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SHORT COMMUNICATION

Sialylated *N*-glycans mediate monocyte uptake of extracellular vesicles secreted from *Plasmodium falciparum*-infected red blood cells

Hila Ben Ami Pilo[1] | **Sana Khan Khilji**[2,3] | **Jost Lühle**[2,3] | **Karina Biskup**[4] | **Bar Levy Gal**[5] | **Irit Rosenhek Goldian**[6] | **Daniel Alfandari**[1] | **Or-Yam Revach**[1] | **Edo Kiper**[1] | **Mattia I. Morandi**[1] | **Ron Rotkopf**[7] | **Ziv Porat**[5] | **Véronique Blanchard**[4] | **Peter H. Seeberger**[2,3] | **Neta Regev-Rudzki**[1] | **Oren Moscovitz**[2]

[1]Faculty of Biochemistry, Department of Biomolecular Sciences, Weizmann Institute of Science, Rehovot, Israel

[2]Department of Biomolecular Systems, Max-Planck-Institute of Colloids and Interfaces, Berlin, Germany

[3]Institute of Chemistry and Biochemistry, Freie Universität Berlin, Berlin, Germany

[4]Institute of Laboratory Medicine, Clinical Chemistry and Pathobiochemistry, Charite University Medicine Berlin, Berlin, Germany

[5]Flow Cytometry Unit, Life Sciences Core Facilities, Weizmann Institute of Science, Rehovot, Israel

[6]Department of Chemical Research Support, Weizmann Institute of Science, Rehovot, Israel

[7]Bioinformatics Unit, Life Sciences Core Facilities, Weizmann Institute of Science, Rehovot, Israel

Correspondence
Neta Regev-Rudzki, Faculty of Biochemistry, Department of Biomolecular Sciences, Weizmann Institute of Science, Rehovot, Israel.
Email: neta.regev-rudzki@weizmann.ac.il
Oren Moscovitz, Department of Biomolecular Systems, Max-Planck-Institute of Colloids and Interfaces, Berlin, Germany.
Email: oren.moscovitz@mpikg.mpg.de

Hila Ben Ami Pilo, Sana Khan Khilji and Jost Lühle contributed equally to this work.

Abstract

Glycoconjugates on extracellular vesicles (EVs) play a vital role in internalization and mediate interaction as well as regulation of the host immune system by viruses, bacteria, and parasites. During their intraerythrocytic life-cycle stages, malaria parasites, *Plasmodium falciparum* (*Pf*) mediate the secretion of EVs by infected red blood cells (RBCs) that carry a diverse range of parasitic and host-derived molecules. These molecules facilitate parasite-parasite and parasite-host interactions to ensure parasite survival.

To date, the number of identified *Pf* genes associated with glycan synthesis and the repertoire of expressed glycoconjugates is relatively low. Moreover, the role of *Pf* glycans in pathogenesis is mostly unclear and poorly understood. As a result, the expression of glycoconjugates on *Pf*-derived EVs or their involvement in the parasite life-cycle has yet to be reported.

Herein, we show that EVs secreted by *Pf*-infected RBCs carry significantly higher sialylated complex *N*-glycans than EVs derived from healthy RBCs. Furthermore, we reveal that EV uptake by host monocytes depends on N-glycoproteins and demonstrate that terminal sialic acid on the *N*-glycans is essential for uptake by human monocytes. Our results provide the first evidence that *Pf* exploits host sialylated *N*-glycans to mediate EV uptake by the human immune system cells.

1 | INTRODUCTION

Malaria is a vector-borne parasitic disease that affects over 200 million people worldwide and is caused by the *Plasmodium* parasite, transmitted by female mosquitoes of the genus Anopheles sp. (Carrera-Bravo et al., 2021). There are five known species of the parasite that cause infection, and *P. falciparum* is the deadliest due to its prevalence, virulence, and drug resistance (Kappe

et al., 2010). *Plasmodium* undergoes three life phases: exo-erythrocytic (host liver), erythrocytic (host red blood cells (RBCs)), and sporogony (mosquito vector's gut) (Kappe et al., 2010). During various life stages of the parasite, *Plasmodium* exploits host and parasite glycans for their survival (Kappe et al., 2010). Glycans denote the carbohydrate part of molecules that consist of monosaccharides linked via glycosidic bonds to proteins, lipids, nucleic acids, and additional monosaccharide building blocks. As found on the cell surface and the extracellular matrix (ECM), glycans are engaged and modulate most cell-cell and cell-ECM interactions (Varki et al., 2017). Moreover, the glycocalyx is the initial contact point for different cell-pathogen interactions, such as bacteria (Formosa-Dague et al., 2018), viruses (Li et al., 2021), and parasites (Veríssimo et al., 2019), including *Plasmodium* (Goerdeler et al., 2021). Thus, various glycans on host and parasite cells contribute to malaria pathogenesis and play a significant role in parasite-host interactions. For example, the highly abundant free and protein-coupled glycosylphosphatidylinositol (GPI) glycolipids on *Plasmodium* membrane serve as a proinflammatory toxin and contribute to infection severity (Gowda et al., 1997; Schofield & Hackett, 1993). However, to date, only a few glycoconjugates have been identified on *Plasmodium* parasites. These include short, truncated *N*- and *O*- glycosylations (Bushkin et al., 2010; Swearingen et al., 2017), C-mannosylation (Hoppe et al., 2018), *O*-fucosylation (Swearingen et al., 2017), and α-Gal antigen (Galα1–3Galβ1–4GlcNAc-R) (Yilmaz et al., 2014). Importantly, although shown to be essential for different aspects as protection against malaria transmission (Yilmaz et al., 2014) and colonization of the mosquito midgut (Lopaticki et al., 2017), current understanding of parasite glycan contribution to its survival is still limited.

Nevertheless, similar to other pathogens, the parasite exploits host glycans at different life-stages. For instance, terminal glycosylations of the different blood group antigens are utilized by *Plasmodium* to invade RBCs, and their presence dictates higher (Cserti & Dzik, 2007; Fry et al., 2008; Uneke, 2007) or lower (Pathak et al., 2016) *Plasmodium* susceptibility. Moreover, glycosaminoglycans (GAGs) such as heparan sulphate, chondroitin sulphate, and hyaluronic acid that occupy cell membranes and the extracellular matrix are also essential for *Plasmodium* infection. GAGs facilitate iRBCs adherence to brain endothelial cells (Jurzynski et al., 2007) and human placenta (Chotivanich et al., 2012), as well as parasitic invasion of hepatocytes and RBCs (Frevert et al., 1993; Pancake et al., 1992; Rogerson et al., 1995).

The sialic acid-dependent RBC invasion by *Plasmodium* has been studied for decades (Camus & Hadley, 1985; Orlandi et al., 1992; Sim et al., 1994). Erythrocyte-binding Antigen 175 (EBA-175) forms essential contacts with Neu5Ac(α2–3)Gal on the terminal end of O-glycans expressed on Glycophorin A (Sim et al., 1994), while EBA-140 interacts with sialic acid on Glycophorin C on the RBC surface (Lobo et al., 2003; malpede et al., 2013). In addition, an identified *N*-glycan motif on the Glycophorin C recognized by one variant of EBA-140 demonstrates the importance of sialylation of both *N*- and *O*-glycans of host cells for *Plasmodium* cytoadherence and invasion (Mayer et al., 2006).

Extracellular Vesicles (EVs) are produced across almost all the living kingdoms (Carrera-Bravo et al., 2021; Malda et al., 2016; Osteikoetxea et al., 2016; Pegtel et al., 2010; Schorey et al., 2015; Wortzel et al., 2019) including parasites (Carrera-Bravo et al., 2021; Montaner et al., 2014; Ofir-Birin & Regev-Rudzki, 2019). They contain a cornucopia of different molecules and transfer functional signals to target cells. As an intracellular obligate parasite, *P. falciparum* exploits the mechanism of EV release during its blood-stage cycle to assure successful development and survival (Dekel et al., 2021; Mantel et al., 2016; Mantel et al., 2013; Ye et al., 2018). More EVs are secreted during *Plasmodium* infection than in healthy conditions, as is also seen in other diseases such as cancer (Macedo-Da-Silva et al., 2021; Sampaio et al., 2017). EVs secreted by *Pf*-infected-RBCs (iRBCs) serve as fundamental tools for delivering key components that enable the parasites to sense and regulate their surroundings from within the iRBC (Babatunde et al., 2020; Ofir-Birin & Regev-Rudzki, 2019; Ofir-Birin et al., 2021; Ye et al., 2018). Indeed, these EVs deliver cargo to different host cells (Sisquella et al., 2017; Toda et al., 2020; Ye et al., 2018),including immune, endothelial, and healthy as well as iRBCs in order to promote parasite growth (Mantel et al., 2016; Regev-Rudzki et al., 2013; Sisquella et al., 2017; Ye et al., 2018). Recent findings demonstrate that these EVs play important roles in the malaria immune-evading mechanism (Hosseini-Beheshti & Grau, 2018; Ofir-Birin & Regev-Rudzki, 2019; Ofir-Birin et al., 2021) and in disease pathogenesis (Antwi-Baffour et al., 2020; Neveu et al., 2020; Toda et al., 2020). While several parasitic proteins were already identified to play a role in EV production (Avalos-Padilla et al., 2021; Regev-Rudzki et al., 2013), the research on EV uptake mechanism is still at infancy, and the key components mediating the internalization of the vesicles from iRBCs are still unknown.

EVs are rich in glycans that play an important role in biogenesis, cargo recruitment, and different pathologies, including parasitic infections (Dagenais et al., 2021; Carregari et al., 2018; Gavinho et al., 2020; Macedo-Da-Silva et al., 2021; Mcnamara & Dittmer, 2020; Rodrigues et al., 2018; Toledo et al., 2020). Changes in cell surface glycans are a hallmark for disease, and it stands to reason that these changes also reflect at the EV level (Macedo-Da-Silva et al., 2021; Moremen et al., 2012; Nishida-Aoki et al., 2020; Sampaio et al., 2018; Tan et al., 2020; Xu et al., 2018). Furthermore, glycoconjugates are involved in the uptake of EVs by recipient cells (Williams et al., 2018). For example, internalization of *Schistosoma mansoni schistosomula* EVs by host-dendritic cells (Kuipers et al., 2020; Van Die & Cummings, 2017), is mediated by complex-type *N*-glycans, carrying Lewis motifs and oligomannose glycans. Interestingly, although carrying a similar glycosylation profile, *Schistosoma*-derived EVs exhibited differences in the relative abundance of glycans compared to the parasite cell surface glycans (Kuipers et al., 2020).

The involvement of sialylated glycoconjugates in EV uptake by various cells is under investigation (Royo et al., 2019; Saunderson et al., 2014; Shenoy et al., 2018; Shimoda et al., 2017). Desialylation abolished the inhibitory effects of exosome-GD3 glycolipids on T cell activation in ovarian cancer (Shenoy et al., 2018). Similarly, a significant decrease in EV uptake was achieved by

interference of sialic acid-binding immunoglobulin-type lectin (Siglec) receptors by Siglec-3 targeting antibody and free monosaccharide inhibition *in vitro* (Shimoda et al., 2017), as well as *in vivo*, with murine gene knockouts of Siglec-1 (Saunderson et al., 2014). In contrast, EV de-N-glycosylation and desialylation have been shown to increase their uptake by various cell lines (Williams et al., 2019), showing the importance of studying uptake mechanisms for individual cells and organisms. EV biodistribution is also seen to be altered *in vivo* due to changes in sialylation (Royo et al., 2019).

Here, we present the first study on glycosylation in EVs isolated from *Pf*-iRBCs (*Pf*EVs) and suggest a critical role of host glycans on EVs in malaria pathogenesis. By lectin binding to EV lysates, we showed differences in glycan content between EVs derived from uninfected and *Pf*-iRBCs. Furthermore, trimming *N*-glycans from the EV surface revealed high levels of sialylated complex *N*-glycans on *Pf*EVs which are absent from healthy EVs. Given that, unlike EVs from iRBCs, EVs derived from healthy uninfected RBCs (uRBCs) are less taken up by THP1 monocytes (Sisquella et al., 2017), we speculated that sialylated *N*-glycan contribute to *Plasmodium*'s interaction with the host immune system, as seen in various diseases such as cancer (Büll et al., 2014), bacterial (Severi et al., 2007), and additional parasitic infections (Freire-De-Lima et al., 2012). To test this hypothesis, we shaved sialic acid off the surface of *Pf*EVs and analysed their uptake by human monocytes. We further examined EV uptake after preincubating the monocytes with free sialylated N-glycopeptides (SGPs) extracted from chicken egg yolk (Seko et al., 1997). Notably, in both cases, the uptake of *Pf*EVs by monocytes was significantly lower, supporting a sialylated *N*-glycan-dependent uptake. As *Pf* parasites do not possess the genetic machinery to synthesize full length complex *N*-glycans (Samuelson et al., 2005), we provide the first evidence of host sialylated *N*-glycan interception by *Pf* parasites for interactions with host immune cells via EVs.

2 | RESULTS

2.1 | *Pf*-derived EVs express surface *N*-glycans

The involvement of membrane glycoconjugates in the uptake of *Pf*EVs by host immune cells had not been extensively studied. Therefore, we set out the system to explore the glycobiology aspects behind *Pf*EVs. Due to lack of reports characterizing surface glycans on *Pf*EVs or their glycoconjugates cargo, we first isolated EVs from healthy uRBC (hEVs) and *Pf*-iRBCs (*Pf*EVs) as was previously described (Regev-Rudzki et al., 2013) and characterized them by lectin staining and mass spectrometry (Figure 1a). At first, we lysed both EV samples and inspected by western-blot analysis using plant lectins that recognize different glycan epitopes (Table S1). We observed significantly higher binding to *Pf*EV glycoconjugates for most lectins tested, including WGA, SNA, ECA, UEA, MMA, and RCA. The mannose-binding lectin Concanavalin A (ConA) showed similar binding to both hEVs and *Pf*EVs, indicating equal levels of mannose glycoconjugates (Figure 1b and Table S2). The results indicated a higher level of different glycoconjugates in lysates of *Pf*EVs (Figure 1b). Importantly, examining the different glycan epitope specificities of the binding lectins (sialic acid, Gal(β1-4)GlcNAc, fucose) suggested a higher expression of complex *N*-glycan-coupled glycoconjugates in *Pf*EV lysates. In order to validate complex *N*-glycan presence on EV surface, we incubated the EVs with peptide-N 4-(N-acetyl-β-glucosaminyl) asparagine amidase F (PNGase F) to release *N*-glycans from EV surface glycoproteins enzymatically. The released *N*-glycans were isolated and analyzed using MALDI-TOF-MS. Interestingly, in contrast to high-mannose and complex-type *N*-glycans detected in both samples, qualitative and quantitative changes of the *N*-glycome were observed in *Pf*EVs compared with non-infected EVs (Figure 1c). The additional peaks between *m/z* 2500–3250 observed in infected EVs were further characterized by LIFT-MS/MS analysis that revealed the presence of complex sialylated *N*-glycans carrying both the *N*-acetylated (purple diamond) as well as non-acetylated (white diamond) sialic acid variants (Figure S1 and S2). Sialylated *N*-glycans were solely measured in *Pf*EVs. Moreover, *Pf*EVs carried *N*-glycans bearing bisecting N-acetylglucosamine and core-fucosylation, similar to recently identified findings on human erythrocytes surface (Bua et al., 2021). Hybrid-type *N*-glycans were not identified in infected or non-infected EVs.

These results indicate a significantly higher level of complex sialylated *N*-glycans in *Pf*EVs as compared with hEVs (Figure 1c).

2.2 | *N-glycans* on *Pf*-derived EV proteins mediate uptake by host monocytes

Encouraged by our results, we set to corroborate the involvement of *N*-glycans on secreted *Pf*EVs in the uptake mechanism by host immune cells as human monocytes. Isolated EVs from uninfected or *Pf*-infected RBCs were labeled with thiazole orange, RNA-specific fluorescent stain, as was previously shown (Ofir-Birin et al., 2018; Sisquella et al., 2017) and treated with PNGase F to remove *N*-glycans from the membrane glycoproteins. As a control, EVs were treated with the serine protease proteinase K (PK) to completely digest surface proteins. Following enzymatic digestion, EV concentration was measured using Nanosight (NTA) analysis, and uptake by monocytes (THP-1 cells) was quantified using Imaging Flow Cytometry (IFC) as previously described (Sampaio et al., 2018) (Figure S4). Atomic force microscopy (AFM) imaging and Nanosight (NTA) measurements indicated no change in shape and structure of the PNGase F treated-EVs and confirmed their integrity (Figure 2a lower panel and Figure S3,

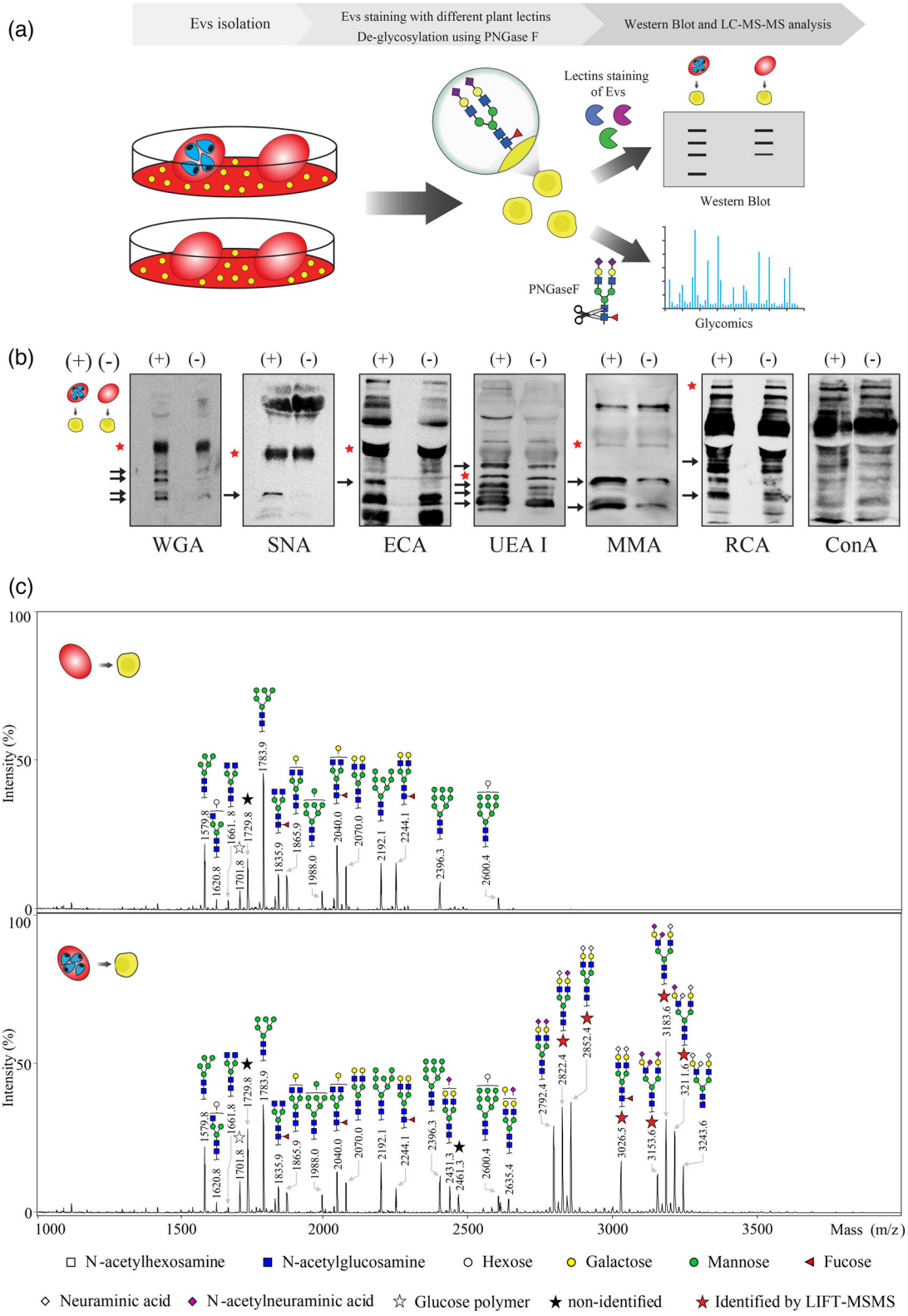

FIGURE 1 **Sialylated *N*-glycans are present on the cell surface of *Pf*-derived EVs.** **(a)** Schematic overview of the workflow for detecting N-glycosylations on *Pf*-infected RBCs -derived EVs. **(b)** Western-blot staining of hEVs and *Pf*EVs. Lectin abbreviations and binding specificities are depicted in Table S1. Black arrows indicate staining differences between *Pf*EVs (left) and control hEVs derived from non-infected RBCs (right), red stars indicate reference bands used for quantification. **(c)** MALDI-TOF mass spectra of permethylated *N*-glycans isolated from (upper panel) hEVs and (lower panel) *Pf*EVs. Measurements were carried out in the positive-ionization mode. All molecular ions are present in their sodiated $[M+Na]^+$ form. Blue squares, GlcNAc; Green circle, Man; Yellow circle, Gal; light blue diamond, Neu5Gc; Purple diamond, Neu5Ac, Red triangle, Fucose. Red stars indicate structures characterized by LIFT-MS/MS analysis

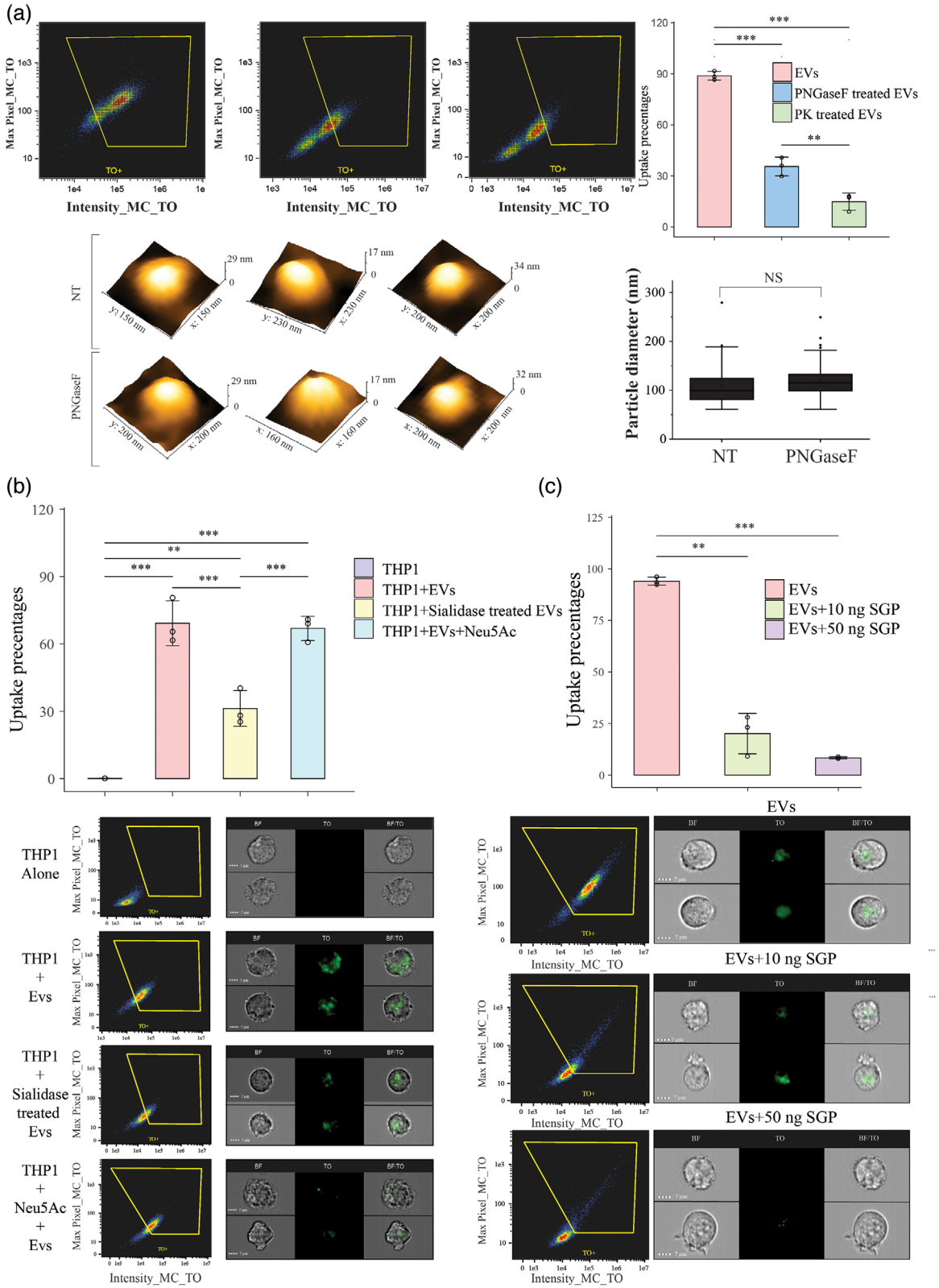

FIGURE 2 **Sialylated *N*-glycans mediate PfEV uptake by monocytes. (a)** *N*-glycan digestion by PNGase F reduces *Pf*EV uptake by monocytes (THP-1 cells). *Pf*EVs were labeled by Thiazole orange (TO) and treated with either PNGase F or Proteinase K as control. The treated EVs were then incubated with monocytes (THP-1 cells) and their uptake was measured using Imaging Flow Cytometry (IFC) (upper panel). Graphs show the percentage of positive labeled cells, gated according to cells treated with unlabeled EVs. Representative results out of three experiments are shown. Error Bars represent standard deviation, statistical analysis was done using ANOVA-One Way ($F2,6 = 209.1$ $p < 0.0001$) and Tukey HSD. The integrity of non-treated EVs and EVs treated with PNGase F was validated using Atomic force microscopy (AFM). Lower panel, representative AFM 3D images of control and PNGase F treated EVs. EV size analysis is presented in a Box plot, the black dots represent outliers and the black horizontal line represents the median. **(b)** Sialidase treatment reduces *Pf*EV uptake by

left and middle panels). The uptake of non-treated EVs was measured by IFC and exhibited significant uptake level by THP-1 cells (Figure 2a upper panel). In contrast, *N*-glycan removal from EV surface proteins by PNGase F resulted in significant inhibition of the uptake that was comparable to PK digestion (Figure 2a upper panel). These results indicate a role of *N*-glycans in *Pf*EV uptake by THP-1 cells.

2.3 | Sialic acid on *N*-glycans regulate uptake of *Pf*-EVs by human monocytes

To better characterize the role of *N*-glycans in the interaction of *Pf*-derived EVs with recipient THP-1 cells, we treated *Pf*EVs with neuraminidase (Sialidase) that trims α2–3/6/8 sialic acid from all EV surface glycans. Nanosight (NTA) measurement confirmed the integrity of the sialidase treated *Pf*EVs post treatment (Figure S3, right panel). Notably, sialidase treatment resulted in a substantial reduction of PfEV uptake by THP-1 cells as observed by IFC analysis (Figure 2b). Interestingly, presaturating the monocytes with a high concentration of free sialic acid monosaccharides (N-acetylneuraminic acid, Neu5Ac) did not alter *Pf*EV uptake in comparison to control non-treated EVs, pointing toward the need for a sialylated-*N*-glycan component for *Pf*EVs uptake (Figure 2b). Next, to further demonstrate the need for an intact sialylated-*N*-glycan in *Pf*EV uptake, we purified sialylated complex *N*-glycopeptides (SGPs) from egg yolks (Alagesan & Kolarich, 2019) (Figure 2c and S5). We then pretreated THP-1 cells with elevated SGP concentrations prior to incubation with *Pf*EVs. As shown in Figure 2c, increase in SGPs induced a gradual decrease in *Pf*EV uptake, indicating saturation of *Pf*EV- binding receptor(s) on THP-1 cell surface (Figure 2c).

In summary, our results provide the first indication that the parasite can utilize host *N*-glycans machinery for parasite-host interactions throughout *Pf*EV uptake.

3 | DISCUSSION

EVs derived from *Pf*-infected red blood cells (iRBCs) play an important role in malaria infection and pathogenesis. They were shown to mediate cell-cell communication between iRBCs via transfer of genetic material and, by this, contribute substantially to parasite differentiation and transmission (Ankarklev et al., 2014; Mantel et al., 2013; Neveu et al., 2020; Regev-Rudzki et al., 2013). Furthermore, it was shown that EVs from *Pf*-iRBCs interact with the host's immune system to alter the innate immune response (Mantel et al., 2013; Sampaio et al., 2018; Sisquella et al., 2017). However, the molecular mechanism underlying EV interaction with immune cells is poorly understood. In particular, the role of EV surface glycans in interactions with host factors remains unstudied, although they serve as part of the first line of contact between the vesicles and the immune system. We provide the first *in-vitro* evidence that glycosylation patterns of EVs derived from *Pf*-iRBCs are altered and that those distinct glycans mediate interaction with the host's immune system cells.

To characterize EV glycosylation patterns, we performed both lectin-based detection of glycans via western blot and mass spectrometry-based glycomics. The western blot results show both the occurrence of additional glycoconjugates and higher exposure of pre-existing glycoconjugates in *Pf*-iRBC-derived EVs. Staining with different lectins indicates that terminal sialic acids (WGA, SNA, MMA), α-L-fucose (UEA I), and Gal(β1-4) GlcNAc repeats (ECA, RCA) are more prominent in parasite-derived EVs (Figure 1b). High-mannose structures (ConA) did not show a significant difference in expression compared to healthy EVs. This glycosylation pattern indicates the increased occurrence of complex, sialylated, and core-fucosylated *N*-glycans on the surface of *Pf*EVs. These findings are supported by the mass spectrometry results, which prove the occurrence of additional complex, sialylated *N*-glycans in *Pf*EV in contrast to hEVs (Figure 1c).

Furthermore, the role of sialylated glycans on interaction with immune cells was examined by measuring the uptake of EVs by human monocytes. Monocytes were shown to interact with *Pf*EVs by internalization and downstream transcriptional changes (Ofir-Birin et al., 2021; Sampaio et al., 2018; Sisquella et al., 2017), but the mechanism of uptake is yet to be elucidated. We provide the first evidence that altered EV glycosylation is critical for the uptake mechanism by monocytes, as PK- and PNGase F-treated EVs showed a significant reduction in internalization (Figure 2a). Furthermore, sialidase-treated EVs showed a similar reduction in uptake, even though sialic acid monosaccharide alone did not inhibit interaction (Figure 2b). On the other hand, treatment of the monocytes with purified glycopeptides bearing complex, sialylated *N*-glycans was shown to inhibit the uptake

monocytes. *Pf*EVs were labeled by TO and were treated with Neuraminidase (sialidase). The stained EVs were incubated with monocytes and with sialic acid-treated monocytes and their uptake was measured using IFC. Graphs show the percentage of TO-labeled positive cells, gated according to cells treated with unlabeled EVs, and representative images of recipient monocytes. Representative results from three experiments are shown. Error Bars represent standard deviation, statistical analysis was done using ANOVA-One Way ($F_{3,8} = 67.12$ $p < 0.0001$) and Tukey HSD. **(c)** Sialylated *N*-glycopeptides block *Pf*EV uptake by monocytes. *Pf*EVs were labeled by TO and incubated with monocytes or sialylated *N*-glycopeptides treated monocytes and their uptake was measured using IFC. Graphs show the percentage of labeled cells, gated according to cells treated with unlabeled EVs. Representative results from three experiments are shown. Error Bars represent standard deviation statistical analysis was done using ANOVA-One Way ($F_{2,6} = 193$ $p < 0.0001$) and Tukey HSD. Significant code: $p < 0.05$,"*," $p < 0.01$, "**," $p < 0.001$,"***"

in a concentration-dependent manner (Figure 2c). These findings suggest the interaction of *Pf*EVs with human monocytes to be dependent on complex *N*-glycans, while terminal sialic acid is mandatory for efficient EV uptake.

Our results show that EV glycosylation in *Pf* infection is altered toward a higher abundance of complex, sialylated *N*-glycans. Moreover, those glycans mediate *Pf*EV uptake in contrast to EVs derived from healthy uRBCs, that are less internalized (Sisquella et al., 2017). These findings suggest that EV glycosylation plays a vital role in host-pathogen interactions during malaria infection and, furthermore, propose an additional mechanism of immune alteration exploited by the parasite. Immune escape mediated by increased expression of sialylated glycoconjugates is a long documented phenomenon exploited by cancer cells (Büll et al., 2014) and different bacterial pathogens (Severi et al., 2007). Immune system inhibition is executed by expressing sialylated glycan structures on proteins and lipids that prevent recognition by different immune system cells by binding inhibitory sialic acid-binding immunoglobulin-type lectins (Siglecs). Increased exposure of highly sialylated *N*-glycans on *Pf*EVs supports the hypothesis that *Pf* exploits a similar mechanism to dampen the host immune response by interaction with host cells and downstream immune modulation. This possible mechanism of EV-mediated molecular mimicry by *Pf* was proposed previously at the protein level (Armijos-Jaramillo et al., 2021), but no evidence for the involvement of glycans in this process has been provided until now. However, altered EV glycosylation was reported in other diseases to dramatically influence EV-cell interactions, impacting receptor interaction and cellular uptake (Dusoswa et al., 2019; Whitehead et al., 2020; Williams et al., 2019). In addition, sialic-acid-dependent uptake through Siglec-3 on HeLa cells of EVs derived from mesenchymal stem cells was reported (Shimoda et al., 2017). Interestingly, in contrast to our results, a decrease in EVs uptake was reported when preincubating the cells with anti-Siglec-3 mAb and increased concentration of free sialic acid. Nevertheless, uptake was not entirely obliterated, while in our case, incubation with SGP showed a drastic decrease in internalization of EVs supporting the involvement of a complex sialylated N-glycoprotein conjugate and not just sialic acid in EV uptake.

Pf can obtain GlcN and GlcNAc monosaccharides from external sources for its survival (Chi et al., 2020; Gomes et al., 2019), but uptake of full-length *N*-glycans by *Pf* parasites is not yet reported. In addition, although *Pf* can express most subunits of the oligosaccharyl transferase complex (OST) needed to link *N*-glycans to proteins (Gomes et al., 2019), evidence for OST expression or synthesis of full length complex *N*-glycans or sialic acid has not been provided yet (Goerdeler et al., 2021; Macedo et al., 2010). Moreover, due to lack of suitable mannose and glucose Asn-linked glycans (Alg) glycosyltransferases to elongate the precursor *N*-glycan during its synthesis in the Endoplasmic Reticulum, the parasites' *N*-glycans repertoire is limited to short structures composed of two GlcNAc moieties at most (Bushkin et al., 2010; Samuelson et al., 2005).

Even though most of these glycan structures are present on healthy uRBC surfaces (Bua et al., 2021), the higher abundance of complex N-glycans on *Pf*EVs cannot be explained by increased N-glycosylation expression, as mature RBCs lack genomic DNA. Nevertheless, *Pf*-iRBCs were recently reported to present significantly higher levels of high mannose N-glycans on their cell surface due to oxidative stress at the late stages of the intraerythrocytic parasite cycle (Cao et al., 2021). Furthermore, although *Pf* parasites do not possess the genetic machinery to synthesize full length complex N-glycans (Samuelson et al., 2005), host sialylated N-glycoproteins carried or expressed on *Pf*EV membrane are a plausible explanation for our observations. To date, the diverse ways by which *Pf* parasites manipulate their intraerythrocytic surroundings and utilize EVs to ensure their survival are far from fully understood. Nonetheless, *Pf*EVs and hEVs differ in nucleic acids (Sisquella et al., 2017) cytosolic protein cargo (Dekel et al., 2021), and even display distinct membrane lipids profiles (Gulati et al., 2015). Moreover, current knowledge on membrane protein cargo of *Pf*EVs is limited, and significant differences are observed even between EVs derived from substrains as NF54 (Dekel et al., 2021) and 3D7 (Mantel et al., 2013). Interestingly, RBC membrane glycoproteins as the anion transport protein (Band 3), glycophorins, or the Glucose transporter 1 Glut-1 (Cao et al., 2021; Cohen et al., 2009) are also highly abundant in *Pf*EVs (Mantel et al., 2013). We hypothesize that our observed differences in sialylated N-glycan reflect changes in the glycosylated membrane proteins displayed on *Pf*EVs and hEVs. The identity of these glycoproteins, or the exact mechanism by which the parasites manipulate EV membrane N-glycoproteins levels, awaits identification.

Comparing glycosylation patterns of different *Pf*EV subtypes and changes on the surfaces of both healthy and infected RBCs is the first step in understanding glycan expression on EVs. The molecular interaction between EV glycans and monocytes and their implications on monocyte activation needs further characterization. Finally, the surface receptor(s) on monocytes, presumably a sialic acid-binding lectin, remain to be identified.

In conclusion, our results provide the first evidence that altered glycosylation patterns on EVs secreted by *Pf*-iRBCs influence the interaction with the host's immune system in a sialic acid-dependent manner. Increased expression of complex, sialylated *N*-glycans is critical for monocyte EV uptake and might represent a mechanism to exploit host glycans for immune modulation.

4 | MATERIALS AND METHODS

4.1 | Human malaria parasites (plasmodium falciparum)

NF54 *P. falciparum* WT cells (generously provided by Malaria Research Reference Reagent Resource Centre (MR4)) were grown in pooled donor RBCs provided by the Israeli blood bank (Magen David Adom blood donations in Israel) at 4% hematocrit

and incubated at 37°C in gas mixture of 1% O2, 5% CO2 in N2 (Ofir-Birin et al., 2021). Parasites were maintained in complete RPMI medium pH 7.4, 25 mg/ml HEPES, 50 μg/ml hypoxanthine, 2 mg/ml sodium bicarbonate, 20 μg/ml gentamycin, and 0.5% AlbumaxII. *P. falciparum* cultures were tested for mycoplasma once a month using a commercial kit; MycoAlert Plus kit (Lonza). Growth was monitored using methanol fixed Giemsa-stained blood smears (Sisquella et al., 2017).

4.2 | Human monocytes (THP-1 cells)

THP-1 cell line was cultured as previously described (Unterholzner et al., 2010). In brief, cells were grown in complete RPMI 1640+, L-glutamine, and 10% FBS, in a humidified incubator at 37°C, with 5% CO2. Cells were tested for mycoplasma once a month using commercial kit; MycoAlert Plus kit (Lonza).

4.3 | EV isolation

EVs were isolated from NF54 strain. The parasites were highly synchronized, growing in relatively high parasitemia levels (~5%) at 4% hematocrit, 24 h post invasion into the RBCs (Trophozoite stage). Control EVs were harvested from uninfected RBCs cultured under the same conditions. Prior to media collection, cultures were tightly synchronized by using 5% sorbitol, according to standard protocols (Sisquella et al., 2017). EV purification was performed as previously described (Dekel et al., 2021). Briefly, 200–400 ml of parasite growth medium was collected, cellular debris was removed by centrifugation at 413 × g for 5 min, 1650 × g for 10 min (Eppendorf Centrifuge 5804), followed by centrifugation at 15,180 g for 1 h in a SORVALL RC5C PLUS, SLA1500 rotor at 4°C. The supernatant was filtered through a 0.45-μm filter and concentrated down using a VivaCell 100,000 MWCO PES (Sartorious Stedium). The supernatant was then ultracentrifuged at 150,000 × g overnight at 4°C to pellet EVs. The EV pellet was resuspended in PBS−/− and subjected to different measurements and enzymatic treatments. Isolation of EVs derived from uRBCs was done under the same method (without sorbitol synchronization); uRBCs were incubated with *Pf* media for 24 h and the media was harvested for further EV purification as described above.

We have submitted all relevant data of our experiments to the EV-TRACK knowledgebase (Van Deun et al., 2017) (EV-TRACK ID: EV220010).

4.4 | Nanosight Particle Analysis (NTA)

EV size distribution and concentration were measured using Nanoparticle Tracking Analysis (NTA) (Malvern Instruments, Nanosight NS300). Sample size distributions were calibrated in a liquid suspension by the analysis of Brownian motion via light scattering. Nanosight provides single particle size and concentration measurements.

4.5 | Sialic acid treatment for THP-1 cells

Sialic Acid (N-Acetylneuraminic acid A0812, Sigma-Aldrich) at a concentration of 100 μg/ml was added to naïve THP-1 cells, prior addition of *Pf*-EVs. Sialic acid treated cells were incubated for 1 h at 37°C and then were subjected to EV uptake analysis via IFC.

4.6 | Siaylylated N-glycopeptides treatment to THP-1

Siaylated N-glycopeptides were desalted as described below and added in two different concentrations of 10 ng and 50 ng to the THP-1 cells prior addition of the EVs. Treated cells were incubated for 5 min at 37°C and then were subjected to EV uptake analysis via IFC.

4.7 | PNGase F treatment

After suspension of the EV pellet with PBS -/- after the overnight ultracentrifugation, the EV sample was divided into two: 50 μl was used as a control and 50 μl was used for PNGase F treatment. 2.5 μl PNGase F in a concentration of 500,000 U/ml (Peptide -N-Glycosidase F, P0704S, LOT 10055890, Bio Labs) and 10X buffer- glycol buffer 2 (#B3704S, LOT 0041709, Bio Labs) were added to 50 μl sample and incubated for 4 h at 37°C. Next, the EV sample was concentrated using a Vivacell 100 with a 100 kDa

cut-off, according to the manufacturer's protocol and washed three times in 1 ml PBS-/- in order to remove any residuals of the enzyme and the glycans. EV concentration post PNGase F treatment was measured using NTA analysis.

4.8 | Proteinase K treatment

EV pellet was suspended with PBS -/- after the overnight ultracentrifugation and the solution was divided to two: 50 μl were used as untreated control and 50 μl were treated with Proteinase K (Proteinase K from *Tritirachium album*, Cat num 39450-01-6, Lot V016020168-722, Sigma-Aldrich Merck). The enzyme was added in a ratio of 1:1000 to the EV sample following incubation for 10 min at 37°C. Next, the EVs were concentrated using a Vivacell 100 with a 100 kDa cut-off, according to the manufacturer's protocol and washed three times in 1 ml PBS-/- in order to remove any residuals of the enzyme and the glycans. EV concentration post Proteinase K treatment was measured using NTA analysis.

4.9 | Sialidase treatment

EVs were concentrated using a Vivacell (Ofir-Birin et al., 2021), third of the EV sample was treated with 10 μl/ml Sialidase (Neuraminidase, REF 11080752001, LOT 1005025, Roche). The solution was incubated for 30 min at 37°C. The remaining two thirds of the EV sample were used as control and were incubated for 30 min at 37°C. The EV production proceeded to TO staining and an overnight ultracentrifugation step (Sisquella et al., 2017). The next day the EV pellet was resuspended with 100 μl PBS-/- and the EV sample concentration was measured using NTA analysis. As control, not treated EVs were incubated for the same duration of time as treated samples.

4.10 | NTA analysis for vesicle size and concentration

Analysis was conducted using NanoSight NS300 nanoparticle tracking instrument (NTA). This device monitors the the Brownian motion of nanoparticles of 30–1000 nm size, using light scattering. The software then calculates the concentration and size distribution of the nanoparticles. During these measurements, each EV sample (in a 1:1000 dilution) was measured five times for 60 s.

4.11 | AFM analysis

AFM method: Mg modifies mica was prepared as follows; Freshly cleaved mica surface was incubated with 10 mM $MgCl_2$ solution for 2 min than rinsed with 200μlPBS. 50 μl of NT-EVs or PNGase-EVs solution was placed on the Mg modifies mica for 10 min. 50 μl PBS was added to the sample prior to scanning. Images were captured using the JPK Nanowizard III AFM microscope (Berlin, Germany) in QI mode using qp-BioAC-Cl-CB-3 probe, spring constant≈0.06 N/m (Nanosensors). Image analysis was performed using Gwyddion or or JPK-SPM data processing software. Particle size analysis was conducted using grain-analysis in Gwyddion (Nečas, D. & Klapetek, P.Gwyddion: an open-source software for SPM data analysis.Cent. Eur. J. Phys.10, 181–188 (2012). Plots constructed with OriginPro 2018. Image assembled in AdobeIllustrator 2019.

4.12 | EV uptake into monocytes

THP-1 cell (human monocytes) line were cultured overnight in RPMI1640+ L-glutamine (Biological Industries Ltd., Beit Ha'Emek, Israel) and 10% FBS (Biological Industries Ltd., Beit Ha'Emek, Israel). 1–2×10^6 monocytes were plated into 6 well plates a day in advance with 2 ml of complete media. EVs were measured by Nanosight quantification and were added to the cells in a concentration of approximately 6500 EVs per cell. The cells were incubated for 5–15 min at 37°C and washed 3 times with PBS-/-. The pellet was resuspend with 20 μl PBS-/-and left on ice until the IFC. Each sample run by IFC until obtaining 10000–15000 cells.

4.13 | Monitoring EV uptake by IFC

EVs were stained using Thiazole Orange (TO) (Cat number) for RNA staining at a dilution of 1:1000 and left in 37°C for 30 min as was previously described before (Coleman et al., 2012; Ofir-Birin et al., 2018).

4.14 | Multispectral IFC analysis

Cells were imaged using a multispectral IFC (ImageStreamX mark II imaging flow-cytometer: Amnis Corp, Seattle, WA, Part of Luminex). *Pf*-derived EVs, labeled with TO were added to host cells. At least 10×10^4 cells were collected from each sample and data were analysed using the manufacturer's image analysis software (IDEAS 6.3; Amnis Corp). Monocytes were gated for single cells, using the area and aspect ratio features, and for focused cells, using the Gradient RMS feature, as previously described (Sisquella et al., 2017). Cropped cells were further eliminated by plotting the cell area of the bright field image against the Centroid X feature (the number of pixels in the horizontal axis from the left corner of the image to the centre of the cell mask). Vesicle internalization was evaluated using several features, including the intensity (the sum of the background-subtracted pixel values within the masked area of the image) and the max pixel (the largest value of the background-subtracted pixel).

4.15 | Statistical analysis

Uptake percentages were compared between treatments with a 1-way ANOVA (run separately for each date), followed by Tukey's posthoc test. Statistics were done in R, v. 4.1.0.

4.16 | Mass spectrometry analysis

N–Glycans were released from equal amounts of EVs quantified via Nanoparticle Tracking Analysis (NTA) (Malvern Instruments, Nanosight NS300) in 500 μl PBS buffer (pH 7.4) at 37°C overnight using 500 mU of Peptide-N-glycosidase F (PNGase F PRIME ; N-Zyme Scientifics). Released *N*-glycans were purified using C18 cartridges and graphitized carbon columns (both purchased from Alltech, Deerfield, IL). Cartridges were conditioned with 80% ACN containing 0.1% TFA solution and then equilibrated with 0.1% aqueous TFA. Samples were washed with 0.1% aqueous TFA. The desalted *N*-glycans were subsequently eluted with 25% ACN and 50% ACN, each containing 0.1% TFA. Eluates were pooled and evaporated to dryness under reduced atmosphere. Permethylation was performed as published by Wedepohl et al (Wedepohl et al., 2010). After termination of the reaction, permethylated *N*-glycans were extracted by chloroform/water liquid-liquid extraction. Samples were washed with water until the pH of the aqueous phase became neutral and the excess of chloroform was removed under reduced atmosphere. Permethylated *N*-glycans were dissolved in 20 μl 75% aqueous acetonitrile. For MALDI-TOF-MS measurement 0.5 μl of the sample was mixed with 0.5 μl 10 mg/ml super DHB matrix (2-hydroxy-5-methoxy-benzoic acid and 2,5-dihydroxybenzoic acid, 1:9; Sigma-Aldrich), on a ground steel MALDI target (Bruker Daltonics, Bremen, Germany) and used for MALDI-TOF-MS measurement. Spectra were recorded in a reflectron positive mode on an Ultraflex III mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a Smartbeam laser. A glucose ladder was used as internal calibration standard. Spectra were acquired with 25 kV accelerating voltage and 200 Hz laser frequency in a mass range 1000–4000 Da. In total 2000 laser shots were collected for each spectrum. *N*-Glycans were presumed to have N2H3 core structure and the compositions (numbers of H, N, F, S) were manually interpreted based on their *m/z* values. MALDI-TOF-MS spectra were annotated using the GlycoWorkBench software (version 1.1.3480). MALDI-TOF mass spectra were recorded on an Ultraflex III mass spectrometer (Bruker Daltonics) equipped with a Smartbeam laser and a LIFT-MS/MS facility.

4.17 | Purification of sialylated glycopeptides

Sialylglycopeptide (SGP) were purified from EGG yolk powder as previously described (Alagesan & Kolarich, 2019). In short, 250 g of egg yolk powder was suspended in 750 ml of water (1: 3 = W/V ratio) and stirred for 2h at 20°C. 500 ml of Methanol then added, and the mixture was stirred for another 1h at 20°C. After centrifugation at 3000 g/4°C/5 min, 10 ml chloroform were added, and the solution was centrifuged at 3000 g/4°C/10 min. The supernatant was then concentrated using a Rotovap, filtered through 0.2 μm membrane and loaded on a Sephadex G50 (fine) (25 × 935 mm) prewashed with 50 mM ammonium acetate (pH 7). SGP containing fractions were identified by a sugar staining solution on a TLC plate (3.70 mL of p-anisaldehyde in 140 mL of 3.5% H2SO4 in ethanol solution) and a dot blot binding assay using Sambucus nigra lectin III (SNA) lectin that binds the sialic acid(a2-6) galactose epitope. Positive samples were diluted in water and analysed by direct injection into a Xevo G2-XS QTof Quadrupole Time-of-Flight mass spectrometer (Waters), in positive resolution mode. *M/Z* value 956 corresponding to SGP was chosen for further MSMS fragmentation using 10v collision energy.

4.18 | Desalting sialoglycopeptide

The protocol was modified from Selman et al. 2011 (Selman et al., 2011). In short, a ~2.5 cm column with cotton was prepared. The column was equilibrated by washing 5 CV with H2O and 5 CV with 83% ACN. Sialoglycopeptide with high salt was dissolved in 83% ACN by vortexing and pipetted on to the equilibrated cotton column. Flow-through was collected. This was washed 3 × 2 CV with 83% ACN + 0.1% TFA to remove salt. For elution, 3 × 2 CV of H2O was used. To ensure everything eluted, the column was centrifuged once for 1 min at 3000 g after the final elution step. Samples were flash frozen and lyophilized after sugar staining verification (Guberman et al., 2019)

4.19 | Lectin staining of EV glycoproteins

EVs were quantified via Nanoparticle Tracking Analysis (NTA) (Malvern Instruments, Nanosight NS300) as described above. For lysis of EVs, equal amounts of non-infected and infected samples were treated with 5x RIPA buffer (250 mM Tris, 750 mM NaCl, 2.5 % Sodium deoxycholate, 5 % Triton X-100, 0.5 % SDS, pH 8) for 15 min on ice, with frequent vortexing. After centrifugation for 15 min/4°C/20,000 *g* and supernatant removal, the protein mixture was boiled at 95°C/5 min with 6x Laemmli buffer and separated by SDS-PAGE. The gels were blotted on to a 0.2 μm PVDF membrane using Trans–Blot Turbo Transfer System (Bio-Rad) and blocked with 5% BSA in PBS solution before lectin treatment. The different lectins were diluted in 1% BSA lectin buffer (10 mM Hepes, 150 mM NaCl and 0.1 mM $CaCl_2$, 0.1 % Tween-20, pH 7.5) in the following concentrations: FITC-coupled lectins 1:100 (WGA, ConA, UEA I, RCA (Lectin kit 1, Vector Labs, Catalogue Number: FLK-2100), and ECA (Vector Labs, Catalogue Number: FL-1141-5)); biotinylated MMA (Vector Labs, Catalogue Number: B-1265-1) 1:1000; SNA-HRP (bioWORLD, Catalogue Number: 21510932-1) 1:500. This was followed by 3 times wash with PBS-T for 10 min each. Subsequently, the blot was incubated for 1 h at RT with either HRP-coupled secondary antibody, Rabbit Anti-FITC-HRP (abcam, Catalogue Number: ab 19492) or Streptavidin-HRP (BioLegend, Catalogue Number: 405210) diluted in 1 % BSA in PBS-T in the following concentrations: Anti-FITC-HRP 1:5000; Streptavidin-HRP 1:2000. For detection, the blot was developed for 1 min with ECL Western Blotting Reagents (Cytiva) and imaged with LAS-4000 mini Fuji Film. The procedure was repeated for samples from two individual EV purifications. Band intensities of the different lanes were quantified using the software ImageJ (http://rsb.info.nih.gov/ij/). Bands that showed a decrease of band intensity in the non-infected samples were compared with a control band for each lectin.

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AUTHOR CONTRIBUTIONS

Oren Moscovitz and Neta Regev-Rudzki conceived, designed, and supervised the project. All authors acquired, analysed, and interpreted data. Hila Ben Ami Pilo, Sana Khan Khilji, Jost Lühle, Neta Regev-Rudzki and Oren Moscovitz wrote the manuscript, with contributions and revision from all other authors to the final version.

ORCID

Neta Regev-Rudzki https://orcid.org/0000-0001-7252-0193
Oren Moscovitz https://orcid.org/0000-0002-6310-2579

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SUPPORTING INFORMATION

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