Extracellular Vesicles Promote Mycobacterial Killing in Macrophages in Combination with Antibiotics

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Abstract

Mycobacterium tuberculosis (*M.tb*), the causative agent of tuberculosis (TB), has been a major source of human suffering since antiquity. Presently, over 2 billion people are infected by *M.tb* worldwide, leading to an estimated 10 million active TB cases and 1.4 million deaths in 2020. Drug-resistant TB is becoming a major threat in the global TB control. Multidrug-resistant/rifampicin-resistant TB (MDR/RR TB) was diagnosed in an estimated 4.1% of new cases and about 19% of previously treated cases. Among these, approximately 6.2% of cases were extensively drug-resistant TB (XDR-TB). An estimated treatment success rate for MDR/RR-TB and XDR-TB was 54% and 30%, respectively. Treatment for MDR/RR-TB and XDR-TB requires a longer therapeutic duration with less effective, more expensive and toxic drugs, leading to a higher rate of treatment failure and mortality. To stop the global spread of MDR/RR-TB and XDR-TB, new anti-TB drugs or combined regimens are urgently needed. Recently, a combined therapeutic strategy consisting of an adjunct immunotherapy and anti-mycobacterial drugs has been proposed and investigated. In our current study, we found that extracellular vesicles isolated from *M.tb*-infected macrophages synergistically increased *M.tb* clearance in macrophages in combination with moxifloxacin, a key antibiotic against MDR-TB, in *in vitro* cell culture infection model and *in vivo* mouse model. We further demonstrated that extracellular vesicles isolated from *M.tb*-infected macrophages acted by activating host cytosolic RIG-I/MAVS-dependent pathway and LC3-associated *M.tb*-containing phagolysosome maturation in host cells. Our results shed light on the development of extracellular vesicle-based host-directed therapy against tuberculosis in humans.

Introduction

Extracellular vesicles (EVs) are membrane-bound vesicles released by both eukaryotic and prokaryotic cells. These vesicles play an important role in intercellular communication regulating various cellular functions of recipient cells. Based on their origin and size, EVs released by eukaryotic cells are divided into three main categories: exosomes, microvesicles, and apoptotic bodies (Schorey et al., 2015). Previous studies found that *M.tb*-infected macrophages release exosomes and microvesicles carrying *M.tb* PAMPs including mycobacterial proteins, lipids, and nucleic acids. These EV-carrying *M.tb* PAMPs may be detected by PRRs on recipient cells to activate or attenuate cellular responses (Bhatnagar et al., 2007; Giri et al., 2010; Walters et al., 2013; Hare et al., 2015; Singh and Schorey, 2015; Li et al., 2018). More recently, EVs released by *M.tb*-infected human neutrophils were also found to regulate proinflammatory response in recipient cells (Alvarez-Jiménez et al., 2018). EVs from *M.tb*-infected macrophages trigger the TNF-a production in THP-1 human macrophages and naïve mouse bone marrow-derived macrophages (BMMs) (Bhatnagar et al., 2007; Singh et al., 2012). A similar result was detected in Raw 264.7 cells treated with EVs from *M.bovis* BCG-infected macrophages (Walters et al., 2013). In contrast, these vesicles also suppress the expression of major histocompatibility complex (MHC) class II molecules through a TLR2-dependent pathway in mouse BMMs (Singh et al., 2011). In the context of the adaptive immune response, *M.tb* antigens carried in host cell-derived EVs may be delivered to the antigen processing and presentation pathway in recipient cells. EVs from *M.bovis* BCG-infected or *M.tb* culture filtrate protein (CFP)-pulsed macrophages activate an *M.tb* Ag-specific CD4+ and CD8+ T cell response in naïve mice or mice previously vaccinated with *M.bovis* BCG. The EV-vaccinated mice were also protected from a low-dose aerosol *M.tb* infection (Giri and Schorey, 2008; Cheng and Schorey, 2013). The recent identification of mycobacterial RNA within EVs (Singh and Schorey, 2015) suggests that host RNA sensors may also be activated in EV-recipient cells.

In the present study, we found that the transport of *M.tb* RNA to EVs is dependent on the expression of the mycobacterial SecA2 secretion system and that EVs carrying *M.tb* RNA stimulate IFN-β production in recipient BMMs. Moreover, EVs also promote LC3-associated *M.tb* phagosome maturation in a RIG-I/MAVS-dependent pathway. Finally, we found EVs from *M.tb*-infected BMMs work synergistically with antibiotics to decrease bacterial load within infected macrophages and following an in vivo mouse infection and do so in a MAVS-dependent manner.

Results

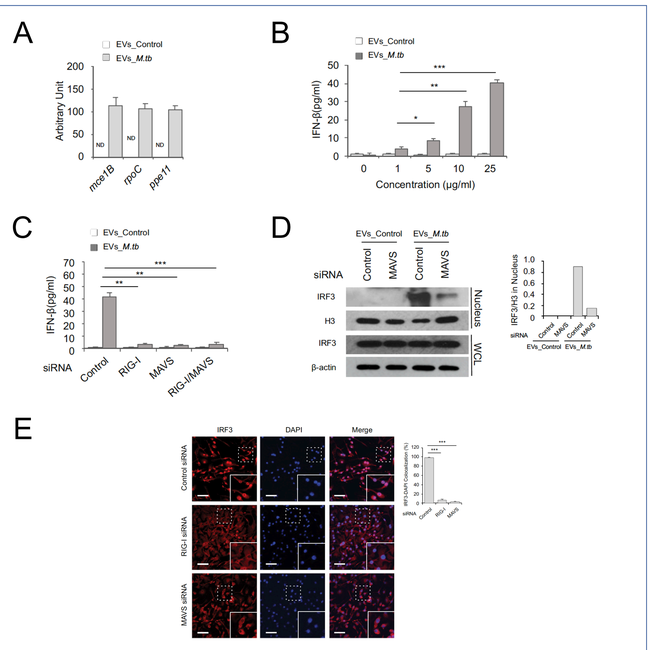


Figure 1. EVs released by *M.tb*-infected macrophages stimulate RIG-I/MAVS-dependent type I interferon expression in host cells.
(A): qRT–PCR analysis for *M.tb* RNA in EVs from uninfected (EVs_Control) or *M.tb*-infected (EVs_*M.tb*) BMMs. ND, not detected. **(B):** qRT–PCR analysis for IFN-β protein level in wild-type BMMs 24 h after treatment with EVs (1 μg/ml equals approximately $3x10^8$ vesicles/ml). **(C):** ELISA analysis for IFN-β protein in either WT or MAVS- or RIG-I-knockdown BMMs treated for 24 h with 10 μg/ml EVs from uninfected or *M.tb*-infected macrophages. Control: negative control siRNA. **(D):** Western blot analysis for IRF3 in WT and MAVS-knockdown BMMs treated for 4 h with EVs from uninfected or *M.tb*-infected macrophages. Histone H3 (H3) and β-actin were used as loading controls for nuclear fraction and whole-cell lysate (WCL), respectively. Densitometry of the Western blots is shown. **(E):** IRF3 nuclear translocation was analyzed in RIG-I- or MAVS-knockdown BMMs. Scale bar, 20 μm. Data shown in (A–C) are the mean ± SD (n = 3 wells per group), and all data shown are representative of three independent experiments (biological replicates). *P < 0.05, **P < 0.01, and ***P < 0.001 by Student's t-test (two-tailed).

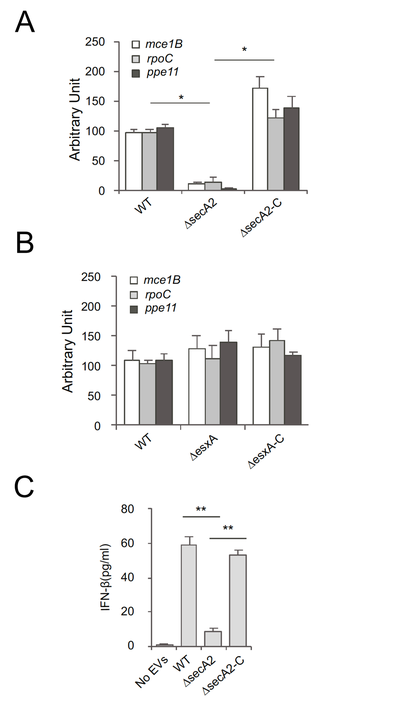


Figure 2. EV-induced type I production in macrophages requires the *M.tb* SecA2 secretion system.
(A and B): qRT–PCR analysis for *M.tb* RNA in EVs from BMMs infected with various *M.tb* CDC 1551 strains **(A)** or Erdman strains **(B)**. **(C):** ELISA analysis for IFN-β protein level in WT BMMs treated for 24 h with EVs isolated from BMMs that were infected with the different *M.tb* CDC 1551 strains. Data shown are the mean ± SD (n = 3 per group), and all data shown are representative of three independent experiments. *P < 0.05, **P < 0.01 by Student's t-test (two-tailed).

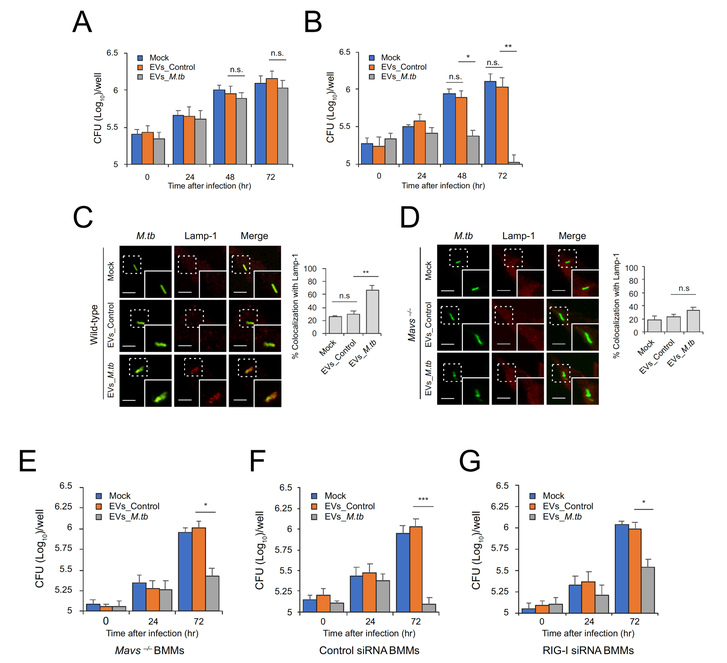


Figure 3. EVs released by *M.tb*-infected macrophages restrict *M.tb* replication in host cells by activating the *M.tb* RNA/RIG-I/MAVS signaling pathway.
(A and B): *M.tb* CFU in WT mouse BMMs pre-treated with EVs minus **(A)** or plus **(B)** IFN-γ. BMMs were treated with EVs from uninfected (EVs_Control) or *M.tb*-infected (EVs_*M.tb*) cells for 0, 24, 48, and 72 h following a 1-h *M.tb* infection. Mock, no EV treatment. **(C and D):** Immunofluorescence microscopy analysis for colocalization of *M.tb* (GFP) with lysosome marker Lamp-1 in WT **(C)** or *Mavs*$^{-/-}$ **(D)** mouse BMMs. Cells were pre-treated for 5 h with EVs from uninfected or *M.tb*-infected macrophages plus IFN-γ and then infected with GFP-expressing *M.tb* for 24 h prior to immunostaining. **(E–G):** *M.tb* CFU in infected *Mavs*$^{-/-}$ **(E)**, control siRNA-treated **(F)**, or RIG-I siRNA-treated **(G)** mouse BMMs pre-treated with IFN-γ plus EVs from uninfected (EVs_Control) or *M.tb*-infected (EVs_*M.tb*) BMMs. CFU was determined immediately after the 1-h infection or 24 and 72 h post-infection. Data shown are the mean ± SD of three independent infections, and all data shown are representative of at least three independent experiments. Scale bars, 5 μm (C and D). n.s., not significant; *P < 0.05, **P < 0.01, and ***P < 0.001 by two-tailed Student's t-test.

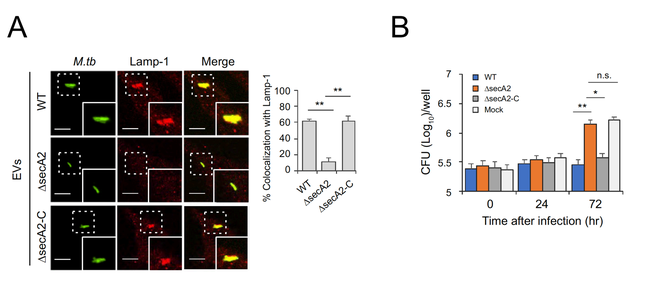


Figure 4. EV-stimulated *M.tb* phagolysosome maturation in BMMs relies on *M.tb* SecA2 secretion system.
(A): Immunofluorescence microscopy analysis for colocalization of *M.tb* with Lamp-1 in WT BMMs pre-treated with EVs from macrophages infected with WT, ΔsecA2, or secA2-complemented (ΔsecA2-C) *M.tb* CDC 1551 strains. The cells were pre-treated with EVs supplemented with recombinant mouse IFN-γ for 5 h and subsequently infected for 24 h with GFP-expressing *M.tb*. **(B):** *M.tb* CFU in WT BMMs pre-treated with recombinant mouse IFN-γ and EVs from macrophages infected with WT, ΔsecA2, or secA2-complemented (ΔsecA2-C) *M.tb* CDC 1551 strains. Data shown in are the mean ± SD of three independent infections, and all data shown are representative of at least three independent experiments. Scale bars, 5 μM (A). n.s., not significant; *P < 0.05 and **P < 0.01 by Student's t-test (two-tailed).

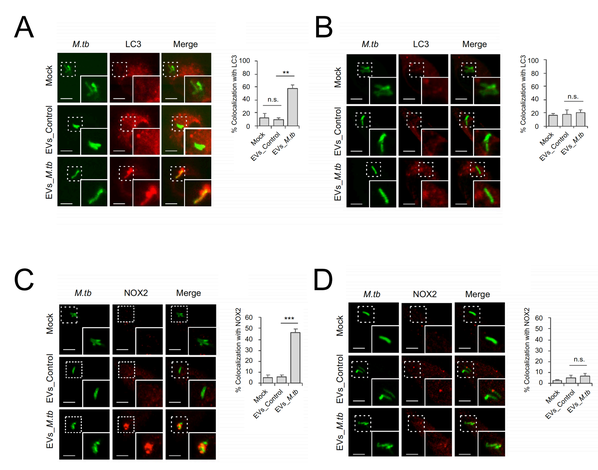


Figure 5. EVs released by *M.tb*-infected macrophages activate LC3-associated phagosome maturation in BMMs via a RIG-I/MAVS-dependent pathway.
(A): Immunofluorescence microscopy and quantitative analysis for colocalization of *M.tb* with marker LC3 in WT BMMs that were left untreated or pre-treated for 5 h with recombinant mouse IFN-γ and EVs from uninfected (EVs_Control) or *M.tb*-infected (EVs_*M.tb*) macrophages, followed by a 24-h infection with GFP-expressing *M.tb*. Mock, untreated. **(B):** Similar to **(A)**, but in *Mavs*$^{-/-}$ BMMs. **(C):** Immunofluorescence microscopy analysis for colocalization of *M.tb* with NOX2 in WT BMMs. **(D):** Similar to **(C)**, but using *Mavs*$^{-/-}$ BMMs. Quantitative data are the mean ± SD of three independent infections, and all data shown are representative of at least three independent experiments. Scale bars, 5 μm. n.s., not significant; **P < 0.01, and ***P < 0.001 by Student's t-test (two-tailed).

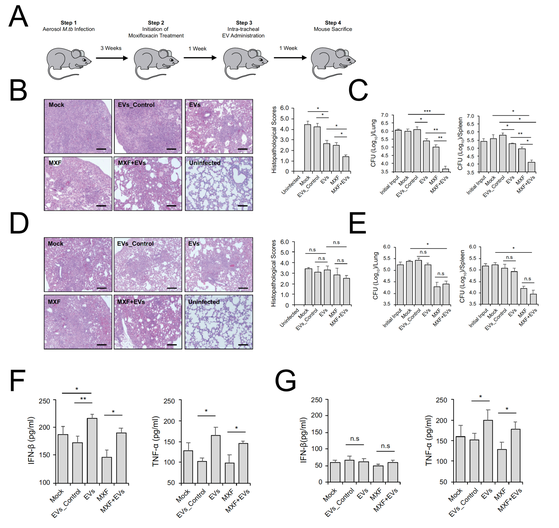
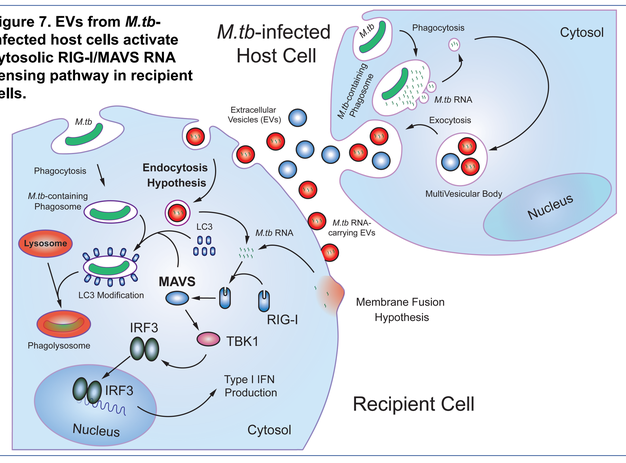


Figure 6. EVs released by *M.tb*-infected macrophages significantly decrease *M.tb* survival in mice when combined with moxifloxacin.
(A): Schematic for EV-based adjunctive immunotherapy and moxifloxacin-based chemotherapy in *M.tb*-infected mice. **(B and D):** Representative histopathological analysis for lung sections of WT **(B)** and *Mavs*$^{-/-}$ **(D)** mice that were infected with *M.tb* and subsequently left untreated (Mock) or treated with EVs from uninfected BMMs (EVs_Control), EVs from *M.tb*-infected BMMs (EVs), moxifloxacin (MXF), or a combination of EVs and MXF (EVs+MXF). **(C and E):** *M.tb* CFU in the lung and spleen of WT **(C)** or *Mavs*$^{-/-}$ **(E)** mice treated with EVs, MXF, or a combination of both. **(F and G):** ELISA analysis for IFN-β and TNF-α protein level in serum from *M.tb*-infected WT **(F)** or *Mavs*$^{-/-}$ **(G)** mice treated with EVs, MXF, or a combination of both. Data shown are representative of two independent experiments. The results in (B–G) are the mean ± SD (n = 4 mice per group). Scale bars, 100 μm (B and D). n.s., not significant; *P < 0.05, **P < 0.01, and ***P < 0.001 by Student's t-test (two-tailed).

Figure 7. EVs from *M.tb*-infected host cells activate cytosolic RIG-I/MAVS RNA sensing pathway in recipient cells.



Conclusion

In summary, we found that the presence of *M.tb* RNA in EVs released from infected macrophages is dependent on the bacteria's SecA2 secretion system. Further, these EVs carrying *M.tb* RNA can activate the host RIG-I/MAVS/TBK1/IRF3 RNA sensing signaling pathway in recipient macrophages, leading to the production of type I IFNs. Additionally, a RIG-I/MAVS-dependent phagosome maturation is induced by EVs from *M.tb*-infected macrophages, resulting in an increased trafficking of *M.tb* into LC3- and Lamp-1-positive vesicles and increased bacterial killing. Finally, we found that EVs can synergize with TB antibiotics to promote bacterial clearance and limit lung pathology suggesting a novel immunotherapeutic approach to treat drug-resistant *M.tb*.

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