



Keywords

Chlamydia trachomatis,
Bacterial Ghosts,
Adjuvant,
Monocytes,
Macrophages,
T Helper Cytokines

Received: July 22, 2014

Revised: August 11, 2014

Accepted: August 12, 2014

THP-1 macrophage cells as a model to study intrinsic adjuvant properties of *Vibrio cholerae* bacterial ghosts during *Chlamydia* infection

M. Stevens¹, D. M^cKeithen^{1,2}, O-R Martinez¹, J. U. Igietseme^{2,3},
C. Black³, Q. He², G. A. Ananaba¹

¹Department of Biological Sciences, Clark Atlanta University, Atlanta, GA, United States

²Department of Microbiology, Biochemistry and Immunology, Morehouse School of Medicine, Atlanta, GA, United States

³National Center for Infectious Disease, Centers for Disease Control and Prevention, Atlanta, GA, United States

Email address

mumbi.stevens@students.cau.edu (M. Stevens)

Citation

M. Stevens, D. M^cKeithen, O-R Martinez, J. U. Igietseme, C. Black, Q. He, G. A. Ananaba. THP-1 Macrophage Cells as a Model to Study Intrinsic Adjuvant Properties of *Vibrio cholerae* Bacterial Ghosts during *Chlamydia* Infection. *American Journal of Microbiology and Biotechnology*. Vol. 1, No. 1, 2014, pp. 1-8.

Abstract

Chlamydia trachomatis is an obligatory intracellular human pathogen responsible for the most common sexually transmitted infections (STIs) worldwide. *Chlamydia* primarily infects the urogenital tract. In men the infections are usually symptomatic versus in women whereby the infections are usually asymptomatic causing severe complications including pelvic inflammatory disease (PID), ectopic pregnancy and infertility. We tested the hypothesis that intrinsic adjuvant properties of *Vibrio cholerae* ghosts (VCG) induce an enhanced immune response to infections. The human monocytic leukemia cell line THP-1, has been established as a useful tool for studying the role of monocytes in the human immune response (1) and for their phagocytosis capacity (2). THP-1 monocytes (Mn) or macrophages (MΦ) were pulsed with VCG with or without interleukin (IL)-10 for 24 hours. IL-10 inhibits nuclear factor kappa B (NF-κB) translocation into the nucleus by blocking inhibitor of kappa B (IκB) kinase activity (3). IL-10 also inhibits NF-κB already found in the nucleus blocking DNA-binding (4) and gene transcription (5). After stimulation, cellular supernatants were assayed for T_h1 and T_h2 cytokine secretion. Mn secretion of T_h1 cytokine tumor necrosis factor-alpha (TNFα) was greater (2200.0 pg/mL) than when cells were primed with IL-10 (1356.0 pg/mL) or secretion by macrophages (1917.0 pg/mL). We concluded that this secretion was significant enough to compliment that which would be secreted when THP-1 Mn or Mφ are pulsed with *Chlamydia* elementary bodies alone (6), enhancing the innate immune response during infection. Cellular supernatants containing T_h1 or T_h2 cytokines were also used to culture *Chlamydia*-infected HeLa cells. Cell viability against the secretory factors contained in the supernatant was measured to determine the effects of the secretory factors on the *Chlamydia*-infected HeLa cells. Cells infected with serovar F and cultured in VCG-pulsed THP-1 Mφ soup were only 8.7% viable after 8 h. This suggests that the immune factors elicited by the VCG signaling pathway

are toxic to *Chlamydia*-infected HeLa cells.

1. Introduction

Chlamydia trachomatis (*C. trachomatis*) is a pestilential intracellular pathogen that causes ocular, genital and respiratory diseases and is a public health anomaly worldwide affecting over 90 million people (7). Repeated infections with *C. trachomatis* lead to serious sequelae, such as infertility (8) in women of childbearing age. Other significant morbidities in women include pelvic inflammatory disease (PID) and ectopic pregnancy (9). In men the sequelae include epididymitis and infertility, however at less severe rates. 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 *Chlamydia* infections worldwide. The persistent magnitude of infectious diseases caused by *C. trachomatis* has made it imperative that a treatment constitutive to elicit long-term protective immunity be developed. In the USA alone more than \$2 billion is spent annually (9) treating these infections. As such, a cost-effective solution is the best alternative to controlling *Chlamydia* and alleviating the severe diseases caused by this infection. Utilizing VCG vectors is our suggested alternative for a cheap prophylactic for controlling *Chlamydia* infection and diseases.

Currently infections caused by *C. trachomatis* are treated with antimicrobial agents, which can affect the host's bacterial ecology hindering the natural course of immunity. The current vaccine against *Chlamydia* does not provide long-term protection. It has been suggested that a multi-subunit prophylactic (9, 10) in the form of a vaccine would provide a protective advantage over current treatments. Recombinant vaccine constructs using Gram-negative bacteria *e.g.*, *Vibrio cholerae* (11), *Escherichia coli* (12), and *Shigella* spp. (13) as bacterial vectors targeting primary antigen presenting cells have been evaluated in previous studies with the intent to simultaneously immunize against multiple pathogens (14). In a preliminary study conducted by our lab, THP-1 monocytes were pulsed with VCG for 6 – 18 h and 24 – 72 h (in two independent experiments) to measure TNF α secretion. TNF α is produced in response to acute injury and plays a role in systemic inflammation. Production of TNF α peaks between 4 and 6 h (15, 16, 17, 18), which has been well documented in the literature. For each independent experiment, we observed a peak in TNF α production at 6 h and 24 h when THP-1 monocytes were pulsed with VCG. After these peaks, there was a significant decrease in TNF α production which could be due to cell necrosis leading to the release of lysosomal proteases and the degradation of TNF α .

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 (1) and their phagocytosis capacity (2). In

the present study we used THP-1 macrophage-like cells to evaluate *Chlamydia* immune responses by stimulating activated THP-1 cells (differentiated, mature monocytes) with the VCG vector to elicit multiple cytokine secretions during a 24 h pulse. Specifically, we have investigated the adjuvant contribution of our ghost vector. IL-10, an anti-T_h1 cytokine (19, 20) produced during genital chlamydial infection (20, 21, 22), is potentially an important local factor that would control T_h1 response against *Chlamydia*. We observed that T_h1 cytokine secretions decreased when macrophage-like cells were primed with IL-10 prior to pulse with VCG, indicating that the inflammatory immune response to the presence of pathogens is controlled by IL-10. We sought to test the hypothesis that intrinsic adjuvant properties of VCG induce an enhanced immune response to *Chlamydia* infection.

Chlamydia trachomatis and *Vibrio cholerae* are both Gram-negative bacteria and have similar envelop structure (Table 1). We 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-specific immune response and signaling pathway, which in turn can enhance the immune response to *Chlamydia* infection.

Table 1. Comparison of structure and property similarity between *V. cholerae* and *C. trachomatis*

| | <i>C. trachomatis</i> | <i>V. cholerae</i> |
|--------------------------|---------------------------|--|
| Property | | |
| Number of serotypes | 18 | 4 |
| Size | 0.20 – 1.3 μ m | 1-3 x 0.5-0.8 μ m |
| Morphology | rod-shaped | comma-shaped |
| Natural host | human | human, water birds, shellfish, fish and herbivores |
| Infection route | contact; epithelial cells | contact; epithelial cells |
| Lipopolysaccharides | Yes | Yes |
| Antibiotic sensitivity | Yes | Yes |
| Survival outside of host | No | Yes |

2. Materials and Methods

2.1. Production of VCG Vectors

Production of the VCG was carried out by gene *E*-mediated lysis as described previously (23). Briefly, *V. cholerae* 01 strain V588 was transformed with the lysis plasmid pDKLO1. Bacteria were grown in brain heart infusion (BHI) broth containing ampicillin (100 ug/mL) and kanamycin (25 ug/mL) at 37°C and after 1 h cell lysis was achieved by adding 3-methyl benzoate (5 mM) to induce gene *E* expression. Lyophilized VCGs preparations were stored at room temperature until use.

2.2. Chlamydia Strains

C. trachomatis strains MoPn and serovar F (generously supplied by Dr. Joseph Igietseme, Centers for Disease Control and Prevention, Atlanta, GA) were grown in McCoy or HeLa cells, respectively. Infection forming units (IFUs) was provided on the package.

2.3. THP-1 Cell Culture

The human monocytic leukemia cell line THP-1 (original stocks generously provided by Dr. Byron Ford, Morehouse School of Medicine, Atlanta, GA and Dr. Joseph Igietseme, Center for Disease Control and Prevention, Atlanta, GA) 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 10⁶ cell/mL.

2.4. HeLa Cell Culture

HeLa cells (generously donated by Dr. Joseph Igietseme, Center for Disease Control and Prevention, Atlanta, GA) were recovered from liquid nitrogen 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 6-well plate at a volume of 1500 uL/well (60 x 10⁴ cells/mL) and set in a humidified incubator under 37°C, 5% CO₂ for the monolayer to adhere - ~48 h.

2.5. Differentiation of THP-1 Monocytes (Mn) Into Macrophages (MΦ)

THP-1 monocytes (Mn) were differentiated into activated macrophages (MΦ) by treating 10 mL of culture with 160 nM phorbol 12-myristate-13-acetate (PMA) for 1 h in a humidified incubator at 37°C, 5% CO₂. After the treatment period, the cellular pellet was collected by centrifugation at 330xg for 10 min then resuspended in 1 mL PMA-free media and brought to a final volume of 10 mL. Cell count was determined by Trypan Blue exclusion using a hemacytometer.

1 mL cell culture aliquots (1.2 x 10⁶ cells/mL) were seeded into a 24-well plate and returned to a humidified incubator at 37°C, 5% CO₂ for 72 h. Cells were washed thereafter and the remaining adherent MΦ were used for experiments.

2.6. Macrophage Secreted Cytokine Assay

Following differentiation of THP-1 Mn into adherent macrophages, culture media was carefully removed by pipet. 1 mL of fresh media containing VCG (10 ug/mL) alone, VCG (10 ug/mL) + IL-10 (5 ug/mL), IL-10 (5 ug/mL) + lipopolysaccharide (LPS; 1 ug/mL), or LPS (1 ug/mL) (positive control) was introduced to designated wells for a treatment period of 24 h while in a humidified incubator at 37°C, 5% CO₂. Culture media served as a negative control. After the treatment period, cellular supernatants were carefully collected by pipet and transferred to a 1.5 mL Eppendorf tube and stored in -20°C until assayed for cytokine concentration.

2.7. Multiplex Assay

Multiplex assay was contracted out to Quansys Bioscience, Logan, UT. The samples were tested for IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-15, IL-17, IL-23, IFNγ, TNFα, and TNFβ by Quansys Biosciences (Logan, UT) Q-Plex Array™ kits for human cytokines. Samples were thawed on ice and diluted into Quansys Human Sample Dilution Buffer prior to loading into preparatory polypropylene low-binding 96-well plates. Standard ELISA incubation steps apply such as initial sample incubation, washing, secondary antibody incubation, washing, incubation with the label and measurement are involved. The label and reporting system used in a Q-Plex Array™ is chemiluminescent. The total protein concentration was determined via absorbance at 280 nm and an extinction coefficient of 1 using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington DE).

2.8. Preparation of THP-1 Macrophage Conditioned Media

A 10 mL culture of THP-1 monocytes was treated with 160 nM PMA for 1 h while in a humidified 37°C, 5% CO₂ incubator to initiate differentiation into adherent macrophages. After 1 h, PMA-treated media was removed by centrifugation at 1500 rpm for 10 mins, and the pellet was resuspended in PMA-free media. 1 - 2 mL aliquots of the activated THP-1 culture was introduced to the wells of a 6-well plate and returned to humidified 37°C, 5% CO₂ incubator overnight. The following day designated wells containing adherent macrophages were treated with either VCG (10 ug/mL) alone, or VCG plus murine T lymphocytes (3 x 10⁶ cells/ml), or VCG plus murine T lymphocytes and EBs (5 MOI), or EBs plus murine T lymphocytes and pulsed 18 – 24h. THP-1 MΦ in culture media served as our negative control. After 24h, cultures were collected by centrifugation as aforementioned, and stored in 1.5 mL Eppendorf tubes at -20°C until use.

2.9. Chlamydia Infection of HeLa Cells and Culture with Supernatants from VCG-Pulsed THP-1 Macrophages

Before infection, two of the HeLa cell monolayers – seeded in a 6-well plate – were pretreated with DEAE-dextran (45 ug/mL) in PBS for 15 min at 37°C; two wells were not pretreated and two wells served as negative controls. Purified EBs of strain MoPn and serovar F were diluted in MEM Earle’s complete media and inoculated onto HeLa monolayers in 1000 uL aliquots at a MOI of 5. Plates were returned to a humidified incubator at 37°C, 5% CO₂ for 24 h. Cells were observed by phase microscopy for infectivity 4 h post infection (PI). After 24 h CT-infected HeLa cell media was removed from cell monolayers carefully by pipet and replaced with conditioned media from THP-1 MΦ. Each HeLa cell monolayer was treated with a different conditioned media solution and negative control contained MEM Earle’s culture media. Cytotoxicity was determined by counting viable cells using a Cellometer® (Nexcelom Biosciences).

3. Results

3.1. T Helper 1 Cytokine Production by Monocytes or Macrophages after Exposure to VCG

We investigated monocytic cells for their uptake of VCG and subsequent production of inflammatory mediators such as TNF α and IFN γ , which are controlling pathways of adaptive immune responses. We used the THP-1 human monocytic leukemia cell line which has been established as a useful tool for studying the role of monocytes in the human immune response (1) and their phagocytosis capacity (2). Cytokine production during the 24 h treatment was assayed by multiplex ELISA using Q-Plex™ technology by Quansys Biosciences’ (Logan, UT) for human cytokines. T_h1 cytokine secretion of TNF (tumor necrosis factor) α was as expected, except when MΦ were primed with IL-10 (Fig. 1). The anti-inflammatory IL-10 attenuated TNF α secretion by more than half. When MΦ were co-pulsed with IL-10 (5 ug/mL) and LPS (1 ug/mL), TNF α cytokine secretion was significantly attenuated (864 pg/mL) versus incubation with LPS alone (1784 pg/mL) (data not shown). This data specifically demonstrates the anti-inflammatory property of IL-10 and its inhibition of nuclear factor kappa B (NF- κ B) activation. When MΦ were co-pulsed with VCG (10 ug/mL) and IL-10 (5 ug/mL), TNF α cytokine secretion was 946 pg/mL (Fig. 1b) versus 1917 pg/mL (Fig. 1a) when IL-10 was not in the treatment.

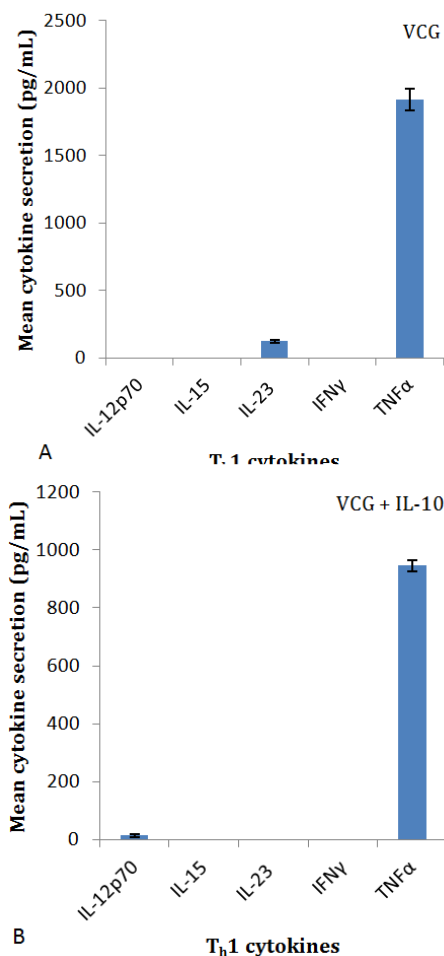


Fig. 1. T helper 1 cytokine secretion by THP-1 macrophages pulsed with VCG (A) or VCG+IL-10 (B). TNF α secretion was significantly higher ($p < 0.05$) than IL-23 or IL-12p70 secretions.

Interferon γ (IFN γ) production was negligible in all treatments (Mn and MΦ) (Table 2). When monocytes were pulsed with VCG (10 ug/mL) alone or VCG (10 ug/mL) and IL-10 (5 ug/mL), IL-23 secretion was suppressed in the co-treatment (Fig. 2). IL-23 is an important part of the (pro-) inflammatory response during infection promoting the up-regulation of MMP9, increasing angiogenesis and reducing CD8+ T-cell infiltration. When IL-10 levels are increased, the functions of IL-23 are suppressed (22, 23). When Mn were pulsed with VCG alone, IL-23 secretion was 2600 pg/mL vs. 585 pg/mL when IL-10 was in the treatment (Fig. 2a and 2b). The same was true for TNF α secretion. When Mn were pulsed with VCG alone, TNF α secretion was 2200 pg/mL vs. 1300 pg/mL when IL-10 was in the treatment (Fig. 2a and 2b).

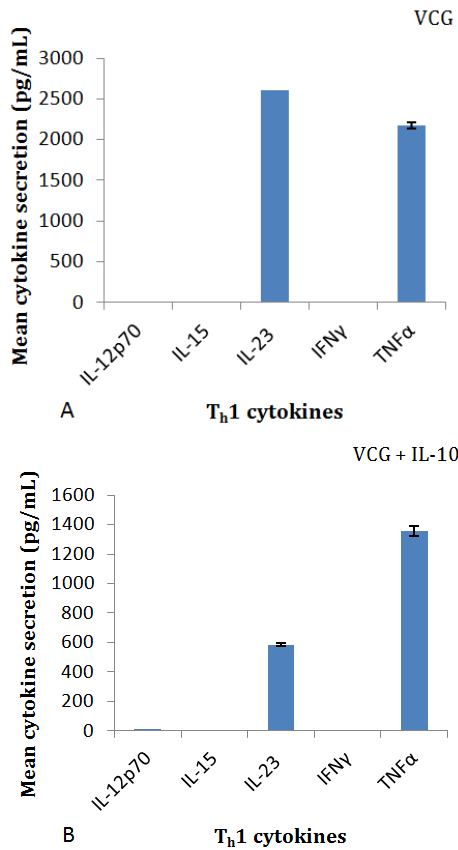


Fig. 2. *T* helper 1 cytokine secretion of THP-1 monocytes pulsed with VCG (A) or VCG + IL-10 (B). IL-10 inhibits NF- κ B activation and nuclear translocation causing attenuation of IL-23 and TNF α secretion ($p < 0.05$).

It has been demonstrated by our lab that certain immune effectors are required in the local genital mucosa for reducing the intensity of and terminating a cervico-vaginal infection, arresting ascending disease, and preventing major complications of the infection (20). These immune effectors are T_H1 cytokines.

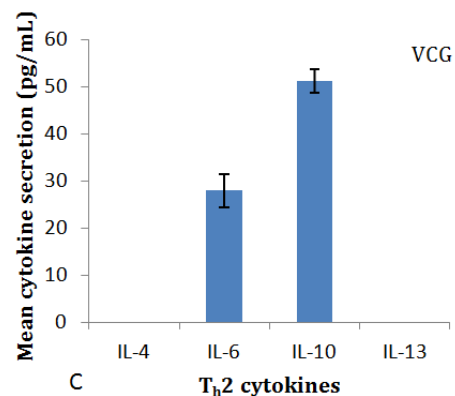
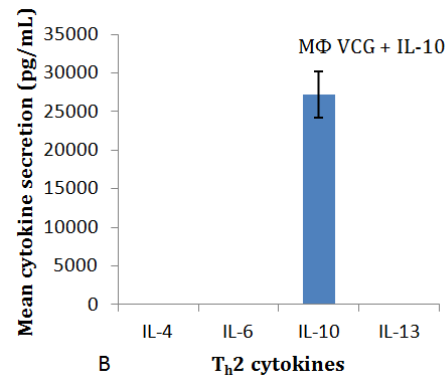
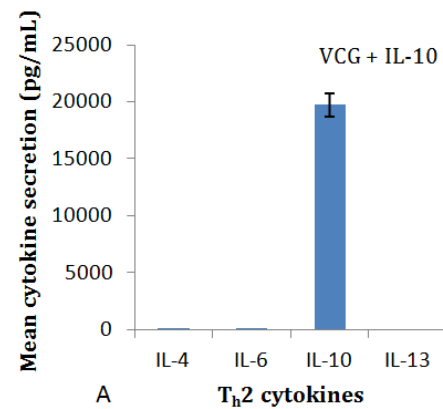
Table 2. Interferon gamma (IFN γ) secretion by THP-1 monocytes (Mn) or macrophages (M Φ)

| IFN γ Secretion | | |
|------------------------|------|----------|
| | Mn | M Φ |
| VCG | <0.9 | <0.9 |
| VCG + IL-10 | <0.9 | <0.9 |
| LPS | <0.9 | <0.9 |

3.2. T Helper 2 Cytokine Production by Monocytes or Macrophages after Exposure to VCG

The level of T_H2 cytokine secretion by monocytes or macrophages following stimulation with VCG for 24 h was assayed by multiplex ELISA using Q-Plex™ technology by Quansys Biosciences' (Logan, UT) for human cytokines. T_H2 cytokine IL-10 secretion levels were highest when cells were

co-pulsed with this cytokine versus when pulsed with VCG alone. Mn (Fig. 3a) secreted 20×10^5 pg/mL vs. M Φ (Fig. 3b) which secreted 27×10^5 pg/mL IL-10 (Fig. 3). T_H2 cytokine secretion of IL-10 and IL-6 was modest by either Mn or M Φ (Fig. 3c and 3d). When Mn were pulsed with VCG, IL-6 secretion was <30 pg/mL and IL-10 was ~50 pg/mL. When M Φ were pulsed with VCG, IL-6 secretion was 85 pg/mL and there was no IL-10 secretion (Fig. 3). This suggests that most of the IL-10 observed in Fig. 3a (since Mn secrete modest amounts of IL-10) and all of the IL-10 observed in Fig. 3b is as a result of the IL-10 in treatment, not produce by the cells.



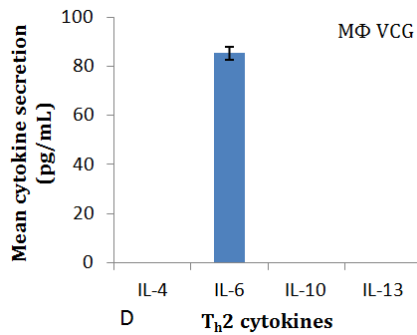


Fig. 3. *T* helper 2 cytokine secretion by THP-1 M Φ or M Φ . M Φ pulsed with VCG + IL-10 (A); M Φ pulsed with VCG + IL-10 (B); M Φ pulsed with VCG (C); and M Φ pulsed with VCG (D). Co-pulsing with IL-10 lead to increased IL-10 concentrations. IL-10 detected may be left over from what was initially added. What is reported does not mean that the cells were producing IL-10 (A and B). IL-4 secretion was ≤ 5.3 pg/mL for all treatment conditions. This is indicative of these cell morphologies lacking a receptor for this cytokine.

3.3. Factors Secreted in Supernatant of VCG-Pulsed THP-1 M Φ are Toxic to *Chlamydia* Infected HeLa Cells

To determine if factors secreted into the supernatant of VCG-pulsed THP-1 M Φ culture were toxic to *C. trachomatis* infected HeLa cells, HeLa monolayers were cultured with the conditioned media for ~24h. After which, cells were counted using a Cellometer[®] (Nexcelom Biosciences) (Fig. 4). HeLa monolayers (1500 μ L) were seeded (6×10^5 cells/mL) in a 6-well plate. Two wells were pretreated with DEAE-dextran (45 μ g/mL) in PBS for 15 min at 37°C. DEAE-dextran was used to sensitize the cells prior to inoculation (30) with VCG-pulsed THP-1 M Φ conditioned media. Untreated wells were inoculated with VCG + T cell-pulsed THP-1 M Φ conditioned media. Two wells served as negative controls and contained MEM Earle's media. HeLa monolayers infected with serovar F were 6% (VCG-conditioned media) viable vs. 34% (VCG/T cells-conditioned media) viable for the two culture conditions. HeLa monolayers infected with MoPn were 46% (VCG-conditioned media) viable vs. 16% (VCG/T cells-conditioned media) viable for the two culture conditions. Our negative control showed significant cell growth (59%) over the culture period. This observation clearly demonstrates that the immune factors secreted by THP-1 M Φ as a result of pulsing with VCG are toxic to CT-infected HeLa cells. Furthermore, this demonstrated VCG has active intrinsic structural properties that stimulate a signaling pathway of its own. The level of toxicity of the M Φ secreted cytokines is relatively immediate based on the morphological changes observed 24 h PI. Infected cells were scored semi quantitatively by evaluation of cell rounding, detachment, and lysis compared with uninfected control cells. Cells infected with MoPn or serovar F strains were examined by phase microscopy and we observed marked cell rounding represented by noticeable 'ballooning' (28) appearance 4 h PI (Fig. 5b - 5e), morphological changes not observed in

uninfected control cells (Fig. 5a). Cells were again examined 8 h after conditioned media was introduced. Morphological changes increased exponentially (Fig. 6) as observed by cell rounding, detachment and ballooning, all indicative of toxic effects of the factors in conditioned media.

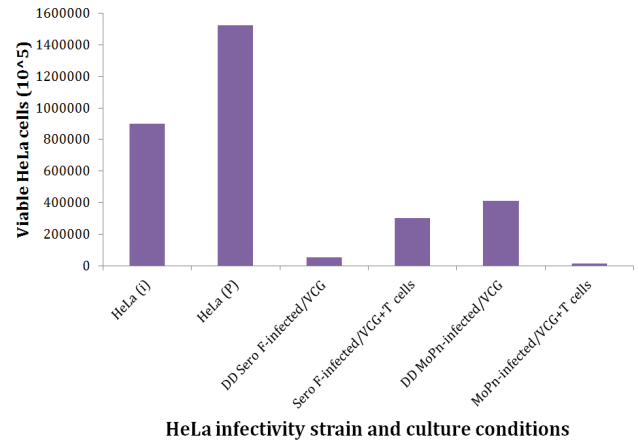


Fig. 4. HeLa cell viability following culture with conditioned media from VCG-pulsed THP-1 macrophages. Factors contained in supernatant were toxic to *Chlamydia*-infected HeLa cells. HeLa (i): Initial cell count; HeLa (P): Post cell count.

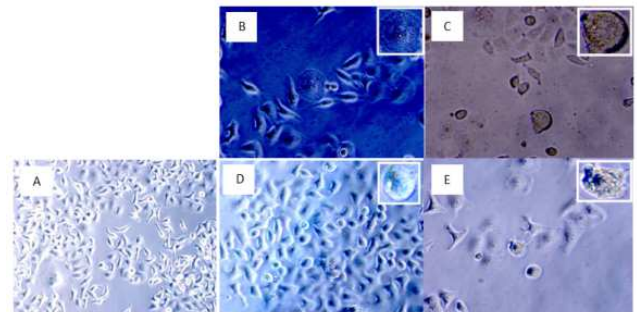


Fig. 5. *Chlamydia*-infected HeLa monolayers. HeLa cell monolayers were examined 4 h PI by phase microscopy. (A) Uninfected. (B) Serotype F infected, pretreated with DEAE-dextran. (C) Serotype F infected. (D) MoPn infected, pretreated with DEAE-dextran. (E) MoPn infected. (Magnification, X20) A pronounced cytopathic effect represented by noticeable rounding is evident with F and MoPn (see insets).

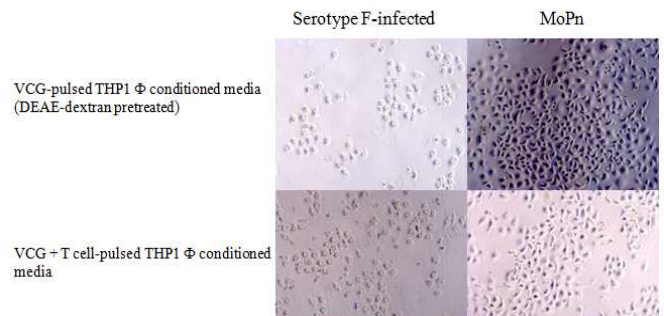


Fig. 6. *Chlamydia*-infected HeLa cell monolayers 8 h after conditioned media introduced. Cytotoxicity of conditioned media was visibly more toxic to F-infected vs. MoPn-infected HeLa. After 8 h, cell rounding in F-infected HeLa was >80% under both conditions (VCG- or VCG + T cells-pulsed) vs. <80% in MoPn-infected.

These morphological changes were most prominent with serovar F-infected HeLa under both conditions – VCG- or VCG + T cell-pulsed THP-1 M ϕ conditioned media. Additionally, this was consistent with what we observed at 4 h PI with serovar F-infected HeLa monolayers. This observation and the cell viability speak to the differences in cytotoxic activity among the two strains (28). At the same MOI, observable infectivity was evidenced by serovar F, whereas with similar levels of MoPn it was not. This implies that MoPn EBs are considerably more toxigenic than EBs of serovar F (28), able to evade innate immune responses more aggressively and effectively to propagate its infectious cycle. However, factors secreted by macrophages pulsed with VCG or VCG/T cells are equitably toxic to CT-infected cells in a relatively short period of time.

4. Discussion

Chlamydia trachomatis is a genetically intractable (29) pathogen that causes severe complications in both men and women. With treatment cost steadily rising, development of a cost-effective prophylactic that provides long-term immunity against re-infection is imperative. Premenopausal women are more susceptible to these infections significantly diminishing their fertility. Many studies prior to this have concluded that using the recombinant ghost platform expressing multiple *Chlamydia* antigens would be the best advantage introduced in recent years for effective treatment of these infections. This would also allow inoculation against a broad-spectrum of infections.

When we compare the structures of *Vibrio cholerae* [ghost] and *Chlamydia trachomatis* [elementary body], both Gram-negative bacteria, the bacterial ghosts (bacteria minus its cytoplasmic contents) retains all major immune stimulating structures of its envelope *e.g.*, lipopolysaccharide (LPS), peptidoglycan, flagellin, and certain lipoproteins – the same characteristics are found on elementary bodies except flagellin and EBs of serotype L1, L2, and L3 lack lipopolysaccharides (28). These pathogen associated molecular patterns (PAMPs) are recognized by Toll-like receptors on the epithelial cells they infect, and initiate a signal that activates an immune pathway. VCG may behave as an adjuvant in several ways for preventing *Chlamydia* infections or reinfection. Used as an adjuvant, VCG can enhance the efficacy of vaccines. With an enhanced immune response, the intrinsic adjuvant properties can significantly reduce the period of time before protective levels of anti-chlamydial IgG are obtained and it may increase the immunogenicity of cells making them more susceptible to clearance by cytotoxic T cells.

In the present study we used THP-1 human monocytic leukemia cells which have been established as a useful tool for studying the role of monocytes in the human immune response (1) and their phagocytosis capacity (2). THP-1 cells have also been shown to have antigen presenting properties when activated, and subsequently acquiring macrophage

morphology. Using this system to observe T helper cytokine secretion by Multiplex assay was an effective approach. We saw significant pro-inflammatory cytokine secretion of IL-23 (Mn) and TNF α (Mn and M ϕ), and equally important, but less secretion of IL-1 β (Mn and M ϕ), IL-12p70 (Mn and M ϕ), and IL-15 (Mn) cytokines. A type 1 response is required for effective control of *Chlamydia* through early suppression of chlamydial growth and removal of extracellular chlamydial elementary bodies during the innate phase. Subsequent effective control, which may involve the induction of persistence, requires adaptive immunity (21). Previous studies from our lab validate that a specific T_h1 response following rVCG multi-subunit vaccine regimen conferred a greater level of protective immunity, including protection from infertility (10).

The paucity of T_h2 cytokine secretion by Mn or M ϕ we observed from our assay was as expected and in an *in vivo* environment would be ideal for effective clearance of chlamydial elementary bodies by T_h1 cytokines. We assayed for IL-4, IL-5, IL-6, IL-10 and IL-13 and all were <150 pg/mL. IL-10, a T_h2 cytokine and regulator of cytokines secreted by Type 1 lymphocytes, attenuated the secretion of T_h1 cytokines by Mn and M ϕ . We believe this mechanism is achieved by preventing NF- κ B translocation into the nucleus, thereby suppressing the expression of these T_h1 cytokines.

We should mention that we did observe the chemotactic and inflammatory cytokine, IL-8, which was secreted by Mn at >7500 pg/mL and exponentially more by M ϕ at >49000 pg/mL. This is the typical response to invasion of foreign particles and infection by microorganisms. IL-8's secretion is higher in the phagocytic macrophages whose response to VCG is to secrete an inflammatory stimulus (*e.g.*, tumor necrosis factor) and attract the chemokine to the infection site.

To address the *Chlamydia*-specific immune response elicited by immune factors secreted by THP-1 M ϕ the VCG signaling pathway produces, we cultured HeLa cell monolayers in the conditioned media from VCG- or VCG/T cell pulsed THP-1 M ϕ . By cell count and observation with phase microscopy, cytotoxicity of the conditioned media was evidenced. HeLa cells infected by serovar F (a human strain of *Chlamydia*) or MoPn (a mouse strain of *Chlamydia*) at the same MOI showed significantly different toxigenic effects. Serovar F-infected HeLa cells showed morphological changes 4 h post infection. Then 8 h after introduction of the conditioned media, >80% of the cells were rounded, detached or ballooning. These morphologies are indicative of cytotoxicity resulting from the immune factors in the conditioned media that were elicited by the VCG signaling pathway. MoPn-infected HeLa cells were considerably more toxigenic than serovar F (28), able to evade innate immune responses more aggressively and effectively to propagate its infectious cycle as evidenced by cell morphology at 4 h and 8 h PI.

Our overall hypothesis is that the effectiveness of *Chlamydia* prevention is via antigen presentation. Development of a vaccine that delivers multiple *Chlamydia*

antigens to induce maximum mucosal and systemic T_h1 skew in addition to accessory antibody responses is clinically significant for optimal microbial clearance and to prevent the complications resulting from *Chlamydia* infection and/or reinfection.

In this study we tested the hypothesis that intrinsic adjuvant properties of VCG induce an enhanced immune response to *Chlamydia* infection. When HeLa cells infected with *chlamydia* were cultured in the conditioned media of VCG-pulsed THP-1 Mφ, we observed marked cell death compared to HeLa monolayers not cultured in the conditioned media. This data confirm our rationalization that the VCG-specific immune response and signaling pathway can enhance the immune response to *Chlamydia*.

Future studies should include an investigation of the antigen presenting function of activated THP-1 cells. THP-1 cells have been demonstrated to acquire dendritic cell (DC) properties upon stimulation with phorbol esters such as PMA (phorbol 12-myristate 13-acetate) (2). The study's aim should include direct contact of THP-1 with stimulated T cells to see if specific cytokine secretion is markedly induced. The current study should be repeated for a shorter stimulation period, say 2, 4 and 6 h, given the precedent on TNFα peak secretion and to see if other T_h1 cytokine secretion peaks equitably. Additionally, HeLa cells should be inoculated with a range of MOIs.

Acknowledgements

This study was supported by grant RO1 AI41231 from National Institutes of Health (NIH) and Grant #1 C06 RR18386 from National Centers for Research Resources, NIH; Title III Grant HBGI #22211K – Enhancing Graduate Students Academic and Professional Development; NSF-CREST Center for Functional Nanoscale Materials (HRD-0630456); MBRS-RISE Grant #5R25GM060414-11; RCMI grant #12RR03062 NCRR-NIH.

We would like to extend a special thank you to Dr. Yusuf Omosun, Morehouse School of Medicine, Atlanta, GA, for providing mouse spleens to isolate splenic T lymphocytes.

References

- [1] Tsuchiya, S. et al., *Int. J. Cancer*: 26, 171-176 (1980).
- [2] Santegoets, SJAM, et al., *Journal of Leukocyte Biology* Vol. 84 No. 6, 1364-1373 (2008).
- [3] Vey, E. et al., *J. of Immunol.* Vol. 149 No.6, 2040-2046 (1992).
- [4] Driessler F. et al., *Clin Exp Immunol* 135, 64-73 (2004).
- [5] Wang P. et al., *J. Biol.Chemistry* Vol. 270 No. 6, 9558-9563 (1995).
- [6] Gervassi, A et al., *Infection and Immunity* 7231-7239 (2004).
- [7] Brunham RC et al., *Nat Rev Immunol.* (2), 149-61 (2005).
- [8] Frankhauser SC et al., *J Immunol.* 192(3), 1079-90 (2014).
- [9] Eko FO et al., *Vaccine* 29, 1472-1480 (2011).
- [10] Ifere GO et al., *J Microbiol Immunol Infect.* 40(3), 188-200 (2007).
- [11] Jalava K. et al., *Expert Rev Vaccines* 2(1), 45-51 (2003).
- [12] Haidinger W. et al., *Appl Environ Microbiol* 69(1), 468-74 (2003).
- [13] Tabrizi CA. et al., *Curr Opin Biotechnol* 15(6), 530-7 (2004).
- [14] Igietseme JU. et al., *Expert Rev Vaccines* 5(6),739-45 (2006).
- [15] Araujo FG. et al., *Antimicrob Agents Chemother.* 46(10), 3327–3330 (2002).
- [16] Kakizaki Y. et al., *Endocr J.* 46(4), 487-96 (1999).
- [17] Xiong Y. et al., *Endocrinology* 133(6), 2568-73 (1993).
- [18] Wang JE. et al., *Infect. Immun.* vol. 68 no. 7, 3965-3970 (2002).
- [19] Moore KW. et al., *Annu Rev Immunol.* 11, 165-90 (1993).
- [20] He Q. et al., *J Immunol* 174(8), 4860-9 (2005).
- [21] Yang, X. et al., *J. Immunol.* 156:4338 (2006).
- [22] Cohen, CR. *AIDS* 25:327 (1999).
- [23] Eko FO. et al., *Vaccine* 21(15), 1694-703 (2003).
- [24] Igietseme JU. et al., *Immunology* 98(4), 510-9(1999).
- [25] Gracey, E. et al., *Nature Reviews Rheumatology* 8, 55-59 (2012)
- [26] Langowski JN. et al., *Nature* 442 (7101), 461–5 (2006).
- [27] Kikly K. et al., *Curr. Opin. Immunol.* 18 (6), 670–5 (2006).
- [28] Belland RJ. et al., *PNAS* Vol. 98 No. 24, 13984-13989 (2001)
- [29] Cocchiari JL. et al., *Cell Microbiol.* 11(11), 1571-1578 (2009).
- [30] Al-Moslih MI et al., *J. gen. Virol.* 18, 189-193 (1973).