CXCL10, BMDM (10^5 cells/well) were infected with infectious particle of *C. trachomatis* (10^4 IFU/well) for two days and then infection media were replaced with media containing Ag-PVP (2.5 µg/mL). Cell-free-supernatants were collected after 24 h following centrifugation at 450 x g for 10 min at 4 ºC and stored at -80ºC until used. These time-points and MOI were found to be the optimum conditions based on our previous studies.

2.5 Cytokines

Cytokine enzyme-linked immunosorbent assays (ELISAs) were used to quantify the concentration of mouse IL-6 and CXCL10 in cell-free supernatant using OPti-EIA kits according to the manufacturer suggested protocol (BD Biosciences).

2.6 RNA extraction and quantitative real time–PCR (qRT-PCR)

Mouse J774 macrophage (3×10^6 cells/well) were infected with live *C. trachomatis* (3×10^5 IFU/well) for 48 h followed by replacement of fresh media containing Ag-PVP (2.5 µg/mL). The total RNA was extracted from the cells pellets using Qiagen RNeasy kit (Qiagen Inc, Valencia, CA) following the manufacture's protocol. qRT-PCR was employed to quantify mRNA gene transcript of TLR2 and NOD2 using TaqMan® RNA-to-C_T™ kit in combination with TaqMan® gene expression assays (Applied Biosystems by Life Technology, Foster city, CA) as reported [8]. Amplification of gene transcripts was performed according to the manufacture's protocol using ABI ViiA™ 7 real-time PCR (Applied Biosystem by Life Technologies) and standard amplification conditions.

2.7 Statistics analysis

All the data are expressed as mean ± SD and data were analyzed by using the two-tailed unpaired

Student's *t*-test and $P < 0.05$ was considered significant.

3 RESULT

3.1 Ag-PVP exhibited less toxicity to bone marrow derived macrophages

Despite the many benefits of nanotechnology certain nanoparticles may cause adverse effects because of their small size and unique properties [5]. Therefore, we determined the toxic effect of Ag-PVP to BMDM. More than 80 % of cells survived at Ag-PVP concentrations ranging from 0.39-3.12 (Figure 1), suggesting that these concentrations are not toxic to BMDM. More specifically it implies that any ensuing anti-inflammatory effect observed at concentrations of 3.12 µg/mL and less of Ag-PVP will not be attributed to increased cell death. Our results also indicate that higher concentration of Ag-PVP is toxic to the BMDM, where the cell viability decreased gradually as we increased the Ag-PVP concentrations (Figure 1).

3.2 Ag-PVP reduced the production level of CXCL10 and IL-6 in *C. trachomatis* infected BMDM

During host pathogen interaction the host innate immune response is the initial responder to clear the pathogen and as a result various cytokines and chemokines are secreted [1-3]. Herein, we investigated whether Ag-PVP would exert its anti-inflammatory effect on primary macrophages in addition to cell lines as we previously reported [6, 7].

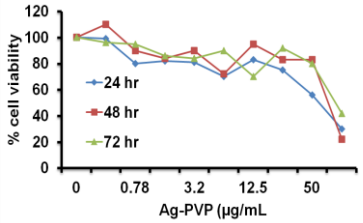

Figure 1: Ag-PVP toxicity profile to BMDM. Percent cell viability was determined after exposing BMDM to various concentrations of Ag-PVP up to 72 hr. Shown are the averages of triplicates values. The experiment was repeated twice.

In comparison to uninfected BMDM, infected BMDM exhibited significant increase in the levels of CXCL10 (chemokine) and IL-6 (cytokine). However, when Ag-PVP was added the levels of CXCL10 and IL-6 were significantly ($P < 0.05$) reduced (Figure 2A and B), corroborating our previous results using mouse J774 macrophages [6, 7]. The data suggests that Ag-PVP is important in regulating IL-6 and CXCL10 in *C. trachomatis*-infected BMDM.

3.3 Ag-PVP decreases the expression of TLR2 and NOD2 on *C. trachomatis* (CT)-infected mouse J774 macrophages.

For most eukaryotic cells, activation of TLR and NOD like receptors triggers the production of inflammatory mediators, which are essential for the clearance of infectious agents [1-2]. Thus, finally we asked whether Ag-PVP is effective in inhibiting the expression level of TLR2 and NOD2. Our qRT-PCR analysis revealed that *C. trachomatis* induced the expression of TLR2 and NOD2 in mouse J774 macrophages, however in the presence of Ag-PVP (2.5 µg/mL) the expression level reduced significantly, suggesting Ag-PVP role in down-regulating TLR2 and NOD2 during host pathogen interaction (Figure 3).

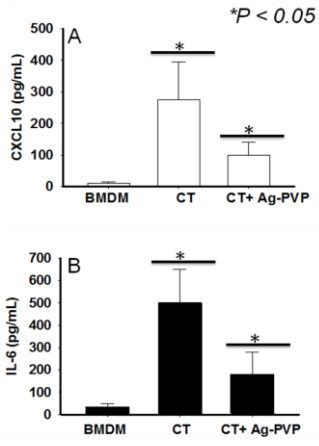

Figure 2: Anti-inflammatory effect of Ag-PVP in *C. trachomatis* infected BMDM. CXCL10 and IL-6 levels were measured by ELISA using specific capture antibodies. * $P < 0.05$ is considered as significant relative to uninfected BMDM and BMDM

exposed to Ag-PVP. The experiment was repeated twice.

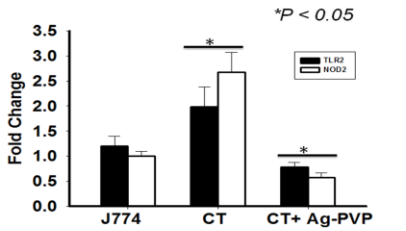

Figure 3: Ag-PVP down-regulates the expression of TLR2 and NOD2. qRT PCR was employed to quantify mRNA gene transcripts of TLR2 and NOD2. Relative fold changes in gene expression were calculated using the equation: $2^{-\Delta\Delta CT}$ where all values were normalized with respect to the housekeeping gene GAPDH mRNA levels. * $P < 0.05$ is considered significant as determined by the two-tailed unpaired student's test.

4 DISCUSSION

Inflammatory mediators released by *C. trachomatis*-infected macrophages initiate inflammation [3]. The inflammation of an infected tissue has several beneficial effects in combating infection. It recruits cells and molecules of innate immunity out of the blood and into the tissues where they are needed to destroy the pathogen directly. In addition, it increases the flow of lymph bearing microbes and antigen-bearing cells nearby lymphoid tissues, where they will activate lymphocyte and initiate adaptive immune responses. However, over production of inflammatory mediators promote infiltration of immune cells that can release protease that damage other cells [1, 3]. Thus, it is important to control excess inflammatory response during *C. trachomatis* infection.

Silver nanoparticles have been investigated as an anti-inflammatory molecule in various infectious models [4, 5]. Previously, we have shown the ability of Ag-PVP to reduce the production levels of IL-6, IL-8, and TNF in an *in vitro C. trachomatis*-infection of mouse J774 macrophages cells line [6, 7]. Now in this study we extend our studies by testing the ability of Ag-PVP on bone marrow derived macrophages. Our MTT assay result indicates less toxicity of Ag-PVP to BMDM when it is used in low dosages (Figure 1). This is an interesting finding because at low concentrations Ag-PV exerts its anti-inflammatory effect very effectively (Figure 2). The inflammatory process is a complex phenomenon induced by different pathways and therefore many hypothetical possibilities can be explored to explain Ag-PVP anti-inflammatory mode of action. In this study we have shown the ability of Ag-PVP (2.5 µg/mL) to reduce the expression of TLR2 and NOD2 in *C. trachomatis*-infected J774 macrophages, suggesting that Ag-PVP reduce the ability of TLR2 and NOD2 to recognize *C. trachomatis.* Although we have not demonstrated here, but unsuccessful recognition of *C. trachomatis* with TLR or NOD may interrupt the downstream inflammatory signaling pathway, which is used by *C. trachomatis* to evoke the production of cytokines and chemokines. Overall, our *ex-vivo* data imply that Ag-PVP is an important therapeutic agent to regulate inflammation during an early *C. trachomatis* infection and further invites direct *in vivo* studies using Ag-PVP for its potential application in medicine.

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