RESEARCH ARTICLE

Proteomic analysis identifies highly antigenic proteins in exosomes from *M. tuberculosis*-infected and culture filtrate protein-treated macrophages

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Exosomes are small 30–100 nm membrane vesicles released from hematopoietic and nonhematopoietic cells and function to promote intercellular communication. They are generated through fusion of multivesicular bodies with the plasma membrane and release of interluminal vesicles. Previous studies from our laboratory demonstrated that macrophages infected with *Mycobacterium* release exosomes that promote activation of both innate and acquired immune responses; however, the components present in exosomes inducing these host responses were not defined. This study used LC-MS/MS to identify 41 mycobacterial proteins present in exosomes released from *M. tuberculosis*-infected J774 cells. Many of these proteins have been characterized as highly immunogenic. Further, since most of the mycobacterial proteins identified are actively secreted, we hypothesized that macrophages treated with *M. tuberculosis* culture filtrate proteins (CFPs) would release exosomes containing mycobacterial proteins. We found 29 *M. tuberculosis* proteins in exosomes released from CFP-treated J774 cells, the majority of which were also present in exosomes isolated from *M. tuberculosis*-infected cells. The exosomes from CFP-treated J774 cells could promote macrophage and dendritic cell activation as well as activation of naïve T cells *in vivo*. These results suggest that exosomes containing *M. tuberculosis* antigens may be alternative approach to developing a tuberculosis vaccine.

Keywords:
Exosomes / Macrophages / Microbiology / *Mycobacterium*

1 Introduction

The release of bioactive vesicles by one cell and its interaction with others expands the mechanisms of intercellular communication. Exosomes are one type of bioactive vesicle that has received considerable attention, as they are derived from the release of intraluminal vesicles upon fusion of multivesicular bodies (MVBs) with the plasma membrane and therefore contain constituents present in the endocytic network [1, 2]. Moreover, exosomes are functionally diverse and known to be released from numerous cell types including cells of hematopoietic and nonhematopoietic origin. Exosome release was originally observed in maturing reticulocytes and appears to function in eliminating

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Abbreviations: BMDC, bone marrow-derived dendritic cells; **CFP,** culture filtrate protein; **CFSE,** carboxy fluoroscein succinimidyl ester; **DC,** dendritic cell; **FDR,** false discovery rates; **FP,** false positive; **MLN,** mediastinal lymph nodes; ***M.tb,*** *Mycobacterium tuberculosis*; **MVB,** multivesicular body; **UT,** untreated

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nonessential proteins from the maturing red blood cells. Later studies demonstrated that exosomes derived from B cells and other antigen-presenting cells were enriched for MHC class II, as well as co-stimulatory molecules like CD86 and could present antigens to sensitized T cells [3]. More recent studies have shown that exosomes carrying tumor antigens can promote antigen-specific T-cell activation and tumor rejection *in vivo* [4].

To better understand exosome function, immunoelectron microscopy, Western blot and mass spectrometry have been used to define exosome composition. These studies have identified common as well as cell-type specific proteins [5]. Moreover, the composition of the excreted exosomes can change when the cell of origin is exposed to different environmental conditions or to foreign material [2]. We found that exosomes released from *Mycobacterium*-infected macrophages carry mycobacterial antigens such as the 19 KDa lipoprotein and lipoarabinomannan [6]. The exosomes, which were released from mycobacterial-infected macrophages, induced a MyD88-dependent proinflammatory response in naïve macrophages [6].

Interestingly, when exosomes isolated from *M. bovis* BCG infected macrophages were administered intra-nasally into naïve mice, they stimulated antigen-specific CD4+ and CD8+ T cells, suggesting that the exosomes contain a number of mycobacterial antigens [7]. However, the mycobacterial proteins present in isolated exosomes which could promote T-cell activation were not defined in this study. Therefore, to address this question, we established the first extensive proteomic analysis of exosomes isolated from *M. tuberculosis* H37Rv-infected macrophages. Our analysis identified 41 mycobacterial proteins including some well-known antigenic proteins such as ESAT-6 (Rv3875), Ag85 complex (Rv3804c, Rv1886c, Rv0129c), MPT64 (1980c) and MPT63 (1926c). We also found that J774 cells incubated with *Mycobactrium tuberculosis* (*M.tb*) culture filtrate proteins (CFPs) released exosomes containing a number of the highly antigenic *M.tb* proteins. Moreover, these exosomes could stimulate macrophage and dendritic cell (DC) activation *in vitro* and activate naïve CD4+ and CD8+ T cells *in vivo*, suggesting a possible use for these exosomes as a TB vaccine.

2 Materials and methods

2.1 Bacterial culture, complement opsonization and *M. tuberculosis* H37Rv infection or CFP treatment of J774 cells

M.tb H37Rv stocks were generated as described [8]. Appropriate concentrations of mycobacteria were suspended in DMEM (Gibco) containing 10% normal horse serum as a source of complement components, followed by a 2 h incubation at 37°C. The mouse macrophage cell line J774 was maintained at 37°C in 5% CO_2 in DMEM supplemented with 10% FBS (Life Technologies), 25 mM Na^+-HEPES, 100-U/mL penicillin and 100 μg/mL streptomycin (BioWhittaker, Walkersville, MD). Infection of macrophages with complement-opsonized BCG was carried out 3–4 h after seeding the cells and infected for 4 h as described [8]. For CFP treatment, J774 cells were seeded in T175 tissue culture flasks and treated with CFP (20 μg/mL) in 20 mL of DMEM supplemented with 10% exosome-free FCS [6]. After 20 h, culture supernatant was harvested for exosome purification.

2.2 Isolation and cultivation of bone marrow-derived DCs

Bone marrow-derived dendritic cells (BMDCs) were harvested from the bone marrow of 6- to 8-wk-old Balb/C mice as described previously [7]. Mature BMDCs were harvested and stored at −140°C. To evaluate the IL-12p40 secretion by BMDCs following exosome treatment, BMDCs were thawed and seeded in 24-well plates at 1×10^5 cells/well in 0.5 mL of exosome-free culture media for 4 h and then treated with CFP exosomes or untreated (UT) exosomes at different concentrations. After 24 h, cell-free culture supernatant was harvested for quantification of IL-12p40 by ELISA according to the manufacture's instructions (Endogen, Woburn, MA).

2.3 Isolation and purification of exosomes

Exosomes were isolated and purified as described previously [9]. Culture supernatants were collected 72 h post-infection from *M.tb* or from uninfected J774 cells. For purification of exosomes from CFP-treated and UT J774 cells, culture supernatants were collected 20 h post-treatment. Exosomes concentration was approximately 2 μg/mL culture supernatant. Culture supernatants were centrifuged at $300 \times g$ for 10 min at 4°C to remove debris. Cleared culture supernatants were filtered through 0.22-μm polyethersulfone filters (Corning, NY, USA). Exosomes are 30–90 nm in diameter and filter freely through 0.22 μm filters. Filtered supernatants were centrifuged at $10\,000 \times g$ for 30 min at 4°C and again at $100\,000 \times g$ for 1 h to pellet the exosomes. Exosomes were further purified using a sucrose gradient where the $100\,000 \times g$ pellet was resuspended in 0.5 mL of 0.25 M sucrose (20 mM HEPES/NaOH, pH 7.2) and layered on top of the linear sucrose gradient (0.25–2.0 M sucrose, 20 mM HEPES/NaOH, pH 7.2). The resulting sample was centrifuged at $100\,000 \times g$ for 15 h. Gradient fractions (7×1.5 mL) were collected from the top of the tube, diluted with 10 mL of PBS and ultracentrifuged at $100\,000 \times g$ for 1 h. The purified exosomes from *M.tb* infected (*M.tb* exosomes), CFP-treated (CFP exosomes), uninfected cells (UI exosomes) or UT cells (UT exosomes) were resuspended in

PBS to a protein concentration of approximately 0.1–0.5 mg/mL as determined by a Micro BCA Assay (Pierce, Rockford, IL). Exosomes were sterilized by filtration through 0.22 μM syringe sterile filter (Millipore, Bedford, MA) and stored at −80°C.

2.4 Immunization of mice with CFP exosomes

Exosomes were administered to mice in three doses at 2 wk intervals *via* an intranasal route. At each time point, C57Bl/6 mice were lightly anesthetized with isoflurane and 30 μL volume containing 25 μg of exosomes from CFP-treated or untreated macrophages, ±30 μg CpG-ODN (Invitrogen) adjuvant in PBS were administered drop-wise to the external nares of mice (15 μL *per* nostril) using a micropipette. As a positive control, BCG was administered as a single dose of 1×10^6 CFU/mouse in 30 μL of PBS *via* intranasal route as described above. Negative control groups received only CpG-ODN adjuvant in PBS.

2.5 Isolation of lymphocytes from spleen and mediastinal lymph node

Spleens from BCG-infected mice were isolated after 30 days of infection. For isolation of spleen and mediastinal lymph nodes (MLN) from exosome-treated or UT animals, mice were sacrificed 2 wk after the final immunization and spleens and MLNs were aseptically removed. The spleens were then perfused with RPMI-1640 using 10 mL syringe fitted with 26G needle to obtain a single cell suspension of splenocytes. MLN cells were separated into single cells using the pistol of a 3 mL sterile dispovan syringe. The splenocytes and MLN cell suspension were then centrifuged at $300 \times g$ for 10 min. The RBCs were lysed by hypotonic shock using 3 mL ACK lysis buffer for 5 min. The cells were then washed thrice with RPMI 1640 to remove lysed RBCs and lysis buffer.

2.6 Isolation of lung lymphocytes

Lung lymphocytes from exosome treated and UT as well as infected mice were isolated as described previously [7] with minor modifications. Pooled lungs were homogenized in 6 mL sterile complete RPMI-1640 in a glass homogenizer and incubated at 37°C for 2 h with type IV collagenase (125–150 U/mL) and DNase I (50–60 U/mL). Digested lung tissue was then pressed through a 70 mm nylon mesh and centrifuged at $300 \times g$ for 10 min. The cell pellet was then resuspended in complete RPMI-1640 media. The cell suspension was layered on a histoplaque and density centrifugation was carried out for 10 min at $300 \times g$ at room temperature. Lung mononuclear cells were obtained from the interface, washed, counted and resuspended at a concentration of 10^6 cells/mL in complete RPMI-1640 media.

2.7 Analysis of memory phenotype ($CD44_{hi}$ and $CD62L_{low}$) on CD4+ and CD8+ T cells

Tri-color flow cytometry was done for analysis of memory phenotype. Two weeks after the final immunization or 30 days after BCG infection, lungs, MLNs and spleen cells were isolated from exosomes-treated and UT animals. Cells were washed with FACS wash buffer and incubated on ice with panel of antibodies: antiCD4-PE, antiCD8-PE, antiCD44-PECy5 and antiCD62L-FITC at the concentration of 0.5 mg/million cells in 50 mL of FACS buffer for 30 min at 4°C. Cells were then washed and fixed with 2% paraformaldehyde and analyzed by flow cytometry. All the analysis was done with an acquisition of 100 000 events. Gating of CD4+ and CD8+ cells in the lymphocyte-rich regions was based on forward-angle light scatter and log Phycoerythrin fluorescence (FL2-H) and then the expression of CD62L (FL1-H) and CD44 (FL4-H) on CD4+ or CD8+ gated lymphocytes was analyzed. Cells stained with isotype control antibodies were analyzed at each time point.

2.8 Electron microscopy

Exosome pellets were resuspended and fixed in PBS containing 2% glutaraldehyde and then loaded onto Formar/carbon-coated electron microscopy grids. The samples were contrasted with uranyl acetate to visualize membrane, and viewed with a Hitachi H-600 TEM microscope (Hitachi, USA).

2.9 Detection of mycobacterial components associated with exosomes

Mycobacterial components in exosomes were detected by Western blot. Briefly, 8 μg of exosomes were loaded into each well onto SDS-PAGE gels, electrophoresed and transferred onto PVDF (Millipore). The membranes were probed with appropriate dilution of monoclonal antibodies against GroES (SA-12), KatG (IT-42), 19 kDa lipoprotein LpqH (IT-12), CFP10 (α-CFP10), Ag85 (α-Ag85), GlnA (IT-58) and SodA (CS-18) of mycobacterial antigens. This was followed by incubation with HRP-conjugated secondary antibodies (1:25 000), and detected using an enhanced chemiluminescence kit (Roche Diagnostic, USA). All antibodies against mycobacterial components were obtained from Colorado State University, Colorado, USA, under TB Vaccine Testing and Research Materials Contract (NIH-NIAID NO1-A1-40091).

2.10 *In-vitro* T-cell assays

Spleens were aseptically removed from CFP treated and control mice 2 wk post-immunization. Single cell suspensions of splenocytes were generated as described above. The

splenocytes suspension was then centrifuged at $300 \times g$ for 10 min. The RBCs were lysed for 5 min in ACK lysis buffer. The cells were then washed thrice with RPMI 1640 to remove lysed RBCs and ACK buffer. Splenocytes from CFP treated and control mice were seeded in 24-well tissue culture plates at 1×10^6 cells/well in 500 μL of RPMI-1640 (supplemented with 10% heat-inactivated FCS, 25 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU/mL penicillin and 100 μg/mL streptomycin) in the presence or absence of CFP exosomes, UT exosomes and CFP at different concentrations for 72 h. Phytohemagglutinin (PHA) (5 μg/mL) was used as a positive control for cell viability. Cell-free culture supernatant was harvested after 72 h, from which IFN-γ and TNF-α levels were analyzed by ELISA, as *per* the manufacturer's instructions using ELISA kit (Bioscience).

2.11 Flow cytometric analysis for carboxy fluoroscein succinimidyl ester dilution and intracellular IFN-γ

For carboxy fluoroscein succinimidyl ester (CFSE) labeling, splenocytes, MLN lymphocytes and lung lymphocytes from CFP exosomes +/−CpG adjuvant, and UT exosomes treated mice were labeled with CFSE (2.5 μM), and 1×10^6 cells/well were seeded in 24-well tissue culture plates in 500 μL of RPMI-1640 (supplemented with 10% heat-inactivated FCS, 25 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU/mL penicillin and 100 μg/mL streptomycin) in the presence or absence of CFP (10 μg/mL). Phytohemagglutinin (5 μg/mL) was used as a positive control for cell viability. The cells were incubated for 72 h at 37°C, 5% CO_2. Dilution of CFSE after 72 h was analyzed by flow cytometry.

For intracellular IFN-γ, lymphocytes from spleen, lungs and MLN were treated with CFP (10 μg/mL) for a total of 12 h with the final 6 h in the presence of 2 μM monensin. These cells were first stained with anti-CD4-PE, and anti-CD8-PE, fixed with 2% paraformaldehyde, permeabilized with permeabilization buffer (Biolegend), and then stained with anti-IFN-γ-FITC (Biolegend) according to the manufacturer's instructions. The labeled cells were washed with FACS buffer and analyzed by flow cytometry.

2.12 Tryptic digest of exosomal proteins for proteomic analysis

To determine the most optimal method for the analysis of exosomal proteins, multiple methods were used, including in-solution digestion and in-gel fractionation prior to digestion. As summarized in Table 1, the in-gel methodology generated the most abundant high-quality spectra. For this reason, exosomes purified from irradiated-*M.tb*-infected and CFP-treated J774 cells were only processed *via* this protocol.

2.12.1 In-solution analysis

A total of 10 μg of each sample was added to breaking buffer to a final volume of 500 μL. Breaking buffer is composed of 50 mL of PBS, pH 7.4 (Gibco) with 1 mM EDTA, 30 μL DNase stock (1 mg/mL) and 30 μL RNase stock (1 mg/mL). Lysis of exosomes was achieved by sonication on ice with a micro-tip probe using five rounds of 60 s pulses with 90 s of rest between. The resultant lysates were denatured in 6 M guanidine hydrochloride, reduced with 10 mM DTT and alkylated in 100 mM iodoacetamide. After overnight micro-dialysis in 10 mM ammonium bicarbonate, the samples were digested with trypsin (1:50, trypsin:lysate) in 10% ACN overnight at 37°C. Digests were dried and resuspended in LTQ loading buffer (3% ACN, 0.1% formic acid in H_2O). All digests were performed in deplasticized tubes to reduce plastic polymer contamination. Briefly, tubes were deplasticized by incubation with 600 μL 60% ACN, 0.1% TFA for 1 h at room temperature, three times.

2.12.2 In-gel analysis

In total, 10 μg of each sample was separated on a 4–20% gradient Tris-Glycine 8 cm × 8 cm mini-gel (Invitrogen). Each lane was fractionated into ten subsamples and subjected to overnight digestion with 0.5 μg of trypsin at 37°C. Digests were stopped with 1/10th total volume of 10% TFA. Peptides were extracted from gel slices using two rounds of 100 μL of extraction solution (60% ACN, 0.1% formic acid) at 37°C for 40 min. The supernatant was then dried and resuspended in LTQ loading buffer (3% ACN, 0.1% formic acid in H_2O).

2.13 MS

An aliquot (1 μL; approximately 50–100 ng/μL) of each sample was used for analysis by MS. Peptides were purified and concentrated using an on-line enrichment column (Agilent Zorbax C18, 5 μm, 5 × 0.3 μm column, Agilent 1100 nanoHPLC). Subsequent chromatographic separation was performed on a reverse-phase nanospray column (Zorbax C18, 5 μm, 75 μm id × 150 mm column). Samples were eluted into a LTQ linear ion trap (Thermo Scientific) using a flow rate of 300 nL/min with the following gradient profile: 0% B for 0–5 min, 0–15% B for 5–8 min, 15–55% B for 8–98 min and 55–100% B for 98–103 min (A = 3% ACN, 0.1% formic acid, B = 100% ACN, 0.1% formic acid). This elongated method has been optimized to separate complex samples, such as whole cell lysate. Mass spectra are collected over a m/z range of 200–2000 Da using a dynamic exclusion limit of two MS/MS spectra of a given peptide mass for 30 s (exclusion duration of 90 s). Compound lists of the resulting spectra were generated using Bioworks 3.3 software (Thermo Scientific) with an intensity threshold of 5000 and 1 scan/group. All samples were run in triplicate.

Table 1. List of proteins identified in exosomes from H37Rv-infected J774 cells by LC-MS-MS

Rv Number	Protein name	# of Unique peptides identified[a]				Secreted?[b]	References[c]
		L	L^{ex}	*G*	G^{ex}		
Rv0129c	Antigen 85-C	0	0	2	4	Predicted/experimental	1, 2, 3, 4
Rv0211[d]	PckA	0	0	1	0	Experimental	4
Rv0234c	GabD1	0	0	2	0	Nd	–
Rv0315	β-1,3-Glucanase precursor	0	0	2	3	Predicted	1
Rv0350	DnaK	0	0	0	2	Predicted/experimental	1, 2, 3, 4
Rv0462	LpdC	0	0	3	3	Predicted/experimental	1, 2, 3, 4
Rv0577	TB27.3	0	0	2	0	Experimental	2
Rv0798c[d]	Cfp29	0	0	1	0	Experimental	2
Rv0896	GltA2	0	0	2	4	Experimental	4
Rv0934	PstS1	0	0	2	3	Predicted/experimental	1, 2, 4
Rv1174c[d]	TB8.4	0	1	0	0	Predicted	1
Rv1270c[d]	LprA	0	0	1	0	Predicted	1
Rv1448c	Tal	0	0	2	2	Predicted/experimental	1, 4
Rv1827[d]	Cfp17	0	0	1	1	Predicted/experimental	1, 2, 4
Rv1837c	GlcB	0	0	2	6	Predicted	1, 4
Rv1860	Mpt32/Apa	0	2	7	4	Predicted/experimental	1, 2, 4
Rv1876	BfrA	0	1	0	1	Predicted	1
Rv1886c	Antigen 85-B	0	2	4	6	Predicted/experimental	1, 2, 3, 4
Rv1906d[d]	Rv1906c	0	1	0	0	Predicted	1
Rv1908c	KatG	0	0	3	2	Predicted/experimental	1, 2, 4
Rv1926c	Mpt63	0	2	3	2	Predicted/experimental	1, 2, 3, 4
Rv1932	Cfp20/Tpx	0	0	2	0	Predicted/experimental	1, 2, 3, 4
Rv1980c	Mpt64	0	5	5	6	Predicted/experimental	1, 2, 3, 4
Rv2031c	HspX	0	4	1	4	Experimental	2, 3, 4
Rv2110d[d]	PrcB	0	0	1	0	Predicted/experimental	1, 2
Rv2220	GlnA1	0	0	6	8	Predicted/experimental	1, 2, 4
Rv2244	AcpM	0	3	1	4	Predicted/experimental	2, 4
Rv2376c	Cfp2	0	4	0	1	Predicted/experimental	1, 2, 4
Rv2467	PepN	0	0	3	0	Experimental	4
Rv2681[d]	Alanine-rich protein	0	1	0	1	Nd	
Rv2780	Ald	0	0	0	5	Experimental	2, 3, 4
Rv2878c[d]	Mpt53	0	0	1	0	Predicted/experimental	1, 2, 4
Rv3036c	TB22.2	0	0	1	1	Predicted/experimental	1, 2
Rv3248c	SahH	0	0	4	5	Predicted/experimental	1, 2, 4
Rv3310[d]	SapM	0	0	1	0	Predicted	1
Rv3418c	GroES	0	5	3	2	Predicted/experimental	1, 2, 3, 4
Rv3587c[d]	Rv3587c	0	0	1	0	Predicted	1
Rv3803c	Mpt51	0	0	3	2	Predicted/experimental	1, 2, 3, 4
Rv3804c	Antigen 85-A	0	0	4	4	Predicted/experimental	1, 2, 3, 4
Rv3841	BfrB	0	0	2	4	Predicted	1
Rv3875	ESAT-6	0	2	0	2	Predicted/experimental	1, 2

a) *L*, liquid digest; *L*ex, liquid digest plus exclusion list; *G*, in-solution digest; *G*ex in-solution digest plus exclusion list.
b) Predicted, contains a signal sequence; Experimental, identified in culture filtrate by proteomic method; Nd, no data published related to secretion.
c) References: 1, [38]; 2, [39]; 3, [40]; 4, This study, Table 2.
d) Single peptide identifications, manual validation.

During the initial injections, all ions were collected; however, due to the abundance of the mouse exosome proteins, an exclusion list was designed to eliminate the collection of the top 25 most abundant ions. This allowed for the collection and fragmentation of less abundant ions that would otherwise be suppressed. A comparison of the peptides identified from the in-solution and in-gel trypsin digests, with and without the use of the exclusion list, is summarized in Table 1. Although in many cases the exclusion list provided the identification of additional peptides, it rarely increased the number of protein identifications. Therefore, in subsequent experiments using exosomes purified from irradiated-*M.tb*-infected and CFP-treated J774 cells, the exclusion list was not used.

Database searching: All tandem mass spectra (.raw files) were extracted by LCQ_DTA.exe (www.matrixscience.com)

for subsequent loading into the MASCOT (Matrix Science, London, UK; version 2.1) [10] MS/MS search form or into BioworksBrowser (version 3.3.1 SP1) for subsequent analysis with SEQUEST (Thermo Finnigan, San Jose, CA; Release 27, rev12). MASCOT was set up to search the a customized database (on 05/06/08) composed of the *M.tb* H37Rv FASTA (Genbank accession ♯;AL123456, R9, 3991 entries) concatenated to the IPI Mouse database (International Protein Index – http://www.ebi.ac.uk/IPI/IPIhelp.html) which contains a total of 222 498 entries. SEQUEST and X!Tandem (Version 2007.01.01.1) were set up to search only the *M.tb* H37Rv database listed above. All searches were performed assuming trypsin digestion, with a fragment ion mass tolerance of 1.00 Da and a parent ion tolerance of 3.00 Da. Oxidation of methionine (+16), iodoacetamide derivative of cycteine (+57) and the acrylamide adduct of cysteine (+70) were specified as variable modifications.

2.14 Calculation of false discovery rates

In order to calculate the false discovery rate (FDR) for each of the data sets, all .raw files were searched against a decoy database. Decoy databases contain a reversed version of the proteins included in either the customized database composed from the *M.tb* H37Rv FASTA file (for SEQUEST), as well as concatenated to the reverse IPI Mouse database (for MASCOT). The resulting files were pooled in Scaffold and the analysis was identical to that described above. The number of proteins matched to the reverse database is as follows: Table 1: 1 in the "L^{ex}" sample, 1 in the "G" sample, 2 in the "G^{ex}" sample and for Table 2: 2 matches in the CFP exosome samples; these numbers are equal to the number of false positives (FPs). The equation for the FDR is FP/(FP+TP), where TP is the number of total positives found in each data set (13 for L^{ex}, 33 for G, 28 for G^{ex} and 29 for the CFP). Therefore, the FDRs are 7.1, 2.9, 6.9 and 6.5% for the data sets L^{ex}, G, G^{ex} and CFP, respectively.

Table 2. List of proteins identified in exosomes from CFP-treated J774 cells

Rv number	Protein name	♯ of Unique peptides identified[a)]	Secreted?[b)]	References[c)]
Rv0129c	Antigen 85-C	3	Predicted/experimental	1, 2, 3
Rv0211[d)]	PckA	1	Nd	–
Rv0350	DnaK	3	Predicted/experimental	1, 2, 3
Rv0363c	**Fba**	3	Predicted/experimental	1, 2, 3
Rv0462	LpdC	2	Predicted/experimental	1, 2, 3
Rv0896	GltA2	2	Nd	–
Rv0934	PstS1	5	Predicted/experimental	1, 2
Rv1355c	**MoeY**	3	Nd	–
Rv1448[d)]	Tal	1	Predicted	1
Rv1827	Cfp17	4	Predicted/experimental	1, 2
Rv1837	GlcB	2	Predicted	1
Rv1860	Mpt32/Apa	5	Predicted/experimental	1, 2
Rv1886c	Antigen 85-B	9	Predicted/experimental	1, 2, 3
Rv1908c	KatG	2	Predicted/experimental	1, 2
Rv1926c	Mpt63	5	Predicted/experimental	1, 2, 3
Rv1932	Cfp20/Tpx	3	Predicted/experimental	1, 2, 3
Rv1980c	Mpt64	10	Predicted/experimental	1, 2, 3
Rv2031c	HspX	7	Experimental	2, 3
Rv2220	GlnA1	6	Predicted/experimental	1, 2
Rv2244	AcpM	3	Experimental	2
Rv2306A[d)]	**Rv2306A**	1	Nd	–
Rv2376c	Cfp2	5	Predicted/experimental	1, 2
Rv2467	PepN	2	Nd	–
Rv2780	Ald	4	Experimental	2, 3
Rv2878c	Mpt53	3	Predicted/experimental	1, 2
Rv3248c	SahH	2	Predicted/experimental	1, 2
Rv3418c	GroES	5	Predicted/experimental	1, 2, 3
Rv3793	**EmbC**	2	Nd	–
Rv3804c	Antigen 85-A	8	Predicted/experimental	1, 2, 3

Bold designates unique to CFP-treated exosomes.

a) All samples identified by in-gel digestion.

b) Predicted, contains a signal sequence; Experimental, identified in culture filtrate by proteomic method; Nd, no data published related to secretion.

c) References: 1, [38] 2, [39]; 3, [40].

d) Single peptide identifications, manual validation.

2.15 Criteria for protein identification

All in-solution and in-gel analyses for each sample were compiled in Scaffold (version 2.02.01, Proteome Software, Portland, OR), in order to validate MS/MS-based peptide and protein identification. The Scaffold program added another level of stringency utilizing the Peptide and Protein Prophet statistical analysis. Proteins and peptides were disqualified below a 90% threshold. [11, 12]. All proteins identified were subjected to manual validation for final confirmation. FPs were identified using a naïve exosome sample having no exposure to *M.tb*.

2.16 Statistical analysis

Wherever applicable, data were analyzed using the Microsoft Excel Software program. Group comparisons were performed by using the unpaired Student's *t*-test.

3 Results

3.1 Characterization and proteomic analysis of exosomes from *M.tb*-infected J774 cells

Exosomes purified from *M.tb*-infected J774 cells were analyzed by EM and shown to have the characteristic spherical or cup-shape appearance and the expected size of 30–100 nm (data not shown). The isolated exosomes were also analyzed to confirm the presence of host protein components known to be present in exosomes (Supporting Information Table 1). As expected, we observed CD81, members of the annexin family, the ATPase α_3 subunit and integrins such as α_M and β_2.

We previously observed that exosomes released from mycobacterial-infected J774 cells could activate naïve CD4+ and CD8+ T cells *in vivo* [7]. To determine the mycobacterial protein components present in exosome that could serve as the antigens to promote the T-cell response, we performed a proteomic analysis of exosomes released from *M.tb*-infected J774 cells. Tryptic digest of exosomal proteins was performed by both in-solution and in-gel to optimize MS, as well as maximize protein identifications. A total of 41 mycobacterial proteins were identified (Table 1, Supporting Information Tables 2 and 3). In this group there were a number of well-known *M.tb* antigens including the antigen 85A, B and C (Rv3804c, Rv1886c, Rv0129c), MPT64 (Rv1980c) and ESAT-6 (Rv3875) [13]. The presence of the 85A was particularly interesting as this protein is part of a subunit vaccine currently in clinical trial [14]. The presence of a number of well-known immuno-dominant *M.tb* antigens in exosomes (highlighted in table) likely explains our previous observation that exosomes from BCG-infected J774 cells can activate naïve T cells when injected intranasally into C57Bl/6 mice [7]. Interestingly, the exosomes could stimulate this T-cell response in the absence of adjuvant, indicating that the purified exosomes have both antigenic and adjuvant properties.

The vast majority of mycobacterial proteins identified on the exosomes are known/predicted to be secreted [15] as summarized in Table 1. Most of the proteins are likely secreted *via* the SecA secretory system but some such as ESAT-6 are released through the newly described type VII secretion system present in mycobacteria [16]. Nevertheless, there are a couple of mycobacterial proteins present in exosomes that are not expected to be secreted including the succinic semialdehyde dehydrogenase (GabD1), an enzyme involved in *M.tb* intermediary metabolism. This suggests that either there is some alternative mechanism for release of these proteins or that some mycobacterial lysis is occurring within the infected macrophage and subsequent transport of these proteins to MVBs and exosomes.

3.2 Western blot analysis of exosomes

To confirm the results obtained through the proteomic analysis, exosomes were probed for a subset of proteins by Western blot. We found that exosomes released from *M.tb*-infected J774 cells but not from uninfected cells contained GroES (Rv3418c), MPT63 (Rv1926c), KatG (Rv1908c), 19 KDa lipoprotein/LpqH (Rv3763), antigen 85 complex (Rv3804c, Rv1886c, Rv0129c), GlnA (Rv2220) and SodA (Rv3846) (Fig. 1). As expected, both exosome populations contained the host protein lysosomal-associated membrane protein-1. With the exception of the identification of LpqH and SodA, the Western blot analysis is consistent with the findings observed through our shotgun proteomic approach (Table 1). Due to the complexity of the exosome samples and the inclusion of murine proteins in the MS analysis, it is likely that the LpqH and SodA peptides were either less susceptible to ionization or suppressed by the abundant host peptides. The benefit of this LC-MS/MS analysis was our ability to identify exosomal proteins without the need for additional detection reagents (*i.e.* antibodies), as well as discovering proteins that were not predicted. In addition, previous studies indicated that CFP-10 and ESAT-6 are secreted from the *M.tb* as a complex [16] and suggest that these proteins might also be in exosomes. Although we observed the presence of CFP-10 by Western blot, we found that exosomes released from infected J774 cells also contain ESAT-6 by LC-MS/MS (Fig. 1, Table 1).

3.3 Exosomes generated from CFP-treated macrophages contain *M.tb* protein antigens

Since our proteomic analysis indicated that the majority of *M.tb* proteins present in exosomes are known to be secreted, we hypothesized that the majority of these exosome proteins result from the release from the mycobacteria and not from mycobacterial lysis within the macrophage. To begin testing this hypothesis, we treated macrophages with proteins

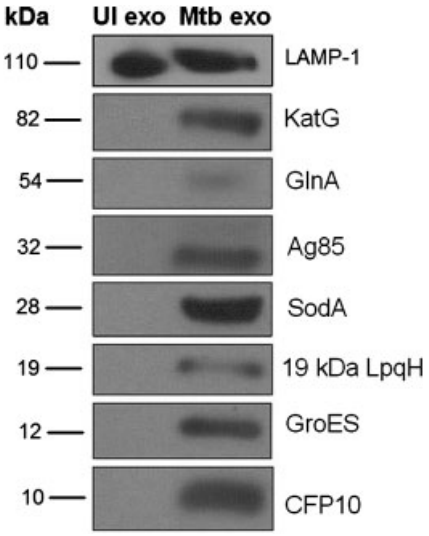

Figure 1. Characterization of mycobacterial proteins associated with exosomes from *M.tb*-infected macrophages. Exosomes isolated from uninfected and 72 h post *M.tb*-infected J774 cells were analyzed by Western blot for the indicated mycobacterial proteins and for the lysosomal-associated membrane protein-1 as a positive control for exosomes.

actively secreted from *M.tb* (*i.e.* the CFPs) with the anticipation that at least some of the mycobacterial proteins will be endocytosed and trafficked to MVBs and exosomes.

This hypothesis is supported by the previous studies where macrophages exposed to *Toxoplasma gondii* protein antigens produced exosomes containing a subset of these proteins [17]. We observed a similar finding as exosomes isolated from CFP-treated J774 cells (CFP-exosomes) contained many of the same proteins we observed in exosomes released from *M.tb*-infected macrophages including the antigen 85 complex, GroES, MPT63 among others (Tables 1 and 2). In fact only six unique proteins were found in CFP-exosomes. This is not surprising since 95% of the proteins found in exosomes from *M.tb*-infected J774 cells have been experimentally shown or computationally predicted to be secreted (Table 1). In summary, 41 mycobacterial proteins were identified in exosomes from *M.tb*-infected J774 cells, whereas 29 were identified from CFP-treated cells (Supporting Information Tables 4 and 5). Of these 29 identified proteins, 25 (86%) were identical to those observed from *M.tb*-infected cells (Fig. 2).

J774 cells were also infected with irradiated *M.tb* to evaluate whether macrophage-mediated degradation of *M.tb* results in significant transport of mycobacterial proteins to MVBs and release in exosomes. Despite the sustained level of exosome release from these cells compared with uninfected or *M.tb*-infected J774 cells, these exosomes were essentially absent of mycobacterial proteins (Fig. 2). This may be due to the limited release of protein and lipid components from irradiated *M.tb* following phagocytosis.

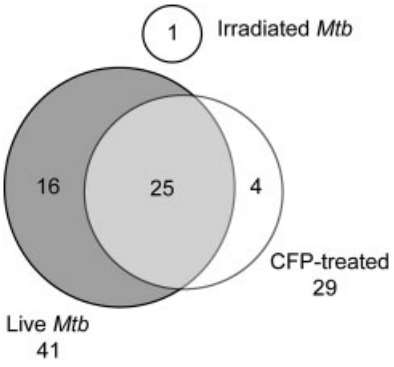

Figure 2. Venn diagram showing overlap between proteins identified in exosomes released by *M.tb*-infected, irradiated *M.tb*-infected and CFP-treated J774 cells.

3.4 Exosomes from CFP-treated macrophages can activate resting macrophages and sensitized lymphocytes

Previously we reported that exosomes isolated from mycobacterial-infected macrophages contain PAMPs and are pro-inflammatory. We have also shown that exosomes isolated from BCG or *M.tb*-infected macrophages induced BMDC activation and maturation [7]. Since the exosomes derived from CFP-treated J774 cells contained a number of mycobacterial components, we hypothesized that they may also stimulate macrophage and DC activation. As shown in Fig. 3, we observed TNF-α and IL-12 production by macrophages and DC, respectively, following treatment with CFP-exosomes.

To evaluate the antigenic potential of CFP-exosomes, splenocytes were harvested from CFP vaccinated mice and *ex vivo* stimulated with exosomes from CFP-treated or UT J774 cells. As shown in Fig. 4, exosomes from CFP-treated J774 cells could stimulate splenocytes to produce TNF-α and IFN-γ. Interestingly, when similar quantities of CFP-exosomes or CFP alone were used, CFP-exosomes promoted a more robust TNF-α response compared with *ex vivo* stimulation with CFP, suggesting either an enrichment of molecules in exosomes that can stimulate TNF-α production by splenic cells (likely macrophages) or more efficient presentation of these molecules to the splenic cells to promote TNF-α production. The similar IFN-γ production by splenocytes treated *ex vivo* with CFP-exosomes compared with CFP alone also suggest that either the exosomes contain the most potent *M.tb* culture filtrate antigens or that the exosomes are more efficient at transferring a limited number of *M.tb* antigens to antigen-presenting cells for presentation to antigen-specific T cells.

3.5 Exosomes from CFP-treated macrophages can activate naïve CD4+ and CD8+ murine T cells *in vivo*

Based on the presence of prominent *M.tb* antigens on CFP-exosomes and our previous studies with exosomes from

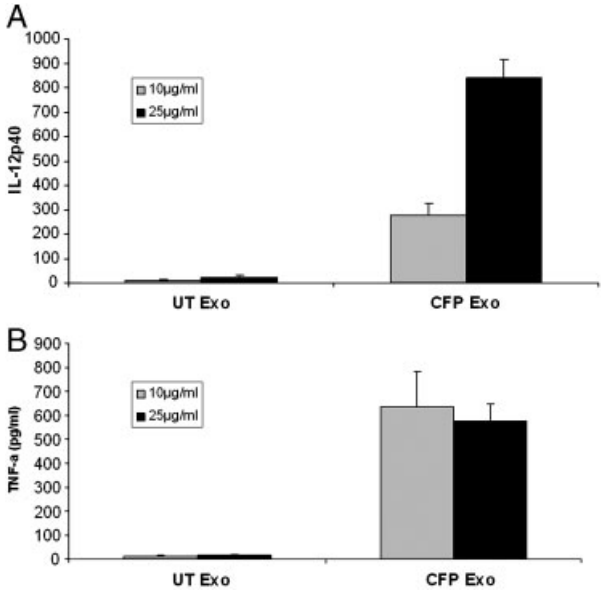

Figure 3. Exosomes from CFP-treated macrophages induce TNF-α and IL-12p40 secretion in naïve macrophages and BMDC, respectively. (A) BMDCs were incubated for 24 h with exosomes from *M.tb* or uninfected (UI) macrophages and the culture supernatants were analyzed for IL-12p40 by ELISA. (B) J774 cells were incubated for 24 h with exosomes from *M.tb* or uninfected (UI) macrophages and the culture supernatant was analyzed for TNF-α by ELISA. Results are representative of at least two independent experiments.

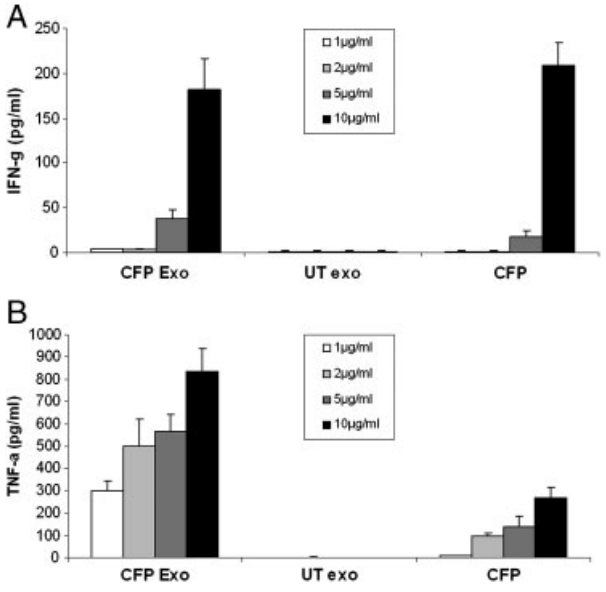

Figure 4. Activation of splenocytes from CFP treated mice by exosomes from CFP-pulsed macrophages. Splenocytes from CFP-treated mice were cultured with CFP, exosomes from UT J774 cells, or exosomes from CFP-treated cells. In total, 72 h post-treatment, culture supernatants were analyzed for IFN-γ (A) and TNF-α (B) by ELISA. Results are representative of at least three independent experiments.

BCG-infected macrophages, we hypothesized that the exosomes from CFP-treated J774 cells could activate naïve CD4+ and CD8+ T cells *in vivo*. To test this prediction, mice were administered intranasally with exosomes from uninfected or CFP-treated cells +/− adjuvant as described in Section 2 and 2 wk after the final vaccination the lungs, MLN and spleens were harvested. Single cell suspensions were stimulated *ex vivo* with CFP and the T cells were analyzed for proliferation, IFN-γ production and memory phenotype. As shown in Fig. 5, both CD4+ and CD8+ T cells isolated from mice treated with CFP-exosomes showed increased production of IFN-γ upon restimulation with CFP and this was evident in all three organs. The adjuvant did not have a significant effect on the number of T cells which were positive for IFN-γ, suggesting that exosomes contain both adjuvant and antigenic properties. This conclusion is supported by the data shown in Fig. 3 which indicate that exosomes from CFP-treated cells can promote DC activation. CD4+ and CD8+ T cells isolated from mice treated with CFP-exosomes but not from mice treated with uninfected exosomes also showed increased proliferation when stimulated *ex vivo* with CFP (Figs. 5D and E). Previous studies indicated that antigen-specific effector memory cells may play an important role in protection against *M.tb* in BCG-vaccinated individuals [18, 19]. Therefore, we addressed whether effector memory cells were produced in mice vaccinated with CFP-exosome and found $CD44^{hi}$, $CD62L^{low}$ CD4+ and CD8+ T cells, which is indicative of effector memory cells, in all three organs (Figs. 5F and G). Together, the data suggest that vaccination of mice with exosomes isolated from CFP-treated J774 cells can induce a profile of T-cell activation often associated with *M.tb* control (*i.e.* IFN-γ production and effector memory cells).

4 Discussion

Previous studies indicate that exosomes isolated from *M.tb*-infected macrophages can have immune modulatory activity [6, 20]. The exosomes activated both the innate and the acquired immune responses suggesting the presences of multiple mycobacterial components. However, in these previous reports only the antigen 85 complex was identified. In this study, an MS proteomic approach was taken to identify the mycobacterial proteins present in exosomes released from *M.tb*-infected J774 cells. A surprisingly high number of mycobacterial proteins (*i.e.* 41) were identified along with a number of expected host proteins. Why the high number and why these particular proteins? Most of the identified proteins are known to be secreted by *M.tb* and some, including the fibronectin attachment protein have been shown previously to be released outside the phagosome [21]. Recent studies have identified two major mechanisms by which proteins are targeted to the intraluminal vesicles of MVBs for subsequent release in

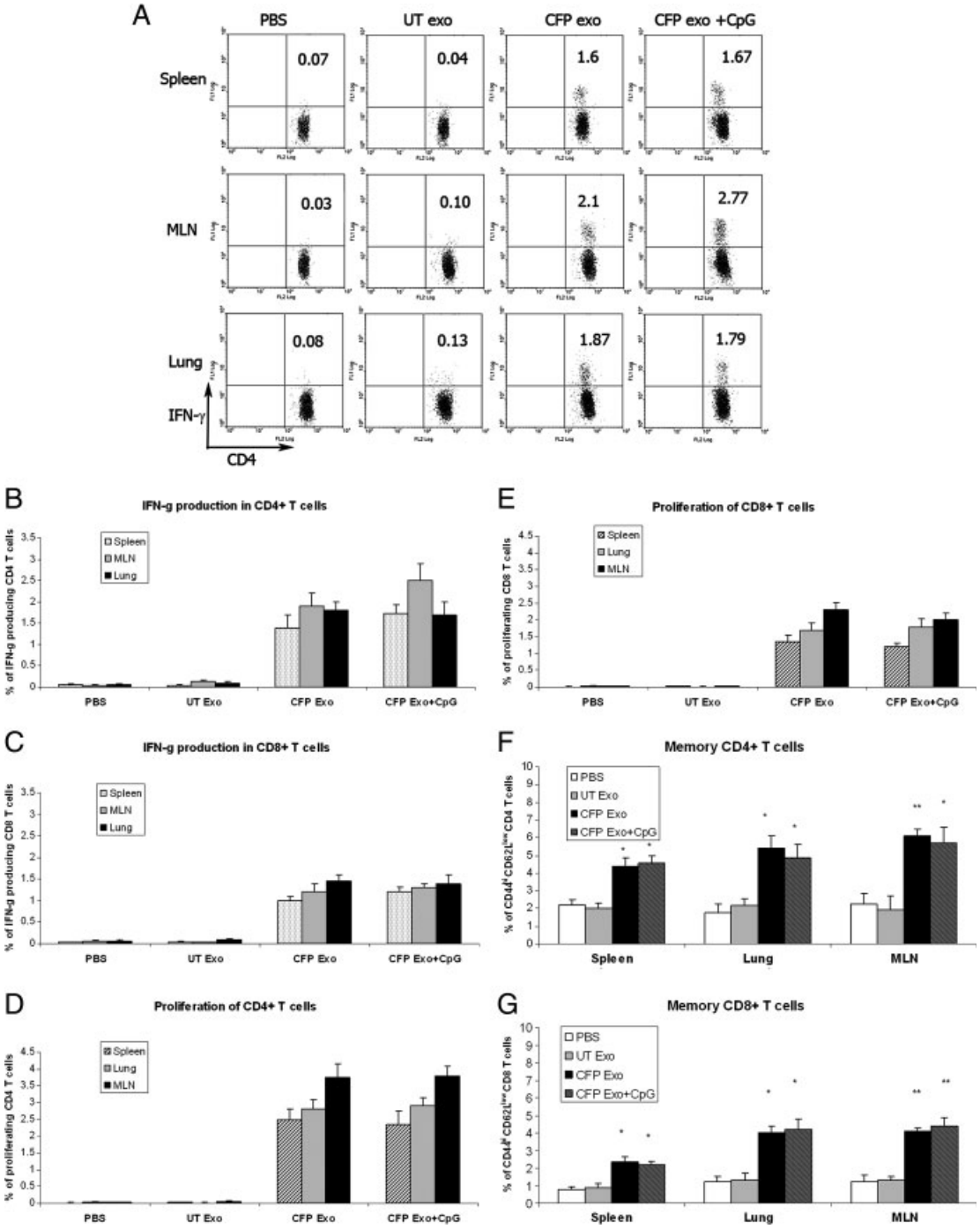

Figure 5. Exosomes from CFP-treated macrophages induce antigen-specific T-cell activation. Lymphocytes from lungs, MLN and spleens were isolated from mice intranasally treated with PBS and exosomes and the isolated cells cultured in the presence or absence of CFP. Cells were stained with anti-CD4-PE, anti-CD8-PE and anti-IFN-γ-FITC and analyzed by flow cytometry (A). Percentage of T cells stained for CD4 and intracellular IFN-γ (B) and CD8 and intracellular IFN-γ (C). Lymphocytes from lungs, MLN and spleens isolated from mice intranasally treated with PBS or exosomes were stained with CFSE (2.5 μM) and then cultured in the presence or absence of CFP for 72 h. Cells were then stained with anti-CD4-PE or anti-CD8-PE and analyzed for CFSE dilution by flow cytometry. Percentage of total CD4+ T cells which showed CFSE dilution (D) Percentage of total CD8+ T cells which showed CFSE dilution (E). Lymphocytes from lungs, MLN and spleens were isolated from mice intranasally treated with PBS or exosomes and cultured in the presence or absence of CFP. Cells were stained with anti-CD4-PE, anti-CD8-PE, antiCD44-PECy5 and antiCD62L-FITC and analyzed by flow cytometry. Expression of effector memory CD4+ T cells ($CD44^{hi}$ and $CD62L^{low}$) (F) or effector memory CD8+ T cells (G). Shown is the percentage of total T cells which were $CD44^{hi}$ and $CD62L^{low}$. Values are means ± SD of two independent experiments. Total lymphocytes were gated using FSC/SSC. Cells were negative for staining with isotype control antibodies. Exo-exosomes, UT, untreated.

exosomes. One involves ubiquitination of proteins which is recognized by the ESCRT (endosomal sorting complex required for transport) machinery for subsequent trafficking and retention of proteins in the intraluminal vesicles [22]. A second mechanism appears to involve aggregation of membrane proteins forming microdomains which may stabilize the exosomal membrane domains [22]. Although we did not find ubiquitination motifs among the different *M.tb* exosome proteins, we could not determine from our analysis whether these proteins are aggregating within the phagosome and subsequently trafficked through the endocytic network into MVBs. In addition to these known pathways, there is some evidence to indicate that there may be ESCRT-independent transport mechanisms [23] but whether some or all of the mycobacterial proteins use these alternative targeting pathways must wait until the process is better defined. This is further complicated by the lack of studies addressing the mechanism of phagosomal protein trafficking to MVBs which may differ from how plasma membrane or endosomal proteins are targeted to MVBs and exosomes.

Independent of how mycobacterial proteins and other components are targeted to MVBs/exosomes, it is clear that this occurs during the course of an *M.tb* infection both *in vitro* and *in vivo* [6]. At the time of this publication, no other study has defined the microbial components present in exosomes following infection with an intracellular pathogen. Some studies with HIV-infected macrophages and DCs suggest that HIV may bud from cells through exosomes or exosome-like vesicles [24, 25]. Other studies have shown that the HIV Gag protein, when expressed in cells, shows preferential targeting to MVBs and release in exosomes [26]. Studies by Raposo and colleagues have shown that prion protein in both its normal and its scrappie conformation are trafficked to MVBs and released in exosomes [27]. Prion diseases are fatal neurodegenerative disorders and the infectious forms involve transmission of the prion protein in its scrappie conformation to a susceptible host. The possibility that exosomes may promote the transfer of prion protein is an intriguing one.

Previous studies have shown that mycobacterial proteins, glycolipids, lipoproteins, *etc.*, can affect the host immune system and can function to either promote an effective immune response or potentially inhibit aspects of this response [28, 29]. To what degree exosomes promote or inhibit immunity will likely depend on what mycobacterial components are present on the exosomes at any given time. The proteomic analysis of exosomes released from infected macrophages as described in this study is the first venture into dissecting their protein composition. Future studies will be needed to define how exosome composition may change over time particularly during the course of an *in vivo* infection and how this correlates with bacterial burden and the stage of infection. The eventual goal is to better understand exosome's role during the course of an *M.tb* infection. Nevertheless, our previous studies demonstrate that exosomes isolated from *M. bovis* BCG-infected J774 cells could activate both naïve CD4+ and CD8+ T cells, suggesting that under some conditions exosomes from infected macrophages can promote an acquired immune response. This also suggests that exosomes with the right subset of *M.tb* components may function as a viable vaccine candidate and the presence of highly antigenic *M.tb* proteins on the isolated exosomes supports this possibility. Members of the 85 complex which are proteins found in exosomes were originally described as a prominent TB antigens in animal models and within infected humans [30, 31]. Subsequent subunit vaccine studies in nonhuman primates showed that this antigen along with another well-known antigen ESAT-6 provides protection against *M.tb* infection [32]. Clinical trials are presently ongoing using an Ag85B-ESAT-6 fusion protein as a subunit vaccine.

Since we found a number of *M.tb* secreted proteins in exosomes released from *M.tb*-infected macrophages, we tested whether incubating macrophages with these CFPs exogenously would produce exosomes containing at least a subset of these proteins. Interestingly, we found 29 *M.tb* proteins on these exosomes. There are approximately 205 *M.tb* proteins in the culture supernatant [15] so our findings that 29 of these including ESAT-6 and the 85 complex are trafficked to MVBs and exosomes suggest an efficient transport mechanism. However, why these specific *M.tb* proteins are targeted is not known. Another interesting observation was that the majority of these exosomal proteins (25 of the 29) were also present in exosomes from *M.tb*-infected macrophages. This indicates that these proteins are targeted to MVBs and exosomes independent of whether they traffic through the phagosome or through endocytic pathway. It is important to note that the *M.tb* is washed prior to the macrophage infection and there is no culture supernatant added to the cells at the time of infection. The ''feeding'' of proteins to DC, B cells or macrophages to produce exosomes containing the added proteins has been performed in the context of tumor antigens [1, 33] as well as microbial antigens from *T. gondii* [34], diphtheria toxin from *Streptococcus pneumoniae* [35] and spike S protein from the SARS-associated coronavirus [36], suggesting that this may be a mechanism to ''engineer'' exosomes to contain specific proteins. However, before this method can reach its full potential, we need a better understanding of how proteins are trafficked to MVBs and exosomes.

We hypothesized that since these exosomes derived from CFP-treated J774 cells contained known *M.tb* antigens, it could function to promote activation of naïve T cells *in vivo* and our data support this hypothesis. The number of CD4+ and CD8+ T cells which produced IFN-γ upon *ex-vivo* stimulation was comparable to our previous studies when exosomes from BCG-infected macrophages were used for vaccination [7]. The same held true for the production of effective memory T cells. The similar levels of T-cell activation induced by exosomes from CFP-treated and BCG-infected macrophages suggest that in our mouse model they

share the major antigens that are inducing the acquired immune response. However, which of these 25 shared *M.tb* proteins are the major contributors to the T-cell response is presently unknown. Nevertheless, the exosomes derived from CFP-treated macrophages have distinct advantages over those generated from *M.tb*-infected macrophages with the major advantage stemming from the removal of a BSL3 pathogen infection from the exosome-generation process. Moreover, like the exosomes from *M.tb*-infected macrophages, CFP-exosomes did not require adjuvant for activation of naïve T cell *in vivo* and could promote DC activation and maturation *in vitro*. In contrast, CFP alone, although capable of inducing T-cell activation when injected into mice, could only do so in the presence of adjuvant [37]. This is an important consideration since only Alum has been approved as an adjuvant for use in humans. Future studies will determine whether the CFP-exosomes when used to vaccinate mice protect against an *M.tb* infection.

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The authors have declared no conflict of interest.

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