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Ubiquitination as a Mechanism To Transport Soluble Mycobacterial and Eukaryotic Proteins to Exosomes

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Exosomes are extracellular vesicles of endocytic origin that function in intercellular communication. Our previous studies indicate that exosomes released from Mycobacterium tuberculosis-infected macrophages contain soluble mycobacterial proteins. However, it was unclear how these secreted proteins were targeted to exosomes. In this study, we determined that exosome production by the murine macrophage cell line RAW264.7 requires the endosomal sorting complexes required for transport and that trafficking of mycobacterial proteins from phagocytosed bacilli to exosomes was dependent on protein ubiquitination. Moreover, soluble mycobacterial proteins, when added exogenously to RAW264.7 or human HEK293 cells, were endocytosed, ubiquitinated, and released via exosomes. This suggested that endocytosed proteins could be recycled from cells through exosomes. This hypothesis was supported using the tumor-associated protein He4, which, when endocytosed by RAW264.7 or HEK293 cells, was transported to exosomes in a ubiquitin-dependent manner. Our data suggest that ubiquitination is a modification sufficient for trafficking soluble proteins within the phagocytic/endocytic network to exosomes. The Journal of Immunology, 2015, 195: 000–000.
also suggests that endocytosed proteins can be recycled back to the extracellular environment by incorporation into exosomes.

**Materials and Methods**

**Bacterial strains and media**

The mouse macrophage cell line RAW264.7 and human HEK293 cells were maintained in DMEM supplemented with 10% FBS, 10 mM sodium pyruvate, and 25 mM HEPES. M. tuberculosis H37Rv was grown in Middlebrook 7H9 broth supplemented with oleic albumin dextrose catalase until midlogarithmic growth phase and frozen down as stocks in growth media plus 15% glycerol. Prior to use, the bacterial stocks were thawed and the mycobacteria were depleted by a brief sonication and passed through a syringe fitted with a 27-gauge needle at least 10 times.

**Small interfering RNA transfections in RAW mouse macrophages**

A total of 5 × 10⁵ RAW264.7 cells was cultured overnight in 6-well plates in DMEM supplemented with 10% FCS. Prior to transfection, cells were washed three times with 1× PBS and replenished with DMEM containing exosome-depleted FCS, produced by spinning the FCS at 100,000 g for 1 h to remove exosomes. The transfection mix consisted of 75 nM target small interfering RNA (sRNA) in 100 μL DMEM containing 10% HiPerfect (Qiagen). siRNA for Tsg101, Hrs, Park2, and control used were siGENOME SMARTpools (Dharmacon). The oligonucleotides used for the knockdowns are as follows.

**Immunoprecipitation of ubiquitinated proteins**

In brief, ubiquitin Ab (P4D1) was conjugated to Sepharose A/G beads. Conjugation mixture containing 2 μg Ab, 40 μL Sepharose A/G beads (Santa Cruz), and 450 μL 1× PBS containing 1% multiple protease inhibitor (MPI) was incubated for 1 h at 4°C. Beads were centrifuged at 1500 × g for 5 min to remove supernatant. Beads were blocked at 4°C for 3 h with cold 1% BSA in 1× PBS containing MPI. Beads were centrifuged at 1500 × g for 5 min to remove supernatant. Exosomes were lysed by incubating with blocking buffer containing 0.1% Triton X-100. Exosome lysate (50 μg) was added to beads and incubated overnight at 4°C. Supernatant was removed, and beads were washed three times in 1× PBS containing MPI. Ubiquitinated proteins were removed from beads by heating for 5 min at 95°C and analyzed by Western blot.

**Plasmids and constructs**

All primers are listed below. WT HspX forward primer: 5'-CTCGAGTTGGC-CACCACTCCTCCC-3'. WT HspX reverse primer: 5'-CATATGGCATGTG-GTGGGACCAGATCTG-3'. Mutant Lys47 HspX reverse primer: 5'-CTGGAAGAGAAATGAGAGGGGCGCTGAGTAGCC-3'. Mutant Lys47 HspX reverse primer: 5'-AGGACGCGGCTCGCCCATGAGAA-3', 5'-UUGCCGCAUGCCUGGUUCC-3', 5'-GCAGCGGCGGCTCGCCCATGAGAA-3', 5'-ACGCGCGGCTCGCCCATGAGAA-3'. Parkin primers: 5'-GGCAUGAUAUUGGGGGCAACUAUCUCAUC-3', 5'-GGCAUGAUAUUGGGGGCAACUAUCUCAUC-3', 5'-GGCAUGAUAUUGGGGGCAACUAUCUCAUC-3', 5'-GGCAUGAUAUUGGGGGCAACUAUCUCAUC-3'. Knockdown was confirmed by Western blot using 10 μg whole-cell lysate.

**M. tuberculosis H37Rv infection or CFP treatment of RAW 264.7 cells and isolation of exosomes**

Confluent layer of RAW264.7 cells was infected with M. tuberculosis H37Rv or left untreated. Prior to infection, the bacteria were complement osonized using normal horse serum for 2 h and infected at a multiplicity of infection that achieved 80% infectivity, as determined by uptake assay (17). The RAW264.7 macrophages were infected with bacteria for 4 h before being washed three times with 1× PBS. The cells were cultured in DMEM supplemented with 10% exosome-depleted FCS. Cell culture supernatants were harvested at 72 h postinfection for exosome isolation. For CFP treatment, RAW264.7 cells were seeded in Ti-175 tissue culture flasks and treated with CFP (20 mg/mL) in 20 mL DMEM supplemented with 10% exosome-depleted FCS. After 16 h, cell culture supernatant was harvested for exosome purification. CFP was purchased from BEI Resources (Manassas, VA) and was originally made by K. Dobos, Colorado State University, as described in the product sheet (catalog NR-14825).

For exosome isolation, culture supernatants were centrifuged at 300 × g for 10 min at 4°C to remove debris. Cleared culture supernatants were filtered through 0.22-μm polyethersulfone filters (Nalgene). Filtered supernatant were centrifuged at 10,000,000 × g for 30 min at 4°C and again at 100,000,000 × g for 1 h to pellet the exosome-enriched vesicle population.

**Sucrose gradient prepared exosomes**

RAW264.7 cells were infected with Mycobacterium smegmatis expressing wild-type (WT) M. tuberculosis HspX or K88R M. tuberculosis HspX for 72 h. Cell culture supernatants were centrifuged at 300 × g for 10 min at 4°C to remove debris. Cleared culture supernatants were filtered through 0.22-μm polyethersulfone filters (Nalgene). Filtered supernatants were centrifuged at 10,000,000 × g for 30 min at 4°C and again at 100,000,000 × g for 1 h to pellet the exosomes. Exosomes were purified on linear sucrose gradient. The exosomes formed a distinct ring based on their density (1.13 and 1.18 g/ml) and were carefully recovered from the gradient. In the absence of a distinct ring, gradient fractions 5, 6, and 7 were collected, pooled, and washed in 1× PBS.

**Western blotting and Abs**

Exosomes (10 μg) were resuspended in 1× PBS with protease inhibitors. The suspension was mixed with Laemmli buffer, heated at 95°C for 5 min, and chilled on ice for 5 min before loading onto SDS gel. Whole-cell lysates were prepared in radioimmunoprecipitation assay buffer. Suspension was mixed with Laemmli buffer and heated at 95°C for 5 min before loading onto SDS gel. Immunoblots probed with Abs for proteins: ubiquitin (P4D1, 1:1,000; Santa Cruz), Tsg101 (C-2, 1:1,000; Santa Cruz), Hrs (V-20, 1:500; Santa Cruz), tubulin (T5293; Sigma-Aldrich), CFP (C192, 1:1,000; American Type Culture Collection), Kat-G (IT-42, 1:20; American Type Culture Collection), His (1:500; Santa Cruz), GroES (SA-12, 1:20; American Type Culture Collection), HspX (IT-20, 1:15; American Type Culture Collection), and Park2 (ABP3038, 1:500; Aviva). Primary Abs were incubated with HRP-conjugated secondary Abs (1:25,000; Pierce) and detected using ECL kit (Pierce).

**Protein expression and purification**

In brief, pellets from 250 mL cultures were dissolved in 10 mL lysis buffer containing 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, and 0.5 M urea with protease inhibitor mixture (Sigma-Aldrich). Resuspended cells were sonicated twice, each for 5 min (with a 0.3-s pulse and 0.7-s rest) at 5-min intervals to prevent overheating, using a Fisherbrand Sonicator at 45% amplitude. The supernatant was collected after centrifugation (30,000 × g) for 30 min at 4°C and loaded onto a nickel affinity Sepharose (Ni-AC) column. After washing with 10-column volumes of 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, and 20 mM imidazole, proteins were eluted in 50 mM Tris-HCl, 100–150 mM NaCl, and 250–500 mM imidazole.

**His-tag removal from recombinant GroES**

His-tag was removed from recombinant GroES by thrombin digestion. Target protein was incubated with 0.5 U/mL thrombin for 4 h at 22°C. Following digestion, thrombin was removed from sample by a benzamidine Sepharose column.

**Treatment of cells with endocytosis inhibitor Dynasore**

RAW264.7 cells were plated at 1 × 10⁶ cells/mL in 6-well plates and allowed to attach overnight. Cells were treated for 30 min with 80 μM Dynasore (Sigma-Aldrich) or equivalent volume of the DMSO vehicle control. Cells were washed with PBS, and fresh culture media was added. Cells were treated with 40 μg/mL His-HspX recombinant protein for 4 h. Cells were again washed with PBS to remove any free recombinant protein, and exosome-depleted culture media was added. After 16 h, exosomes were harvested from cell culture supernatant, as described above, and
whole-cell lysates were generated by the addition of radioimmunoprecipitation assay buffer. Samples were analyzed by Western blot for His-tagged HspX and Lamp-1.

**Transfection of M. smegmatis**

WT and mutant HspX constructs were subcloned into pMV261 transfection plasmid. *M. smegmatis* culture was grown in 7H9 medium until OD reached log-phase growth. Pelleted cells were washed and resuspended in 10% (v/v) glycerol in PBS. Concentrated bacteria were mixed with 100 ng plasmid DNA and electroporated at 2.5 kV, 1000 ohms, and 25 μF in 0.2-cm electroporation cuvettes. Cells were diluted with fresh 7H9 media and incubated for 5 h at 37°C. Following incubation, cells were plated and screened after 3 d.

**Immunofluorescence staining and microscopy**

Cultured RAW264.7 cells were seeded on glass coverslips in 24-well plates. Cells were treated with 40 μg/ml His-tagged HspX for 3 and 6 h. Cells were fixed and stained, and F-actin distribution was visualized by staining with FITC-phalloidin (Molecular Probes). The anti-Lamp1 was obtained from the Developmental Studies Hybridoma Bank, created by the National Institute of Child Health and Human Development, and maintained at the Department of Biology, University of Iowa. The Ab at 1:250 dilution was used to label the Lamp1 protein. Cells were visualized with a Nikon fluorescent microscope coupled to a Bio-Rad MRC 1024 scanning confocal three-channel system.

**Results**

*ESCRT machinery is necessary for exosome biogenesis in macrophages*

Various studies have identified proteins involved in exosome biogenesis in specific cell types; however, there is limited information on how proteins are trafficked to MVB/exosomes in macrophages. This is an important consideration because the published data suggest that different cells may use different mechanisms for MVB biogenesis (7, 8). Therefore, we evaluated the requirement for ESCRT-0 and ESCRT-1 in macrophage exosome biogenesis. Using siRNA knockdown, we targeted Tsg101 and Hrs, the ubiquitin-binding domain of ESCRT-1 and ESCRT-0, respectively. RAW264.7 cells were treated for 48 h with four independent siRNA oligos against either a specific target or with a scramble control. Knockdown efficiency of Tsg101 and Hrs was assayed by Western blot and was >95% for both proteins (Fig. 1A, 1D). No significant difference in cell numbers was observed for the different treatment groups (data not shown). Exosomes were isolated from cell culture supernatants of the transfected macrophages, and protein concentrations were determined using a bicinchoninic acid assay (BCA). Exosome protein concentration was reduced >85% as a result of Tsg101 and Hrs knockdown compared with siRNA scramble control (Fig. 1B, 1E). Because the protein assay cannot discriminate between a decrease in the number of exosomes and a decrease in the amount of protein per exosome, we used particle tracking to quantify the total number of vesicles secreted by the RAW264.7 cells. As shown in Fig. 1C and 1F, we observed a >80% decrease in vesicle concentration from cells transfected with Tsg101- and Hrs-specific silencing RNA oligonucleotides compared with cells treated with scramble oligonucleotides, which matched our BCA data.

**Exosomes from infected macrophages contain ubiquitinated proteins**

The results from the Tsg101 and Hrs knockdown experiments indicate that the ESCRT machinery is required for production of exosomes in macrophages, suggesting that ubiquitination is an important mechanism for protein trafficking to MVBs and exosomes. Therefore, we evaluated exosomes from H37Rv-infected and uninfected cells for the presence of ubiquitinated cargo. As shown in Fig. 2A, exosomes were enriched for ubiquitinated proteins relative to cell lysate, and exosomes from infected macrophages appeared to contain a higher number of ubiquitinated proteins at lower m.w. To determine whether any of the ubiquitinated proteins were mycobacterial in origin, we first isolated and then lysed exosomes from infected macrophages and performed an immunoprecipitation using an Ab that recognizes monoubiquitinated proteins. The pull-down was probed for mycobacterial proteins using a polyclonal Ab made against *M. tuberculosis* CFP, which has previously been shown to recognize many of the *M. tuberculosis* proteins present in exosomes (18). As shown in Fig. 2B, a number of immunoprecipitated proteins were identified using the polyclonal Ab made against the CFP, and this was specific to exosomes from infected cells. To gain some insight into which mycobacterial proteins are pulled down with the ubiquitin Ab, we first analyzed *M. tuberculosis* proteins previously identified in exosomes for predicted ubiquitination sites. KatG, HspX, and GroES were all identified as having a number of lysines that could be ubiquitinated. Therefore,
we probed the proteins from the pull-down for these mycobacterial proteins and found all three to be present (Fig. 2C).

In our previous studies, we determined that exosomes released from macrophages treated with *M. tuberculosis* CFP also contained mycobacterial proteins, sharing much of the same proteins present in exosomes isolated from directly infected cells (4). This suggests that mycobacteria proteins may contain the necessary signal to be trafficked to exosomes upon entry into the cell, whether through phagocytosis or endocytosis. In support of this hypothesis, we found exosomes derived from CFP-treated macrophages also contained ubiquitinated proteins (Supplemental Fig. 1A). Moreover, exosomes released from CFP-treated macrophages when lysed and immunoprecipitated for ubiquitinated proteins showed a number of specific bands when probed with the mycobacterial Ab made against CFP (Fig. 2B).

**Inhibiting ubiquitination blocks trafficking of mycobacterial proteins to exosomes**

Given ubiquitinated proteins could be detected in exosomes, we examined the dependency of ubiquitination for protein sorting in macrophages. Ubiquitination is mediated by the actions of ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3) (19). Previous studies have shown that the drug compound, PYR-41, effectively prevents ubiquitination in the cells through inhibition of ubiquitin thioester bond formation within E1 (20). Treating macrophages with 50 nM PYR-41 resulted in a 30–40% reduction in exosome production as defined by Nanosight and BCA (Fig. 3A, 3B). Furthermore, pretreatment of CFP-treated RAW264.7 cells with PYR-41 resulted in exosomes that lacked mycobacterial proteins detectable by the CFP polyclonal Ab (Fig. 3C).

**Trafficking of GroES and HspX to exosomes requires ubiquitination**

Although the above results suggest ubiquitination is required for mycobacterial protein trafficking to exosomes, it is not clear whether this is due to direct ubiquitination of the mycobacterial proteins or their association with ubiquitinated host proteins. To address this question, HspX and GroES were expressed and purified as His-tag fusion proteins. The purified fusion proteins were added to RAW264.7 cells, and exosomes were isolated 16 h posttreatment. Based on the presence of HspX and GroES in exosomes following treatment of macrophages with CFP, we anticipated that the His-tagged fusion proteins would be endocytosed and trafficked to MVBs and into exosomes. Indeed, we observed both proteins in exosomes following their addition to macrophages, suggesting that each protein has the signal for trafficking to MVBs/exosomes (Fig. 4). We also found that GroES purified from *M. tuberculosis* culture supernatant and His-tagged GroES with the tag removed were also trafficked to exosomes when added to macrophages, indicating that the His-tag was not responsible for the trafficking (Supplemental Fig. 1B). Immunofluorescence staining of macrophages posttreatment with His-HspX indicates its intracellular localization and partial colocalization with the late endosomal/MVB marker LAMP1 (Fig. 4B). Interestingly, when we purified the His-tagged proteins from exosomes using nickel-resin, we were able to detect the protein using a His-tag Ab and an Ab that recognizes monoubiquitinated protein (Fig. 4A). Of note, the 8-kDa shift in protein size observed by Western blot for the fusion protein isolated from exosomes compared with originally purified His-HspX from *E. coli* corresponds to the size expected from a monoubiquitination event. These results suggest that HspX and GroES are endocytosed, ubiquitinated, and trafficked to exosomes. However, to determine whether ubiquitination was necessary, macrophages were pretreated with E1 inhibitor PYR-41, and, as shown in Fig. 4C and 4D, the presences of the His-tagged GroES and HspX in exosomes were lost when cells were treated with ubiquitin inhibitor.

Ubiquitination occurs at lysine residues within proteins; furthermore, it has been shown that mutation of lysine residues can...
inhibit proper ubiquitin tagging and trafficking within a cell (21). To identify which lysine residue on HspX was ubiquitinated and required for exosomal trafficking, we first used an in silico analysis of the HspX protein sequence to identify potential ubiquitination sites. We identified three lysine residues that gave high probability as sites for ubiquitination. Using site-directed mutagenesis, lysine to arginine substitutions were generated in HspX at amino acid positions 47, 58, and 85. Following purification of native and mutated His-tagged HspX, the recombinant proteins were added to naive macrophages and exosomes were purified 16 h posttreatment. Despite similar concentrations for all HspX mutants within the treated macrophages, the K85R HspX mutant was not present in exosomes (Fig. 5A, 5B). Together, these studies suggest that HspX is directly ubiquitinated and that this modification is required for sorting into exosomes.

**Macrophages infected with M. smegmatis-expressing WT or K47R HspX, but not K85R HspX, release exosomes containing the His-tagged HspX fusion protein**

Although our evidence suggests that HspX and GroES are ubiquitinated upon endocytosis by macrophages and that this ubiquitination is required for trafficking to exosomes, it is unclear whether this mechanism extends to trafficking of mycobacterial proteins during a natural infection. Therefore, due to the ease of expressing mycobacterial proteins in the nonpathogenic fast-growing bacteria M. smegmatis, we generated M. smegmatis clones that express either WT, K47R, or K85R HspX. However, prior to evaluating the trafficking of the recombinant protein, it was necessary to determine whether mycobacterial proteins are transported to exosomes during a M. smegmatis infection of RAW264.7 cells as has been observed for other mycobacteria (e.g., Mycobacterium avium, M. tuberculosis, and Mycobacterium bovis bacillus Calmette-Guérin) (3). As shown in Fig. 5C, exosomes derived from M. smegmatis-infected macrophages also contained mycobacterial proteins that could be pulled down with an anti-ubiquitin Ab. Based on these results, we infected RAW264.7 cells with M. smegmatis-expressing WT, K47R, or K85R HspX, and the exosomes from the cell culture supernatant were purified by ultracentrifugation 72 h postinfection. Exosomes and cell lysates were assayed for the presence of the WT and mutant His-tagged HspX proteins by Western blot using the His-tag Ab. As was observed using purified protein, macrophages infected with M. smegmatis expressing the K85R mutant released exosomes that lacked this HspX, whereas bacteria expressing either WT or K47R HspX contained exosomes with the recombinant protein (Fig. 5D). The results were not due to a loss of K85R expression, as WT, K47R, and K85R HspX were found to be present at similar expression levels within infected macrophage cell lysate (Fig. 5E). To confirm that the HspX that we observed was associated with exosomes and not protein aggregates that copurified, we further purified the exosomes released from M. smegmatis-infected macrophages by sucrose gradient and analyzed the exosomes for HspX. Using these highly purified exosomes, we again observed HspX in vesicles released from M. smegmatis expressing the WT, but not the HspX K85R mutant (Supplemental Fig. 2).

** Trafficking of HspX into exosomes requires clathrin-mediated endocytosis**

The data above indicate that ubiquitination of HspX is required for its targeting to exosomes, suggesting that ubiquitination occurs within an endosomal compartment. However, these studies did not directly evaluate the mechanism of HspX uptake. To assess whether HspX is endocytosed and therefore present within an endosomal compartment, we treated cells with Dynasore, an inhibitor of clathrin-mediated endocytosis, and showed that the inhibitor blocked uptake of HspX and consequently blocked transport of HspX to exosomes (Fig. 6).

**Ubiquitin-mediated transport of endocytosed proteins to exosomes is not limited to mycobacterial proteins**

Our results suggest that soluble proteins upon endocytosis by macrophages can be trafficked to MVBs and released on exosomes...

**FIGURE 4.** His-tagged HspX and GroES when added to RAW264.7 cells are ubiquitinated and packaged into exosomes. (A) RAW264.7 macrophages were pulsed with recombinant His-tagged HspX, and exosomes were isolated from culture supernatant 16 h posttreatment. The recombinant protein from lysed exosomes was purified using a Ni-based resin. Also shown is the original His-tagged HspX purified from E. coli. The Ni-column–purified proteins were probed for the presence of ubiquitin and for the His-tagged protein. (B) Confocal microscopy analysis of RAW264.7 macrophages that were pulsed with recombinant His-tagged HspX (Cy5: Lamp-1; Texas Red: poly His; FITC: actin). Arrows indicate staining of His-tagged protein. (C and D) RAW cells were treated with 50 pmol PYR-41 for 2 h or left untreated and pulsed with recombinant His-tagged GroES or HspX, and supernatants were collected after 16 h for exosome purification. Exosomes were probed for His-tagged proteins. Lamp-1 was used as a loading control.
if they are properly ubiquitinated. However, this hypothesis is based on the analysis of just mycobacterial proteins, specifically HspX and GroES. Therefore, to determine whether this mechanism is more broadly applicable to endocytosed proteins, we tested a different soluble Ag, He4, a well-characterized cancer protein. Based on our prediction analysis, He4 contains lysine residues that are potentially ubiquitinated. A His-tagged He4 fusion protein was generated, purified, and added to macrophages using the same protocol as performed for the mycobacterial proteins. The exosomes isolated from the He4-treated macrophages contained the His-tagged protein, and this localization was ubiquitin dependent, as its trafficking was inhibited by macrophage pretreatment with PYR-41 (Fig. 7A). This sorting of endocytosed proteins into exosomes is relatively specific, as His-tagged GFP when added to macrophages was endocytosed, but remained intracellular and was not released via exosomes (Fig. 7B).

FIGURE 5. Lysine 85 on HspX is required for its packaging into exosomes when added exogenously or expressed by M. smegmatis. RAW264.7 cells were seeded in a 6-well plate and treated with 40 μg/ml either purified WT His-HspX, His-HspX K47R, His-HspX K58R, or His-HspX K85R. After a 16-h incubation, culture media was removed and used as the starting material for exosome purification. Purified exosomes and the corresponding cells were lysed to obtain total protein. (A and B) Exosomes (5 μg) and cell lysates (10 μg) were assayed for presence of the WT and mutant His-HspX by Western blot using a polyclonal His-tag Ab. (C) Exosomes from uninfected or M. smegmatis-infected RAW264.7 cells were purified and lysed, and the ubiquitinated proteins were immunoprecipitated. The ubiquitinated proteins were probed for the presence of mycobacterial proteins using the polyclonal Ab made against the CFP of M. tuberculosis. (D and E) RAW264.7 cells were infected with M. smegmatis-expressing WT HspX, K47R HspX, or K85R HspX for 72 h. Exosomes were purified from the culture supernatant, and the exosome lysate (10 μg) and the cell lysate (20 μg) were assayed for presence of the WT and mutant His-HspX proteins by Western blot using a polyclonal His-tag Ab. Lamp-1 was used as a loading control.

FIGURE 6. Trafficking of HspX to exosomes was dependent on clathrin-mediated endocytosis of the recombinant protein. RAW264.7 cells were pretreated with the endocytosis inhibitor Dynasore, followed by the addition of recombinant His-tagged HspX. Cells were washed 4 h post-HspX treatment and incubated for an additional 16 h. Cell lysates were analyzed by Western blot for His-tagged HspX and Lamp-1. Exosomes were isolated from the culture media and probed for His-tagged HspX and Lamp-1 by Western blot. Shown are the results of duplicate experiments.
ubiquitination.
cating that parkin is not the E3 ligase responsible for HspX exogenous to the RAW264.7 cells (Supplemental Fig. 3), indi-
to a diminished incorporation of HspX into exosomes when added parkin expression in RAW264.7 cells. However, this did not lead proteins. We were successful in knocking down may be responsible for the ubiquitination of the mycobacterial ubiquitination activity. Therefore, we hypothesized that parkin characterized for its polyubiquitination function, also has mono-
the mechanisms of how and where Ags are processed and presented.

**FIGURE 7.** His-tagged He4 when added to RAW264.7 or HEK293 cells is packaged into exosomes in a ubiquitin-dependent manner. (A) RAW264.7 cells pretreated with PYR-41 for 2 h of left untreated were pulsed with recombinant He4 protein for 16 h. Exosomes were purified from the culture supernatant, and the exosomal proteins and cell lysates were probed for His-tagged He4. (B) RAW264.7 cells were pulsed with recombinant GFP protein for 16 h. Exosomes purified from the culture supernatant were lysed and along with the cell lysates probed for the presence of His-tagged GFP. (C) HEK293 cells were pulsed with recombinant WT HspX or the K85R HspX. (D) HEK293 cells pretreated with PYR-41 for 2 h or left untreated were pulsed with His-tagged He4. After 16 h, the HEK293 culture media was removed for exosome purification, and the exosomal proteins and the cell lysates were probed for the presence of His-tagged recombinant protein. Lamp-1 served as a loading control. CL, cell lysate; Exo, exosomes.

**Traffic of HspX and He4 into exosomes derived from HEK cells is dependent on ubiquitination**

Our findings indicate that, for at least a subset of soluble proteins, ubiquitination is required for their sorting to exosomes. However, these studies were limited to a macrophage cell line, and whether this ubiquitin-dependent transport applied to other cell types was unknown. Therefore, purified WT or K85R mutant HspX were incubated with HEK293 cells, a human embryonic kidney cell line. As described above, exosomes were purified from the cell supernatant, and the cell lysates were collected as controls for cellular uptake. Similar to the macrophage results, the WT, but not the K85R, HspX was present in exosomes released from HEK293 cells (Fig. 7C). Furthermore, exosomes derived from HEK293 cells treated with He4 contained the recombinant protein, but He4 was absent when cells were pretreated with PYR-41 (Fig. 7D). Therefore, our data suggest that ubiquitin-dependent trafficking of soluble proteins within the endocytic/phagocytic pathway to exosomes is not restricted to macrophages (Fig. 8).

**The E3 ligase parkin is not responsible for the ubiquitination of HspX**

Previous studies identified parkin as an E3 ligase that can poly-
ubiquitinate *M. tuberculosis* (22). Parkin, although primarily characterized for its polyubiquitination function, also has mono-
ubiquitination activity. Therefore, we hypothesized that parkin might be responsible for the ubiquitination of the mycobacterial proteins. We were successful in knocking down >90% of the parkin expression in RAW264.7 cells. However, this did not lead to a diminished incorporation of HspX into exosomes when added exogenous to the RAW264.7 cells (Supplemental Fig. 3), indicating that parkin is not the E3 ligase responsible for HspX ubiquitination.

**Discussion**

As an intracellular pathogen, *M. tuberculosis* has limited exposure to various immune components, including Abs and complement. Nonetheless, the immune system reacts efficiently and appropriately in most cases, as evidenced by >90% of those infected with *M. tuberculosis* never developing clinical disease. A key aspect to our understanding of the immune response to infection is to know the mechanisms of how and where Ags are processed and presented.

Recent evidence suggests that exosomes serve as sources of Ag during infections and may play an important role in T cell activation and Ag cross-priming (23). Exosomes are vesicles of 30–100 nm in size that are secreted from cells of both hematopoietic and non-
hematopoietic origin and in general function in intercellular communication. Previously, we have reported that exosomes modulate both the innate and acquired immune response during a *M. tuberculosis* infection (3) (24) and have shown that exosomes containing TB Ags can be used as an effective vaccine against an aerosolized *M. tuberculosis* infection using a mouse model (5). These results suggest that exosomes may provide a unique approach to TB vaccine development. However, a number of challenges remain, including developing exosomes with specific mycobacterial Ags. Therefore, to bioengineer exosomes as a TB vaccine or as a vaccine in general, we need a better understanding as to how proteins are sorted into exosomes.

We have identified >40 different mycobacterial proteins on exosomes derived from *M. tuberculosis*-infected macrophages and many of the same proteins from CFP-pulsed macrophages (4). Of the mycobacterial proteins identified on exosomes, 95% were experimentally shown to be secreted, soluble proteins (4). In the current study, we showed that host ubiquitination is required for this sorting and demonstrated that HspX and GroES are directly ubiquitinated by macrophages and that this provides the signal necessary for trafficking and loading into exosomes. Surprisingly, this sorting seemed to be independent of entry mechanisms, as proteins were trafficked to exosomes in a ubiquitin-dependent manner whether added as free protein and therefore taken in by an endocytic route or expressed by a mycobacteria that gains entry by phagocytosis. This suggests that, in both cases, the soluble mycobacterial protein gains access to the E1, E2, and E3 ligases. Whether the same ligases are involved is unclear, but the data with the HspX K85R mutant that was absent from exosomes when expressed by *M. smegmatis* or added as exogenous protein suggest a homologous ubiquitination process.

Ubiquitination by E3 ligases predominately occurs within the cytosol (19), and there are a number of cytosolic proteins present within exosomes. Presumably, some of these proteins are targeted through a ubiquitin-dependent/ESCRT-mediated pathway. This hypothesis is supported by the presence of ubiquitinated luminal proteins within exosomes (25). There is even more evidence to
FIGURE 8. An illustration highlighting ubiquitination as a mechanism for the transport of proteins from a phagosome or endosome to a MVB and into the intraluminal vesicles for release in exosomes.

support transport of plasma–membrane receptors to MVBs/exosomes through a ubiquitin-dependent process (26). Our data suggest that endocytosed or phagocytosed proteins can also be trafficked to exosomes. In the context of an infection, we and others have shown that some microbial components, including proteins, are trafficked to exosomes (3, 27). However, none of these studies defined a mechanism of how this targeting was mediated. One possibility is that these Ags, particularly in the context of direct M. tuberculosis infection, gain access to the cytosol, perhaps through pores formed within the phagosome in an ESX-1-dependent manner (28), and are ubiquitinated by the cytosolic E3. However, identifying mycobacterial proteins within the cytosol has been a challenge. Moreover, mycobacterial proteins can be found on exosomes derived from M. bovis bacillus Calmette-Guérin–infected macrophages. This strain lacks a genomic region called RD1, which contains a number of genes essential for ESX-1–mediated protein release from the phagosome (28). In addition, our data using an inhibitor of clathrin-mediated endocytosis indicate that the endocytic process is required for uptake of soluble HspX, and there is no known mechanism that would transport HspX from an endosomal compartment to the cytosol. The retention of HspX in an endosomal compartment is supported by our fluorescent microscopy data.

The second possibility is that soluble Ags are first ubiquitinated within the endocytic network. Interestingly, a number of E3 ligases have been identified within the endocytic network (29, 30) (see Supplemental Table 1 for a more complete list), and most likely more still remain to be defined. Furthermore, proteomic analysis of exosomes has shown that they too contain all the necessary machinery required for ubiquitination to occur, including both E1 and E2 and different members of E3 ligase family, suggesting their propensity for being confined within endocytic compartments (31, 32). Therefore, we hypothesize and our data support that the endocytosed and phagocytosed proteins are sorted into endocytic vesicles that have the machinery for ubiquitination (Fig. 8); however, the E3 ligase responsible has yet to be identified. Due to their ability to target a myriad of proteins, there are many E3 ligases within a mammalian cell. Current estimates suggest as many as 1000 E3 ligases within a single cell (33). Nevertheless, we hypothesized that the E3 ligase Park2 (parkin) may be involved because it has been shown to polyubiquitinate M. tuberculosis (22). However, knockdown of parkin in macrophages did not alter the transport of soluble HspX. Therefore, additional studies into the ligases contained within endocytic compartments are needed. Moreover, how ubiquitinated proteins within the lumen of an endocytic vesicle are recognized by components of the ESCRT machinery, which are cytoplasmic, or by other host components for packaging into exosomes is presently unclear.

It is generally accepted that monoubiquitin of a lysine residue can target a protein to the MVB through both ESCRT-dependent and independent mechanisms (15, 34). Through the use of inhibitors and lysine point mutants, we show that HspX when added to macrophages requires ubiquitination for trafficking to exosomes. Interestingly, this was true for the trafficking of HspX in both macrophages and HEK293 cells, a kidney cell line. These data suggest that endocytosed/soluble proteins that are monoubiquitinated might be trafficked to exosomes as a general mechanism. In light of this observation, we evaluated whether ubiquitination also served to traffic other soluble proteins to the MVB. Our analysis of He4, a soluble cancer Ag, indicated that it behaved similarly to the mycobacterial proteins both in macrophages and HEK293 cells and was trafficked to exosomes in a ubiquitin-dependent fashion. This suggests that endocytosed proteins can be recycled by cells through exosomes, a process not previously described, and this has implications into how normally secreted proteins may be repackaged for extracellular release. However, additional studies are needed to determine how general this mechanism is for endocytosed proteins and what role it may have under physiological and pathological conditions.

In summary, our data indicate that, within the endocytic/phagocytic network, ubiquitination functions as a tag for protein delivery to exosomes. What ligases are responsible for this ubiquitination remains unknown, but most likely involves multiple E3 ligases within endosomal compartments. This previously undescribed mechanism of packaging and release of extracellular proteins through exosomes may have consequences for protein function, as their presence in exosomes may lead to their different distribution and cellular response. To what extent secreted proteins are repackaged for release through exosomes and the functional consequences of this exosome localization remain to be defined.

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Disclosures
The authors have no financial conflicts of interest.

References
Supplementary Figure 1: (A) Raw 264.7 cells were pulsed with *M. tb* culture filtrate proteins (CFP) or left untreated for 16 hours. Following treatment, exosomes were purified from cell culture supernatant. Exosomes from uninfected and CFP-pulsed macrophages (10µg) were probed for the presence of mono-ubiquitin by western blot. (B) Raw 264.7 cells were pulsed for 16 hours with purified GroES or recombinant GroES with the His-tag removed by endopeptidase digestion. Exosomes were purified from cell culture supernatant and probed for the presence of GroES. Loading control; Lamp-1.
Supplementary figure 2: Raw 267.4 cells were left untreated or infected with *M. smegmatis* expressing wild-type *M. tb* HspX, or K85R *M. tb* HspX for 72 hours. Exosomes were purified from the cell culture supernatant by ultracentrifugation followed by flotation on a sucrose gradient. Purified exosomes (10μg) were assayed for presence of the wild-type and mutant His-HspX proteins by western blot using a polyclonal His-tag antibody. Lamp-1 was used as a loading control. pHspX; purified HspX from *E. coli* (positive control).
Supplementary figure 3: The ubiquitin ligase parkin is not responsible for the HspX ubiquitination. (A) Raw 264.7 cells were treated for 24 hours with the siRNA directed against parkin and the cell lysates of treated cells were analyzed for parkin protein expression by western blot. Scrambled siRNAs were used as a control. (B) Raw264.7 cells treated with the siRNA for parkin or the scrambled siRNA were pulsed with His-HspX protein for 16 hours. Exosomes were isolated from cell culture supernatant and the purified exosomes (10ug) were probed for the presence of His-tagged HspX by western blot.
Supplementary Table 1: List of E3 Ligases associated with endosomal compartments with references

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<thead>
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<th>E3 Ligase</th>
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<tr>
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<tr>
<td>Ube3c</td>
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