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Chlamydia Infection-derived Exosomes Possess Immunomodulatory Properties Capable of Stimulating Dendritic Cell Maturation

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Authors' contributions

This work was carried out in collaboration between all authors. Author FOE conceived and designed the study. Authors RSR wrote the protocol, acquired the data and wrote the first draft of the manuscript. Authors RSR, MBH and KR performed the statistical analysis. Authors FOE, JUI, MK, YO, MBH and MDP managed the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The aim of this study was to characterize exosomes derived from *Chlamydia muridarum*-infected and normal healthy untreated mouse oviduct epithelial cells (MOEC) and evaluate their immunomodulatory properties.
Methodology: Exosomes were purified from *C. muridarum*-infected and uninfected MOEC and analyzed using the NanoSight nanoparticle tracking analysis system. The concentration of cytokines and chemokines associated with exosomes and secreted by exosome-activated bone marrow-derived dendritic cells (BMDCs) was assessed using the Bio-Plex cytokine assay kit in

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combination with the Bio-Plex Manager software. Also, the exosome-stimulated dendritic cell expression of co-stimulatory surface and major histocompatibility complex class II (MHC-II) molecules was assessed by FACS analysis.
Results: The results showed that the concentration of cytokines (IL-6, IL-10, IL-12, IFN-γ and TNF-α) and chemokines (KC (CXCL1), MCP-1 (CCL2), MIP-1α (CCL3), Rantes (CCL5) and Eotaxin (CCL11)) secreted by BMDCs pulsed with *Chlamydia* infection-derived exosomes (IDEX) was significantly higher ($p < 0.05$) than that secreted by BMDCs pulsed with uninfected control-derived exosomes (CDEX). Furthermore, exosomes purified from *Chlamydia*-infected MOEC significantly upregulated ($p < 0.05$) the dendritic cell expression of CD86 and MHC-II, molecules associated with DC activation and maturation, compared to those from uninfected cells.
Conclusion: The results indicate that *Chlamydia* IDEX possess immunomodulatory properties capable of stimulating dendritic cell activation and maturation. Further studies will delineate their potential use as immunomodulators or as vaccine delivery vehicles.

Keywords: Exosomes; Chlamydia; infection; proinflammatory cytokines; immunomodulation.

ABBREVIATIONS

ANOVA: Analysis of variance; APC: Antigen-presenting cells; BMDC: Bone marrow derived dendritic cells; BSA: Bovine serum albumin; CD: Cluster of differentiation; DC: Dendritic cells; ELISA: Enzyme-linked immunosorbent assay; ESCRT: Endosomal sorting complexes required for transport; FACS: Fluorescence-activated cell sorting; FBS: Fetal bovine serum; FITC: Fluorescein isothiocyanate; HSP: Heat shock protein; IFN: Interferon; IL: Interleukin; ISEV: International Society for Extracellular Vesicles; KC: Keratinocyte chemoattractant; LGT: Lower genital tract; MAP: Mitogen-activated protein; MCP: Monocyte chemoattractant protein; MHC-II: Major histocompatibility complex class II; MIP: Macrophage inflammatory protein; MOEC: Mouse oviduct epithelial cells; NaN3: Sodium azide; NFKB: Nuclear Factor Kappa Beta; PBS: Phosphate-buffered saline; PE: Phycoerythrin; RANTES: Regulated on Activation, Normal T Expressed and Secreted; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; STD: Sexually transmitted disease; Th: T-helper cells; TNF: Tumor necrosis factor; UGT: Upper genital tract.

1. INTRODUCTION

Chlamydiae are obligate intracellular bacteria that infect both humans and animals, causing a variety of diseases. In fact, *Chlamydia trachomatis* is the leading cause of bacterial sexually transmitted diseases (STDs) worldwide [1]. In females, *Chlamydia* targets the apical surface of polarized superficial columnar epithelial cells that are found lining the endocervix and upper reproductive tract. Majority of chlamydial infections, especially in females, are asymptomatic and if left untreated, may ascend to the upper genital tract (UGT) causing severe irreversible sequelae that include pelvic inflammatory disease, ectopic pregnancy, and infertility [2]. Previous studies indicate that chlamydial pathogenesis is a consequence of innate and adaptive host immune responses to ongoing or repeated infections. However, minimal pathology has been shown to develop in the UGT after clearance of the microbe from the lower genital tract [3]. The mechanism of ascending chlamydial disease is not fully understood although recent studies suggest that *Chlamydia*-specific CD8+ T cells induce the UGT pathology via the production of TNF-α [3]. Resolution of a chlamydial infection involves the induction of an initial inflammatory response characterized by a marked infiltration of innate immune cells followed by an adaptive immune response characterized by infiltrating lymphocyte subpopulations, including B cells, CD4+ T cells and CD8+ T cells [4].

Exosomes are small EVs, 30-100 nm in diameter, produced and secreted by a variety of cells including immune cells [5,6]. They are present in all major bodily fluids, including blood, urine, bile, saliva, semen, and cerebrospinal fluid [7,8]. They are involved in cell-to-cell communication by conveying lipids, proteins and RNAs, including messenger RNAs (mRNAs) and microRNAs (miRNAs) to recipient cells [9,10]. Recent evidence suggests EVs play a contrasting role in the regulation of immune responses, whereby an immune response may be enhanced or suppressed by EVs depending on their cell of origin and its functional state [11]. For example, while exosomes in milk and

colostrum inhibit T cell activation in vitro [10,12], exosomes released from Mycobacterium or Toxoplasma infected macrophages induce the secretion of proinflammatory cytokines by recipient macrophages [13]. Additionally, tumor-derived exosomes have been shown to induce anti-tumor cell-mediated immune responses in dendritic cells [14].

The role of *Chlamydia* infection-derived exosomes in the regulation of immune responses is currently unknown. We hypothesized that exosomes released from *Chlamydia*-infected cells possess immunomodulatory properties capable of stimulating the maturation of antigen presenting cells, such as dendritic cells. In this study, we characterized exosomes derived from *Chlamydia* infected and normal healthy untreated mouse oviduct epithelial cells (MOEC) and evaluated their ability to induce dendritic cell activation. Our results showed that bone marrow-derived dendritic cells (BMDC) treated with exosomes derived from *Chlamydia* infected MOEC secreted significantly higher ($p < 0.05$) levels of cytokines such as IL-1β, IL-6 and TNFα and chemokines (KC, MCP-1, MIP-1α) compared to healthy untreated MOEC, suggesting a proinflammatory role for infection-derived exosomes. Furthermore, exosomes purified from *Chlamydia*-infected MOEC significantly upregulated ($p < 0.05$) the dendritic cell expression of CD86 and MHC-II, molecules associated with DC activation and maturation, compared to those from uninfected cells. These results suggest that *Chlamydia* infection-derived exosomes possess immunomodulatory properties capable of inducing dendritic cell activation and maturation.

2. MATERIALS AND METHODS

2.1 *Chlamydia* Strain and Stock

Chlamydia muridarum (laboratory stock), formerly *C. trachomatis* agent of pneumonitis strain (MoPn), was employed in these studies. Chlamydial stock for infecting epithelial monolayers was prepared in HeLa cells by the standard tissue culture cultivation methods as previously described [15].

2.2 Cell Culture

C57BL/6 mouse oviduct epithelial cells (MOEC; Bm1.11) were generously provided by Raymond Johnson (Yale University School of Medicine, New Haven, CT) and grown in 1:1 Dulbecco's modified Eagle medium (DMEM):F12K (Gibco, Grand Island, NY) supplemented with 10% exosome free FBS (System Biosciences, Palo Alto, CA) and 1% L-glutamine/ streptomycin/penicillin (Quality Biological, Gaithersburg, MD). The mouse epithelial cell line (Bm1.11) employed in the studies was selected on the basis of a previous report of expected epithelial morphology and functional activity; expression of cytokeratins and IFN-□-inducible expression of major histocompatibility complex (MHC) class II [16]. Moreover, we have established that the *C. muridarum* strain employed in these studies grows efficiently in this cell line (unpublished data).

2.3 *C. muridarum* Infection of MOEC (Bm1.11)

Cells grown to 90% confluence were infected as described previously [17] but with slight modifications. Cells were infected with 5 IFU of *C. muridarum* elementary bodies (EBs)/cell in 50 ml of culture medium without centrifugation (a multiplicity of infection, MOI of 5) and incubated without agitation at 37°C for 3 h in the presence of 5% CO_2. Sham-infected flasks (0 h) received an inoculum-equivalent volume of sucrose-phosphate-glutamic acid buffer (SPG buffer) without *C. muridarum* in the medium. After gently washing three times with phosphate-buffered saline (PBS) (Corning, Manassas, VA) to remove unbound *Chlamydia*, the cells were replenished with fresh media and incubated further at 37°C for 48 h in 5% CO_2.

2.4 Exosome Isolation

Exosomes were isolated from MOEC culture supernatants by differential centrifugation as described previously [18] with slight modifications. Briefly, 50 ml of *C. muridarum*-infected and sham-infected normal healthy MOEC at 48 h postinfection were centrifuged at 2000 g for 10 min at 4°C to remove cellular debris. The supernatant was again centrifuged at 10,000 g for 30 minutes at 4°C to remove smaller contaminants. The resultant supernatant was transferred to plastic reusable 30-ml BD-tubes (BD Biosciences, Franklin Lakes, NJ) and centrifuged at 200,000 g for 2 h at 4°C to isolate exosomes. The exosome pellet was resuspended in 300-500 μl of PBS.

2.5 Western Blot Analysis

Immunoblotting analysis was used for confirmation of exosomes and was performed

essentially as described previously [19]. Briefly, isolated exosomes were lysed in reducing sample buffer ([0.25 M Tris HCl (pH 6.8), 40% glycerol, 8% SDS, 5% 2-mercaptoethanol and 0.04% bromophenol blue] (BioRad, Hercules, CA) and heated at 95° for 5 minutes. Proteins were separated by SDS-PAGE and transferred electrophoretically to nitrocellulose membrane in a trans-blot cell (Bio-Rad, Hercules, CA). After blocking for 1 h in 5% non-fat dry milk in PBS containing 0.5% Tween-20 (PBS-T) (Sigma-Aldrich, St. Louis, MO) to minimize non-specific protein binding, the membrane was incubated with rabbit monoclonal antibodies to the tetraspanin (CD63) and heat-shock protein 70 (HSP70) proteins (Thermo Scientific, Rockford, IL) diluted 1:500 in 3% blocking buffer. Subsequently, the membrane was incubated with HRP-conjugated goat anti-rabbit IgG and bound antibody was detected by chemiluminescence using the Pierce ECL Western Blotting kit (Thermo Scientific, Rockford, IL) and visualized by the ImageQuant LAS4000 biomolecular imager (GE, Fairfield, CT).

2.6 Proteomic Profiling of *C. muridarum* Infected MOEC-Derived Exosomes

Exosome samples were prepared for mass spectrometry analysis via in gel digestion. Briefly, exosomes from control-MOEC or from infected – MOEC were loaded into wells of a criterion gel (Bio-Rad, Hercules, CA) and separated via electrophoresis. Following separation and staining with Coomassie blue (Bio-Rad, Hercules, CA), the bands were excised and stored in clean (rinsed with mass spec grade Acetonitrile) Eppendorf tubes for transportation or storage. Bands were washed three times in 100% acetonitrile for at least 10 min per wash to remove any stain. After the last wash the bands were placed in a Speed-Vac and dried for 20 min. The dried bands were rehydrated in trypsin digest buffer (50mM Ammonium Bicarbonate). Bands were first reduced in DTT 10 mM at 56°C for at least 30 min and alkylated with 15 mM Iodoacetic Acid for 30 min at room temperature in the dark. Samples were then digested with mass spec grade trypsin 20 ng/ul for 4 hours at 37°C. Just before analysis, the sample is acidified by the addition of Formic acid to 0.1%. Bands from the 1D gel were analyzed using a 60 min LC/MS run on a Multimate 3000 nanoflow HPLC. The sample was loaded onto a trap (C18 PepMap 100, 300 µm x 5mm, Thermo Fisher Scientific Inc., Wilmington, DE) at 5 µl/min for 5 min. The multiport valve was then switched and the trap was eluted onto a reverse phase column using a gradient of acetonitrile in water 5%-30% over ten minutes at a flow rate of 200 nl/min. Eluate from the column was directly sprayed into an LTQ ion trap mass spectrometer using nanospray. Spectra are collected using X calibur 2.2 software (Thermo Fisher Scientific Inc., Wilmington, DE) using a threshold setting of 200. Spectra were searched against a mouse and *Chlamydia* Swiss-Prot database using Proteome Discoverer 3.1 software (Thermo Fisher Scientific Inc., Wilmington, DE). The percolator selection algorithm was used, set to a 5% false discovery rate to select the best database hits. Proteins were validated and identified at 95% minimum protein group probability using the ProteoIQ software (Premier Biosoft, Palo Alto, CA).

2.7 NanoSight Exosome Analysis

Exosomes were analyzed on the NanoSight LM10 instrument equipped with a 405 nm laser on the light scatter mode for quantification and scatter distribution according to the manufacturer's protocols (Malvern Instruments, Malvern, UK). The instrument was calibrated for nanoparticle size and quantity using standardized nanoparticle dilutions. Particle movement was tracked by the NTA software (version 2.3, NanoSight) with low refractive index corresponding to cell-derived vesicles. Each track was then analyzed to get the mean, mode, and median vesicle size together with the vesicle concentration (in millions) for each size. Exosome protein concentration was determined using the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE) according to the manufacturer's instructions. All samples were analyzed in triplicates.

2.8 Determination of Acetylcholinesterase (AChE) Activity in MOEC-Derived Exosomes

AChE activity was measured spectrophotometrically in a 96-well microtiter plate. MOEC-derived exosomes (50 µl) or 200 µl of AChE standards (0.0, 0.98, 1.96, 3.91, 7.81, 15.63, 31.25, 62.5, 125, 250, 500, 1,000 and 2,000 µUnits/ml) were placed in triplicates in designated wells of a 96-well plate. About 200 µl of a reaction mixture containing 0.5 mM 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB) (Sigma Aldrich, St. Louis, MO) and 1 mM Acetylcholine substrate [prepared by diluting acetylcholine iodide (Sigma Aldrich, St. Louis, MO)] in PBS (Corning, Manassas, VA) were then added and

plates were incubated at room temperature for 20 minutes. Control wells included all reagents but without substrate. The kinase activity of Acetylcholinesterase was measured at 450 nm using the SpectraMax M4 Microplate Reader. The experiment was repeated twice.

2.9 Determination of Apoptosis

Apoptosis of exosome-treated MOEC was determined using the Annexin V detection assay kit (Biolegend, San Diego, CA) according to the manufacturer's guidelines. Briefly, exosomes isolated from infected and uninfected MOEC were co-cultured with epithelial cells for 24 h. Following incubation, the cells were collected in PBS (Gibco, NY, NY) and washed twice with wash buffer (0.1% NaN_3, 2% BSA in PBS). The pelleted cells were resuspended in 100 µl of Annexin V buffer (Biolegend, San Diego, CA) and stained for 15 min at room temperature with FITC-Annexin V and PE-7AAD in the dark. The cells were centrifuged and resuspended in Annexin V binding buffer and cell death was assessed using the EMD Millipore's Guava® easyCyte 8HT Benchtop Flow Cytometer in combination with the InCyte application software.

2.10 Dendritic Cell (DC) Isolation and Culture

Bone marrow-derived dendritic cells (BMDC) were generated from the bone marrow of 6-week-old C57BL/6 mice as described previously [20]. Briefly, bone marrow progenitor cells were obtained by flushing out marrow from the femurs and tibias of mice with 2 ml RPMI-1640 medium. Red blood cells were lysed with ammonium chloride solution. Following washing with RPMI-1640, murine recombinant IL-4 and GM-CSF were added to stimulate proliferation and maturation of the DCs. Purified DCs were plated at a concentration of 1 x 10^6 cells/ml in 24-well plates and pulsed with exosomes purified from *C. muridarum*-infected and sham-infected normal healthy MOEC for 24 h. Fc receptor sites on DCs were blocked by FACS buffer (2% BSA and 0.1% NaN_3 buffered in PBS) at 4°C for 10 min before treatment with exosomes. All incubations were performed in a humidified 37°C, 5% CO_2 incubator.

2.11 Fluorescent Antibody Staining and Flow Cytometry

Harvested BMDCs pulsed with exosomes from *C. muridarum*-infected and sham-infected MOEC were stained with monoclonal antibodies against CD11c, CD86, CD40 and 1Ab (MHC-II) conjugated with either PE- or FITC (Pharmingen, San Diego, CA). Controls were incubated with isotype-matched irrelevant antibodies. Staining was carried out in PBS containing 5% FCS. After washing twice, cells were fixed with 1% paraformaldehyde and surface marker expression was analyzed in triplicates by flow cytometry using the Millipore Guava EasyCyte Cytometer in combination with the InCyte software (EMD Millipore, Hayward, CA). Marker expression was assessed on gated CD11c cells.

2.12 Quantification of Cytokine Concentration by Cytokine ELISA

The concentration of cytokines and chemokines associated with exosomes purified from *C. muridarum*-infected and sham-infected normal healthy MOEC as well as from supernatants of BMDC cultures pulsed with exosomes was assessed by the Luminex cytokine ELISA assay using the Cytokine Mouse Magnetic 13-Plex Panel (BioRad, Hercules, CA) according to the manufacturer's instructions. The concentration of cytokines and chemokines was acquired in duplicates from independent experiments using the Bio-Plex Manager software version 4.1 (BioRad, Hercules, CA). The mean and SD of all replicate cultures were calculated.

2.13 Statistical Analysis

All experiments were independently repeated twice. Statistical significance was assessed using the GraphPad Prism 5 software (GraphPad Software, Inc. La Jolla, CA). The graphical data is presented as the mean $\pm$ standard deviation of independent measurements. The statistical significance of the difference between two groups was evaluated by Student's t-test and between more than two groups by one-way ANOVA. The results were considered significant when $^*P < 0.05$ or $^{**}P < 0.01$.

3. RESULTS

3.1 Characterization of Isolated Exosomes

Size determination of the exosomes from both infected and uninfected cells performed with the NanoSight nanoparticle tracking system showed a size distribution where majority of the exosomes were $\leq$ 100 nm suggesting pure populations of exosomes. (Fig. 1A). The concentration (number of particles/ml) showed

significant differences ($P \leq 0.05$) between exosomes released from *C. muridarum*-infected and uninfected MOEC at 48 h post-infection (Fig. 1B). *C. muridarum* infection decreases the release of exosomes from MOEC.

3.2 Western Immunoblotting Analysis

Protein expression was detected by SDS-PAGE and immunoblotting analysis using monoclonal antibodies to CD63 and HSP70. The antibodies reacted specifically with the exosome proteins CD63 and HSP70 as indicated by specific bands at the approximate molecular masses of 50 and 70 kDa, respectively (Fig. 2). This result confirms that exosomes isolated from *C. muridarum*-infected (IDEX) and uninfected (CDEX) MOEC are, as expected, enriched in CD63 and HSP70.

3.3 MOEC-Derived Exosomes Have Induced AChE Activity

The MOEC-derived exosomes were further characterized by analyzing the Acetylcholinesterase (AChE) activity. As Acetylcholinesterase is enriched in exosomes, AChE activity is an indirect measure of exosome presence. High AChE activity indicates a high concentration of exosomes. The result showed both infected and uninfected MOEC-derived exosomes express high levels of AChE activity confirming that the isolated particles are indeed exosomes (Fig. 3). However, the AChE activity of exosomes from uninfected MOEC was significantly higher ($P < 0.05$) compared to that of exosomes from infected cells.

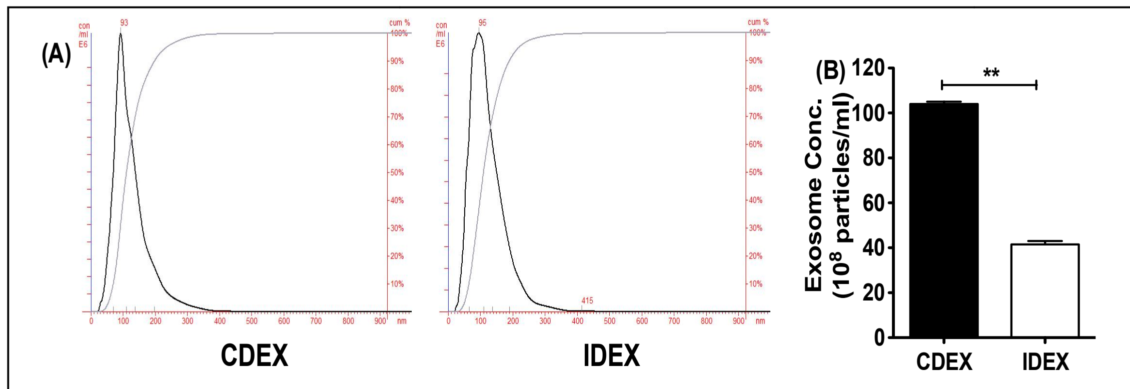

Fig. 1. Characterization of exosomes isolated from *Chlamydia*-infected and uninfected mouse oviduct epithelial cells (MOEC)

Isolated exosomes were analyzed on the NanoSight LM10 instrument. The concentration and particle size of infection-derived exosomes (IDEX) and uninfected control-derived exosomes (CDEX) were determined using the NanoSight nanoparticle tracking analysis system (NTA 2.3). (A) The data shows the particle size distribution (≤ 100 nm) of exosomes derived from three independent experiments. (B) Exosome concentration was also determined by the NanoDrop Spectrophotometer and expressed as number of particles/ml

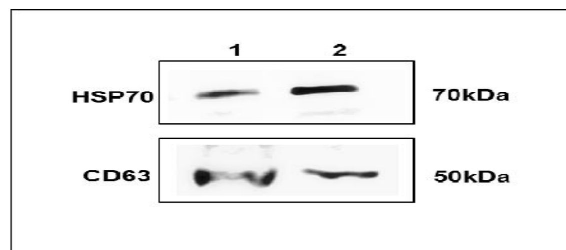

Fig. 2. Detection of CD63 and HSP70 by Western immunoblotting analysis

Exosomes isolated from Chlamydia-infected and uninfected MOEC were separated by SDS-PAGE, subjected to western immunoblotting analysis and probed for expression of the exosome markers, CD63 and HSP70 using anti-CD63 and anti-HSP70 monoclonal antibodies. Lanes 1 and 2: Exosomes isolated from uninfected and Chlamydia-infected MOEC, respectively

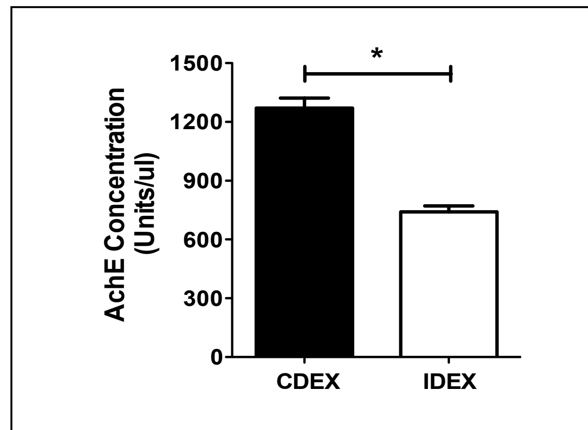

Fig. 3. The AChE activity of exosomes derived from *Chlamydia* infected and uninfected MOEC

The AChE activity of exosomes derived from C. muridarum infected and uninfected MOEC was measured spectrophotometrically. Results, generated simultaneously with a standard curve, display data sets corresponding to absorbance values as mean concentrations (µUnits /ml) ± standard deviations and represent the mean values from triplicate wells. The AChE activity of control wells without substrate served as background and the mean was deducted from each treatment mean. The figure shows data from one of two independent experiments with similar results. Significant differences between experimental groups were evaluated at (p < 0.05)*

3.4 *C. muridarum* Infection-Derived Exosomes Induced Apoptosis in MOEC

Apoptosis of MOEC treated with IDEX and CDEX for 24 h was determined using the Annexin V detection assay and analyzed by flow cytometry. The results show that 60% of the MOEC treated with IDEX were apoptotic compared to 28% of those treated with CDEX indicating that *C. muridarum* infection increases the ability of exosomes to induce apoptosis in MOEC (Fig. 4).

3.5 *C. muridarum* Infection-Derived Exosomes Express a Distinct Protein Profile

Given the mounting evidence for the ability of exosomes to package pathogen-derived proteins following infection, exosomes from *C. muridarum* infected and uninfected MOEC were analyzed on mass spectrometry for proteomic profiling to investigate if *C. muridarum* infection-derived exosomes packaged chlamydial proteins. While a large number of host proteins were commonly expressed in both IDEX and CDEX samples, only IDEX expressed specific chlamydial proteins. Chlamydial proteins packaged in IDEX included those involved in an array of metabolic and synthesis pathways. The virulence associated plasmid protein 6 (Pgp6) and probable outer membrane proteins were also identified (Table 1).

3.6 Concentration of Cytokines Associated with Exosomes

The concentration of cytokines and chemokines associated with exosomes isolated from *C. muridarum*-infected (IDEX) and uninfected (CDEX) MOEC was assessed by cytokine ELISA. The results showed that *C. muridarum* infection inhibited the packaging of cytokines (Fig. 5A) and chemokines (Fig. 5B) by exosomes isolated from MOEC. Notably, exosomes isolated from uninfected MOEC showed significantly higher (P < 0.05) levels of the proinflammatory cytokine, TNF-a as well as the chemokines KC, MCP-1 and RANTES compared to exosomes from *C. muridarum*-infected cells (Fig. 5).

3.7 MOEC-Derived Exosomes Induce Dendritic Cell Production of Cytokines

The ability of exosomes isolated from *Chlamydia*-infected (IDEX) and uninfected (CDEX) MOEC to stimulate dendritic cells to secrete cytokines and chemokines was also evaluated by cytokine ELISA. Significantly higher (P < 0.05) levels of proinflammatory cytokines (IL-1β, TNF-α, IL-12p70 and IL-6) were secreted by BMDCs pulsed for 24 h with exosomes isolated from

C. muridarum-infected cells compared to those pulsed with exosomes from uninfected cells (Fig. 6A). In addition, BMDCs pulsed with *C. muridarum* infection-derived exosomes secreted significantly higher ($p^* < 0.05$) levels of chemokines, including Eotaxin, KC, MCP-1 and MIP1-α, compared to DCs pulsed with exosomes derived from uninfected cells (Fig. 6B). The results indicate that *Chlamydia* infection enhances the ability of MOEC-derived exosomes to induce the production of cytokines by BMDCs.

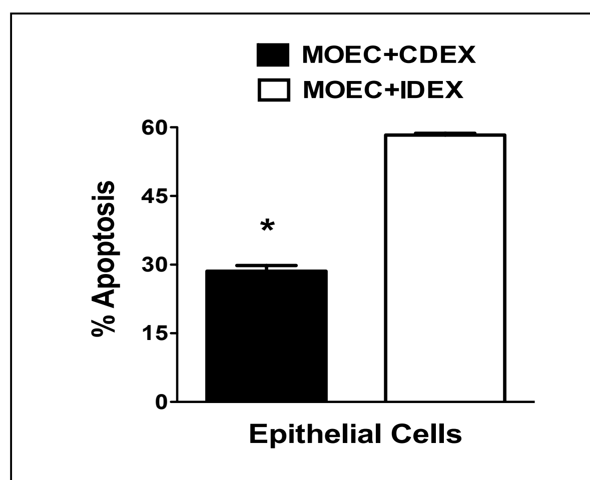

Fig. 4. Detection of apoptosis in MOEC treated with exosomes derived from *Chlamydia* infected and uninfected MOEC

Apoptosis of MOEC exposed to IDEX and CDEX was determined using the Annexin V detection assay and analyzed by flow cytometry. The data is shown as rate (%) apoptosis for MOEC treated with IDEX and CDEX. Significant differences between experimental groups were evaluated at ($p^* < 0.05$).

Table 1. List of chlamydial proteins identified in exosomes derived from MOEC-infected cells

***Chlamydia muridarum* proteins in IDEX**			
6-phosphogluconolactonase	Delta-aminolevulinic acid dehydratase	Probable outer membrane protein (PmpB, C, D, F, G and H)	Ribose-5-phosphate isomerase A
Holo-[acyl-carrier-protein] synthase	Heat-inducible transcription repressor HrcA	Glutamyl-tRNA(Gln) amidotransferase subunit A	DNA-directed RNA polymerase subunit beta
Chaperone protein ClpB	Translation initiation factor IF-2	Virulence plasmid protein pGP6-D-related protein	DNA-directed RNA polymerase subunit beta'
Probable ATP-dependent Clp protease ATP-binding subunit	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase (flavodoxin)	Transcription elongation factor GreA	Protein translocase subunit SecA
Probable periplasmic serine endoprotease DegP-like	3-deoxy-manno-octulosonate cytidylyltransferase	DNA gyrase subunit A	Glutamate--tRNA ligase
Chromosomal replication initiator protein DnaA 2	Pyruvate kinase	DNA mismatch repair protein MutL	Uncharacterized protein
Chaperone protein DnaJ	Elongation factor 4	PNP_ Polyribonucleotide nucleotidyltransferase	Transaldolase
DNA polymerase III subunit alpha	Apolipoprotein N-acyltransferase	Primosomal protein N'	tRNA (guanine-N(1)-)-methyltransferase

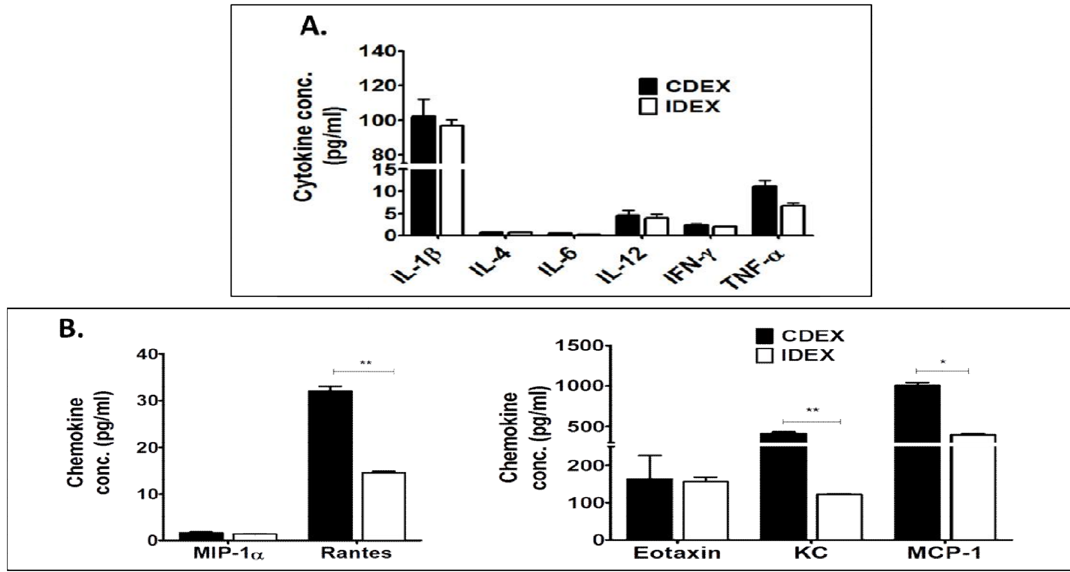

Fig. 5. Th1/Th2 cytokines and chemokines associated with exosomes isolated from *Chlamydia*-infected oviduct epithelial cells

The concentration Th1/Th2 promoting cytokines and chemokines associated with IDEX and CDEX was assessed by cytokine ELISA using the BioRad Bio-Plex cytokine assay kit in combination with the Bio-Plex Manager software. (A) The concentrations of Th1/Th2-promoting cytokines (IL-1β, IL-4, IL-6, IL-12, IFN-γ and TNF-α) associated with exosomes from Chlamydia infected and uninfected epithelial cells are shown as the mean values (+ S.D.) for triplicate cultures for each experiment. (B) The concentrations of chemokines [KC (CXCL1), MCP-1(CCL2), MIP-1α (CCL3), RANTES (CCL5) and Eotaxin (CCL11)] associated with exosomes from Chlamydia-infected and uninfected epithelial cells are shown as the mean values (+ S.D.) for triplicate cultures for each experiment. The results are from two independent experiments. Significant differences between experimental groups were evaluated at ($p^* < 0.05$) or ($p^{**} < 0.01$)

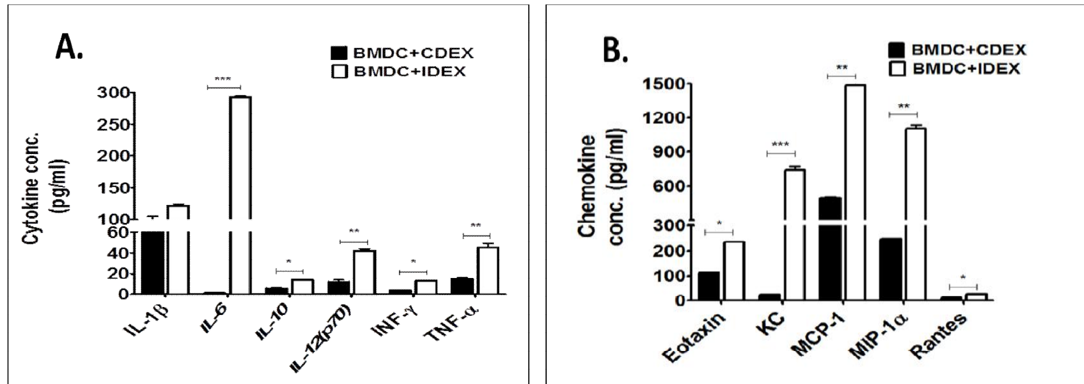

Fig. 6. Activation of cytokine and chemokine secretion by BMDCs pulsed with *Chlamydia* infection-derived exosomes

BMDCs were isolated from mice by established procedures as described in the materials and method section and pulsed for 24 h with exosomes isolated from Chlamydia-infected and uninfected MOEC. Culture supernatants were collected and assayed for levels of Th1/Th2 promoting cytokines and chemokines by cytokine ELISA using the Bio-Plex cytokine assay kit in combination with the Bio-Plex Manager software. (A) The levels of Th1/Th2-promoting cytokines produced by BMDCs after treatment with exosomes are shown as the mean values (± S.D.) for triplicate cultures for each experiment. (B) The concentration of chemokines [KC (CXCL1), MCP-1 (CCL2), MIP-1α (CCL3), Rantes (CCL5) and Eotaxin (CCL11)] produced by BMDCs after treatment with exosomes are shown as the mean values (± S.D.) for triplicate cultures for each experiment. The results are from two independent experiments. Significant differences between experimental groups were evaluated at ($p^* < 0.05$), ($p^{**} < 0.01$) or ($p^{***} < 0.001$)

3.8 MOEC-Derived Exosomes Induce DC Expression of Co-Stimulatory Molecules *in vitro*

To assess the impact of exosomes on DC maturation, *Chlamydia* IDEX and uninfected CDEX were cultured with BMDCs for 24 h and expression of the surface molecules, CD86 and MHC II was evaluated by FACS analysis. The results showed that almost 40% of the cells stained positive for the dendritic cell marker, CD11c. The levels of expression of CD86, MHC II (I-Ab) and CD40 by BMDCs stimulated with exosomes from *C. muridarum*-infected MOEC were significantly higher ($P < 0.05$) than those expressed by cells stimulated with exosomes from uninfected cells (Fig. 7). Also, BMDCs expressed high levels of CD40 irrespective of exosome source. These results indicate that *Chlamydia* infection leads to up-regulation of expression of the co-stimulatory molecules associated with DC activation and maturation.

4. DISCUSSION

Extracellular vesicles (EVs), including exosomes have been reported to play contrasting roles in the regulation of immune responses, such that an immune response may be enhanced or suppressed depending on the cellular origin and functional state of the EV [11]. In this study, we tested the hypothesis that *Chlamydia* IDEX possess immunomodulatory properties capable of stimulating the maturation of mouse BMDCs.

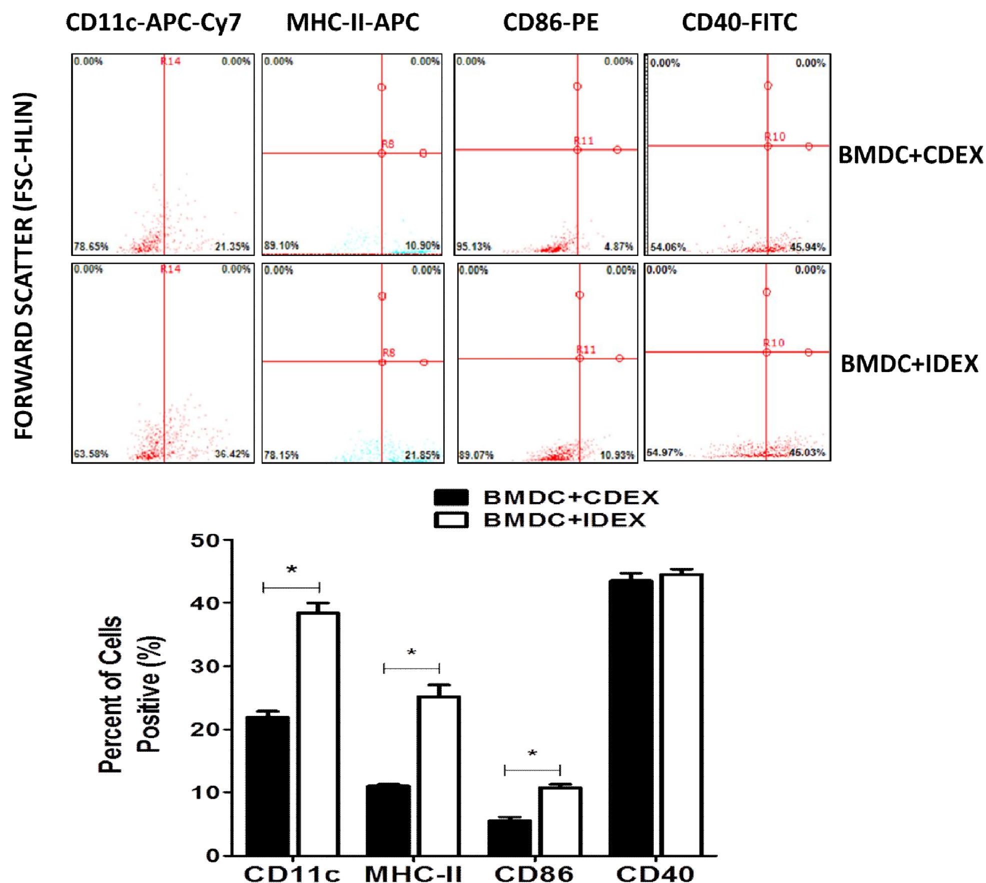

Fig. 7. Activation of BMDC co-stimulatory marker expression

BMDCs were isolated from mice by established procedures as described in the materials and method section. Harvested cells were pulsed for 24 h with exosomes isolated from Chlamydia-infected and uninfected MOEC, stained with conjugated monoclonal antibodies against CD11c, CD40, CD86 and 1Ab or isotype-matched controls, and quantified in triplicate by flow cytometry. The data shows the percentage number of cells expressing the indicated co-stimulatory marker. The results are from two independent experiments. Significant differences between experimental groups were evaluated at ($p^* < 0.05$)

Isolated exosomes, characterized according to the guidelines established by the International Society for Extracellular Vesicles (ISEV), were enriched in the endosome-associated tetraspanin protein, CD63 and heat-shock protein 70 (HSP70) matching the characteristics of typical exosomes [21,22]. We also characterized the concentration of exosomes released from *Chlamydia*-infected cells by NanoSight nanoparticle tracking. The finding that *Chlamydia* infection resulted in a decrease in exosome release by MOEC suggests that *Chlamydia* usurps physiological exosome biogenesis, trafficking or budding machinery. *Chlamydia* is an energy parasite that lacks the full repertoire of metabolic genes and therefore obtains ATP from the cytosol of the host cell to provide energy for metabolic functions [23]. The redirection of host ATP for *Chlamydial* intracellular survival and metabolic activities possibly results in less energy available for exosome processing, and thereby resulting in less exosomes being released from host cells.

Since the content of exosomes determines their effect on the cells with which they come in contact, we next evaluated the protein content of exosomes isolated from *Chlamydia*-infected MOEC. Exosomes from both infected and uninfected cells contained host-derived proteins that are essential in metabolism, protein synthesis, and other regulatory pathways. While some of the host-derived proteins overlapped and were common to both IDEX and CDEX, other proteins were unique to each group. Chlamydial proteins that play direct roles in inflammation and induction of immune responses were highlighted. The Pgp6 protein is encoded by the conserved chlamydial cryptic plasmid, which has been found to play a role in virulence and loss of the plasmid is associated with reduction in oviduct pathologies [24] and inability to induce a TLR2 response [25]. The Pgp6 plasmid-encoded protein lacks homology to other proteins of known function and has been identified as essential for maintaining the growth of *Chlamydia* within host cells. [26]. The packaging of Pgp6 by *Chlamydia* IDEX suggests the ability of exosomes to transport the protein from cell to cell. Several polymorphic membrane proteins (Pmp) were also identified in infection-derived exosomes. The Pmp proteins are a family of membrane bound surface proteins that resemble autotransporters of the type V secretion system some of which are expressed throughout the chlamydial developmental cycle and play an important role in pathogenesis [27, 28]. A number of the Pmp proteins, including PmpD [29] and PmpG [30] are highly conserved and immunogenic and have been shown to be significant vaccine targets.

Following their release from secreting cells, exosomes like other EVs, carry a plethora of molecules that influence their mode of action when they come in contact with target cells. Intracellular pathogens, such as *Chlamydia* are known to directly alter host cells in order to replicate and survive [31]. Thus, the manipulation of host genes, protein expression and protein loading into vesicles may be essential for its growth and survival. The Endosomal Sorting Complexes Required for Transport (ESCRT) sort ubiquitinated proteins into exosomes. Interestingly, the interaction with ESCRT has also been shown to be necessary for the chlamydial lifecycle [32]. This hijacking of the host ESCRT machinery for microbial survival likely accounts for the decrease in the concentration of proteins associated with exosomes derived from *Chlamydia* infected MOEC. It is also possible, however, that an ESCRT-independent mechanism could be involved in the disruption of protein loading into exosomes [33,34]. Our data showed slight decreases in cytokines and significant decreases in the concentration of chemokines, Rantes, KC and MCP-1 associated with IDEX (Fig. 5). This result is in line with our finding that *Chlamydia* infection changes the repertoire of exosomes, and the concentration of certain proteins and possibly other molecules associated with exosomes. Conversely, in Fig. 6, BMDCs stimulated with IDEX significantly increased the secretion of cytokines IL-6, IL-10, IL-12, IFN-γ and TNF-α, and chemokines Eotaxin, KC, MCP-1, MIP-1α and Rantes. The increase in secretion suggests that IDEX are capable of inducing the secretion of cytokines and chemokines by BMDC. The difference in the concentration of cytokines and chemokines shown in Fig. 5 (IDEX) and Fig. 6 (BMDC stimulated with IDEX) indicates that IDEX package small amounts of cytokines and chemokines, but they also package other factors capable of inducing BMDC maturation. It is then possible that exosomes serve as a vehicle for the dissemination of chlamydial antigens to naïve cells. A previous study investigated the azithromycin-enhanced extra-inclusion vesicles in chlamydiae-infected cells and reported the existence of multiple mechanisms for escape of chlamydial antigens from the protected inclusion niche in mucosal epithelial cells [35]. The trafficking of chlamydial

antigens to the endoplasmic reticulum of infected epithelial cells via inclusion membrane vesicles has also been reported [36]. Another study investigated the expression of tissue factor (TF) and release of TF-containing microparticles from an endothelial cell line infected with *C. pneumoniae* [37].

Although EVs derived from antigen-presenting cells have been involved in both innate and acquired immune responses, tumor and stem cell derived EVs have been shown to exert an inhibitory effect on immune responses by carrying immunomodulatory effectors, such as transcriptional factors, non-coding RNA, and cytokines [11]. In addition, stem cell-derived EVs have been reported to impair dendritic cell maturation and to regulate the activation, differentiation, and proliferation of B cells [11]. Based on these findings, we tested the hypothesis that exosomes released from *Chlamydia*-infected cells possess immunomodulatory properties capable of stimulating the maturation of antigen presenting cells, such as dendritic cells. Our results showed exosomes purified from *Chlamydia* infected MOEC stimulated DCs to secrete high levels of proinflammatory cytokines and chemokines, validating our hypothesis. It is likely that the delivery of Pmp proteins to BMDC via IDEX is responsible, at least in part, for the observed secretion of high levels of proinflammatory cytokines and chemokines via NFKB activation. In a previous study, macrophages infected with the intracellular pathogens, *Mycobacterium tuberculosis* and *Toxoplasma gondii* released exosomes containing pathogen-associated molecular patterns (PAMPs) that stimulated naïve macrophages to secrete high concentrations of proinflammatory cytokines [13]. Moreover, genital infection of mice with *C. muridarum* results in the production of high concentrations of proinflammatory cytokines and chemokines [25]. While IFN-γ and IL-12 have been associated with protective immunity against genital chlamydial infection [38], TNF-α production contributes to upper genital tract pathology following vaginal chlamydial infection in mice [39]. TNF-α is a potent chemoattractant for neutrophils and acts as a key transcription factor in the NFKB and MAP kinase pathways triggering inflammation, immune cell proliferation and apoptosis [40].

Further evaluation of the functional significance of treating BMDCs with exosomes showed that BMDC treated with IDEX expressed significantly higher ($p \leq 0.05$) levels of CD86 and MHC-II, molecules known to be associated with DC activation and maturation, compared to BMDCs treated with CDEX. We previously showed that both *Chlamydia* and chlamydial proteins stimulate dendritic cell activation leading to the production of proinflammatory cytokines and expression of co-stimulatory molecules and toll-like receptors [29,41,42]. CD86 (or B7.2) is a protein expressed on antigen-presenting cells (APC) that provides co-stimulatory signals necessary for T cell activation and survival. CD86 works in tandem with CD80 to prime APC for antigen presentation to T cells. It is worthy of note that *Chlamydia* infection of BMDCs or co-culture of BMDCs with UV-irradiated chlamydial elementary bodies upregulates the BMDC expression of CD86 and MHC-II [41]. The MHC is a set of cell surface proteins essential for the induction of acquired immunity. Their main function is to bind to antigens derived from pathogens and display them on the cell surface for appropriate T-cell recognition. The upregulated expression of these surface molecules by DCs following exposure to *Chlamydia* IDEX suggests that exosomes package and transport factors, likely the Pmp proteins, able to activate BMDCs and thus regulate immunity. Immune activation may promote clearance of chlamydial infection or induce pathology.

5. CONCLUSION

This study demonstrates that exosomes released from *Chlamydia*-infected mouse oviduct epithelial cells stimulate dendritic cells to secrete proinflammatory cytokines and activate the upregulated expression of surface molecules associated with dendritic cell maturation. *Chlamydia* infection alters the dynamics of release and protein repertoire of exosomes. The protein content of exosomes from *Chlamydia* infected cells may therefore play a pivotal role in the activation and maturation of dendritic cells. These results highlight the immunomodulatory potential of *Chlamydia* infection-derived exosomes. Further studies will delineate their potential use as immunomodulators or as vaccine delivery vehicles.

CONSENT

It is not applicable.

ETHICAL APPROVAL

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The Institutional Animal Care and Use Committee (IACUC) of Morehouse School of Medicine approved the study protocol (Protocol Number: 16-15).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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