Exosomes Released from Infected Macrophages Contain Mycobacterium avium Glycopeptidolipids and Are Proinflammatory*

Received for publication, March 15, 2007, and in revised form, June 13, 2007 Published, JBC Papers in Press, June 25, 2007, DOI 10.1074/jbc.M702277200

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*This work was supported by Grants AI056979 and AI052439 from the NIAID, National Institutes of Health (NIH). The H4A3, H4B4, 1D4B, and ABL2 monoclonal antibodies developed by J. Thomas August and James E. K. Hildreth were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD, NIH and maintained by the University of Iowa, Dept. of Biological Sciences. The anti-GPL antibodies were a generous gift from Patrick Brennan and John Spenser, Colorado State University of Iowa, Dept. of Biological Sciences. The anti-GPL antibodies were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD, NIH and maintained by the University of Iowa, Dept. of Biological Sciences. The anti-GPL antibodies were a generous gift from Patrick Brennan and John Spenser, Colorado State University. The MyD88 suggesting that released exosomes contain Mycobacterium avium-expressed TLR ligands. Our studies are the first to demonstrate that exosomes isolated from mycobacteria-infected macrophages can induce a proinflammatory response, and we hypothesize that exosomes play an important role in immune surveillance during intracellular bacteria infections.

Mycobacterium avium is a major opportunistic pathogen in HIV-positive individuals and is responsible for increased morbidity and mortality in AIDS patients. M. avium express glycopeptidolipids (GPLs) as a major cell wall constituent, and recent studies suggest that GPLs play an important role in M. avium pathogenesis. In the present study we show that M. avium-infected macrophages release GPLs, which are trafficked from the phagosome through the endocytic network to multivesicular bodies. Prior studies have shown that multivesicular bodies can fuse with the plasma membrane releasing small 50 to 100 nm vesicles known as exosomes. We found that M. avium-infected macrophages release exosomes containing GPLs leading to the transfer of GPLs from infected to uninfected macrophages. Interestingly, exosomes isolated from M. avium-infected but not from uninfected macrophages can stimulate a proinflammatory response in resting macrophages. This proinflammatory response is dependent on Toll like receptor (TLR) 2, TLR4, and MyD88 suggesting that released exosomes contain M. avium-expressed TLR ligands. Our studies are the first to demonstrate that exosomes isolated from mycobacteria-infected macrophages can induce a proinflammatory response, and we hypothesize that exosomes play an important role in immune surveillance during intracellular bacteria infections.

Mycobacteria have a long history as infectious organisms and are the etiologic agents of numerous human diseases. One such disease is caused by the Mycobacterium avium complex (MAC),2 which consists of three mycobacterial species; M. avium, Mycobacterium intracellulare, and Mycobacterium scrofulaceum. MAC is one of the most common opportunistic pathogens found in AIDS patients particularly in individuals with low CD4+ T cells (~50–100 cells/mm3) and is associated with increased morbidity and mortality in these patients (1). M. avium appears to account for the majority of infections in AIDS patients. This may reflect the ubiquitous nature of M. avium in the environment, where it can be found both in water and soil. Moreover, pulmonary MAC infections appear to have increased in the United States over the past half century in non-AIDS patients (2). At present it is unclear if this reflects an increased incidence of infection or increased ability to recognize MAC-infected individuals.

M. avium is an intracellular pathogen that resides within the phagosome of a host macrophage. Upon phagocytosis, M. avium, like other species in the genus Mycobacterium, inhibit phagosome maturation and thereby limit their exposure to hydrolytic enzymes present within a mature phagolysosome (3). This is an essential component of mycobacterial pathogenesis. Pathogenic mycobacteria also modulate macrophage signaling responses and thereby limit the ability of macrophages to produce or respond to immune modulators. The capacity of mycobacteria to alter these macrophage functions requires the expression of specific mycobacterial surface components. For example, mannosylated lipoarabinomannan (LAM) expressed by M. tuberculosis has been shown to both inhibit phagosome maturation and to block interferon-γ-mediated activation of macrophages (4, 5). Although M. avium express a single mannosylated form of mannosylated LAM, the major surface molecule, which accounts for 10% of the total extractable lipid from the cell wall, is the glycopeptidolipids (GPLs) (6). The high expression levels and its presence on the cell surface make the M. avium GPLs an excellent candidate for altering host microbicidal function. Moreover, our data indicates that GPLs are a major M. avium virulence factor as the absence/alteration of
GPL Trafficking in *M. avium*-infected Macrophages

GPL significantly attenuates the virulence of *M. avium* (7, 8). However, it is not clear how GPL can modulate the immune response and therefore accentuate the survival of *M. avium* in the host.

The GPLs consist of a tripeptide amino alcohol core modified with an amide-linked fatty acid, a methylated rhamnose and a 6-deoxytalose (9). The *M. avium* GPLs can be further modified in the length and composition of sugars attached to the 6-deoxytalose residue. The GPLs are non-covalently attached to the cell wall of mycobacteria. Interestingly, studies by Vergne et al. (10) showed that GPLs can interact with host membranes and suggests that GPLs could promote mycobacteria survival by interfering with the membrane-mediated functions of the host cells. There is also evidence showing that GPLs accumulate inside infected cells (11–13). Moreover, due to the extensive movement of membrane within the endocytic pathway, the mycobacterial GPL could be trafficked to other cellular compartments and modulate cellular functions.

In the present study, we hypothesized that the mycobacterial GPL are released from the *M. avium* surface and trafficked throughout the cell as has been shown for mannosylated LAM and other mycobacterial lipids (14). However, our studies indicate that GPLs are more restrictive in their distribution; with the majority of the GPL trafficking to a distinct compartment within the endocytic pathway designated the multivesicular body or MVB. Prior studies have shown that fusion of MVBs within the endocytic pathway designated the multivesicular body or MVB. Prior studies have shown that fusion of MVBs within the endocytic pathway designated the multivesicular body or MVB. Prior studies have shown that fusion of MVBs within the endocytic pathway designated the multivesicular body or MVB. Prior studies have shown that fusion of MVBs within the endocytic pathway designated the multivesicular body or MVB. 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**EXPERIMENTAL PROCEDURES**

*M. avium* Isolation and Culture—BALB/c and C57BL/6 mice were purchased from Harlan (Mutant Mouse Regional Resource Center, Indianapolis IN). TLR2−/− (C57BL/6 background) and TLR4−/− (BALB/c background) mice were purchased from Jackson Laboratory (Bar Harbor, ME). MyD88−/− mice (C57BL/6 background) were generously provided by Dr. Soon-Cheol Hong, Indiana University Medical School.

Bone marrow-derived macrophages (BMMs), used in all experiments, was isolated from 6- to 8-week-old BALB/c mice as previously described (16). The macrophages were used 7–14 days after isolation or frozen after 7 days of culture in freezing media (50% Dulbecco’s modified Eagle’s medium, 40% fetal bovine serum, and 10% endotoxin-tested Me2SO (Sigma). Thawed or fresh macrophages were cultured on non-tissue culture plates for 3–7 days and then re-plated at 3 × 10⁶ cells per 35-mm tissue culture plate. The cells were allowed to adhere for 24 h prior to treatments with mycobacteria or inhibitors. The plates were then incubated at 37 °C in 5% CO₂ for various times. The infection was done for 4 h, and then the media on the cells was replaced with fresh media. All tissue culture reagents were found to be negative for the endotoxin contamination by the E-Toxase assay (Sigma).

The mouse macrophage cell line J774 was maintained at 37 °C in 5% CO₂ in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Invitrogen), 25 mM HEPES, 100 units/ml penicillin, and 100 μg/ml streptomycin (BioWhittaker, Walkersville, MD).

**Bacteria Culture**—To generate the *M. avium* 2151 variant stocks, the bacteria were grown on Middlebrooks 7H11 plates for 20–30 days. The different morphotypes of *M. avium* 2151 were then resuspended in freezing media (Middlebrooks 7H9, 10% glycerol, glucose, oleic acids, Tween 20, and NaCl (Ref. 16). All the stocks were quantitated by the serial dilutions. *M. avium* 101 and *M. avium* A5 were also prepared as described for *M. avium* 2151. Infection assays evaluated by fluorescence microscopy was performed on each stock of mycobacteria to determine the infection ratio required to obtain ~80% of the macrophages infected. The amount of mycobacteria required to obtain this infection rate varied between species and batch preps of mycobacteria. However, the results were consistent across the different mycobacteria-to-macrophage ratios when the infection levels were kept constant.

**Complement Opsonization**—Appropriate concentration of mycobacteria were suspended in macrophage culture media containing 10% horse serum as a source of complement components and incubated for 2 h at 37 °C (16).

**Antibodies and Immunofluorescence Staining**—The immunofluorescence staining and confocal microscopy conducted as previously described (17). Briefly, infected cells were fixed in 2% paraformaldehyde (Sigma) in PBS. Fixed cells were permeabilized with 0.02% Triton X-100 (Sigma) and washed with PBS, 1% bovine serum albumin, and 0.02% gelatin (Sigma). The anti-mouse monoclonal antibody against serovar-specific 1, 2, and 4 glycopeptidolipids, the anti-rat monoclonal antibody against LAMP1 (1D4B, The Developmental Studies Hybridoma Bank, University of Iowa), the anti-rat monoclonal antibody against LAMP2 (ABL2, The Developmental Studies Hybridoma Bank), the anti-glucose-regulated protein (Gnp-78, QED Bioscience Inc, San Diego, CA), the anti-rab-11 (Zymed Laboratories Inc.), and the anti-MHCII (Lab Vision). The bisalkylaminoanthraquinone fluorophore Draq5 (Ex: 646 nm; Em: 681 nm, Axxora), which stains DNA, was used to visualize the nucleus.

**TLC Immunostaining**—The TLC immunostaining of GPLs on TLC was performed according to the method of Watarai et al. (18) with slight modifications. Briefly, alkali-stable GPLs were separated by TLC using Silica Gel-60 plates (EM Science) with chloroform/methanol/water (30:8:1, v/v) as the developing solvent. The GPLs were visualized using 2-naphthol/sulfuric acid as the spray reagent. The dried plate was soaked for 1 min in a 0.02% solution of polysorbactymethacrylate (Glycotech, MD) dissolved in acetone, allowed to air dry, and then blocked by incubation in PBS containing 10% heat-inactivated horse serum at 37 °C for 2 h. The plate was then rinsed with PBS containing 0.1% Tween 20 (washing buffer) and incubated with anti-GPL monoclonal antibody in PBS for 2 h at room temper-
nature. Following this, the plate was washed thrice with washing buffer and probed with horseradish peroxidase-conjugated anti-mouse immunoglobulin G antisera (Amersham Biosciences) at room temperature for 2 h. The plate was washed, and the bound Abs was detected using SuperSignal West Femto enhanced chemiluminescence reagents (Pierce).

Isolation of Exosomes and Apoptotic Vesicles—The fetal calf serum used in the cell culture media for exosome isolation was centrifuged at 100,000 × g, 15 h to remove any contaminating exosomes from the media. J774 cells were infected with *M. avium* 2151 SmT for 4 h and washed extensively to remove extracellular bacteria. After 72 h, the culture medium was collected and centrifuged twice at 300 × g, 10 min to remove whole cells, followed by centrifugation at 1200 × g for 10 min to remove any bacilli. The supernatant from the previous spins was centrifuged at 10,000 × g for 30 min. This was followed by ultracentrifugation at 100,000 × g for 1 h. The resulting pellet following 100,000 × g centrifugation was further purified by sucrose gradient. Apoptotic vesicles were purified by consecutive centrifugations as described previously (19, 20). Briefly, apoptosis was induced by fetal calf serum deprivation (21, 22), and culture supernatant was collected 72 h post-treatment. The culture supernatant was centrifuged at 800 × g (15 min), 1800 × g (15 min), and 25,000 × g (20 min), and the remaining supernatant was spun at 100,000 × g (1 h) to pellet small apoptotic vesicles. Apoptotic vesicles were coated onto latex beads as described below and labeled with antibodies for analysis by flow cytometry or fluorescence microscopy.

Sucrose Density Gradient Centrifugation—For further purification of exosomes, the 100,000 × g pellet was resuspended in 1 ml of 2.5 mM sucrose, 20 mM Heps/NaOH, pH 7.2. A linear sucrose gradient (2–0.25 mM sucrose, 20 mM Heps/NaOH, pH 7.2) was layered on top of the exosomes suspension in a tube, and the sample was centrifuged at 100,000 × g for 15 h. Gradient fractions (7 × 1 ml) were collected from the top of the tube, diluted with 3 ml of PBS, and ultracentrifuged at 100,000 × g for 1 h.

Caspase 3 Inhibitor Treatment and Staining of Apoptotic Cells—Cells were stained for annexin V as per manufacturer’s instructions (Annexin V-FITC apoptosis Detection kit II, Calbiochem, WI). Briefly, cells were washed with binding buffer and stained with annexin V (1:40) for 10 min at room temperature. Cells were washed with binding buffer and stained with propidium iodide (1 μg/ml) and analyzed by confocal microscopy. For caspase inhibition, the caspase-3-specific inhibitor Ac-DEVD-CHO (Calbiochem) was added to macrophages at a final concentration of 50 μM 1 h before infection and maintained for the duration of assay (23–25).

Electron Microscopy—Exosome pellets were resuspended and fixed in phosphate buffer containing 2% glutaraldehyde and then loaded on Formvar/carbon-coated electron microscopy grids. The samples were contrasted in uranyl acetate and viewed with Hitachi H-600 transmission electron microscope.

Labeling with N-Rh-PE—The fluorescent phospholipids analog N-Rh-PE was inserted into the plasma membrane as previously described (26). Briefly, an appropriate amount of lipid was resuspended in ethanol. The ethanol solution was injected with a Hamilton syringe into serum-free Dulbecco’s modified Eagle’s medium while vigorously vortexing. This mixture was then added to the cells already infected with *SmT M. avium* 2151, and they were incubated for 60 min at 4°C. Subsequently, the medium was removed, and the cells were extensively washed with PBS. Labeled cells were fixed after 3 h of incubation at 37°C and immediately analyzed by confocal microscopy.

Analysis of Uninfected Bystander Cells—BMMs were infected with FITC-labeled *SmT M. avium* 2151, and, after 4 h of infection, an equal number of uninfected BMMs labeled with the cell tracker dye 7-amino-4-chloromethylcoumarin (10 μM for 60 min) were added. Cells were examined by fluorescence microscopy for the presence of GPL in uninfected bystander cells.

Coupling of Exosomes or Apoptotic Vesicles to Latex Beads—The purified exosomes or apoptotic vesicles (30 μg) were incubated with 4-μm diameter aldehyde/sulfate latex beads (Interfacial Dynamics) for 15 min at room temperature. This was followed by the dilution with PBS, and binding reaction was continued for another 2 h. The reaction was stopped by addition of 100 mM glycine. Vesicle-coated beads were then washed three times in PBS and stained with specific antibodies.

Western Blot Analysis—For Western blots, equal concentration of protein from cell lysates or exosomes, as quantitated by the Micro BCA Protein Assay, were loaded on 10% SDS-PAGE gels, electrophoresed, and transferred onto polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membranes were probed for INOS (BD Transduction Laboratories) or total p38 (Cell Signaling) as described (16). Blots containing 10 μg of exosome material were probed using antibodies against the *M. tuberculosis* LAM (1:500 dilution) or host hsp70 (R&D, 1/1000 dilution).

Flow Cytometric Analysis of Exosomes and Apoptotic Vesicles—For flow cytometric analysis, beads coated with exosomes were labeled with the following FITC monoclonal antibodies: Grp78 (QED Biosciences Inc., San Diego, CA), LAMP1 (1D4B, The Developmental Hybridoma Bank), and LAMP2 (ABL2, the Developmental Studies Hybridoma Bank), MHCII (MACS, Auburn, CA), CD81 (Biolegend), and CD86 (Biolegend). The exosome-coated beads were also incubated with anti-GPL antibody and probed with FITC-tagged anti-mouse secondary antibody (Biolegend). Apoptotic vesicles were labeled with Annexin V (Calbiochem) or FcγRII/III (eBioscience). Exosomes or apoptotic vesicle-coated beads were incubated for 1 h with each primary antibody (1:100 dilution), followed when necessary by a 30-min incubation with FITC-conjugated secondary antibody (Biolegend) at 1/100 dilution, washed, and analyzed on Cytomics FC500 MPL Flow Cytometer (Beckman Coulter, FL).

Macrophage Incubation with Exosomes or Apoptotic Vesicles—Thawed or fresh macrophages were cultured on non-tissue culture plates for 3–7 days and then re-plated at 1 × 10⁵ cells per well in 24-well tissue culture plate. The cells were allowed to adhere for 24 h prior to the addition of exosomes or apoptotic vesicles. The BMMs in culture media minus antibiotics were incubated with isolated vesicles for 24 h. The culture supernatant was harvested at indicated times for subsequent cytokine/chemokine analysis.
ELISA—The levels of TNF-α and RANTES secreted into the culture medium by exosome-treated macrophages were measured using the ELISA kits from BD Pharmingen and eBioscience, respectively. Culture media collected from the macrophages were analyzed for cytokines according to manufacturer’s instructions, and the cytokine concentrations were determined against standard curves.

**Statistical Analysis**—Data obtained from independent experiments were analyzed by a one-tailed Student t test. Differences were considered significant for \( p < 0.05 \).

**RESULTS**

Our prior studies established that GPLs are *M. avium* virulence factors (7, 8). Moreover, GPLs are shown to accumulate inside the cell and have a long half-life inside a phagosome (11). To determine if GPLs can enter the endocytic pathway, we followed the trafficking of GPL inside infected cells using anti-GPL monoclonal antibodies. The specificity of the antibody was confirmed by TLC immunostaining comparing extracted lipids from *SmT* *M. avium* 2151, which express serotype 2 GPLs to an *Rg* isolate, which lacks GPL expression (28). As shown in Fig. 1A, we observed a single band only in lanes containing lipid extract from *SmT* *M. avium* 2151. This serotype-2 anti-GPL antibody was used to characterize the distribution of GPLs in *SmT* *M. avium* 2151-infected macrophages at 4, 24, 48, 72, and 96 h post infection. The number of cells staining positive for GPLs was quantitated over time and indicated a gradual increase in the percentage

![FIGURE 1](image-url)
of total GPL-positive macrophages between 4 and 96 h post-infection (Fig. 1, C and D). Interestingly, this increase appeared to be due to enhanced staining of uninfected macrophages with the anti-GPL monoclonal antibodies suggesting transfer of GPLs from infected to uninfected cells (Fig. 1 D). To further confirm this cell-to-cell transfer of GPLs, BMMs were infected with M. avium 2151 for 48 h and pulsed with N-Rh-PE. Infected and stained cells were visualized by confocal microscopy. Nuclei were labeled with Draq5. Coincident staining appears yellow in the merged images. Images are representative of three separate experiments (scale = 10 μm).

Analysis of SmT M. avium 2151-infected bone marrow-derived macrophages by fluorescence microscopy revealed significant release and trafficking of glycopeptidolipids. As noted above, mycobacteria are maintained within phagosomes that do not mature to phagolysosomes in non-activated macrophages. However, it is less clear whether released mycobacterial components can enter the late endosomal/lysosomal pathway or whether they can accumulate in specific compartments within infected cells. There are recent reports indicating that some mycobacterial lipids, including phosphoinositolmannoside (PIM) can enter distinct compartments in the endocytic pathway (14). Other mycobacterial lipids such as LAM show a more diffuse staining pattern in M. tuberculosis-infected macrophages (14). Previous studies looking at the trafficking of hydrazide-labeled mycobacterial lipids also reported these distinct membrane organelles carrying mycobacterial lipids, which were subsequently identified as MVBs (29).

To elucidate the route taken by GPL after release from mycobacterial phagosome and to characterize the GPL-positive compartment, we studied the colocalization of GPL with different endocytic markers, including LAMP1 (late endosomes), LAMP2 (late endosomes), and MHC class II (MIIC compartment). Studies with these markers showed significant colocalization with GPL (Fig. 2A and data not shown), indicating that GPLs traffic to one of the late compartments within the BMMs endocytic pathway. Moreover, the GPL-positive compartments were found negative for the Golgi marker Grp78 (Fig. 2A) and transferrin (data not shown). Based on these results and previous published studies, we hypothesized that the GPLs are trafficked predominately to the MVB. To further test our hypothesis, we looked for whether N-rhodamine-phosphatidylethanolamine (N-Rh-PE) colocalized with the GPL. N-Rh-PE when added to cells is efficiently internalized via endocytosis and has been shown to traffic specifically to MVBs in reticulocytes (30) and RAW 264.7 cells (31). N-Rh-PE added to the M. avium 2151-infected cells showed marked colocalization with GPL (Fig. 2B). Together the data indicate that GPLs are released from M. avium-containing phagosomes, enter the endocytic pathway and localize, at least in part, to MVBs within infected cells.

MVBs can be trafficked toward the plasma membrane where they can fuse and release the intraluminal vesicles (ILVs) known as exosomes into the extracellular environment. However, this phenomenon of the transporting, docking, and fusion of MVBs with the plasma membrane is not well understood. Recent studies by Colombo and colleagues demonstrate the recruitment of Rab11 to MVBs and its role in the docking and fusion of MVB with the plasma membrane (31). As shown in Fig. 3, our results indicate significant colocalization between Rab11 and GPL by 48 h post mycobacterial infection. However, at earlier times (24 h) only limited colocalization between

FIGURE 2. GPLs localize to multivesicular bodies in SmT M. avium 2151-infected macrophages. A, cells infected for 48 h with SmT M. avium 2151 were fixed, permeabilized, and probed using antibodies against LAMP-1, MHC class II, GPL, and Grp78. B, BMMs were infected with M. avium 2151 for 48 h and pulsed for 4 h with N-Rh-PE. Infected and stained cells were visualized by confocal microscopy. Nuclei were labeled with Draq5. Coincident staining appears yellow in the merged images. Images are representative of three separate experiments (scale = 10 μm).
Rab11 and GPL was observed. This is in agreement with previously published data showing that Rab11 is involved in the trafficking of MVB toward the plasma membrane but not in the initial biogenesis of MVB (31). However, it is worth noting that many factors in addition to Rab11 play a role in the docking/fusion of MVBs with the plasma membrane.

To determine whether infected macrophages release exosomes upon fusion of MVBs with the plasma membrane and whether the exosomes contain GPLs, we collected culture supernatant from *M. avium* 2151-infected J774 cells 3 days post infection. Parallel experiments were run with the uninfected J774 cells. Exosomes were purified from the culture supernatants by differential centrifugation and sucrose gradient as described (15). The purified exosomes were examined by whole mount transmission electron microscopy. Transmission electron microscopy analysis of exosomes prepared from *M. avium*-infected and uninfected J774 macrophages revealed homogenous populations of ~50–100 nm vesicles (Fig. 4A). Exosome preparations appeared essentially devoid of any cell debris or chromatin material. We also tested other sucrose gradient fractions for vesicles by transmission electron microscopy (data not shown). However, no vesicles in the size range of exosomes were detected in the other isolated fractions.

To further assess our exosome preparation, the protein composition of the isolated vesicles was evaluated. The purified vesicles from *M. avium*-infected and uninfected J774 macrophages were coated on latex/aldehyde beads and probed for proteins known to be present on exosomes including Lamp1 and Lamp2 (lysosomal membrane proteins), MHC1 and MHC2 (antigen presentation), CD81 (tetraspannins), and CD86 (co-stimulatory molecule). As shown in Fig. 4B, the exosomes released from both uninfected and *M. avium*-infected cells were positive for Lamp1, Lamp2, MHC1, MHC2, CD86, and CD81 but lacked the Golgi marker grp78. The presence of hsp70, another marker exosome was also confirmed by Western blotting (Fig. 4C). Also, as expected, the exosomes isolated from *M. avium*-infected but not from uninfected cells contained GPL (Fig. 4B). This further supports our hypothesis that GPLs are trafficked to MVBs and subsequently released extracellularly as part of exosomes. Therefore, this defines one plausible mechanism by which GPLs and possibly other mycobacterial components can be transferred from infected to uninfected cells. Indeed, a similar mechanism has been proposed by David Russell and colleagues for the transfer of PIM2, mycoside B, and other mycobacterial lipids to uninfected macrophages (14). We also found LAM to be present on the exosomes isolated from infected but not uninfected J774 cells (Fig. 4C). To test whether exosomes purified from *M. avium* 2151-infected macrophages can transfer GPLs to uninfected cells, we added 10 μg of purified exosomes to BALB/c BMMs. The macrophages were fixed 24 h post-treatment and probed using the antibody against GPL serovar 2. As shown in Fig. 4D, serovar 2 GPLs could be detected in the macrophages treated with exosomes isolated from *M. avium* 2151-infected but not from uninfected BMMs.

To determine if the trafficking to MVBs and release on exosomes was specific to serovar 2 GPLs, we also studied the trafficking and release of GPLs from BMMs infected with *M. avium* serovar 1 and serovar 4 strains. Localization studies showed significant colocalization between N-Rh-PE and GPLs in macrophages infected with *M. avium* 101 (serovar 1) and *M. avium* A5 (serovar 4), indicating trafficking of these GPLs to MVBs (data not shown). We also added exosomes, purified from the culture supernatants of *M. avium* 101- and *M. avium* A5-infected J774 cells, to BMMs, and in both cases, GPL was detected in the exosome-treated cells (Fig. 4D). This suggests that the trafficking of GPLs to the MVBs and their release on exosomes is independent of GPL serotype.

These data above as well as work by David Russell and colleagues suggest that mycobacterial components, including GPL, LAM, PIM2, trehalose mono- and dimycolate, and phenolic glycolipids among others are released from phagosomes during a macrophage infection (14, 29). Interestingly, some of these lipids have been shown to induce a proinflammatory response when introduced to uninfected macrophages. For example, PIM2 coated on microspheres could induce TNF-α and MCP-1 in interferon-γ primed BMMs or thioglycollate-elicited peritoneal macrophages (32). To determine if exosomes containing these mycobacterial lipids could induce a proinflammatory response, BALB/c BMMs were treated with exosomes, and the culture supernatants were assayed for cytokines and chemokines. As shown in Fig. 5 (A and B), BMMs treated with exosomes from infected cells induced increased levels of TNF-α and RANTES in a dose-dependent manner. This response was specific as exosomes isolated from uninfected macrophages stimulated little or no TNF-α or RANTES.
TLR provides a means to recognize pathogen-associated molecular patterns and play a vital role in stimulating the host immune system (for review see Ref. 33). GPL, PIM2, and other mycobacterial glycolipids have been shown to stimulate macrophages through the TLRs (34, 35). To investigate whether the exosome-mediated macrophage activation was dependent on TLRs and its associated adaptor molecule MyD88, we exposed C57BL/6 wild type (WT), BALB/c WT, TLR2−/− (C57BL/6 background), TLR4−/− (BALB/c background), and MyD88−/− (C57BL/6 background) BMMs to exosomes purified from uninfected and SmT M. avium 2151-infected J774 cells. Macrophage culture supernatants were collected 24 h post exosome treatment and assayed for TNF-α. A significant decrease in TNF-α levels was observed in macrophages lacking TLR2 or TLR4 (data not shown). In addition, macrophages that lacked the MyD88 adaptor molecule were completely unresponsive to exosome treatment in the context of TNF-α production (Fig. 5C). Interestingly, the C57BL/6 BMMs appeared to be more responsive to exosome stimulation relative to BALB/c BMMs (Fig. 5, compare A to C).

Our data suggest that exosomes isolated from M. avium-infected J774 cells are proinflammatory. However, interpretation could be complicated by the potential contamination of our exosome preparation with apoptotic vesicles. Previous studies have shown M. tuberculosis-infected macrophages release apoptotic vesicles with antigen-stimulating activity (19, 20). To evaluate whether apoptotic vesicle were involved in the observe response, we first tested whether apoptosis is occurring in the infected macrophage population by staining cells with annexin V, which stains surface-exposed phosphatidylserine, and propidium iodide, which is retained in late apoptotic cells (36). As shown in Fig. 6A, both M. avium-infected as well as uninfected J774 cells stained negative for both annexin V and propidium iodide. However, J774 cells induced to undergo apoptosis by serum starvation stained positive for both. The infection of the J774 cells was confirmed by LAM staining (data not shown). To further exclude the possibility that apoptotic vesicles are responsible for the proinflammatory activity, we treated infected macrophages with the caspase 3 inhibitor Ac-DEVD-CHO and isolated exosomes from these infected macrophages. The exosomes isolated from M. avium-infected cells showed similar TNF-α and iNOS production whether isolated from Ac-DEVD-CHO-treated or untreated J774 cells (Fig. 6, B and C). Moreover, apoptotic vesicles isolated form serum-starved J774 cells failed to induce TNF-α or iNOS production (Fig. 6, B and C). Finally, we observed minimal staining for FcγRIII and annexin V on exosomes isolated from M. avium-infected J774 cells, similar to the proinflammatory pattern seen in untreated J774 cells.
levels seen on exosomes from uninfected cells. In contrast, apoptotic vesicles isolated from serum-starved J774 cells showed strong staining for both markers (Fig. 6D). Together, the data indicate that exosomes and not apoptotic vesicles are responsible for the proinflammatory activity.

The partially decrease in TNF-α production when using either TLR2- or TLR4-deficient BMMs suggest that the ability of exosomes isolated from M. avium-infected BMMs to stimulate a proinflammatory response is dependent on multiple mycobacterial components. To determine if GPLs is one of the major stimulatory molecules, we isolated exosomes from macrophages infected with GPL-sufficient SmT and GPL-deficient Rg M. avium 2151. Interestingly, the exosome isolated from Rg M. avium 2151-infected macrophages induced TNF-α production comparable to the levels induced in macrophages treated with exosomes from SmT M. avium 2151 (Fig. 7). This suggests that the GPLs present on exosomes are not functioning as major stimulators or that other mycobacterial components present on exosomes from Rg 2151-infected macrophages can compensate for the loss of GPLs.

**DISCUSSION**

The mycobacterial cell wall consists of a highly complex array of distinctive lipids, glycolipids, and proteins; many of which have been shown to have immune modulatory activity. Some of these modulatory functions can be attributed to the interaction of the mycobacteria surface components with the host macrophages during phagocytosis and likely involves pattern recognition receptors such as the TLRs. In addition, many mycobacterial components are also shed during the course of an infection. *In vitro* studies have shown that some of the shed mycobacterial components such as PIM when added to macrophages can induce TNF-α production (37). Trehalose dimycolate is another mycobacterial glycolipid released during an M. tuberculosis infection, which can promote macrophage activation when added to macrophages (38). Interestingly, these glycolipids as well as others have been shown to be transported from the mycobacteria phagosome to other cellular compartments and can be released extracellularly from infected macrophages (14). Our studies with M. avium and GPLs support the observation that mycobacterial glycolipids can be shed from phagosome and transported to other compartments within an infected cell. Moreover, we have found that GPLs are transported predominately to MVBs by 24 h post-infection with M. avium.

MVBs are endosomes that mature by gradually accumulating numerous small vesicles (ILVs) through invagination of the limiting membrane. In most cell types the MVBs are incorporated into the lysosomal compartment. However, in recent years several other functions have been attributed to MVBs, including the down-regulation of signal transduction by plasma membrane receptors (39). Interestingly, MVBs have also been shown to be an alternative secretory pathway initiated by the fusion of MVBs with the plasma membrane (40). This fusion
FIGURE 6. Exosomes and not apoptotic vesicles are responsible for the proinflammatory activity. A, J774 cells were infected with M. avium for 4 h. As controls macrophages were left uninfected or were starved of fetal calf serum to induce apoptosis. Macrophages were fixed and stained for annexin V (green) and propidium iodide (red). Nuclei were stained using Draq5. Fluorescence images are representative of two independent experiments (bar = 10 μm). B, exosomes were prepared from J774 cells infected with M. avium and treated with 50 μM of the caspase-3 inhibitor Ac-DEVD-CHO or left untreated. As a control, apoptotic vesicles were also purified from serum-deprived J774 cells. Exosomes or apoptotic vesicles were added to separate, uninfected J774 cells, and 24 h post-treatment, culture supernatants were assayed for TNF-α by ELISA. Data are representative of two independent experiments and are expressed as mean ± S.D. for duplicate wells. C, cell lysates obtained 24 h post-treatment with exosomes or apoptotic vesicles were analyzed for iNOS expression by Western blot. Total p38 was used as a loading control. Results are representative of two separate experiments. D, sulfate/aldehyde latex beads coated with exosomes or apoptotic vesicles were probed by flow cytometry for annexin V binding or FcγRII/III and the percentage of beads positive for the markers shown. Results are representative of two independent experiments. UI, uninfected J774 cells; SmT, J774 cells infected with SmT M. avium 104.
event leads to extracellular release of the ILVs as exosomes, which can be subsequently endocytosed by the neighboring cells (41).

The release of exosomes through fusion of MVBs with the plasma membrane occurs in many cell types (39, 42). Proteins expressed on exosomes can vary between cell types, but there are some commonalities particularly among antigen-presenting cells (43). B lymphocyte- and DC-derived exosomes display abundant MHC class I and II molecules, co-stimulatory molecules CD80 and CD86, and adhesion molecule ICAM-1 (intercellular adhesion molecule 1) (15). The flow cytometric analysis of exosomes isolated from the M. avium-infected macrophages showed the presence of GPL along with the other markers known to be present on the exosomes, including MHC class I and II and CD86. Moreover, the immunofluorescence studies indicate that GPLs can be transferred from infected to uninfected BMMs through exosomes. These results further support our hypothesis that release of GPLs from an M. avium-infected BMM occurs at least in part through exosomes.

Previous studies by a number of research groups have shown that exosomes can function to modulate an immune response, including immune stimulation and immune suppression (44, 45). DC-derived exosomes also show potent capacity to generate anti-tumor immune responses in vivo (46). Tumor-derived exosomes, which are enriched in tumor antigens, are a novel source of antigens for promoting CTL cross-priming (47). Immature DCs secrete exosomes, which transfer functional MHC-peptide complexes to other DCs (48). Consistent with a stimulatory role in the immune response, exosomes secreted by DCs can elicit T-cell activation in vivo (49). Exosomes secreted by epithelial cells, despite their lack of co-stimulatory molecules, have also been shown to induce humoral immune responses (27).

Prior studies suggest that a number of other mycobacterial factors, including 19-kDa protein, mycolates, and phenol glycolipids, are present on small 50–100 nm extracellular vesicles, which likely constitute exosomes (29). Although the immunogenic properties of purified mycobacterial lipids has been well documented, their role during an infection and how they may interact with cells when released from the mycobacteria is less understood. Studies by David Russell and colleagues have shown that some of the mycobacterial lipids present on these vesicles can elicit an immune response (32). However, these studies were carried out with lipids coated on microspheres and therefore do not reflect the natural route of their release. We initiated studies to define the immunogenicity of mycobacterial lipids in the context of a natural infection by isolating exosomes from mycobacteria-infected macrophages. Interestingly, our studies showed that exosomes isolated from infected cells induced significant TNF-α and RANTES production by BMMs.

Mycobacterial products released by the sequestered bacilli, including GPLs, LAM, PIM, and the 19-kDa lipoprotein can contribute to macrophage activation through pattern recognition receptors. The initial recognition of these mycobacterial pattern-associated molecular patterns by the innate immune system through TLR, and other pattern recognition receptors can contribute to triggering of the host immune response. Because exosomes are believed to contain pattern-associated molecular patterns, which can induce a proinflammatory response, it was possible that the observed macrophage response to exosomes was dependent on TLRs. To test this hypothesis, we treated TLR2−/−, TLR4−/−, or MyD88−/− murine macrophages with exosomes derived from M. avium-infected macrophages. The induction of proinflammatory response was found to be dependent on the TLRs as illustrated by the complete failure of MyD88-deficient macrophages to respond to exosomes.

Our results indicate that the mycobacterial components present on exosomes are released extracellularly and can interact with the neighboring cells through Toll-like receptors. Following exposure to the exosomes these uninfected cells respond by producing proinflammatory molecules like TNF-α and RANTES. This illustrates a previously undefined mechanism by which cells infected with intracellular pathogens can release pattern-associated molecular patterns to “alert” the immune system of an underlying infection and could function in conjunction with cytokines to promote a protective immune response. Importantly, we show that the proinflammatory response is not due to apoptotic vesicles, which have also been shown to contain immunogenic mycobacterial components (19).

Although our studies demonstrate a role for exosomes in promoting macrophage activation, we do not yet know the specific mycobacterial ligand(s) responsible for this response. Our studies with exosomes isolated from GPL-deficient Rg M. avium 2151 indicate that the lack of GPL on exosomes does not increase or decrease the stimulatory activity of the exosome. This suggested that either GPL does not play an important role in macrophage stimulation by exosomes or perhaps exosomes contain more than one ligand that is sufficient to induce macrophage activation. Therefore, the lack of GPL may be compensated by other mycobacterial factors such as PIMs, 19-kDa lipoprotein, or the mycolates. Future studies will be needed to address the mycobacterial pattern-associated molecular patterns present on exosomes that promote the proinflammatory response and their potential role in modulating the host response in vivo.

In summary, our studies demonstrate that GPLs are released from the mycobacteria-containing phagosome and are selec-
tively trafficked to the MVBs. Consistent with the recently proposed role for MVBs, we found that exosomes carrying GPL are released from the mycobacteria-infected cells. Our studies show for the first time that exosomes isolated from mycobacteria-infected macrophages can induce a proinflammatory response and thereby modulate the overall immune response of the host.

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