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1 **Microparticles released from *Mycobacterium tuberculosis*-infected human macrophages contain increased levels of the type I interferon inducible proteins**
2
3 **including ISG15**

4

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19

20 **Abbreviations:**

21 IFN, interferon; MP, microparticle; TB, tuberculosis

22

23 **Keywords:** macrophage, microparticle, *Mycobacterium tuberculosis*, proteome

24

25

Abstract

Microparticles (MP) are small membranous particles (100-1000 nm) released under normal steady-state conditions and are thought to provide a communication network between host cells. Previous studies demonstrated that *Mycobacterium tuberculosis* (*M.tb*) infection of macrophages increased the release of MPs, and these MPs induced a proinflammatory response from uninfected macrophages *in vitro* and *in vivo* following their transfer into uninfected mice. To determine how *M.tb* infection modulates the protein composition of the MPs, and if this contributes to their proinflammatory properties, we compared the proteomes of MPs derived from *M.tb*-infected (TBinf-MP) and uninfected human THP-1 monocytic cells. MP proteins were analysed by GeLC-MS/MS with spectral counting revealing 68 proteins with statistically significant differential abundances. The 42 proteins increased in abundance in TBinf-MPs included proteins associated with immune function (7), lysosomal/endosomal maturation (4), vesicular formation (12), nucleosome proteins (4) and antigen processing (9). Prominent among these were the type I interferon inducible proteins, ISG15, IFIT1, IFIT2, and IFIT3. Exposure of uninfected THP-1 cells to TBinf-MPs induced increased gene expression of *isg15*, *ifit1*, *ifit2*, and *ifit3* and the release of proinflammatory cytokines. These proteins may regulate the proinflammatory potential of the MPs and provide candidate biomarkers for *M.tb* infection.

1. Introduction

Extracellular vesicles are small (0.05-1 μm) particles shed from the membrane of cells under normal steady-state conditions and in response to stimuli such as cellular stress [1]. In contrast to their original description as “platelet dust” [2], an increasing number of important cellular functions have been attributed to these vesicles, including intercellular communication, trafficking of molecular cargo (e.g. protein, nucleic acid), apoptosis, homeostasis and cellular waste disposal [1, 3-5].

Extracellular vesicles are broadly classified according to their size and biogenesis. Exosomes are small vesicles, ~50-100 nm diameter, formed within the cell and released into the extracellular milieu via exocytosis [1]. Microparticles (MPs) (100-1000 nm) are released via budding from the cell membrane under a regulated mechanism influenced by multiple stimuli, including Ca^{2+}, ATP and cell specific factors (e.g. complement proteins) [6]. MPs contain proteins from the cell membrane, and increasing evidence demonstrates that MPs also contain actively packaged molecules, although the mechanism for this process is not well understood [3, 4]. Increased production of extracellular vesicles has been associated with a number of pathological states, including cancer and infectious diseases, such as tuberculosis (TB) [5, 7]. Deciphering the biological roles these vesicles play may lead to an improved understanding of different pathologies, as well as the discovery of potential pharmacological targets or biomarkers for diagnosis and monitoring of disease progression.

TB remains a major threat to human health worldwide with an estimated 8.6 million new cases and 1.3 million deaths in 2012 alone [8]. A major challenge to TB

healthcare is that current diagnostic tests rely heavily on the presence of *Mycobacterium tuberculosis (M.tb)* bacteria within patient sputum. The development of novel diagnostic tests for TB infection, which are not reliant on the isolation and culturing of bacteria, are urgently required. Extracellular vesicles shed into the blood could form the basis of a clinical diagnostic test due to the easy, non-invasive nature of a blood test. We have previously shown [7] using both *in vitro* and *in vivo* studies that macrophages infected with *M. tb* increase the release of MPs, and that these vesicles are proinflammatory both *in vitro* and *in vivo*.

To determine what proteins may mediate or contribute to the proinflammatory nature of the MPs derived from *M. tb*-infected macrophages, we profiled and compared the proteomes of MPs derived from *M. tb* infected and uninfected human monocytic THP-1 cells. Proteins with increased abundance included the Type I interferon inducible proteins ISG15, IFIT1, IFIT2, and IFIT3. These data suggest a possible role for ISG15 and IFITs in the host response to *M. tb* infection, potentially influenced by the type I interferon signalling pathway. Additionally, the findings of this study provide candidate biomarkers of *M. tb* infection for further investigation.

1 **2. Materials and Methods**

2 **2.1. Cell and Bacterial culture**

3 Human myelo-monocytic THP-1 cells were cultured and differentiated by the
4 addition of 100 nM PMA (Sigma-Aldrich) for 48 h. *M. tuberculosis* H37Rv was
5 cultured in supplemented Middlebrook 7H9 to mid-logarithmic phase [7]. Bacterial
6 counts were determined by culturing samples for 3 weeks on Middlebrook 7H11-
7 supplemented agar [7].

8

9 **2.2. *M. tuberculosis* H37Rv infection of THP-1 cells**

10 Differentiated THP-1 cells were cultured at 2×10^7 cells per flask for sufficient yields
11 of MPs, while cultures for RT-PCR were 5×10^5 cells. Cells were infected with *M. tb*
12 H37Rv for 4 h at a multiplicity of infection (MOI) of 1 or left uninfected, washed
13 twice to remove extracellular bacteria, cultured for 4 and 24 h for RT-PCR and
14 Western blotting experiments, and 72 h for MP isolation.

15

16 **2.3. MP isolation**

17 Isolation, quantitation and size distribution of MPs were performed according to the
18 established protocol of Walters et al [7] (Methods S1). Proteins were extracted from
19 MPs by resuspension and lysis in RIPA buffer with protease inhibitors for proteomic
20 or western blot analysis. For determination of functional effects of MPs on naïve
21 THP-1 cells, MPs were resuspended in triple filtered PBS, and the MP concentration
22 determined as previously described [7].

23

24 **2.4. GeLC-MS/MS of *M.tb* infected and uninfected THP-1 derived MPs**

Protein concentrations of MP samples were estimated by BCA protein assay (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. A total of 20 µg of protein from *M.tb*-infected THP-1 cell-derived MPs (TBinf-MP) and uninfected THP-1 cell derived MPs (UI-MP) were separated by SDS-PAGE (Methods S1). Each gel lane was cut into 12 individual gel slices of equal size, were reduced, alkylated and trypsin digested overnight at 37ºC. Peptide samples were concentrated and desalted using prefabricated microcolumns containing Poros R2 resin (Perseptive Biosystems, Framingham MA) [9]. Peptides were subjected to reversed phase LC−MS/MS using either an automated Agilent 1100 nanoflow LC system (Agilent Technologies, Santa Clara CA) coupled to a QSTAR Elite mass spectrometer (Applied Biosystems) or an Ultimate 3000 nano-HPLC and autosampler system (Dionex, Amsterdam, Netherlands) coupled to an LTQ-Orbitrap Velos mass spectrometer (Thermo Fischer Scientific).

Analysis using the 1100/ QSTAR Elite MS instrumentation setup was conducted on a total of six biological replicates. Peptides were loaded onto a trapping column (Agilent Zorbax 300SB-C18, 0.3 mm x 5 mm, 5 µm, 300 Å), washed with buffer A (0.1% v/v formic acid (FA)) and subsequently loaded onto an analytical column (Agilent Zorbax 300SB-C18, 0.1 mm x 150 mm, 3.5 µm, 300 Å pore size). Peptides were eluted over 120 min using a gradient of 5-90% buffer B (0.1% v/v FA, 100% acetonitrile (ACN)) at a nanoflow rate of 300 nL/min. The eluent was subjected to positive ion nanoflow ESI MS/MS in an information dependent acquisition mode (IDA). MS survey scans were performed over the *m/z* range of 400-1800 (three scans), with the 3 most intense ions sequentially subjected to MS/MS scans. Further

analysis to achieve greater proteomic coverage depth using an Ultimate 3000 nano-HPLC /LTQ-Orbitrap Velos MS setup was performed as previously described [10].

Peak lists were extracted from QSTAR Elite data using Analyst (version 2.0, Applied Biosystems) or from Orbitrap velos data by Mascot Daemon/extract_msn (Matrix Science, Thermo; London, UK), using the default parameters. All MS/MS data were submitted to Mascot (Matrix Science, London, UK; version 2.4.0) and X! Tandem (The GPM, thegpm.org; version CYCLONE (2010.12.01.1)) and searched against the SwissProt_2013_09 database (selected for *Homo sapiens*, 20272 entries), with trypsin as the proteolytic enzyme and a single possible missed cleavage. Fragment ion mass tolerances of 0.20 Da or 0.40 Da, and parent ion tolerances of 0.20 Da or 4 ppm were selected for QSTAR and Orbitrap data respectively. Oxidation of methionine, carbamidomethyl of cysteine and propionamide of cysteine were specified in Mascot as variable modifications. Glu->pyro-Glu of the n-terminus, ammonia-loss of the n-terminus, gln->pyro-Glu of the n-terminus, oxidation of methionine and carbamidomethyl of cysteine were specified in X! Tandem as variable modifications.

Scaffold (version Scaffold_4.0.5, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they exceeded specific database search engine thresholds. Mascot identifications required at least ion scores must be greater than both the associated identity scores and 30, 40 and 50 for, doubly, triply and quadruply charged peptides. X! Tandem identifications required at least -Log(Expect Scores) scores of greater than 4.0. Protein identifications were accepted if they contained at least 2 identified peptides. Proteins that contained similar peptides and could not be differentiated

based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters. Proteins were annotated with GO terms from gene_association.goa_human (downloaded 08/07/2013) [11]. Spectral counting was performed to determine differentially abundant proteins with the inverted β-binomial test applied to QSTAR-MS data to determine statistical significance for paired data [12]. Only pairs containing count data in both paired members could be included in the test and hence paired data containing a zero count were excluded from the test. Data were filtered for proteins with a fold change > 1.5 or < -1.5 and a p-value < 0.05. Where a protein was uniquely detected in either the infected or uninfected THP-1 derived MP then it was required to have been detected in at least 2 replicates to be considered for further analysis. The additional biological replicate analysed by LTQ Orbitrap-MS was aligned to further corroborate statistically significant results from the QSTAR-MS analysis. Bioinformatic analysis was aided with online resources including KEGG pathway analysis (www.genome.jp/kegg/pathway) [13], STRING (version 9.1, www.string-db.org) [14], Vesiclepedia (version 2.1, www.microvesicles.org) [15] and Exocarta (version 4.1, www.exocarta.org) [16]. All mgf and raw data files associated with this manuscript can be accessed at http://goo.gl/yZrrKd.

2.5. Treatment of uninfected THP-1 cells with UI-MP or TBinf-MP

Differentiated THP-1 cells were stimulated with UI-MP or TBinf-MP at a ratio of 5 MP's per cell and cultured for 4, 24 and 48 h. Culture supernatants were harvested and cytokines measured by Cytometric bead array (BD Biosciences) according to the manufacturer's instructions using an LSRFortessa flow analyzer (BD Biosciences)

[7]. Cells were harvested in 1 mL Trisure reagent (Bioline, Australia) for RNA purification and RT-PCR.

2.6. Quantitative reverse transcriptase real-time PCR

Gene expression of *isg15*, *ifit1*, *ifit2*, *ifit3* and *ip10* was determined by RT-PCR (Methods S1).

2.7. Western blot analysis

The presence of ISG15 protein in uninfected and *M. tb* infected THP-1 cells was assessed by Western blot (Methods S1).

3. Results

3.1 Macrophage microparticle proteome in response to *M. tb* infection

THP-1 cells were infected with *M. tb* H37Rv for 72 h. Microparticles were purified from the culture supernatants, and their proteomes analysed by a GeLC-MS/MS proteomic approach. UI-MP and TBinf-MP isolations were demonstrated to have similar size distributions (flow cytometry, Fig. S1; SEM [7]) and therefore proteomic analysis was conducted normalising samples by protein concentration. Briefly, microparticle proteins were separated by 1D-SDS-PAGE, gel lanes sliced into bands of equal size, proteins digested with trypsin, and analysed by RPLC-MS/MS using two mass spectrometry platforms. Six paired biological replicates were analysed by the 1100/ QSTAR Elite MS platform with the resulting data compiled and validated in Scaffold (Tables S3 and S4). We applied the inverted β-binomial test described by Pham and colleagues [12] to determine significant differences in the paired spectral count data. A total of 521 proteins (465 protein groups) were identified, with the

majority (476 proteins) present in both MP samples with 25 unique to the TBinf-MP sample and 20 unique to the UI-MP sample (Fig. S2A). 24 proteins were significantly increased in abundance, 18 proteins uniquely identified in the TBinf-MP, while 14 proteins were decreased in abundance in the TBinf-MP and 12 proteins uniquely identified in the UI-MP (Fig. S2C). Bioinformatic analysis revealed increased representations of proteins within the TBinf-MP including the lysosome proteins CD63 (LAMP3), LAMP2, L-amino-acid oxidase IL4I1 and sialin. Increased immune function proteins included HLA-A, fibronectin FN1, MHC antigen H13A and type I interferon (IFN)-inducible proteins, ISG15, IFIT1, IFIT2 and IFIT3. A total of ten proteins associated with vesicular formation or trafficking were increased in abundance in the TBinf-MP, including Rab-5C, Rab-7A and Rab-14, which are known to be critical for immunity to *M. tb* [17, 18] (Fig. 1, Table 1). An additional ten proteins associated with protein maturation and antigen presentation, including six members of a large chaperone multiprotein complex; PDIA6, PDIA4, HSPA5, HSP90B1, PPIB and Erp29 and antigen presentation-related proteins, PDIA3 and CANX, were also increased in abundance. Histone proteins, HIST1H2BD, HIST1H4A, H2AFV and H2AFY, were all identified at increased abundance in TBinf-MP and may correlate to an increase in nucleic acid content in the MPs.

Proteins observed at decreased abundances in TBinf-MP compared to UI-MP largely clustered into protein groups associated with the cytoskeleton (7), membrane proteins (5) and vesicle formation (3) (Fig. S3, Table S7). Decreased abundances observed may be a result of increased MP release and absent *de novo* synthesis of these proteins. Membrane proteins, including CD109, SLC4A7, APOE and MFGE8, displayed decreased abundances in the TBinf-MP of the greatest magnitude.

To complement our approach we performed one additional paired analysis on an Ultimate 3000 nano-HPLC /LTQ-Orbitrap velos MS platform to improve the depth of proteome coverage. We identified a total of 1198 proteins (1018 groups) with a minimum of two unique peptides detected (Table S5 and S6). Proteins identified unique to the TBinf-MP totaled 175, with 334 unique to the UI-MP and 689 proteins identified in both. To focus attention on differentially abundant proteins, likely to be present in biologically relevant abundances, we further filtered data for proteins with a minimum of 6 spectra, and considered those differentially abundant with spectral count fold changes of > 1.5 or < -1.5 (Fig. S2B). A total of 710 proteins were identified common to both TBinf-MP and UI-MP, with 75 proteins increased in abundance and 157 with decreased abundance in TBinf-MP, while 18 were unique to TBinf-MP and 58 unique to UI-MP (Fig. S2D). While the number of protein identifications from the Orbitrap-velos analysis was increased compared to that of the QSTAR analysis, the data sets were largely complementary as the percentages of proteins assigned to GO terms (biological process, cellular component, molecular function) were consistent (Fig. S4). Similarly, proteins displaying significant differential abundance in the QSTAR analysis showed similar changes in abundance in the Orbitrap (Table 1, S2). The Orbitrap analysis did detect IFIT1 and ISG15 in the UI-MP, although these were still more abundant in the TBinf-MP. Two additional type I IFN inducible proteins, IFITM3 and PLSCR1, were detected at increased abundance in the TBinf-MP.

3.2 *M. tb* infection of macrophages induces increased type I IFN inducible gene expression and protein abundance

We next determined whether the increased abundances of type I IFN inducible proteins observed in the TBinf-MP correlated with increased abundance in the infected THP-1 cells, from which they were derived. THP-1 cells infected with *M. tb* were harvested at 4 and 24 h. Increased transcripts for multiple type I IFN inducible proteins, including *ifits*, *isg15* and *ip10* (Fig 2A), were detected. In addition, *M. tb* infection induced a 2-fold increase in free-ISG15 protein abundance and a 4-fold increase in ISGylated protein abundance at 24 h (Fig. 2B, 2C)

3.3 Microparticles from *M. tb* infected macrophages induce expression of type I IFN inducible genes and cytokines in uninfected macrophages

We have previously demonstrated that MPs are involved in intercellular communication between macrophages [7]. Therefore we investigated whether TBinf-MPs induced gene expression of the IFN inducible genes *ifit1*, *ifit2*, *ifit3*, *ifitm1/3*, *isg15* and *ip10* upon uptake by recipient cells. THP-1 cells were stimulated with purified MPs at a 5:1 MP:cell ratio for 24 hr. Statistically significant increases in transcript for *ifit1*, *ifit2, ifit3, ifitm1/3* and *isg15,* but not *ip-10*, were observed (Fig 3A). We also measured the release of IP-10 protein and two common pro-inflammatory cytokines, IL-8 and MIP-1α, in the supernatants of the MP stimulated cells. The addition of TBinf-MP, but not UI-MPs, significantly increased the release of all three cytokines within 24 h of stimulation (Fig 3B). Interestingly, this included the release of considerable amounts of IP10, despite the fact that there was no increase in *ip10* mRNA transcript at this time. This suggests that these cells may have either increased translation of *ip10* mRNA, increased IP10 protein stability or an intracellular store of IP10, which is readily secreted without the need for further gene expression of IP10.

4. Discussion

Extracellular microvesicles, including exosomes and MPs, represent a novel source of biomarkers for multiple disorders, including cancers and infectious diseases. Our analysis of the MP proteome following *M.tb* infection provides evidence for an important functional role in innate immunity and has highlighted promising biomarker candidates for further studies.

Analysis of the MP proteome following *M.tb* infection identified 68 differentially abundant proteins, including several type I IFN inducible proteins, the IFITs and ISG15. Very little is known about the role of ISG15 in *M.tb* infection, however patients with mutations in *isg15*, leading to a loss of ISG15 protein, are highly susceptible to mycobacterial disease [22]. ISG15 is highly expressed in lymphocytes, monocytes and the lung and is induced by both IFN-α and IFN-β [23]. We also observed the induction of *isg15* with IFN-γ (data not shown). ISG15 is present both within cells and extracellularly, and can exist as a free form or conjugated to other proteins via an ubiquitination-like three-enzyme pathway, termed ISGylation [23]. Our data demonstrates that *M.tb* infection induced an increased abundance of both free and conjugated ISG15, suggesting a potential role for both in TB immunity. Free ISG15 can stimulate the proliferation of NK and T cells, the secretion of IFN-γ, and cell-mediated killing [24]. Furthermore, investigations in human patients with an *isg15* mutation have attributed an important role for free intracellular ISG15 in the prevention of IFN-α/β-dependant autoinflammation [25]. As the mature ISG15 protein lacks a defined secretion signal, MP release may provide an alternate means of secretion for ISG15. ISGylation of proteins has been linked with the regulation of signal transduction pathways, including JNK and NF-κB [26]. While it appears

1 ISGylation is not as common as other types of PTMs (e.g. phosphorylation), there are
2 still over 300 proteins that are candidates for ISGylation, and these are involved in
3 diverse functions from central metabolism, RNA processing and protein translation,
4 to chromatin remodelling and cytoskeletal organisation, suggesting that the impact of
5 ISGylation may be widespread [27-29]. While an essential role for ISG15 and
6 ISGylation in anti-viral immunity has been demonstrated [30][31], the role of ISG15
7 in immunity to bacterial infection has largely been unexplored. A recent study,
8 however, demonstrated that modulation of ISGylation is critical for resistance against
9 *Salmonella typhimurium* and virulent *M.tb*, but not against the less virulent vaccine
10 strain, *Mycobacterium bovis* BCG [32]. Interestingly, a number of ISGylation
11 candidates are also type I IFN inducible proteins, including the IFIT proteins, which
12 we observed at increased abundance in the TBinf-MP [27].
13
14 Similar to ISG15, the IFIT protein family in humans (IFIT1, IFIT2, IFIT3 and IFIT5)
15 have been largely studied in the context of antiviral immunity [33]. Their gene
16 expression is rapidly induced by type I IFNs or by stimulation with PAMPs, and the
17 type of IFIT and the kinetics of expression are dependant on the cell and tissue type
18 [34]. IFITs mediate antiviral immunity by inhibiting viral mRNA translation through
19 multiple steps or by sequestering viral RNA, resulting in the inhibition of viral
20 replication [33]. While increased *ifit* gene expression has been observed in *M.tb*
21 infection, no functional role in anti-bacterial immunity or role in MP biology has been
22 determined [19-21].
23
24 Alignment with a compendium of previously identified microvesicle components
25 (www.vesiclepedia.com), including viral infection studies, revealed that this is the

first study to identify ISG15 and IFITs present in microvesicles [15]. This suggests that their presence in MPs is possibly due to a specific characteristic of, or stimulus to, the cell of origin (*M.tb* infection) leading to increased expression within the cell and consequently in the MP. Whether the presence of ISG15, IFIT1, IFIT2 and IFIT3 proteins in the TBinf-MPs are due to the increased synthesis in the cell of origin alone, or also involves active packaging into MPs will require further investigation. As type I IFN inducible proteins may have an important role in the innate immune response to *M.tb*, we hypothesised that TBinf-MPs may mediate a signal inducing their biosynthesis in distal cells. Exposing uninfected THP-1 cells to TBinf-MP, indeed, resulted in the induction of *isg15*, *ifit1*, *ifit2*, *ifit3* and *ifitm1/3* gene expression, in addition to the increased release of pro-inflammatory cytokines (IL-8, MIP-1α and IP-10).

This study contributes further evidence for the important role of IFNs in the innate immune resistance to bacterial pathogens, and particularly *M.tb*. Type II IFN, IFN-γ, is crucial for protection against *M.tb* infection, with mutations within the IFN-γ-signalling pathway lead to severe susceptibility to *M.tb* infection in humans and mice [35, 36]. Type I IFNs have roles in anti-viral immunity and modulatory functions in innate and adaptive immunity [37], though their role in *M.tb* infection is less clear. IFN-α/β have been observed to have an antagonistic effect on IFN-γ signalling and to down-regulate *ifngr* expression leading to more severe disease [38]. Type I IFN signalling may be a critical link between the host and virulent *M.tb* pathogen. The secretion of some mycobacterial products by virulent *M.tb* strains may induce type I IFN inducible genes and this may be associated with more severe disease [39]. The interaction between *M.tb* and the host type I IFN signalling pathway is crucial in

determining successful infection, and further elucidation of this pathway may yield novel therapeutic targets.

A major challenge to the control of TB is the effective diagnosis of *M.tb*-infected individuals. The presence of type I IFN inducible proteins in MPs derived from *M.tb* infected cells correlates well with recent transcriptomic studies of patient blood samples [19-21]. Recent studies have produced transcript sets to distinguish active TB, from latent TB infection (LTBI) and other respiratory diseases [19, 21]. A consistent finding was the profound increase in type I inducible gene expression associated with active TB, including ISG15 and IFITs [19, 21]. Incorporating detection of a type I IFN inducible signature at both transcript and protein level may be an effective biomarker for active TB infection and for LTBI patients with potential risk of reactivation.

Our analysis of the MP proteome derived from *M.tb* infected cells provides candidate biomarkers for TB diagnosis, including type I IFN inducible proteins (ISG15, IFITs) and phagosome-associated proteins (Fig. 4). All require further evaluation in blood samples from patients with active TB. Further characterisation of the nucleic acid content of MPs and the molecular events dictating the MP-induced proinflammatory response may reveal key insights into the innate immune response to *M.tb*, as well as additional candidate biomarkers of TB disease status.

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Figure 1. Protein-protein interaction and functional clustering of proteins displaying increased abundances in TBinf-MP. Cluster analysis was performed using String (v9.1) with the additional assistance of KEGG and gene ontology data. Distinct clusters of proteins associated with immune function, endosomes and lysosomes, vesicular formation, protein maturation and antigen presentation, histone proteins, mitochondria, and ribosomal proteins.

Figure 2. *M. tb* infection of THP-1 cells leads to increased gene expression of *isg15*, *ifit1*, *ifit2*, *ifit3, ifitm1/3* and *ip10* (A), and increased free-ISG15 protein and ISG15-conjugated proteins (B and C). A representative western blot is shown (B) with the combined results of densitometry graphed (C). Analyses were performed on biological duplicates with three technical replicates (n=6). Statistical significance was determined by one-way ANOVA. *, $p < 0.05$, **, $p < 0.0001$.

Figure 3. MPs derived from *M. tb*-infected THP-1 cells induce *isg15*, *ifit1*, and *ifit2* gene expression (A) and proinflammatory cytokine production (B). PMA differentiated THP-1 cells were stimulated with TBinf-MP or UI-MPs at a 5:1 MP:cell ratio to determine gene expression of *isg15*, *ifit1*, *ifit2*, *ifit3*, *ifitm1/3* and *ip10* and IL-8, IP-10 and MIP-1α cytokine expression (CBA analysis). Analysis was performed on biological duplicates with three technical replicates (n=6). Statistical significance was determined by one-way ANOVA. *, $p < 0.01$, **, $p < 0.001$, ***, $p < 0.0001$.

1 **Figure 4.** Model of intercellular signaling mediated by MPs following *M.tb* infection.
2 1) Infection of the macrophage by *M.tb* induces expression of type I IFN inducible
3 genes (i.e. *isg15*, *ifits*) and arrest of the phagosome that aids bacterial survival and
4 results in retention of Rab GTPases (Rab5, Rab14), CD63 and LAMP2; 2) MPs
5 contain proteins indicative of the infected cell of origin; 3) MPs mediate signaling to
6 recipient cells (macrophages plus other cell types, e.g. T cells) to enhance the
7 response to *M.tb* infection; 4) In recipient macrophages, MPs induce type I interferon
8 inducible gene expression (i.e. *isg15*, *ifits*) and release of proinflammatory cytokines/
9 chemokines.

1 **Table 1: Proteins with increased abundances in MPs derived from *M. tb* infected**
2 **THP-1 cells.** Spectral counting analysis was performed on six biological replicates on
3 the QSTAR Elite. Spectral counts for LTQ-Orbitrap velos correspond to a single run.

Protein ID	QSTAR Elite Analysis				LTQ-Orbitrap Velos Analysis				
	TB MP Tot. Spec. Cnts[a,b]	UI MP Tot. Spec. Cnts	p value	Fold	TB MP Tot. Spec. Cnt	UI MP Tot. Spec. Cnt	Fold	Prev. ID in EV's?[c]	Put. ISG15 conj.?[d]
Immune Function									
HLA-A	(31,25,38,9,23,32)	(11,11,28,12,15,30)	0.012	1.58	157	128	1.23	Y	N
FN1	(1,0,58,33,3,25)	(0,0,13,20,0,13)	0.017	2.66	nd	nd	na	Y	N
IFIT1	(7,8,2,1,7,1)	(0,0,0,0,0,0)	na	+	17	4	4.25	**N**	**Y**
IFIT2	(2,3,0,0,4,0)	(0,0,0,0,0,0)	na	+	10	0	+	**N**	**Y**
IFIT3	(6,5,1,3,8,0)	(0,0,0,0,0,0)	na	+	20	0	+	**N**	**Y**
ISG15	(5,4,0,2,6,0)	(0,0,0,0,0,0)	na	+	11	6	1.83	**N**	**Y**
HM13	(1,1,1,2,2,1)	(0,0,0,0,0,0)	na	+	3	3	1.00	**N**	N
IFITM3	nd	nd	na	na	17	12	1.42	Y	N
PLSCR1	nd	nd	na	na	9	4	2.25	Y	**Y**
Lysosomal associated									
CD63	(10,12,16,7,14,2)	(4,4,10,7,9,0)	0.018	1.72	19	11	1.73	Y	N
LAMP2	(8,8,3,4,4,2)	(3,3,1,3,3,1)	0.021	2.10	16	11	1.45	Y	N
IL4I1	(2,1,0,0,4,0)	(0,0,0,0,0,0)	na	+	12	0	+	**N**	N
SLC17A5	(2,2,0,1,0,0)	(0,0,0,0,0,0)	na	+	2	0	+	**N**	N
Vesicular formation and trafficking									
MYOF	(6,3,1,2,8,3)	(1,1,1,0,2,4)	0.038	2.46	27	38	-1.41	Y	**Y**
ANXA6	(5,6,9,15,4,4)	(1,1,5,5,2,7)	0.015	2.16	44	30	1.47	Y	**Y**
RAB5C	(11,15,9,13,7,9)	(10,6,1,6,8,6)	0.025	1.80	18	19	-1.06	Y	N
RAB7A	(15,15,15,15,19,10)	(11,9,1,9,15,7)	0.019	1.79	19	14	1.36	Y	N
PDCD6	(11,11,15,8,9,5)	(7,5,7,4,10,4)	0.025	1.63	11	12	-1.09	Y	N
CLTC	(36,35,40,34,29,93)	(15,14,47,39,14,84)	0.049	1.46	52	27	1.93	Y	**Y**
RAB14	(31,24,26,31,31,16)	(27,17,10,14,29,18)	0.028	1.43	16	12	1.33	Y	N
ANXA4	(37,35,39,32,17,11)	(21,23,36,18,18,12)	0.022	1.36	31	19	1.63	Y	N
Protein maturation and antigen presentation									
PDIA3	(9,7,12,16,12,10)	(1,1,4,5,2,4)	>0.001	4.08	29	12	2.42	Y	N
CANX	(1,2,4,5,1,8)	(0,0,1,2,0,2)	0.017	3.97	16	4	4.00	Y	N
PDIA6	(8,6,8,10,5,7)	(2,1,3,4,2,3)	>0.001	3.09	14	6	2.33	Y	N
HSPA5	(14,15,11,11,11,16)	(2,3,6,3,4,11)	>0.001	2.90	67	28	2.39	Y	N
P4HB	(16,17,6,12,8,9)	(4,5,2,7,2,7)	>0.001	2.64	33	20	1.65	Y	N
HSP90B1	(12,12,10,10,16,5)	(7,7,9,6,8,7)	0.033	1.51	39	22	1.77	Y	N
PPIB	(4,3,5,6,5,1)	(3,3,1,1,3,1)	0.047	2.01	6	5	1.20	Y	N
ERP29	(5,3,1,4,4,0)	(0,0,0,0,0,0)	na	+	14	3	4.67	Y	N
PDIA4	(0,1,1,1,3,0)	(0,0,0,0,0,0)	na	+	11	14	-1.27	Y	N
Nucleasome/ Nucleus associated									
HIST1H2BD	(19,20,23,26,6,9)	(1,1,7,7,1,7)	>0.001	4.59	27	6	4.50	Y	N
HIST1H4A	(11,10,24,17,12,9)	(1,1,9,4,2,4)	>0.001	4.11	33	0	+	Y	N
H2AFV	(5,7,10,17,1,7)	(0,0,4,4,0,3)	0.008	3.35	10	0	+	Y	N
H2AFY	(0,0,2,3,0,1)	(0,0,0,0,0,0)	na	+	8	0	+	Y	N
Other									
ANXA5	(52,51,62,51,24,23)	(34,35,60,33,29,20)	0.034	1.28	45	36	1.25	Y	**Y**
VDAC1	(4,4,8,9,2,5)	(1,0,6,6,1,2)	0.047	1.84	15	8	1.88	Y	N

4 a. Tot. spec cnts, total spectral counts
5 b. Six paired replicate spectral counts are displayed in brackets for the QSTAR analysis
6 c. Previously identified according to Vesiclepedia (www.microvesicles.org) [15]
7 d. Put. ISG15 conj., Putative ISG15 conjugate proteins were identified by Zhao et. al (2005) [27] or
8 Giannakopoulos et al. (2005) [28].
9

1 **Supporting Information**

File Name	**File Format**	**Description**
SuppInfo_MethodsS1_TableS1_S2	.docx	Methods S1. MP isolation, quantitation and protein extraction, SDS-PAGE, Western blot analysis, Quantitative reverse transcriptase real-time PCR Table S1. qRT-PCR primers Table S2. Proteins decreased in abundance in the TBinf-MP
SuppInfo_FiguresS1_S2_S3_S4	.pptx	Figure S1. Flow cytometric size analysis of MPs indicates no size difference between MPs derived from uninfected (UI-MP) and *M.tb*-infected THP-1 cells (TBinf-MP). Figure S2. Total proteins identified by mass spectrometry approaches and differentially abundant proteins Figure S3. Protein-protein interaction and functional clustering of proteins displaying decreased abundances in TBinf-MP Figure S4. Gene ontology classifications of identified proteins from both mass spectrometry approaches display highly comparable profiles according to biological process, cellular compartment and

		molecular function
SuppInfo_TableS3	.xlsx	Table S3. Protein report for MP proteome QSTAR Elite MS data analysis
SuppInfo_TableS4	.xlsx	Table S4. Peptide report for MP proteome QSTAR Elite MS data analysis
SuppInfo_TableS5	.xlsx	Table S5. Protein report for MP proteome LTQ-Orbitrap velos MS data analysis
SuppInfo_TableS6	.xlsx	Table S6. Peptide report for MP proteome LTQ-Orbitrap velos MS data analysis
SuppInfo_TableS7	.xlsx	Table S7. Proteins decreased in abundance in the TBinf-MP

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