Linkage between endosomal escape of LNP-mRNA and loading into EVs for transport to other cells

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RNA-based therapeutics hold great promise for treating diseases and lipid nanoparticles (LNPs) represent the most advanced platform for RNA delivery. However, the fate of the LNP-mRNA after endosome-engulfing and escape from the autophagy-lysosomal pathway remains unclear. To investigate this, mRNA (encoding human erythropoietin) was delivered to cells using LNPs, which shows, for the first time, a link between LNP-mRNA endocytosis and its packaging into extracellular vesicles (endo-EVs: secreted after the endocytosis of LNP-mRNA). Endosomal escape of LNP-mRNA is dependent on the molar ratio between ionizable lipids and mRNA nucleotides. Our results show that fractions of ionizable lipids and mRNA (1:1 molar ratio of hEPO mRNA nucleotides:ionizable lipids) of endocytosed LNPs were detected in endo-EVs. Importantly, these EVs can protect the exogenous mRNA during in vivo delivery to produce human protein in mice, detected in plasma and organs. Compared to LNPs, endo-EVs cause lower expression of inflammatory cytokines.
RNA-based therapeutics, which function by either silencing pathological genes through delivery of siRNA or expressing therapeutic proteins through the delivery of exogenous mRNA to cells, hold great potential for the treatment of various diseases. RNA therapy provides potential new treatment options in multiple diseases and has been tested in clinical trials of several diseases including cancer, infectious diseases, and various inherited genetic diseases. However, RNA therapy also faces substantial challenges. For instance, RNA is highly unstable in extracellular fluids because of the presence of nucleases and the fact that mRNA/siRNA needs to be taken up by the right cells and must be able to escape the endosomes to be translocated into the cytosol, for protein expression or gene silencing to occur. The mRNA modifications can increase stability to some extent; however, the transport of mRNA/siRNA to the cytoplasm of recipient cells requires safe and efficient delivery vehicles.

Lipid nanoparticles (LNPs), currently represent the most advanced platform for RNA delivery, which have now advanced into human clinical trials, and their mRNA delivery safety profiles have been evaluated in humans and non-human primates. LNP-mediated mRNA delivery has been tested in preclinical studies of Fabry disease (X-linked lysosomal storage disease) in non-human primates and disease models of Friedreich’s ataxia and methylmalonic acidemia, metabolic and behavioral abnormalities in a murine model of citrin deficiency, preclinical and clinical trials of immunogenicity for protection against Zika and influenza viruses. However, their limited capacity to undergo endosomal escape limits the use of LNPs as RNA delivery vehicles, as only a small fraction of RNA efficiently escapes endosomes to reach the cytoplasm of cells. Despite the fact that, a major proportion of LNPs (95%) is endocytosed (taken up) by cells within half hour, it is estimated that <2% of the siRNA administered via LNPs escapes the endosomes to reach the cytosol. Thereafter, the fate of endocytosed LNPs for example, how and why endosomal escape of LNP-delivered RNA is only in small amounts to reach the cytoplasm, is not completely understood.

Extracellular vesicles (EVs) are a heterogeneous population of nano- and micro-sized vesicles, including microvesicles, exosomes and several other EV populations classified by ISEV. The best described EVs are the exosomes (40–120 nm), which originate from the endosomes and are secreted through the exocytosis pathway. The components of cells could be sorted into the intraluminal vesicles of the late endosomes (also called multivesicular bodies; MVBs). Exosomes are then released into the extracellular environment upon fusion of MVBs with the plasma membrane. EVs can be isolated by several methods, but differential ultracentrifugation remains a gold standard method for processing large volumes of cultured supernatants.

In 2007, we showed for the first time that EVs contain a substantial amount of RNAs, and that EVs transport RNA between cells as a mechanism of genetic exchange. Since EVs act as endogenous carriers for the transfer of RNA between cells, these vesicles could be tailored as siRNA delivery vehicles. EVs can mediate inter-organ communication and deliver cellular cargo between various organs. Therefore, EVs are promising in vivo delivery carriers for siRNA-based therapies. However, because of their small size, inserting exogenous mRNA into EVs aiming for expressing new proteins remains challenging.

In the current study, we investigated the intracellular fate of LNP-delivered modified mRNA encoding human erythropoietin (hEPO protein), as well as the ionizable cationic lipid components (DLin-MC3-DMA and DLin-DMA) of LNPs. As stated above, <2% of the siRNA administered via LNPs escapes the endosomes, we hypothesized that remaining part of the LNP materials, i.e. hEPO-mRNA and ionizable lipids, which are localized to endosomes, can be incorporated into intraluminal vesicles of the late endosomes, and could subsequently be secreted into the extracellular environment as EVs. EVs (endosomal and plasma membrane origin) secreted after the endocytosis of LNP-mRNA are referred endo-EVs.

The present study shows that LNP components (mRNA and ionizable lipids) are partly incorporated into endo-EVs, and that the exogenously delivered mRNA is detected at a molar ratio of 1:1 (mRNA nucleotides: ionizable lipids). i.e. the mRNA should be neutrally charged by ionizable cationic lipids to enable mRNA escape, from negatively charged endosomal membrane, to reach the cytoplasm. Most importantly, these endo-EVs protect exogenous mRNA during in vivo transport to organs, and deliver the intact hEPO-mRNA to the cytoplasm of recipient cells. What comes more important is that the delivered mRNA is functional and produces human EPO protein in mice. Although the systemic delivery of both EVs and LNPs cause the expression of proinflammatory cytokines in mice, the expression levels induced by EVs are not as much higher as LNPs did.

We believe that our data could impact the production of alternative biological vehicles for the delivery of mRNAs to express proteins which are absent in the host with genetic disorders, as exemplified herein by the delivery of hEPO mRNA encoding hEPO, a secretory protein important for treating various anemic disorders.

Results
Characterization of LNPs. The LNP formulations were characterized with respect to several biological parameters including loading efficiency, average size, polydispersity index (PI) and molar ratio between individual components of LNPs (Supplementary Table 1). The efficiency of hEPO-mRNA constructs in LNPs, defined as efficiency of encapsulation (EE) was 93–97%, and the average size of LNPs with mRNA (LNP-mRNA) varied between 82–90 nm. The concentration of hEPO-mRNA in LNPs was 0.1 mg/mL. The molar percentage ratios between individual components of LNPs using the ionizable lipid DLin-MC3-DMA or DLin-DMA were as follows: ionizable lipid/cholesterol/DSPE/DMPE-PEG: 50/38.5/10/1.5. The chemical structures of the two ionizable lipids are presented in Supplementary Fig. 1a. LNPs containing DLin-MC3-DMA mRNA lipids are defined as MC3-LNPs in this study, whereas LNPs containing DLin-DMA ionizable lipids are defined as DD-LNPs.

Delivery of hEPO mRNA to cells via LNPs. The delivery of mRNA encoding human erythropoietin (hEPO protein) to cells was investigated using two different formulations of LNPs i.e. DD-LNPs and MC3-LNPs. The efficacy of mRNA delivery was examined by determining the intracellular amount of hEPO mRNA and the amount of hEPO protein produced.

The hEPO mRNA (100 µg) was transferred to cells via LNPs. After 96 h of LNP administration, hEPO mRNA was quantified in the lysates of recipient cells, and the hEPO protein was quantified both in cell lysates and the supernatants of cell-conditioned media. The results demonstrated that both formulations of LNPs could deliver hEPO mRNA to cells (Fig. 1a) and cause the production of hEPO protein (Fig. 1b, c). Compared to DD-LNPs, MC3-LNPs delivered significantly higher amounts of hEPO mRNA to cells, and accordingly produced higher amounts of hEPO protein. As hEPO is a secretory protein, it was mostly detected in the extracellular fractions (Fig. 1c) compared with the levels detected in cell lysates (Fig. 1b). Nanoparticles cause cellular stress and activate the autophagic-lysosomal pathway, which in current study may depend on the chemical composition of LNPs, as well as the
Fig. 1 Delivery of hEPO mRNA to cells via LNPs and analysis of endo-EVs. MC3-LNPs and DD-LNPs containing 100 μg of hEPO-mRNA were transferred to human epithelial (HTB-177) cells. Untreated cells and cells treated with empty LNPs (without hEPO mRNA) were used as controls. a Amount of hEPO mRNA detected in originally formulated LNPs (control, n = 5). b Amount of hEPO protein detected in cells. Untreated (n = 3), DD-LNPs (w/o mRNA) (n = 6), MC3-LNPs (w/o mRNA) (n = 3), DD-LNPs + hEPO-mRNA (n = 7), and MC3-LNPs + hEPO-mRNA (n = 5). c Amount of hEPO protein detected in the supernatant of cultured cells. Untreated (n = 3), DD-LNPs (w/o mRNA) (n = 6), MC3-LNPs (w/o mRNA) (n = 3), DD-LNPs + hEPO-mRNA (n = 5), and MC3-LNPs + hEPO-mRNA (n = 3). d Percentage of hEPO mRNA detected in the cytosol of cells relative to the total amount of mRNA administered (100 μg) to cells via LNPs. Untreated (n = 10), DD-LNPs (w/o mRNA) (n = 6), MC3-LNPs (w/o mRNA) (n = 3), DD-LNPs + mRNA (n = 8), and MC3-LNPs + mRNA (n = 7). e Hypothetical presentation of the endosomal escape of hEPO mRNA of LNPs into the cytoplasm and translation into protein, versus loading of hEPO mRNA into endo-EVs. f Total amount of hEPO mRNA quantified in endo-EVs isolated from LNP-treated cells. Untreated (n = 3), DD-LNPs (w/o mRNA) (n = 3), MC3-LNPs (w/o mRNA) (n = 3), DD-LNPs + mRNA (n = 10), and MC3-LNPs + mRNA (n = 5). g Molar concentrations of ionizable lipids and hEPO-mRNA (ionizable lipids per hEPO mRNA nucleotides) in originally formulated LNPs (control, n = 1), which contains 3 moles of ionizable lipids per 1 mole of mRNA nucleotides. h Molar concentration of ionizable lipids and hEPO-mRNA of mc3-EVs (n = 7). i Molar concentration of ionizable lipids and hEPO-mRNA of dd-EVs (n = 6). j Stoichiometric comparison between LNPs and endo-EVs regarding molar ratio (mole/mole) of ionizable lipids per hEPO mRNA nucleotides. Red circles (dd-EV) and blue circles (mc3-EV). Ionizable lipids and hEPO-mRNA of mc3-EVs (n = 7) each. Ionizable lipids and hEPO-mRNA of dd-EVs (n = 6) each. Data are presented as scatter dot plots including the mean (bars) and standard deviation (SD) of the number (n) of biologically independent samples specified for each panel. MC3-LNP and DD-LNP groups (a–d, f) were compared using the unpaired two-tailed Student’s t-test. **p < 0.01, ****p < 0.0001 and ns = not significant. Source data are provided as a Source Data file.
Characterization of EVs. Assessment of the effect of hEPO mRNA loaded LNPs on cell-derived EVs showed that the total RNA content of EVs from cells treated with mRNA-loaded MC3-LNPs was higher than EVs from cells treated with mRNA-loaded DD-LNPs or untreated cells (Supplementary Fig. 2a). Total EV protein is slightly increased after MC3-LNP treatment (Supplementary Fig. 2b).

Additionally, EVs were characterized for their size and concentration. The mean ± SEM of the EVs mode size (measured from triplicate samples) was 116.4 ± 9 nm from MC3-LNP-treated cells and 100.2 ± 4 nm from untreated cells (Fig. 2a, Supplementary Fig. 2c, d). The mean ± SEM concentration of EVs was 5.81 × 10^{11} ± 2.25 × 10^{10} particles/ml from untreated cells, and 1.25 × 10^{12} ± 1.54 × 10^{11} particles/ml from MC3-LNP-treated cells.

Previous studies have used siRNA delivery and showed that only a small fraction of the siRNA (<2%) escapes from endosomes. However, in the present study we investigated the fate of LNP-delivered mRNA and other components of LNPs after their uptake by cells, which showed that <1% of the administered mRNA by LNPs was detected in the cytosol of LNP-treated cells (Fig. 1d). Moreover, mRNA delivered via MC3-LNPs undergoes endosomal escape at approximately two-fold higher rate than that delivered by DD-LNPs.

We hypothesized that there could be a link between endocytosis (uptake of LNPs) and exocytosis, wherein, some of the LNP components could be packaged into EVs originated from endosomes. EVs (endo-EVs) derived from LNP-treated cells are deﬁned by qPCR. The results showed that endo-EVs contained hEPO mRNA, and the mRNA content of EVs from cells treated with mRNA-loaded MC3-LNPs was higher than EVs from cells treated with mRNA-loaded DD-LNPs or untreated cells (Supplementary Fig. 1d). By contrast, the total amount of intracellular proteins remained unchanged after LNP (MC3- or DD) treatment (Supplementary Fig. 1c). Total amount of intracellular proteins increased after treatment with mRNA-loaded DD-LNPs compared with untreated cells or cells treated with empty DD-LNPs (Supplementary Fig. 1e).

Characterization of EVs derived from LNP-treated cells. a Nanoparticle tracking analysis for size distribution and concentration of EVs from untreated cells (Left side panel) and from MC3-LNP-treated cells (Right side panel). For each graph (one representative replicate), a table is provided, including mean and mode sizes, SD, and D-values (D10, D50, D90) and particle concentration. b EV-mRNA protection assay against RNase A. The hEPO-mRNA qPCR data is represented as scatter dot plot and mean SD of n = 3 biologically independent samples. c Cy5 mRNA in CD63/CD9 positive EVs. CD63+ EVs were stained against CD9 antibody and analyzed by FACS for Cy5 mRNA detection. The sole beads incubated with PBS instead of EVs are negative control. Approximately 96% of immunoprecipitated EVs (50 µg assay) from untreated cells are positive for CD63 and CD9, but they are negative for mRNA. By contrast, ~88% of immunoprecipitated EVs (50 µg assay) from LNP-treated cells are positive for CD63 and CD9, and 26% EVs contain mRNA that is secreted after the endocytosis of LNP-mRNA. The percentage of CD63/CD9 positive EVs containing Cy5 mRNA is presented in the upper right quadrant. The FACS dot plots represent Cy5 mRNA (y-axis) vs. CD9 (x-axis). One out of two biological replicates is shown. Source data are provided as a Source Data file.

modified mRNA. We therefore, investigated the effects of the LNPs on cells. Compared with untreated cells, the treatment with mRNA-loaded LNPs increased the generation time (retarded cell growth), which remained unaffected when cells received empty LNPs (LNPs without hEPO mRNA) (Supplementary Fig. 1b). LNP treatment had no effect on the amount of total RNA per cell (Supplementary Fig. 1c). Total amount of intracellular proteins remained unchanged after LNP (MC3- or DD) treatment (Supplementary Fig. 1d). By contrast, the total amount of extracellular proteins increased after treatment with mRNA-loaded DD-LNPs compared with untreated cells or cells treated with empty DD-LNPs (Supplementary Fig. 1e).

**Fig. 2** Characterization of endo-EVs derived from LNP-treated cells. a Nanoparticle tracking analysis for size distribution and concentration of EVs from untreated cells (Left side panel) and from MC3-LNP-treated cells (Right side panel). For each graph (one representative replicate), a table is provided, including mean and mode sizes, SD, and D-values (D10, D50, D90) and particle concentration. b EV-mRNA protection assay against RNase A. The hEPO-mRNA qPCR data is represented as scatter dot plot and mean SD of n = 3 biologically independent samples. c Cy5 mRNA in CD63/CD9 positive EVs. CD63+ EVs were stained against CD9 antibody and analyzed by FACS for Cy5 mRNA detection. The sole beads incubated with PBS instead of EVs are negative control. Approximately 96% of immunoprecipitated EVs (50 µg assay) from untreated cells are positive for CD63 and CD9, but they are negative for mRNA. By contrast, ~88% of immunoprecipitated EVs (50 µg assay) from LNP-treated cells are positive for CD63 and CD9, and 26% EVs contain mRNA that is secreted after the endocytosis of LNP-mRNA. The percentage of CD63/CD9 positive EVs containing Cy5 mRNA is presented in the upper right quadrant. The FACS dot plots represent Cy5 mRNA (y-axis) vs. CD9 (x-axis). One out of two biological replicates is shown. Source data are provided as a Source Data file.

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levels were 10-fold higher in endo-EVs secreted from MC3-LNP-treated cells compared to DD-LNP-treated cells (Fig. 1f). Of particular note, cells treated with hEPO mRNA-loaded LNPs contained both hEPO mRNA and hEPO protein (Fig. 1a–c), however, EVs derived from such LNP-treated cells contained hEPO mRNA (Fig. 1f), but not the hEPO protein.

To determine whether the interaction between LNPs and EVs could also have occurred independent of cells and whether mRNA was transferred to EVs outside of cells, the LNPs containing hEPO mRNA were mixed with EVs isolated from the supernatant of untreated cell cultures. After 2 h of incubation, EVs were re-isolated and tested for the presence of hEPO mRNA. The results showed that EVs were negative for hEPO mRNA when directly mixed with LNPs in the absence of cells (Supplementary Fig. 3), indicating that LNPs do not transfer hEPO mRNA directly to EVs outside of cells, but processed inside the cell. This is further supported by the difference between mRNA nucleotides and ionizable lipids ratio in originally formulated LNPs (1:3, see methods) and detected in endo-EVs (1:1) (Fig. 1g–j), which would be expected to be the same (3:1) if direct fusion of LNPs and EVs had occurred.

Additionally, to show that mRNA is located/protected inside EVs, the mc3-EVs containing hEPO-mRNA were exposed to RNase treatment, total RNA was isolated from EVs and hEPO-mRNA was quantified by qPCR. The results showed that, despite the efficient endonucleolytic activity of the RNase (shown on EV free RNA), only a 2 Ct fold-change decrease in the hEPO-mRNA content was observed in the RNase treated mc3-EVs than untreated mc3-EVs (Fig. 2b, Supplementary 2e). Although it is not possible to exclude that a small portion of the delivered mRNA could be attached to the external membrane layer of the EVs, most hEPO-mRNA is protected from the endonucleolytic activity.

To further show that LNP-mRNA is carried by EVs, the mc3-EVs pellets isolated from MC3-LNP-treated cells were immunoprecipitated with CD63 antibody, and examined for the presence of Cy5 mRNA in EVs. The FACS analysis showed that approximately 96% of EVs isolated from untreated cells were positive for CD63 and CD9, whereas at 48 h, 40% of cells were Cy5 mRNA positive (Supplementary Fig. 5b). T-cells displayed the lowest uptake, with maximum 14% positive for Cy5 mRNA at 5 h, with a decrease to 17% of T-cells positive for Cy5 mRNA at 5 h, with a decrease to 17% of T-cells positive for Cy5 mRNA at 5 h, with a decrease to 17% of T-cells positive for Cy5 mRNA at 5 h, with a decrease to 17% of T-cells positive for Cy5 mRNA at 5 h, with a decrease to 17% of T-cells positive for Cy5 mRNA at 5 h.

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Delivery of mRNA to epithelial and immune cells via endo-EVs. After having detected mRNA in endo-EVs, we investigated whether endo-EVs could transport the exogenous mRNA to recipient cells, acting as RNA delivery vehicles.

For tracking the mRNA uptake by recipient cells, a labelled mRNA (Cy5 mRNA) was delivered via endo-EVs to four different cell types; HTB-177, B-cells, T-cells and monocytes. Immune cells are generally difficult to transfected with RNA using other delivery vehicles. Peripheral blood mononuclear cells (PBMCs) isolated from buffy coats of healthy humans and HTB-177 cells were separately incubated with dd-EVs containing Cy5 mRNA. After incubation, PBMCs were harvested and stained with monoclonal antibodies (mAbs) against surface markers for B-cells, T-cells and monocytes. Flow cytometric analysis detected Cy5 mRNA in recipient cells as early as 5 h after EV-mediated mRNA delivery (Supplementary Figs. 5a–d, 6).

At 24 h of EV delivery, 70% of the HTB-177 cells were positive for Cy5 mRNA, whereas at 48 h, 40% of cells were Cy5 mRNA positive (Supplementary Fig. 5a). Compared to HTB-177 cells, the uptake in B-cells was lower, with 6% of B-cells positive for Cy5 mRNA at 5 h, and 30% and maximum 40% positive cells after 24 and 48 h, respectively (Supplementary Fig. 5b). T-cells displayed lower uptake, with maximum 14–17% of T-cells positive for Cy5 mRNA during the evaluation period (Supplementary Fig. 5c). By contrast, monocytes exhibited the highest uptake, as 71% of monocytes were positive for Cy5 mRNA at 5 h, with a decrease to 60 and 40% positive cells at 24 and 48 h, respectively (Supplementary Fig. 5d).

After having confirmed that EVs can deliver mRNA to different cell types, we investigated whether endo-EVs could transport the functional mRNA i.e. exogenous hEPO mRNA to express exogenous hEPO protein in recipient cells. The mc3-EVs and dd-EVs containing hEPO mRNA were transferred to HTB-177 cells. Analysis revealed that hEPO protein is expressed in cell lysates and is secreted in the supernatants of recipient cells in vitro (Supplementary Fig. 5e–g). The hEPO mRNA delivered by mc3-EVs produced higher amounts of hEPO protein than dd-EVs (Supplementary Fig. 5g).

We examined whether EV-based delivery acts differently than LNPs on cellular behavior. The results showed that EV-mediated delivery does not affect the cell generation time or cellular protein amounts, regardless if EVs carry hEPO mRNA or are without hEPO mRNA (Supplementary Fig. 5h–j).

Delivery of human EPO mRNA to mice via endo-EVs. Since endo-EVs could deliver hEPO mRNA to cells in vitro and cause the production of hEPO protein in recipient cells, we investigated whether these EVs could deliver hEPO-mRNA to cells in vivo and produce human protein in mice. First, the cross-reactivity of mouse EPO protein against human EPO antibodies was examined
A parallel experiment was performed with MC3-LNPs to examine hEPO mRNA delivery by LNPs. C57BL6/NCrI mice were injected intravenously with a single dose of MC3-LNPs (1.5 µg of hEPO mRNA per mouse), and the production of hEPO protein was examined in plasma. The hEPO protein was detectable in plasma after 2 h of LNP-mediated hEPO mRNA delivery (Supplementary Fig. 8). The hEPO mRNA and hEPO protein were detectable in five organs, heart, lung, liver, spleen and kidney (Supplementary Fig. 9). Among hEPO mRNA-positive organs, the spleen showed the highest amount of hEPO mRNA, which persisted for 96 h, followed by the kidney, in which hEPO mRNA persisted for 24 h. By contrast, most of the hEPO protein was detected in the liver. In the heart and lung, both hEPO mRNA and protein were detected at 5 h after injection. In the thymus, pancreas and brain, the levels of hEPO mRNA and hEPO protein were comparable to those in the corresponding negative samples (PBS).

Comparison between EVs and LNPs for the delivery of hEPO mRNA to mice. The MC3-LNP formulations based on DLin-MC3-DMA used in the present study are the most potent lipids currently used in clinical trials. Therefore, we sought to compare how efficiently EVs deliver hEPO mRNA and their biodistribution, compared to MC3-LNPs using the same dose of mRNA. In the lung and liver, the amount of hEPO protein produced was comparable between the two delivery vehicles. However, LNP-based hEPO mRNA delivery resulted in a higher protein production in the spleen and, to some extent, in the heart, than EV-based mRNA delivery. The plasma concentration of hEPO protein was higher in LNP-delivered than in EV-delivered mRNA-treated mice (Fig. 5).

Additionally, the assessment of organ weight showed that MC3-LNPs and mc3-EVs had no effect on tissue weight (Supplementary Fig. 10).

Expression of inflammatory cytokines against systemic delivery of EVs and LNPs. DLin-MC3-DMA ionizable lipids are important components in the formulation of most advanced LNPs; however, they are partially immunogenic to recipient cells and elicit immune responses in the host. Since EVs had a lower amount of ionizable lipids than LNPs (1/3), we investigated whether EVs were accordingly less immunogenic to recipient mice. The secreted levels of eight inflammatory cytokines were measured in the plasma of mice at 5 and 24 h of LNPs and EVs injection. Our results indicate that although the systemic delivery of both EVs and LNPs cause the expression of proinflammatory cytokines including IL-6, IP-10, RANTES, MCP-1, and KC in mice, the expression levels induced by EVs were not as much higher as LNPs did (Fig. 6). This indicates that EVs might be better tolerated by the recipient mice, since EVs contain fewer ionizable lipid molecules per mRNA nucleotides (1: 1 molar ratio) compared to LNPs (1: 3 molar ratio).

Discussion

LNPs represent one of the most potent RNA delivery vehicles in vivo, and are currently being tested in human clinical trials. However, the fate of the RNA and the other components of LNPs inside cells remains unclear.

In the present study, we showed that the part of the LNP-hEPO mRNA and ionizable lipid components of LNPs, which had not been dissociated/escaped into cytoplasm and which had not been degraded in early endosomes were packed into endo-EVs and secreted outside the cell. The experiment was performed 13 times (seven times with MC3-LNPs and six times with DD-LNPs), and the results consistently showed that EVs contained one mole of...
indicated that LNPs do not fuse with EVs outside the cell, but together with the results of the cell free mixture of EVs and LNPs, the ratio between them remained the same (1:1). These results showed that LNPs resulted in a 6-fold higher amounts of hEPO protein than DD-LNPs for the same dose of hEPO mRNA delivered to cells.

Moreover, the delivery of Cy5-labelled mRNA to the three primary immune cells, including B-cells, T-cells, and monocytes via endo-EVs indicates a high potential for transferring genetic materials (mRNAs) to human blood cells, such as B-cells, which are difficult to transfect.

We compared the efficacy of LNPs and endo-EVs for the delivery of mRNA to eight different organs and peripheral blood. After LNP or endo-EV delivery in vivo, the translation kinetics of hEPO mRNA in plasma and organs at different time points revealed that the hEPO mRNA transferred to mice via endo-EVs is functional. Because mice lack this form of EPO (hEPO), the EPO ELISA kit used could effectively distinguish between mouse and human EPO. The protein is produced from exogenous mRNA, which the recipient mice lack. Compared to endo-EVs, LNPs led to a higher production of hEPO in the organs analyzed in this study, and the most noticeable difference was observed in the plasma and spleen. Quantification of plasma hEPO protein showed that LNPs resulted in a 6–8-fold higher hEPO production than endo-EVs.

The differences in hEPO expression between endo-EVs and LNPs may be due to specific factors, e.g. that not all EVs contain hEPO mRNA. Some EVs taken up by recipient cells may lack hEPO mRNA, as not all EVs secreted from cells in standard preparations carry RNA (one copy of mRNA per 10,000 EVs) and are, therefore, individually some EVs are unlikely to be functional as vehicles for RNA-based delivery.

Since endo-EVs contained a three-fold lower level of ionizable lipids (which, despite being important compounds for LNP manufacturing, can be toxic) per mRNA than LNPs, we expected that endo-EVs should elicit a milder immune response than LNPs. The expression levels of eight different cytokines in the plasma of mice confirmed our expectation that endo-EVs induce less inflammatory cytokine responses than LNPs upon transferring an equal dose of hEPO mRNA to mice.

Higher levels of pro-inflammatory cytokine responses elicited by LNPs compared to endo-EVs could be related to several factors, e.g. (i) endo-EVs delivered 1/3 of the toxic synthetic ionizable lipids of LNPs, (ii) in contrast to LNPs, EVs are natural biological products, and might be better tolerated by the host, and (iii) the routes of cellular uptake differ between EVs and LNPs, which could behave differently to autophagic-lysosomal pathway. The advantage of using EVs for mRNA delivery would be that

ionizable lipids per mole of hEPO mRNA nucleotides. This differed from LNPs, which contained three moles of ionizable lipids per mole of hEPO mRNA nucleotides. Despite variation in the levels of EPO–mRNA and ionizable lipids in endo-EVs, the molar ratio between them remained the same (1:1). These results together with the results of the cell free mixture of EVs and LNPs indicated that LNPs do not fuse with EVs outside the cell, but rather processed in the endosomal pathway and are secreted in endo-EVs.

We show that LNPs can transfer exogenous hEPO mRNA to cells and produce new protein, i.e. hEPO in vitro both in the cytosol and secreted in the culture supernatant, and in vivo in organs and in the blood in secreted form (hEPO protein is a secretory protein). MC3-LNPs are more effective for delivering functional hEPO mRNA to cells and produce approximately 8-fold higher amounts of hEPO protein than DD-LNPs for the same dose of hEPO mRNA delivered to cells.

The advantage of using EVs for mRNA delivery would be that
compared to synthetic products, EVs are biological products and might elicit a milder immune response in the host.

In the case of LNPs, cellular uptake is mediated by endocytosis, which could activate the cells’ autophagic-lysosomal pathway. Accumulating evidence indicates that endocytosis of nanoparticles generates autophagosomes, and their subsequent fusion with lysosomes leads to the digestion of their content. Autophagosomal–lysosomal activation shapes the cellular immunity as a defence mechanism against foreign particles, by which innate immune effectors elicit inflammatory responses. In the present study, the uptake of LNPs caused an increased cytokine release. The routes of EV uptake differ from those of LNPs and are not likely to elicit the autophagy–lysosomal pathway, as they release their content into the cytoplasm probably without undergoing lysosomal trapping. Additionally, because of their small size, EVs can escape from rapid phagocytosis, and steadily carry and deliver RNA in circulation, passing through the vascular endothelium to the target cells.

Once endocytosed, LNP-encapsulated mRNA must escape from endosomes to reach the cytoplasm of recipient cells for translation into protein. It was suggested that late endosome/lysosome formation is essential for the functional delivery of exogenous mRNA. We examined the linkage between endocytosed LNP-mRNA and secretion via EVs, because the intraluminal vesicles of late endosomes (MVBs) are secreted into the extracellular milieu as EVs.

We introduce a hypothetical mechanism explaining the fate of LNP endosomes and how part of the LNP-mRNA could be translocated to the cytoplasm or loaded (intracellularly) into the luminal vesicles of multivesicular endosomes and secreted from the cell via endo-EVs. This is consistent with two independent experimental observations. First, we showed that the molar ratio of ionizable lipid to mRNA nucleotide in EVs was 1:1, whereas that inside LNPs was 3:1. Second, the mRNA loading efficiency was correlated with intracellular protein expression, i.e. the loading efficiency was significantly higher using MC3-LNPs than DD-LNPs. This suggests that LNPs added to cells are endocytosed and fuse with the endosomal membrane, where the pH is neutral (~7.4), the LNP surface becomes positively charged and interacts with the negatively charged endosomal membrane.

Fig. 5 Comparison of hEPO protein levels in murine blood and organs. The ability of mc3-EVs and MC3-LNPs to produce hEPO protein upon injection of equal doses of hEPO mRNA (1.5 µg) into mice was compared. In most organs, the amount of hEPO protein was comparable between LNPs and EVs except for the spleen, which showed a significant difference in protein production followed by the heart (less significant difference). The most significant difference was observed in the plasma levels of hEPO protein, which were considerably higher for MC3-LNPs than for mc3-EVs. Data are presented as the mean (bars) and standard deviation (SD) n = 4 independent animals at each time point except for LNPs and EVs at 5 h for plasma analysis (n = 8). EV and LNP groups were compared for each organ or plasma at each time point using the unpaired two-tailed Student’s t-test. p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. Source data are provided as a Source Data file.
Fig. 6 Cytokine analysis in mouse plasma after mc3-EV and MC3-LNP delivery. Mice were intravenously injected with 100 μL of mc3-EVs or MC3-LNPs containing 1.5 μg of hEPO-mRNA (per mouse). The concentrations of eight pro-inflammatory cytokines including IL-6 (a), IP-10 (b), RANTES (c), MCP-1 (d), KC (e), IL1-β (f), TNF-α (g), and IFN-γ (h) were determined in mouse plasma after 5 and 24 h of mc3-EV, MC3-LNP or PBS injection. The levels of pro-inflammatory cytokines were significantly higher in mice receiving LNP injection than in those receiving EV injection. White squares: PBS, black circles: EVs and black triangles: LNPs. Data are presented as the mean (bars) and standard deviation (SD) n = 4 independent animals at each time point. Statistical analysis was performed using one-way ANOVA, followed by Sidak’s multiple comparisons test. Significant differences are shown as p-values. Source data are provided as a Source Data file.
vesicles (Fig. 7a, step 4b) packaged with mRNA. These vesicles are then released into the extracellular environment upon the fusion of MVBs with the plasma membrane and are released outside the cell as EVs (Fig. 7a, step 5). We argue that the stoichiometric ratio between ionizable lipid and mRNA nucleotides should be neutrally charged (1:1) to enable mRNA escape from the endosome engulfing and reach the cytoplasm.

Initially, we and other researchers used external loading methods such as EV electroporation to directly incorporate siRNA into EVs (in the absence of cells), which were used as RNA delivery vehicles. Nevertheless, the incorporation of large-sized mRNAs into EVs using internal methods (loading via cells) was not achieved previously. When DNA plasmids (vectors) are transfected into cells via viruses or liposomes, their transcript RNAs can be detected in EVs secreted by transfected cells. In this model, the DNA plasmid transfected into cells must first be translocated to the nucleus and transcribed into RNA, which then enters the cytoplasm via a nuclear pore complex (functional RNA). A small fraction of this vector RNA expressed in the nucleus can be detected in EVs.

Here we present an alternative model to load exogenous mRNA into EVs, where no extra route (i.e. nuclear route) is needed. Our method exploits the direct link for transport of molecules between endocytosis of LNPs containing mRNA and
Fig. 7 A hypothetical mechanism explaining the fate of LNP endosomes. a step 1, 2: after the endocytosis of LNPs, lysosomes fuse with early endosomes and cause the acidification of the endosomal environment (pH 5.5–6.2). a step 3: the surface of LNPs is positively charged, drawing LNPs to the inner membrane of the endosomes, which is negatively charged.57,58. This enables the lipid components of LNPs to fuse with the endosomal membrane, allowing the mRNA translocation to the water phase outside the endosomes. Only the mRNA when neutrally charged by ionizable cationic lipids (ratio 1:1 mRNA: lipid) can cross the endosomal membrane. RNA:lipid ratio other than 1:1 would theoretically be unable to cross the endosomal membrane. b in acidic environment (pH 5.8 or 6.6), the mRNA is slightly released from LNPs, whereas at neutral pH (~7.4), the mRNA and lipids are dissociated (The data are shown as standard error of the mean of three replicates). a step 4a: part of the LNP-mRNA that escapes the endosomal membrane and localizes to the cytoplasm could be dissociated from the ionizable lipids because the pH of the cytoplasm is neutral, consistent with the results shown in b. By contrast a step 4b: when LNP-mRNA is transported to the cytoplasmic side of the endosomal membrane, intraluminal vesicles are formed by invagination of the endosomal membrane, and a portion of the LNP-mRNA could be incorporated into these vesicles. a step 4a and 4b: since only a 1:1 ratio (neutral) can cross the endosomal membrane and become incorporated into luminal vesicles of endosomes, endo-EVs contained a 1:1 ratio of hEPO mRNA and ionizable lipids. a step 5: the luminal vesicles are then released into the extracellular environment upon the fusion of multivesicular endosomes with the plasma membrane.

Since LNPs with the same ionizable lipids used in this study are currently being utilized in clinical trials and endo-EVs contained hEPO mRNA acquired after the endocytosis of LNPs and delivered to other cells, we postulate that a similar scenario may occur in individuals administered with LNPs, suggesting that part of the mRNA delivery is achieved by such EVs exocytosis, where endo-EVs acquire different LNP molecules, such as mRNA and ionizable lipids, directly from the endosomal pathway (Figs. 1e, 7a).

LNPs with the same ionizable lipid used in the present study are currently being tested in human clinical trials. As shown in the present study, a similar scenario may occur in humans: when mRNA is delivered via LNPs, the LNP alone may not deliver mRNA to all cells that express the protein; part of the RNA delivery may be achieved via endo-EVs secreted by cells that internalize the LNPs (Fig. 7c). Additional studies are needed to determine how much of the LNP-delivery is actually achieved by the LNPs’ own contribution and not from the endo-EVs originating from LNP-treated individuals.

Materials and methods

Formulation and characterization of LNPs. DLin-MC3-DMA and DLin-DMA LNPs containing modified hEPO-mRNA (858 nucleotides) (5mC, Ψ) (Trilink) were prepared by precipitating the mRNA with four different lipid components as described previously59,60. These components consist of an ionizable lipid; DLin-MC3-DMA or DLin-DMA, which is ionizable (cationic) at low pH, two helper lipids (DSPC and Cholesterol) and a PEGylated lipid (DMPE-PEG2000). A solution of hEPO-mRNA in water was prepared by mixing mRNA dissolved in MilliQ-water, 100 mM citrate buffer pH 3 and MilliQ-water to give a solution of 50 mM citrate. Lipid solutions in ethanol (99.5%) were prepared with a composition of four lipid components [Ionizable lipid:Cholesterol: DSPC:DMPE-PEG2000] 50:38:3:10.15 mol% and a total lipid content of 12.5 mM. The mRNA and lipid solutions were mixed in a NanoAssembler (Precision Nanosystems) microfluidic mixing system at a volume mixing ratio of Aq:EtOH = 3:1 and a constant total flow rate of 12 mL/min. At the time of mixing, the ratio between the nitrogen atoms on the ionizable lipid (positive charge) and phosphor atoms on the mRNA chain was 3:1. The ionizable lipid and phosphor atoms on the mRNA chain was 3:1. If the rate of 12 mL/min. At the time of mixing, the ratio between the nitrogen atoms on the ionizable lipid (positive charge) and phosphor atoms on the mRNA chain was 3:1. If the ionizable lipid and phosphor atoms on the mRNA chain was 3:1. If the ionizable lipid and phosphor atoms on the mRNA chain was 3:1.

The mixture was then dialyzed over night at 4 °C against PBS (pH7.4). The volume of the PBS buffer was 650 μL, and after dialysis for 35 min at 4 °C, followed by centrifugation at 4 °C. The supernatant was collected and the RNA concentration was interpolated against the standard curve generated hEPO mRNA in epithelial cells (HTB-177 cells) was seeded at a density of 3 × 10⁵ cells/175 cm² flask in 30 mL of medium growth. After incubation (adaptation) for 24 h, the cells were treated with 1 mL of DD- or MC3-LNPs containing 100 μg of hEPO mRNA/flask in the presence of 1% human serum (Sigma Aldrich), which was administered in three different doses; Day (1) 200 μL LNPs (20 μg mRNA), day (2) 400 μL LNPs (40 μg mRNA), day (3) 400 μL LNPs (40 μg mRNA). Cells treated with equal volume (200 μL, 400 μL, 400 μL) of corresponding empty-DD or empty-MC3 LNPs (without mRNA), as well as untreated cells were used as negative controls.

Detection and quantification of hEPO mRNA in epithelial cells. Total RNA from HTB-177 cells was isolated using miRCURY™ RNA isolation kit-Cell and Plant (Exiqon) according to the manufacturer’s instructions. Total RNA was quantified by Qubit 2.0 fluorometer (Thermo Fisher Scientific) and the RNA quality (230/260 ratio) was assessed using NanoDrop 1000 (Thermo Fisher Scientific). Based on RNA yield, 0.25 to 1 μg of total cellular RNA was converted into cDNA using high-capacity cDNA kit (Thermo Fisher Scientific). 100 ng of cDNA was used for hEPO mRNA quantification using TaqMan probe assay (Applied Biosystems; assay ID Hs01071979_m1) on ViiA™ 7 instrument (Thermo Fisher Scientific) according to the manufacturer’s instructions. To generate the standard curve, 2 μg of pure hEPO mRNA was reverse transcribed and the resultant cDNA was serially diluted (ten-fold) to prepare seven standards (highest point: 100 ng) which were run in technical triplicate. Cellular cDNA was used for hEPO mRNA analysis whose absolute quantification was interpolated against the standard curve with minimal R² > 0.975. GAPDH (assay ID Hs02758991_g1) was used as internal control.

Isolation of extracellular vesicles. EVs were isolated from conditioned culture medium of LNP-treated cells and negative controls. Briefly, to remove cell debris, the cultured medium was centrifuged at 3000 × g for 15 min at 4 °C on a 4K15 centrifuge (Sigma) and the resultant supernatant was collected and ultracentrifuged at 60,000 × g for 35 min at 4 °C, followed by filtration through 0.2μm filters to obtain EVs with diameter below 200 nm. Finally, the filtered supernatant was ultracentrifuged using Optima L-100 XP ultracentrifuge with 70Ti rotor (Beckman Coulter) at 120,000 × g for 70 min at 4 °C to pellet EVs. The EV pellets were resuspended in 50–80 μL of PBS. EVs secreted after the endocytosis of LNPs were defined as endo-EVs.

Characterization of EVs by total RNA and protein content. EVs were quantified based on their total protein concentration and total RNA. 2 μl of EV suspension incubated together with 2 μl of M-PEI Mammalian Protein Extraction Reagent (Thermo Fisher Scientific), were sonicated on an Ultrasonic cleaner (VWR) according to manufacturer’s protocol. Total RNA from EVs was isolated using miRCURY™ RNA isolation kit-Cell and Plant (Exiqon) according to the manufacturer’s instructions. Total RNA was
quantified by Qubit 2.0 fluorometer (Thermo Fisher Scientific) and the RNA quality (230/260 ratio) was assessed using NanoDrop 1000 (Thermo Fisher Scientific).

**Characterization of EVs for size and concentration.** The mc3-EVs (i.e. endo-EVs isolated from MC3-LNP-treated cells) and untreated EVs were assessed for their size (nm) and concentration (particles/ml) by LM10 (Malvern Panalytical) equipped with a Hamamatsu C11440-50B/A11893-02 camera. Before the analysis, the particles were diluted 500 times in 0.1 M PBS (Sigma) to reduce the number of particles in the field of view below 180 frame. Three independent measurements (biological replicates) were performed in scatter mode. Measurement readings for each EV-sample were taken in five captures for 60 s each at 25 frames per second (fps), at adjusted camera level (10–16) and detection threshold (5–15) depending on the individual sample and manual monitoring of temperature, bright field, and laser jumps distance were set to auto. The readings, acquisition and data analysis were performed using the NanoSight Fluorescent NTA LM10 software version 3.3 (Malvern Panalytical).

**Detection of LNP-derived exogenous hEPO-mRNA in EVs.** The hEPO mRNA in endo-EVs after LNP administration and in corresponding negative controls was quantified using qPCR. Based on RNA yield, 0.25 to 1 µg of total EV RNA was converted into cDNA using high-capacity cDNA kit (Thermo Fisher Scientific). Hundred nanograms of cDNA was used for hEPO mRNA quantification using TaqMan probe assay (Applied Biosystems; assay ID Hs02758991_g1) on a 15 instrument (Thermo Fisher Scientific) according to the manufacturer’s instructions. To generate the standard curve, 2 µg of pure hEPO mRNA was reverse transcribed and the resultant cDNA was serially diluted (ten-fold) to prepare seven standards (highest point: 100 ng) which were run in technical triplicate. EV cDNA was used for hEPO mRNA analysis whose absolute quantification was interpolated against the standard curve with minimum R² = 0.975. GAPDH (assay ID Hs02758991_g1) was used as internal control.

**Detection of EV markers and mRNA in CD63 / CD9 positive EVs.** HTB-177 cells were treated with MC3-LNPs containing 100 µg of Cy5 mRNA (Trilink) as described above. Untreated cells were included as control. After 96 h, the total EVs were isolated by UC (pre-enrichment) and quantified. After pre-enrichment, the CD63 / CD9 positive EVs were isolated by using an affinity-based method and evaluating the ability of Cy5 mRNA to bind first place, exosomes were isolated using Human CD63 isolation/detection reagent for cell culture medium (Thermo Fisher Scientific). Two different concentrations covering the expected sample concentrations with good linearity of the RNase A activity (Thermo Fisher Scientific) were tested. 100 µg of total RNA from EVs was isolated using miRCURY™ RNA isolation Kit-Cell and Plant (Exiqon). The hEPO-mRNA was quantified by qPCR to assess the effect of the RNase A on hEPO-mRNA content if present outside of EVs. The experiments were performed in triplicate.

**Gradient UPLC for the analysis of ionizable lipids in EVs.** Fraction of EVs was used to examine the presence of LNP-derived ionizable lipids in EVs. Five to ten microliters of each EV sample i.e. mc3-EVs and dd-EVs (obtained from MC3- and DD-LNP-treated cells, respectively) was diluted 50 times with PBS and further diluted 1 + 1 with a mixture of 2%w/v of Triton® X-100 in Tris/EDTA buffer. The samples were incubated at 37 °C for 30 min and then injected on Acquity UPLC® LC coupled to a Single Quad Detector, SQD (Waters, Milford). The analytical column was a Waters Acquity UPLC® CSH C18, 1.7 µm, 2 × 100 mm, kept at 60 °C. The flow rate was 0.15 mL/min using a mobile phase of 0.1% formic acid in water (A) and 0.1% formic acid in an equal mixture of acetonitrile and isopropanol (B). A gradient run was applied where 10% B at 0.0 min was increased to 85% B at 1.0–5.0 min and kept at 85% B to 7.5 min. A washing step of 99% B at 7.6–9.5 min was included in the gradient run. Then 10% B was applied for conditioning from 9.6 min to 12.0 min. The separation between main peak of Triton X-100 and the cationic lipids was good under these conditions with retention time of Triton X-100 at 5.1 min. DLin-DMA 6.3 min and of DLin-MC3-DMA at 6.5 min. Quantification was made using external standard solutions of DLin-DMA and DLin-MC3-DMA dissolved in ethanol 99.5% for at least five different concentrations covering the expected sample concentrations with good linearity of each standard curve. The SQD was run using electrospray, positive mode and tuned using auto tune with a solution of DLin-MC3-DMA. Recording of the cationic lipids was made using Single Ion Recording (SIR) at M+1 for each cationic lipid.

Finally, the molar ratio of ionizable lipid per hEPO mRNA nucleotides (ionizable lipid: mRNA) was determined in both EVs and LNP samples. The experiments were performed in at least six biological replicates both for mc3- and dd-EVs and their corresponding LNP samples.

**hEPO protein quantification.** After LNP treatment, the cell-conditioned supernatant was collected and saved for hEPO protein detection. The total cellular proteins were extracted from cell lysate using 500 µL of M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) in the presence of 1% halot protease inhibitor cocktail (Thermo Fisher Scientific). Briefly, cells were gently digested on a three-dimensional Bio-rotor for 10 min at 4 °C and centrifuged at 14,000 × g for 10 min to pellet the cell debris and the resultant supernatant (containing proteins) was transferred to a new tube. In parallel, the cultured supernatant was centrifuged at 3000 × g for 15 min at 4 °C on a 4K15 centrifuge (Sigma) to remove cell debris and EVs were isolated. To generate EV protein extracts, 2 µL of EV suspension was incubated together with 2 µL of M-PER Mammalian Protein Extraction Reagent and was sonicated on an Ultrasonic cleaner (VWR) for 5 min at 54 °C. Total proteins from all samples (conditioned supernatant, cell lysate and EV lysate) were quantified by Qubit 2.0 fluorometer (Thermo Fisher Scientific). To detect hEPO protein, the Erythrophagocytosis ELISA Kit (STEMCELL Technologies, cat. no. 16030) was used according to the manufacturer’s instructions. Fifty microliters of total proteins solution was used and hEPO protein levels were calculated according to the relative standard curve as mU/mL. The concentration was converted into fg/mL using the conversion (119nmU = 1 ng) and normalized to the total number of cells.

**Effects of LNPs on cell growth, RNA, and protein content.** To determine the effect of LNPs on cellular behavior and their tolerance against LNPs treatment, the cell generation time, cellular total RNA, total amount of intracellular proteins and secreted proteins were calculated after DD- or MC3-LNP treatment period of 96 h. The effect of LNPs on EVs was also examined by quantifying EVs, total RNA and protein amount of EVs after the treatment of LNP. RNA from isolated EVs was quantified by qPCR. The cell generation time (G), the time (in hours) to double the population of cells was calculated based on the difference between the number of cells at the beginning and at the end of the treatment interval (delta tcells i.e. AN using the following formula:

\[ G = t/n \]

\[ n = \log(n. cells post – administration) – \log(n. cells pre – administration) + \log2 \]

The variation of total RNA in cells and in EVs as well as total protein in EVs, total proteins in cells and in cultured supernatant were normalized to the corresponding AN

**hEPO mRNA delivery to human epithelial cells via EVs.** The HTB-177 cells were seeded at a density of 5 × 10^4 cells/175 cm² flask and cultured in RPMI-1640 complete medium. Six hundred micrometres of mc3-EVs (700 ng hEPO mRNA)
that were isolated from MC3-LNP-treated cells and 600 μg of dd-EVs (1100 ng hEPO mRNA) isolated from DD-LNP-treated cells were dissolved in RPMI-1640 medium and different doses of these EVs were transferred to recipient cells in independent experiments over 2 days: day 1, 300 μg and day 2, 300 μg (in two time separated doses of 150 μg each after 8 h). Empty EVs (without hEPO mRNA) and EVs from untreated cells were delivered to recipient cells as controls. After 48 h, cells and cultured supernatant were collected; total RNA was isolated and hEPO mRNA and hEPO protein were evaluated by qPCR and ELISA, respectively. The experiment was performed in two independent biological replicates.

Cyanine 5 mRNA delivery to cells via endo-EVs. One mL of DD-LNPs containing fluorescent Cy5 mRNA was delivered to HTB-177 cells in different doses (200, 400, 4000 μL) with the exception that 1 mL of LNPs contained 76 μg of fluorescent Cy5 mRNA/flask (which in the case of hEPO mRNA was 100 μg/mL). Ninety-six-well plate containing conditioned medium (super- natant) was harvested and used for EV isolation. Empty DD-LNPs and untreated cells were used as controls. HTB-177 cells and immune cells such as B-cells, T-cells and monocytes purified from PBMCs were seeded at a density of 2 × 10^5 cells/well and cultured in 200 μL of culturing medium in 96-well round bottom plates and incubated Staphylococcus aureus Enterotoxin A stimulation for 24 h at 37 °C. The supernatant, 78 μg of dd-EVs containing Cy5 mRNA in 25 μL PBS solution were delivered to recipient cells. As control assays, the empty dd-EVs and EVs from untreated cells were delivered to cells or cells left untreated. After 5, 24, and 48 h of EV treatment, cells were harvested and stained for surface with BV421 monoclonal antibodies (mAbs) against CD19 (B-cells), CD3 (T-cells) and CD14 (monocytes; Invitrogen–Dickinson Biosciences) which were diluted 1:20. Cells were acquired on a FACS-Verse (BD Biosciences), Cy5 mRNA was detected based on fluorescence in each cell type and the data were analyzed using FlowJo software (TreeStar Inc.). The gating strategy for cells by FACS analysis is represented in Supplementary Fig. 1b, c.

Detection of human EPO protein in mouse tissues. Total protein from organs was extracted using M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) in the presence of 1% BAA protease inhibitor cocktail (Thermo Fisher Scientific) following the manufacturer’s instruction. Briefly, 20–70 mg of tissue were lysed in 200–350 μL of lysis buffer (depending on tissue weight) with addition of proteases inhibitors (Thermo Fisher Scientific) in the Tissue LyserII (Qiagen) for 3–5 min at the maximum speed (30 Hz) and centrifuged at 10,000 × g for 4 min at 4 °C to deplete tissue debris. The experimental supernatant was used for protein quantification by Qubit 2.0 fluorometer (Thermo Fisher Scientific). Fifty microliter of total protein were analyzed for hEPO protein detection using Erythropoetin ELISA kit (STEMCELL Technologies, cat. no. 01630) according to manufacturer’s instructions. The amount of hEPO protein (ng) in each organ was normalized to the relative organ weight (g).

Cytokine analysis in mouse plasma. After intravenous administration of MC3-LNPs and mc-EVs, the plasma concentrations of mouse cytokines were measured by EMD Millipore’s MILLPLEX® MAP Mouse Cytokine magnetic bead kit (#MCTMAG-70K, Merck KGaA, Darmstadt) for the simultaneous quantification of IL-6, KC, MCP-1, RANTES, TNFα, IFNγ, IL-1β, and IP-10. The samples were first diluted 1:2 with Assay buffer and then, together with standards and QCs placed in a 96-well plate. A solution containing beads were added. The beads were magnetic microspheres each of which was coated with a specific antibody. The mixture was incubated over night at 4 °C and the reaction mixture was then incubated with Strepavidin-PE conjugate to complete the reaction on the surface of each microsphere. The plate was read on analyzer Bio Rad Luminex 200®. Each individual microsphere was identified and the result of its bioassay was quantified based on fluorescent reporter signals. The concentration was measured using Median Fluorescent Intensity data using 5-parameter logistic curve-fitting method.

Effect of hEPO mRNA release from LNPs. MC3-LNPs, containing hEPO mRNA of concentration 0.011 mg/mL, were incubated in 10 mM citric acid–Na2HPO4 buffer solutions with 150 mM NaCl of various pH environments (pH 7.4, 6.6, and 5.8) at 37 °C under quiescent conditions. The total amount of mRNA was measured at time zero using 0.125 mM TritonX-100 (WV Proteomics Grade) and 0.125 mM Sodium Dodecyl Sulfate (Sigma) in the RiboGreen assay to be able to calculate the fraction of mRNA released. To assess the fraction of mRNA released from LNPs at various pH environments, the free amount of mRNA was analyzed with Quant-it RiboGreen RNA Reagent Assay kit (Thermo Fisher Scientific) using a Perkin Elmer LS55 Luminescence Spectrometer (ex: 480 nm, em: 525 nm).

In vivo transfer of hEPO mRNA via EVs and MC3-LNPs. Experimental procedures were approved (ethical application number 83-2015) by the Regional Laboratory Animal Ethics Committee of Gothenburg, Sweden. All procedures conform to the Swedish Animal Welfare Act and regulations SVF 2012: 26. C57BL6/NCrI female mice (n = 36), 9–10 weeks of age, were purchased from Charles River Laboratory, Germany and housed in the animal facility at Astra Zeneca, Mölndal, Sweden. Mice were kept in groups of four mice per cage under standard conditions (21 °C RT, 12:12 h light-dark cycle, 45% humidity) and were fed standard laboratory diet. Seven to eight mice were housed in each cage. Mice were kept frozen until the day of use. Six weeks after injection of LNPs, the mice were randomly divided into 9 groups of 4 mice each according to their weight. Each group was injected with a single dose of 1 μL of MC3-LNPs or mc-EVs (300–500 μg) and 2 μL of hEPO mRNA was systemically injected into the tail vein of each mouse (10 μg/mouse). One mL of DD-LNPs containing SFH3 31439-010, 1100 mg of hEPO mRNA, were incubated with Streptavidin-PE conjugate to complete the reaction on the surface of each magnetic microsphere. The plate was read on analyzer Bio Rad Luminex 200®. Each individual microsphere was identified and the result of its bioassay was quantified based on fluorescent reporter signals. The concentration was measured using Median Fluorescent Intensity data using 5-parameter logistic curve-fitting method.

Detection of human EPO mRNA in mouse organs. Total RNA from organs was isolated using the RNasy kit (Qiagen) according to manufacturer’s recommendations. 10–50 mg of tissue were lysed in RLT buffer (600 μL) in the Tissue LyserII (Qiagen) for 3–4 min at the maximum speed (30 Hz) and centrifuged at 10,000 × g for 3 min at 20 °C to deplete tissue debris. Subsequently, the supernatant was transferred to columns and further processed. RNA was quantified via Qubit 2.0 fluorometer (Thermo Fisher Scientific) and the quality was assessed using NanoDrop 1000 (Thermo Fisher Scientific) by measuring 260/230 ratio. Based on RNA yield, between 0.5 and 1 μg of total RNA was converted into cDNA. 100 ng of cDNA was used for hEPO mRNA quantification using TaqMan probe assay as described above. The amount of hEPO mRNA in each organ (ng) was normalized to the relative organ weight (g).

Statistical analysis. The statistical analysis was performed by GraphPad Prism v.7 (GraphPad Software). The in vitro data were analyzed by unpaired two-tailed Student’s t-test, except for the effects of LNPs administration on HTB-177 growth, RNA and cell total protein amount, which were analyzed by one-way ANOVA followed by Tukey’s multiple comparison test (significant p-value < 0.05). The levels of cytokines in murine plasma were analyzed using unpaired two-tailed Student’s t-test, while the levels of cytokines in mice plasma were analyzed using one-way ANOVA followed by Sidak’s multiple comparisons test. The level of significance of p-values are indicated as follows: *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

Data availability

The source data for Figs. 1a–d, 2a, 2b, 3, 4a–5, 6a–h as well as for Supplementary Figs. 1b–e, 2a–e, 3, 5e, 7, 8a, 10a–h and Supplementary Table 1 are provided as a “Source Data” file. Other data are available from the corresponding author upon reasonable request.

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Author contributions
H.V. and L.L. conceived the study. M.M., M.N., A.P., A.A., A.C., M.N., M.H., P.S., S.J., J.L., T.K., T.J., and S.O. performed the experiments. M.M., M.N., A.P., A.A., A.C., M.N., M.H., P.S., S.I., M.S., J.L., T.K., I.L., M.B., T.J., P.S., S.O., L.L., and H.V. analyzed the data. M.N., M.M., L.L., and H.V. wrote the paper. All authors reviewed, edited, and approved the final version of the paper.

Additional information
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