Factors secreted by THP-1 macrophage-like cells pulsed with *Vibrio cholerae* ghost (VCG) prevent infection by *Chlamydia pneumoniae* (MoPn)

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Citation  

Abstract  
Infections with the obligatory pathogen *Chlamydia trachomatis* (CT) are frequently oligosymptomatic or asymptomatic. Immune-evasion mechanisms of CT are primarily why the infection remains subclinical in a high proportion of individuals – 70-90% of women and 30-50% of men. For the better part of the last two decades, Eko et al. have been studying the use of bacterial ghosts as an effective delivery vehicle for vaccination against *Chlamydia* and for the induction of increased protective immunity. In the present study we investigated the hypothesis that factors contained in [*Vibrio cholerae* ghosts-pulsed] THP-1 macrophage conditioned media prevent *Chlamydia* infection forming units (IFUs) development. When treated with phorbol esters such as phorbol myristate acetate (PMA), THP-1 monocytes differentiate into macrophages and acquire dendritic cell properties upon stimulation by immunostimulatory cytokines. The supernatant from VCG-pulsed-THP-1 macrophages (MΦ) was used to culture *Chlamydia pneumoniae* (MoPn) infected HeLa cells at a multiplicity of infection (MOI) of 5. IFUs were quantified using immunofluorescence microscopy after monolayers were stained with the Pathfinder® *Chlamydia* Culture Confirmation System (Bio-Rad Labs). HeLa cells were cultured in MoPn-conditioned media or in MoPn-Earle’s minimum essential media (EMEM), the latter serving as our positive control. HeLa cells cultured in MoPn-conditioned media were infected only 5.87% vs. 94.1% infection of HeLa cells cultured in MoPn-EMEM. These results show strong evidence that the immunostimulatory factors induced by VCG confer protective immunity against the development cycle of *Chlamydia*.

1. Introduction  
*Chlamydia trachomatis* (CT) is an obligatory intracellular pathogen that is responsible for infections of the urogenital epithelium (1). Infections with the obligatory pathogen
are frequently oligosymptomatic or asymptomatic (2). CT pathological manifestations continue to be a significant healthcare and economic burden for developed and developing nations, affecting over 90 million people (3) worldwide. There are 15 serotypes of C. trachomatis with serovars D-K being responsible for infections of the urogenital tract and serotypes D-F accounting for >60% of these type of Chlamydia infections worldwide.

Primary infection of the urogenital epithelium usually manifests as urethritis in men or cervicitis in women (4). Chronic infection, associated with persistence of the bacteria in host cells, recurrent infection, or re-infection are much more dangerous (5) and may result from lack of treatment or improper therapeutic management. Treatment costs in the United States using antimicrobial agents have been between $2 – 4 million per year in the last decade (6). Antibiotic therapy can severely impede the body’s ability to fight the infection naturally (7), not to mention the morbidities that result from the side effects antibiotics deliver. Because of the Chlamydia pathogen’s sophisticated immune-evasion mechanisms, the infection remains subclinical in a high proportion of infected individuals. Many efforts are being made through studies to understand the immunobiology of Chlamydia spp., which is essential to developing a vaccine against the infection that will induce protective immunity (3). Bacterial ghost offer a cost effective vehicle for delivering antigen with the added caveat of intrinsic adjuvanticity. Bacterial ghosts elicit a humoral immune response with a significant specific IgG antibody titre (8) followed by an influx of plasma cells and lymphocytes associated with protection against further challenge (9). With this and other strong precedents in mind we hypothesized that factors secreted by VCG-pulsed-THP-1 macrophages prevents the development of Chlamydia infection forming units.

The bacterial ghosts system has been introduced as an efficient delivery system of Chlamydia antigen in addition to its adjuvanticity (9, 10, 11, 12). The intrinsic adjuvant properties of the bacterial ghosts system mediate an innate immune response by eliciting the secretion of proinflammatory cytokines. Since lipopolysaccharides (LPS) and peptidoglycans are known adjuvants and bacterial uptake by macrophages is highly effective, immunization with low doses of bacterial ghosts will stimulate the immune system very efficiently, as seen in a protective mucosal response by aerosol application of ghosts in pigs, vibriocidal antibodies in mice, and humoral and cellular immunity to membrane anchored target proteins in rabbits and mice (13). In the present study HeLa cells were infected with Chlamydia pneumoniae (MoPn) elementary bodies suspended in supernatant (conditioned medium) from THP-1 macrophages that had been pulsed with Vibrio cholerae ghosts. Factors contained in this conditioned medium were inhibitory to Chlamydia IFUs and significantly reduced infection by the pathogen. HeLa cells cultured in MoPn-conditioned medium developed IFUs at a rate of 5.87% vs. 94.1% IFU development in HeLa cells cultured in MoPn-Earle’s minimum essential medium. THP-1 cells are a cell line derived from human acute monocytic leukemia cells and have been established as a useful tool for studying the role of monocytes in human immune response and their phagocytosis capacity (7). The two morphologies of THP-1 cells, monocytes and macrophages, have 21 cell surface markers in common; 23 markers are uniquely expressed on THP-1 monocytes; 20 markers are uniquely expressed on THP-1 macrophages (14).

2. Materials and Methods

Production of Vibrio cholerae ghosts

Production of Vibrio cholerae ghosts was carried out by gene E-mediated lysis as described previously (7, 15). Lyophilized VCGs preparations were stored at room temperature until use.

Chlamydia stock

Chlamydia pneumoniae (MoPn) elementary bodies (EBs) (original stocks generously donated by Dr. Joseph Igiezeme, (Centers for Disease Control and Prevention, Atlanta, GA) were recovered from -80°C storage. Infection forming units (IFUs) was provided on the package.

Cell culture

The human monocytic leukemia cell line THP-1 was recovered from liquid nitrogen stored laboratory stocks, thawed and cultured in medium consisting of RPMI 1640 (Atlanta Biologicals, Lawrenceville, GA), supplemented with 200 mM glutamine (Gibco – Life Technologies, Grand Island, NY), 10% fetal bovine serum (FBS - Atlanta Biologicals, Lawrenceville, GA), 100 mM sodium pyruvate (Gibco – Life technologies, Grand Island, NY), 100 U/mL penicillin and 100 ug/mL streptomycin (Atlanta Biologicals, Lawrenceville, GA), and 0.5% HEPES (Atlanta Biologicals, Lawrenceville, GA) in a humidified incubator at 37°C, 5% CO₂. Cells were passaged every 72 h, maintaining a culture concentration of <2 x 10⁸ cells/mL. HeLa cells were recovered from liquid nitrogen laboratory stocks and thawed in a 37°C water bath followed by careful handling under strict aesthetic conditions in a germ-free culture hood. Thawed cells were transferred to a 15 mL centrifuge tube with Minimum Essential Media (MEM) Earle’s complete (Hyclone, Thermo Scientific) with 1% L-glutamine, 1% Pen/Strep (100 U/mL; 100 ug/mL), 0.1% fungizone, 10% heat-inactivated FBS, 1% HEPES, 1% nonessential amino acids, and 0.1% gentamycin. To remove freezing preservatives and collect cellular pellet, culture was centrifuged for 5 min at 1000 rpm and 4°C. The supernatant was discarded and the pellet was resuspended in MEM Earle’s complete media and transferred to a 24-well plate with coverslips at a volume of 1 mL/well (1.87 x 10⁶ cells/mL) and set in a humidified incubator under 37°C, 5% CO₂ for the monolayer to adhere - ~48 h.

Conditioned media – VCG-pulsed-THP-1 macrophages

THP-1 monocytes were treated with PMA (160 nM) for 1 h to initiate differentiation into monocyte-derived
macrophages (MdM). After 1 h, PMA was removed by centrifugation at 1500 rpm for 10 min at room temperature. The activated pellet was suspended in PMA-free media and placed in a 75 cm² culture flask then set in a humidified incubator under 37°C, 5% CO₂ for the monolayer to adhere -72 h. After 72 h, the MdM monolayer, now adhered, was given fresh media and pulsed with VCG (10 µg/mL) for 4 – 6h. After 6 h, the conditioned media was collected by pipet and stored in a 15 mL centrifuge tube and kept in a humidified incubator under 37°C, 5% CO₂ until use.

Treatment of HeLa monolayers with MoPn-conditioned media

MoPn elementary bodies were thawed at room temperature then suspended in warm (37°C) conditioned media or Earle’s MEM. Earle’s complete media used to culture HeLa cell monolayer was carefully removed by aspiration then MoPn-conditioned media or MoPn-EMEM was added to designated wells at a volume of 200 ul. (MOI 5). The MoPn-EMEM culture served as the positive control. HeLa cell monolayers were also cultured in MoPn-free-conditioned media or EMEM alone serving as negative controls. Plate was centrifuged at 2000 rpm for 1 h at room temperature then set in a humidified incubator under 37°C, 5% CO₂ for 1 h. After 1 h all treatments were carefully removed by aspiration and 1 mL warm cycloheximide media (1:1000) was added. Plate was returned to the humidified incubator under 37°C, 5% CO₂ for 24 – 48 h. Cycloheximide media was carefully removed by aspiration and 1 mL ethanol (100%) was added to each well for 10 min to fix the cells. After 10 min the ethanol was removed by aspiration then in a dark, germ-free hood Pathfinder® Chlamydia Culture Confirmation System (Bio-Rad Laboratories, Redmond, WA) monoclonal antibody was added to cover the monolayer and left in an unlit area to incubate at room temperature for 30 mins. After 30 min the mAb was removed by aspiration and 1 mL PBS was added to each well. The coverslips were removed and placed cell side down onto one drop of Permount® (Fisher Scientific, Fair Lawn, NJ) on a microscope slide and viewed under fluorescent microscopy.

Statistical Analysis

The number of inclusion forming units enumerated following HeLa cell infectivity with MoPn was compared by Student’s t test. The level of significance was judged at p<0.001.

3. Results

Factors contained in conditioned media prevent infection forming unit (IFU) development

To determine if factors secreted into VCG-pulsed-THP-1 MΦ supernatant prevent the development of Chlamydia infectious forming units, HeLa cell monolayers were cultured with MoPn-conditioned media at an MOI of 5. Following the Pathfinder® Chlamydia Culture Confirmation System (Bio-Rad Laboratories, Redmond, WA) protocol, infected HeLa cells were viewed under fluorescent microscopy at 40X magnification (Fig. 1) using Axiovision software 4.8.2 with a Zeiss Axio Imager.z1 fluorescence microscope. HeLa cells cultured in MoPn-EMEM developed 16,486 IFUs, p<0.001 (Fig. 1b). Remarkably, HeLa cells cultured in MoPn-conditioned media developed only 968 IFUs (Fig. 1a), leading to the conclusion that factors secreted into VCG-pulsed-THP-1 MΦ supernatant are inhibitory to Chlamydia inclusion forming units and significantly reduce infection.

Table 1. Cytokines and chemokines determined by Multiplex assay.

<table>
<thead>
<tr>
<th>Cytokine/Chemokine</th>
<th>Level (pg/mL)</th>
<th>Level (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>351.4±0.53</td>
<td>1917.13±79.5</td>
</tr>
<tr>
<td>IL-6</td>
<td>85.26±2.64</td>
<td>125.41±11.83</td>
</tr>
<tr>
<td>IL-8*</td>
<td>66529.2±3211.85</td>
<td>22.68±4.07</td>
</tr>
<tr>
<td>IL-17</td>
<td>22.68±4.07</td>
<td>66529.2±3211.85</td>
</tr>
<tr>
<td>IL-23</td>
<td>125.4±11.83</td>
<td>351.4±0.53</td>
</tr>
<tr>
<td>TNFα</td>
<td>1917.13±79.5</td>
<td>1917.13±79.5</td>
</tr>
</tbody>
</table>

VCG elicit secretion of IL-1β, IL-6, IL-8, IL-17, IL-23, and TNFα by human THP-1 macrophages. THP-1 MΦ were pulsed with VCG for 24 h and the supernatant was assayed for cytokine concentration.

Figure 1. Factors in conditioned media prevent Chlamydia inclusion forming units (IFUs). HeLa cells were cultured in Chlamydia MoPn-conditioned media (supernatant of VCG-pulsed-THP-1 macrophages) or MoPn-Earle’s MEM (positive control) and following the Pathfinder® Chlamydia Culture Confirmation System.

Figure 2. Enumeration of Chlamydia inclusion forming units. HeLa cell monolayers were infected with Chlamydia pneumonia (MOI 5) and cultured in Earle’s MEM (EMEM) or conditioned media (CM) of VCG-pulsed THP-1 macrophages. IFUs in HeLa cells cultured in EMEM were 16,486 IFUs vs. cells cultured in CM 968 IFUs. The level of significance was statistically judged high at p<0.001.

Chlamydia inclusion forming unit development was significantly decreased in HeLa cells cultured in MoPn-conditioned media which contained soluble factors with
antichlamydial activity (968±12.3 IFUs) vs. HeLa cells cultured in MoPn-EMEM (16, 486±338.0 IFUs).

Table 2. Comparison of structure and property similarity between V. cholerae and C. trachomatis

<table>
<thead>
<tr>
<th>Property</th>
<th>C. trachomatis</th>
<th>V. cholerae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of serotypes</td>
<td>18</td>
<td>4</td>
</tr>
<tr>
<td>Size</td>
<td>0.20 – 1.3µm</td>
<td>1-3 x 0.5-0.8µm</td>
</tr>
<tr>
<td>Morphology</td>
<td>rod-shaped</td>
<td>comma-shaped</td>
</tr>
<tr>
<td>Natural host</td>
<td>human</td>
<td>human, water,birds, shellfish, fish and herbivores</td>
</tr>
<tr>
<td>Infection route</td>
<td>contact;</td>
<td>contact;</td>
</tr>
<tr>
<td>Lipopolysaccharides</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Antibiotic sensitivity</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Survival outside of host</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

4. Discussion

C. trachomatis is an obligate intracellular pathogen with sophisticated immune-evasion mechanisms. This property is primarily why the infection remains subclinical in a high proportion of infected individuals (3). Of the vast amount of research that has gone into studying the immunobiology of infections with Chlamydia spp., most conclude that an efficacious chlamydial vaccine (14) would be the best approach for effective control of infection and significantly reduce the healthcare burden.

In a previous study conducted by our lab, we evaluated the factors secreted by THP-1 macrophages when pulsed by VCG (Table 1). These factors are cytokines and chemokines that were identified by Multiplex assay. IL-1β is a member of the IL-1 gene family of cytokines. It is produced by activated macrophages and is an important mediator of the inflammatory response (16). IL-1β is up-regulated in THP-1 MΦ versus THP-1 Mn. IL-6 functions in inflammation and the maturation of B cells. IL-6 is primarily produced at sites of acute and chronic inflammation, where it is secreted into the serum and induces a transcriptional inflammatory response (17). IL-8 belongs to a family of small, structurally related cytokines similar to platelet factor 4 (an antigen in heparin-induced thrombocytopenia). It is produced by phagocytes and mesenchymal cells exposed to inflammatory stimuli (e.g., interleukin-1 or tumor necrosis factor) and activated neutrophils inducing chemotaxis, exocytosis and the respiratory burst. IL-8 is produced in several tissues upon infection and inflammation and is thought to be the main cause of neutrophil accumulation (18). IL-6 and IL-8 are up-regulated in THP-1 MΦ versus THP-1 Mn. IL-17 is expressed by T helper type 17 cells and other lymphocytes. IL-17 can also be produced by several other innate immune cell types, one of these being macrophages. IL-17 plays key regulatory roles in host defense and inflammatory diseases. IL-17 derived from innate and adaptive sources may fight against pathogen invasion at different phases and locations of infection, which may add further complexity and a safeguard to the defensive immune response (19). IL-17 is up-regulated in THP-1 MΦ versus THP-1 Mn. IL-23 is a member of the IL-12 family of cytokines with pro-inflammatory properties. Its ability to potentely enhance the expansion of T helper type 17 (Th17) cells indicates the responsibility for many of the inflammatory autoimmune responses. IL-23 is a key participant in central regulation of the cellular mechanisms involved in inflammation. Both IL-23 and IL-17 form a new axis through Th17 cells, which has evolved in response to human diseases associated with immunooactivation and immunopathogeny, including bacterial or viral infections and chronic inflammation (20). IL-23 is down regulated in THP-1 MΦ versus THP-1 Mn. TNFα is an acute phase protein which initiates a cascade of cytokines and increases vascular permeability, thereby recruiting macrophage and neutrophils to a site of infection. TNFα secreted by the macrophage causes blood clotting which serves to contain the infection. TNFα induces neutrophil proliferation during inflammation and functions in the immune response to bacterial, and certain fungal, viral, and parasitic invasions as well as its role in the necrosis of specific tumors (21). Regulation of TNFα in both THP-1 MΦ and THP-1 Mn is comparable.

Chlamydia trachomatis and Vibrio cholerae are both Gram-negative bacteria and have similar envelop structure (Table 2). In a recent study, Stevens et al. rationalized that the target epithelial cells have Toll-like receptors that can be stimulated by VCG bacterial vector. Furthermore, this epithelial cell stimulation leads to an induction of a VCG-mediated immune response with a cytokine profile similar to Chlamydia immune response (7). We predict the mechanism of action by these macrophage-derived cytokines to prevent IFUs is very much like that which was observed by Dessus-Babus et al. (2002) and Shirey et al. (2006). Although a Th1 response appears to be crucial for the resolution of chlamydial infection (22, 23), the role of TNFα (24) is still unclear. In a recent study, Dessus-Babus et al. (24) showed that chlamydial infection may be the result of soluble factors with antichlamydial activity. This conclusion was made after they cocultivated PMA dTHP-1 cells (seeded in plastic wells below the inserts) with freshly inoculated polarized HeLa cells, and chlamydial growth was evaluated 48 hpi by a direct count of inclusions. The presence of dTHP-1 cells consistently induced a strong reduction in the number of serovar E inclusions (53%/±2%) vs. the control. Furthermore, the size of the inclusions was much smaller in the cocultures with serovar E vs. the control cultures and there was a decrease in the infectious progeny. Cytokine analysis by ELISA revealed significant concentrations of TNFα, IL-1β, IL-6 and IL-8 in the coculture supernatants in Dessus-Babus’ study (24). Shirey et al. observed increased expression of IL-1R and TNFR upon infection of HeLa cells with C. psittaci. Additionally, they observed increased receptor expression in both infected and uninfected populations, and concluded that changes in receptor expression in response to chlamydial infection could be cytokine mediated (25). In our study we believe the cytokines in the conditioned media bind to their receptors on the HeLa cell surface preventing entry of the infectious MoPn and thus the development of IFUs. TNFα and IL-1β antichlamydial activity has been
previously reported (25, 26). Increases in expression of these cytokine receptors by Chlamydia may be due to stimulation of TLRs resulting in the activation of NF-κB (25). The mechanism of these cytokines along with IL-6, IL-8, IL-17 and IL-23 to prevent IFU development could involve those observed by Dessus-Babus and Shirey. Furthermore, Chlamydia has a nutrient require for tryptophan (Trp), and it is very much likely that the antichlamydial activity of these factors leads to degradation of Trp and therefore an environment that is not conducive for chlamydial development.

In the present study HeLa cells were cultured in conditioned media in which we suspended C. pneumoniae elementary bodies (MOI 5). The results were remarkable in that the aforementioned chemokines and cytokines contained in the conditioned media significantly prevented the development of infection forming units in the HeLa cell monolayer. In contrast, HeLa cells cultured in Earle’s MEM in which C. pneumoniae EBs (MOI 5) were suspended had exponential IFU development (Figure 2). These data demonstrate that the immune response elicited by VCG intrinsic adjuvant properties is inhibitory to Chlamydia IFUs and may significantly aid in the prevention of infection by the bacterium.

Acknowledgements

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