Extracellular vesicles from malaria-infected red blood cells

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Extracellular vesicles from malaria-infected red blood cells: not all are secreted equal

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Extracellular vesicles (EVs) mediate the transfer of molecules between cells and play diverse roles in host–pathogen interactions. Malaria is an important disease caused by intracellular *Plasmodium* species that invade red blood cells and these red blood cells release EVs. The EVs from infected cells have diverse functions in the disease and an obstacle in understanding how they exert their functions is that multiple EV types exist. In this issue of EMBO reports, Abou Karam and colleagues use sophisticated biophysical techniques to isolate and characterize two EV subpopulations produced by red blood cells infected with *Plasmodium falciparum* (Abou Karam *et al*, 2022). The authors show that these EV subpopulations have distinct sizes, protein content, membrane packing, and fusion capabilities, suggesting that EV subpopulations from infected cells could target different cell types and subcellular locations. This work underscores the concept that understanding EV heterogeneity will go hand in hand with understanding EV functions.

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See also: **P Abou Karam *et al*** (2022)

Extracellular vesicles (EVs) are small lipid bilayered structures released by cells into the environment, where they can be taken up by other cells as a form of cell-to-cell communication that impacts normal and disease physiology. There has been an explosion of interest in studying the functions of EVs in a range of disease contexts, with excitement that harnessing or inhibiting EVs could help diagnose and treat disease. This also extends to infectious diseases, since many pathogens exploit EVs as a communication mechanism to move their molecules into host cells, to signal to other pathogens, or to alter the host environment to favor pathogen survival. In the case of malaria, EVs derived from red blood cells infected with the *Plasmodium* species have been shown to activate immune cells, communicate information between parasites, and alter a range of host cell types to enable infection (reviewed in Opadokun & Rohrbach, 2021). Work in the last 5 years from various biological systems has shown that multiple EV types can be simultaneously released from cells, which have different functional properties. An obstacle in the field is separating the different EV types; studying a heterogeneous pool of EVs has been equated to trying to do immunology before there was the capacity to separate cell types using technologies such as Fluorescence-Activated Cell Sorting (FACS).

There are at least three classes of EVs, which are generated by distinct biogenesis pathways: those derived from the endosomal system (exosomes), those that bud from the plasma membrane (microvesicles), and those that derive from cells undergoing apoptosis (apoptotic bodies) (Phillips *et al*, 2021). Extensive work in the last decade has shown that EVs derived from different biogenesis pathways cannot always be distinguished by size, or even specific protein markers (Kowal *et al*, 2016). Furthermore, EVs generated through one biogenesis pathway can also include multiple subpopulations with distinct properties (Kowal *et al*, 2016; Willms *et al*, 2016). While this sophistication in EV biology is exciting, it also represents a massive technical challenge that has led to the invention and/or adaption of more diverse methods to characterize EVs (Phillips *et al*, 2021).

In this issue of EMBO Reports, Abou Karam and colleagues take advantage of recent advancement in EV isolation methods to separate and characterize two EV subpopulations generated from red blood cells infected with the protozoan parasite *Plasmodium falciparum (Pf)* (Abou Karam *et al*, 2022). These parasites are the causative agent of malaria, which represents a huge global public health burden. The EVs released from infected cells (termed "*Pf* -iRBC-EVs") have previously been shown to contribute to malaria-associated clinical symptoms, including severe disease cerebral malaria (reviewed in Sampaio *et al*, 2017). This could be linked to the EV role in manipulating immune cells; however, *Pf*-iRBC-EVs have also been shown to play a role in parasite cell–cell communication, promoting differentiation to sexual forms or gametocytes which has implications in *Plasmodium* transmission from the human host to the mosquito vector (reviewed in Babatunde *et al*, 2020). Given the so-far unexplained diversity in functional effects induced by malaria-derived EVs, it is plausible that different EV subpopulations underpin different functions. Indeed, recent findings indicate that EVs produced by red blood cells infected with different life stages of

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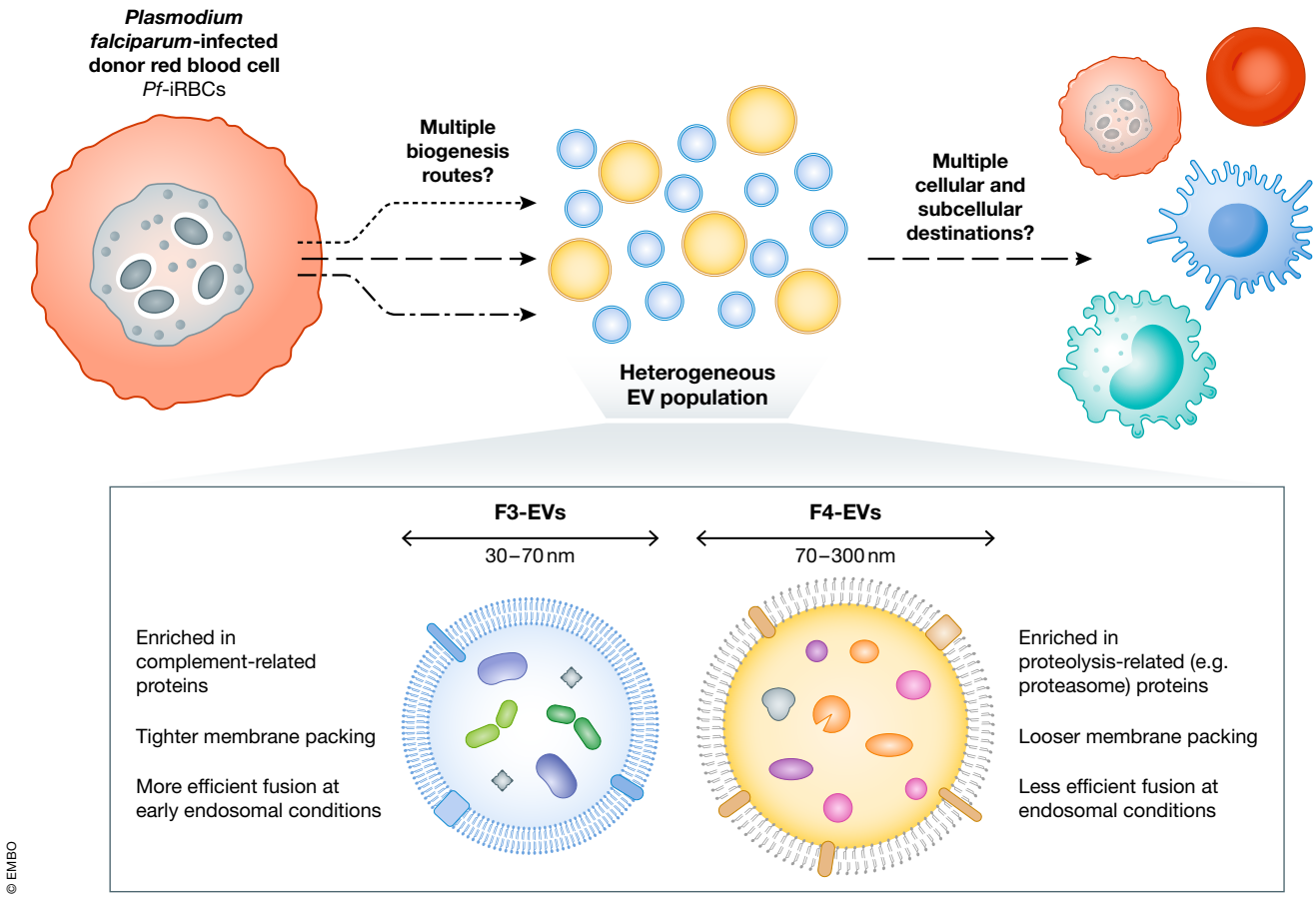

Figure 1. *Plasmodium falciparum*-infected red blood cells produce at least two subpopulations of extracellular vesicles

Using different biophysical techniques, Abou Karam *et al* (2022) characterize two distinct extracellular vesicle (EV) subpopulations with different sizes, protein content, membrane biophysical properties, and membrane fusion capabilities. The smaller F3-EVs (30–70 nm) had more densely packed lipid membranes and demonstrated better fusion capability to early endosomal conditions as compared to the larger F4-EVs (70–300 nm), suggesting that each EV subpopulation may traffic to different recipient cells or subcellular locations, where they could mediate divergent functions in the host–pathogen interaction and disease.

P. falciparum contain different protein cargos (Opadokun *et al*, 2022).

In this study, Abou Karam *et al* (2022) employed a sophisticated combination of techniques to separate and characterize EVs generated by *Pf*-infected red blood cells. They used asymmetrical flow field-flow fractionation (AF4), a method that separates extracellular nanoparticles based on their hydrodynamic size, (Phillips *et al*, 2021), to identify two EV subpopulations (Fig 1) with distinct size ranges of 30–70 nm (F3-EVs) and 70–300 nm (F4-EVs). The sizes of the *Pf*-iRBC EVs were further confirmed using atomic force microscopy (AFM) and cryo-transmission electron microscopy. Liquid chromatography with tandem mass spectrometry (LC–MS-MS) of these subpopulations identified 132 proteins in total in both fractions, 23 of which were *P. falciparum*-derived proteins, and 109 of which were human, including proteins that may mediate vesicle fusion (Abou Karam *et al*, 2022). Of these, 66 proteins had differential abundance in the two EV fractions: 6 *P. falciparum* proteins and 60 human proteins, demonstrating that F3-EVs and F4-EVs contain different protein cargos that include both parasite- and human-derived factors. Complement-associated proteins were enriched in F3-EVs, and proteolysis-related proteins including proteasome subunits were enriched in F4-EVs, potentially describing a mechanism by which EV subpopulations may induce different phenotypic effects in recipient cells (Fig 1).

The two EV subpopulations also had distinct membrane characteristics, as demonstrated by Laurdan staining, which is sensitive to the polarity and fluidity of membranes, and atomic force microscopy puncture analysis, which measures the force required to punch small holes in the EV membrane and serves as a readout for the mechanical properties of the membrane (Abou Karam *et al*, 2022). Both assays indicated that the membranes of smaller F3-EVs had denser lipid packing compared to F4-EVs, which could impact EV tolerance to different subcellular environments and could impact their fusion properties. A Förster resonance energy transfer (FRET)-based membrane mixing assay was also used to investigate membrane fusion properties and showed that maximal fusion probability for both EV subpopulations occurs under conditions (pH and membrane makeup) that mimic the plasma membrane. Both EV subpopulations demonstrate preferential fusion to the plasma membrane, with F3-

EVs demonstrating better fusion capability to early endosomal conditions as compared to F4-EVs (Abou Karam *et al*, 2022). These differences in EV characteristics suggest that each EV subpopulation could have different targeting properties, in terms of recipient cells as well as subcellular compartments.

This work provides a potential explanation for how different EV subpopulations could show different specificities in uptake, trafficking, and stability in recipient cells, which could also underpin different phenotypic responses. While the study by Abou Karam demonstrated that *Pf*-iRBCs generate EV subpopulations with distinct protein cargos and distinct lipid properties, it remains to be elucidated how this is achieved, and how this affects trafficking to recipient cells or subcellular compartments. The need for further characterization of EV subpopulations interfaces with reports in the last year that propose additional diverse mechanisms by which EVs from *Pf*-iRBC could impact malaria infections: from priming naïve RBCs to enable parasite invasion (Dekel *et al*, 2021) to regulating cytokine levels in immune cells (Ofir-Birin *et al*, 2021). It will be timely to investigate the EV biogenesis mechanisms that differentiate EV subpopulations and the role of the parasite factors in driving this, since *P.falciparum* release EVs while growing inside their organelle-free host cells. Due to the diversity of phenotypic effects induced by EVs during *Plasmodium* infections (Babatunde *et al*, 2020), this model provides the potential to link EV heterogeneity with measurable phenotypic outcomes, representing a valuable tool for further investigation of the role of EV heterogeneity in disease outcomes.

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Disclosure and competing interests statement

The authors declare that they have no conflict of interest.

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