Regulatory roles of extracellular vesicles in immune responses against *Mycobacterium tuberculosis* infection

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**Abstract**

Extracellular vesicles (EVs) are cystic vesicles naturally released by most mammalian cells and bacteria. EV contents include proteins, lipids, and nucleic acids. EVs can act as messengers to transmit a variety of molecules to recipient cells and thus play important regulatory roles in intercellular signal transduction. EVs, released by either a host cell or a pathogen, can carry pathogen-associated antigens and thus act as modulators of immune responses. EVs derived from *Mycobacterium tuberculosis* (*Mt*)-infected cells can regulate the innate immune response through various pathways, such as regulating the release of inflammatory cytokines. In addition, EVs can mediate antigen presentation and regulate the adaptive immune response by transmitting immunoregulatory molecules to T helper cells. In this review, we summarize the regulatory roles of EVs in the immune response against Mt.

**Key Words:** Extracellular vesicles; Exosomes; *Mycobacterium tuberculosis*; Infection; Antigen; Immune regulation

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**Core Tip:** Extracellular vesicles (EVs) are nanoscale membrane-bound structures released by mammalian cells and bacteria and play essential regulatory roles in intercellular signal transduction and the immune response. In this review, we discuss...
Extracellular vesicles (EVs) are nanoscale membrane-bound structures released by mammalian cells and bacteria. They contain proteins, lipids, and nucleic acids. EVs can be categorized into four types according to their biological origin, release pathway, size, and content: Exosomes, microparticles, microvesicles, and apoptotic bodies. Exosomes are mainly formed through the fusion of multivesicular bodies with the plasma membrane and the extracellular release of intracavitary vesicles, with a diameter of 30–100 nm and a buoyancy density of 1.13-1.19 g/mL. Exosomes are cup-shaped under a transmission electron microscope and characterized by the expression of CD63 and CD61 markers such as TyA, C1a, and CD35. Microvesicles are larger than exosomes and 100–1000 nm in diameter. They originate from the outer bud of the cytoplasmic membrane and carry selectins and integrins on the surface. The diameter of apoptotic bodies is 1-5 μm, and these EVs are the result of the disintegrated membrane of apoptotic cells[9]. Accumulated studies have indicated that EVs can be taken up by recipient cells and subsequently release their content to regulate gene expression in the recipient cells[9, 10]. Therefore, EVs are critical regulators of various biological processes, such as embryonic development, angiogenesis, and the immune response. This review aims to summarize the research progress on the regulatory role of EVs in the immune responses against Mycobacterium tuberculosis (Mtb).

INTRODUCTION

Tuberculosis (TB) is one of the major lethal infectious diseases caused by Mycobacterium tuberculosis (Mtb). According to the World Health Organization’s global TB report of 2020, approximately 10 million people were infected with Mtb in 2019, causing approximately 1.4 million deaths[1]. Although several public health measures have been taken to prevent the spread of Mtb, the situation is concerning[2]. Drugs-resistant Mtb, especially rifampicin-resistant Mtb, has become one of the deadliest pathogens in the world[2,3]. Therefore, the development of novel anti-Mtb reagents or vaccines is urgent and essential. Investigating the molecular mechanism of the immune response against Mtb is of great value because this information is the basis for preventive and therapeutic approach developments.

Extracellular vesicles (EVs) are nanoscale membrane-bound structures released by mammalian cells and bacteria. They contain proteins, lipids, and nucleic acids. EVs can be categorized into four types according to their biological origin, release pathway, size, and content: Exosomes, microparticles, microvesicles, and apoptotic bodies. Exosomes are mainly formed through the fusion of multivesicular bodies with the plasma membrane and the extracellular release of intracavitary vesicles, with a diameter of 30–100 nm and a buoyancy density of 1.13-1.19 g/mL. Exosomes are cup-shaped under a transmission electron microscope and characterized by the expression of CD63 and CD61 markers such as TyA, C1a, and CD35. Microvesicles are larger than exosomes and 100–1000 nm in diameter. They originate from the outer bud of the cytoplasmic membrane and carry selectins and integrins on the surface. The diameter of apoptotic bodies is 1-5 μm, and these EVs are the result of the disintegrated membrane of apoptotic cells[9]. Accumulated studies have indicated that EVs can be taken up by recipient cells and subsequently release their content to regulate gene expression in the recipient cells[9, 10]. Therefore, EVs are critical regulators of various biological processes, such as embryonic development, angiogenesis, and the immune response. This review aims to summarize the research progress on the regulatory role of EVs in the immune responses against Mtb.

SUMMARY OF THE IMMUNE RESPONSE AGAINST MTB

After being inhaled into the respiratory tract, Mtb is first recognized by antigen-presenting cells (APCs) resident in the lungs, including alveolar macrophages, pulmonary macrophages, and DCs[11]. The pattern-recognition receptors expressed on the APC surface sense pathogen-associated molecular patterns and endocytose Mtb to form a phagolysosome. Simultaneously, the innate immune response is initiated,
and several inflammatory cytokines are released to promote the clearance of \textit{Mtb} in APCs. Subsequently, the APCs migrate to the lymph nodes and initiate an adaptive immune response through antigen presentation and cytokine secretion[12-14].

However, \textit{Mtb} has a variety of immune evasion strategies[15]. For example, it can inhibit the acidification and maturation of phagolysosomes \textit{via} multiple virulence factors, such as protein tyrosine kinase[16], protein tyrosine phosphatase[17], and lipoarabinomannan (LAM)[18]. \textit{Mtb} can also affect the adaptive immune response by regulating antigen presentation. For example, \textit{Mtb} lipoarabinomannan mannose can bind to CD209 on DCs and inhibit DC maturation[19], promote the release of interleukin (IL)-10, reduce the synthesis of IL-12, and ultimately inhibit the release of interferon-γ (IFN-γ) by T helper (Th) cells[20]. The immune evasion mechanisms of \textit{Mtb} are beyond the scope of this review; for more details, please refer to the review by Lerner \textit{et al}[11].

\section*{EVS RELEASED BY HOST CELLS CONTAIN M\textit{TB}-ASSOCIATED ANTIGENS}

Some \textit{in vitro} experiments have indicated that \textit{Mtb} infection can increase the exosome yield and alter the protein composition of EVs released by host cells. For example, the exosome yield of mouse macrophages increases approximately two-fold after \textit{Mtb} infection[21]. However, not all types of cells produce increased amounts of exosomes after \textit{Mtb} infection. For example, Diaz \textit{et al}[22] found that the exosome yields of THP-1-derived macrophages infected with \textit{Mtb} or left uninfected were comparable.

\textit{Mtb} infection can alter the protein profile of exosomes released by a host cell. A study applying liquid chromatography-tandem mass spectrometry revealed that there were 355 host proteins in the exosomes released by \textit{Mtb}-infected macrophages. Most of the proteins were membrane proteins, and 41 of the proteins, including Hsp90, vimentin, Coronin 1 C, and moesin, were increased after \textit{Mtb} infection[22]. In addition, \textit{Mtb} itself can also release vesicles, which are termed bacterial vesicles (BVs)[23,24]. BVs are rich in \textit{Mtb} antigens, such as SodB, EsxN, and Ag85b[23,24]. The protein composition of BVs is different from that of \textit{Mtb} itself[23]. Furthermore, there is great overlap between the protein profile of vesicles released by \textit{Mtb}-infected cells and BVs in aseptic culture[22,23,25-27]. Several studies have confirmed that exosomes released by \textit{Mtb}- or \textit{Mycobacterium bovis} (\textit{M. bovis}) BCG-infected cells contain \textit{Mtb}-related antigens and thus are potentially immunogenic. For example, THP-1 cells infected with \textit{M. bovis} BCG can release exosomes containing \textit{Mtb} 19-kDa lipoprotein and LAM, two \textit{Mtb} antigens[25]. J774 cells stimulated with \textit{M. bovis} BCG also release exosomes containing the Ag85 complex[26].

In addition, \textit{Mtb} DNA was also observed in exosomes released by \textit{Mtb}-infected RAW264.7 cells[29]. Notably, a study revealed that there are two subtypes of EVs (mostly exosomes) released by \textit{Mtb}-infected mouse macrophages. One subtype expressed CD69 and CD9 but did not contain \textit{Mtb} antigens, while the other expressed \textit{Mtb} antigens, such as lipomannan (LM) and LAM[27]. These two subtypes of exosomes could be separated by sucrose density gradient centrifugation. These findings were validated by a subsequent study[24].

Notably, no \textit{Mtb} antigens have been observed in exosomes released by mouse macrophages infected with heat-inactivated or γ-ray-inactivated \textit{Mtb}[27,30], while the exosomes produced by J774 cells treated with \textit{Mtb} culture filtrate protein 10 were shown to contain \textit{Mtb} antigens[30]. In addition, heat-inactivated \textit{Mtb} cannot release BVs when cultured alone[31]. Taken together, these findings imply that the \textit{Mtb} antigens in \textit{Mtb}-infected cell-released exosomes are induced by live \textit{Mtb} or \textit{Mtb} secreted proteins rather than mycobacterial lysis within the infected cell.

A previous study revealed that the \textit{Rab27a} gene is essential for the synthesis of mammalian exosomes[32]. \textit{In vitro} experiments indicated that \textit{Rab27a} knockout could decrease the exosome yield and the content of \textit{Mtb} proteins in exosomes[21]. Furthermore, compared with wild-type mice, \textit{Rab27a} knockout mice had decreased serum exosome levels after \textit{Mtb} infection[21]. The bacterial load was also shown to be increased in \textit{Rab27a} knockout mice, suggesting that exosomes participate in the immune response against \textit{Mtb}[21]. In another study, Bhatnagar \textit{et al}[25] infected mice with \textit{M. bovis} BCG and found that the exosomes in the bronchial lavage fluid contained both human components (Hsp70) and \textit{Mtb} proteins, such as LAM and 19 kDa lipoproteins.
Extracellular vesicles in infection. Since IFN-γ is mainly produced by Th1 cells during infection in the spleen, indicating that exosomes promote the adaptive immune response against bacterial load and a significantly reduced activated mice released decreased amounts of exosomes and consequently had an increased plasma membrane.

As mentioned earlier, Rab27a is a key regulator of the fusion of exosomes and the plasma membrane. Two studies compared the microRNA profiles of exosomes released by cells infected with Mtb or M. bovis BCG-infected J774 cells, THP-1 cells, and RAW264.7 cells can trigger mouse macrophages to release inflammatory cytokines, such as IL-1β, IL-6, IL-12p70, tumor necrosis factor-α (TNF-α), and regulated upon activation normal T cell expressed and secreted factor, and upregulate the expression of iNOS[25,30,31,34]. Exosomes from the serum of mice infected with M. bovis BCG can also promote the expression of inflammatory factors in mouse macrophages[34]. The induction of IL-1β and IL-6 is mediated by Toll-like receptor (TLR) 2, while the release of IL-10 and CCL3 is independent of TLR2[31]. The induction of TNF-α is also MyD88 and TLR4 dependent[25]. In addition, exosomes can promote the phosphorylation of p38 and IκB in mouse bone marrow-derived macrophages[25], suggesting that p38 and IκB are also involved in the production of inflammatory cytokines.

IFN-γ can enhance the clearance of Mtb in three ways: (1) Promoting the clearance of intracellular pathogens by supporting macrophages to enhance the response to reactive oxygen species or reactive nitrogen[35]; (2) Promoting the adaptive immune response by enhancing the expression of major histocompatibility complex II (MHC II) [36]; and (3) Promoting an autophagic response against pathogens[37]. The exosomes released by Mtb-infected RAW264.3 cells can inhibit the upregulation of MHC II and CD64 induced by IFN-γ in uninfected mouse macrophages through TLR2 and MyD88 [24,38]. This inhibitory effect of exosomes is associated with the cargo Mtb lipoprotein, as exosomes produced by RAW264.7 cells infected with lspA knockout (unable to synthesize lipoprotein) Mtb fail to inhibit CD64 expression induced by IFN-γ[38].

The expression of miR-18a is increased in Mtb-infected RAW264.3 cells[39], while the expression of miR-20b-5p is decreased[40]. These two microRNAs can regulate the survival, apoptosis, and proliferation of macrophages[39,40]. Both of these microRNAs were also found to be elevated in exosomes released by Mtb-infected RAW 264.3 cells, but it remains unknown whether these exosomes can be taken up by uninfected macrophages. Two studies compared the microRNA profiles of exosomes released by cells infected with Mtb or M. bovis BCG or left uninfected and verified many differentially expressed microRNAs[29,41]. Bioinformatic analysis showed that these differentially expressed microRNAs are involved in the regulation of multiple signaling pathways, including central carbon, fatty acid, and sugar metabolism[42], but whether these microRNAs can regulate the immune response remains unclear.

REGULATORY ROLE OF EVS IN THE IMMUNE RESPONSE AGAINST Mtb

As mentioned earlier, Rab27a is a key regulator of the fusion of exosomes and the plasma membrane[32]. Smith et al[21] found that Mtb-infected Rab27a gene-deficient mice released decreased amounts of exosomes and consequently had an increased bacterial load and a significantly reduced activated CD4+ T cell population in the spleen, indicating that exosomes promote the adaptive immune response against Mtb in vivo. Furthermore, exosomes promote the T cell response during Mtb mouse infection. Since IFN-γ is mainly produced by Th1 cells during Mtb infection[43], these...
studies suggest that exosomes are involved in the adaptive immune response against *Mtb* and can promote an antigen-specific T cell response[21].

The exosomes secreted by *M. bovis* BCG-infected J774 macrophages can enhance the expression of CD83, CD86, IL-12p40, and MHC II in mouse bone marrow-derived dendritic cells (BMDCs) and thus promote BMDCs mutation[26]. The release of IL-12p40 by DCs can promote the Th1 response[44,45]. Therefore, exosomes containing *Mtb* antigens can promote a subsequent Th1 response via DCs. In addition, *Mtb* itself can also release EVs (termed BVs) in culture, and these vesicles can upregulate the expression of CD86, MHC I, and MHC II in mouse BMDCs, which thereby enhances the release of IL-2 by Ag85-specific T cells[24]. Ramachandra *et al*[46] found that macrophages infected with *Mtb* could release EVs containing MHC II, including microvesicles and exosomes, in which ATP greatly enhanced the release of vesicles. Microvesicles and exosomes have the ability to present *Mtb* peptide–MHC II complexes to T cells[46]. These results suggest that innate immune cells can deliver *Mtb* antigens to T cells outside the infected site by releasing microvesicles and exosomes.

The exosomes released from *M. bovis* BCG-infected J774 cells can promote the proliferation of T cells and upregulate the expression of CD69[26], which is a marker of T cell activation[47]. In addition, these exosomes can directly enhance IFN-γ release from CD4+ and CD8+ T cells in *M. bovis* BCG-immunized mice[26]. These biological functions can be further enhanced in the presence of DCs[26]. *In vivo* studies have indicated that treating mice with exosomes can increase the proportion of spleen effector T cells (CD62L-low, CD44-high)[26]. These findings were further validated by subsequent studies. Exosomes from *Mtb* antigen-treated cells (*Mtb* CFP-treated J744 cells[30] or *Mtb* CFP-treated RAW264.7 cells[48]) can also activate T cells from *Mtb* antigen-immunized mice and enhance T cell production of IFN-γ[26,30] and IL-12[48]
in vitro. Interestingly, adjuvants have little effect on the production of IFN-γ and IL-12 [26,30], indicating that these exosomes may contain some types of substances similar to adjuvants. Furthermore, these exosomes can induce the production of memory T cells in mice [26,30] and reduce the susceptibility of mice to Mtb [48].

Athman et al. [49] found that EVs released by macrophages from mice infected with Mtb and Mtb BVs could directly inhibit the anti-CD3 and anti-CD28 antibody-induced activation of naive T cells and effector T cells. This inhibitory effect was mainly attributed to Mtb antigens in the EVs, including LAM, LM, PIM1/2, and PIM6. Previous studies have shown that these Mtb antigens can inhibit the activation of T cells, which represents one of the immune evasion mechanisms of Mtb [50]. EVs can transmit Mtb antigens to T cells and promote the expression of GRAIL [49], a negative regulator of T cell activation [51,52]. Therefore, EVs can regulate the adaptive immune response against Mtb in at least two ways: Modulating the antigen presentation process and directly regulating T cells.

**CONCLUSION**

In recent years, several studies have been performed to explore the characteristics and potential biological functions of EVs in the immune response against Mtb. However, our understanding of the immunomodulatory role of EVs in Mtb infection is still in its early stages. The regulatory roles of EVs in the immune response against Mtb are summarized in Figure 1. The EVs released by Mtb-infected host cells contain Mtb-related proteins and nucleic acids, which establishes the foundation for a regulatory role in the immune response against Mtb. EVs regulate both the innate and adaptive immune responses against Mtb through various pathways. Therefore, EVs may represent a key factor in the development of an Mtb vaccine.

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