# Explicating the cellular uptake, trafficking and biodistribution mechanism of cell derived vesicles, a unique therapeutic messenger

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## INTRODUCTION

Cell derived vesicles (CDVs) are membraneous, nanosized vesicles, which have recently emerged as an important carrier in many therapeutic areas. CDVs are produced by extruding cells through a series of membrane filters with different pore sizes. Increasing number of studies have demonstrated the potential role of CDVs in cell to cell communication. In addition, CDVs produced from certain types of immune cells carry molecules crucial for recognizing specific target cells, such as tumors, on their surface and were proposed as a novel carrier for anticancer medicine. However, the exact mechanism and route of cellular uptake remain largely unresolved, becoming one of the most challenging hurdles in the development and therapeutic applications of CDVs. In this study, we aimed to investigate the cellular uptake mechanism of CDVs produced from natural killer (NK) cells using a manufacturing platform recently developed. *In vitro* uptake assay was performed to provide precise insights into how CDVs exert its effect at the cellular level.

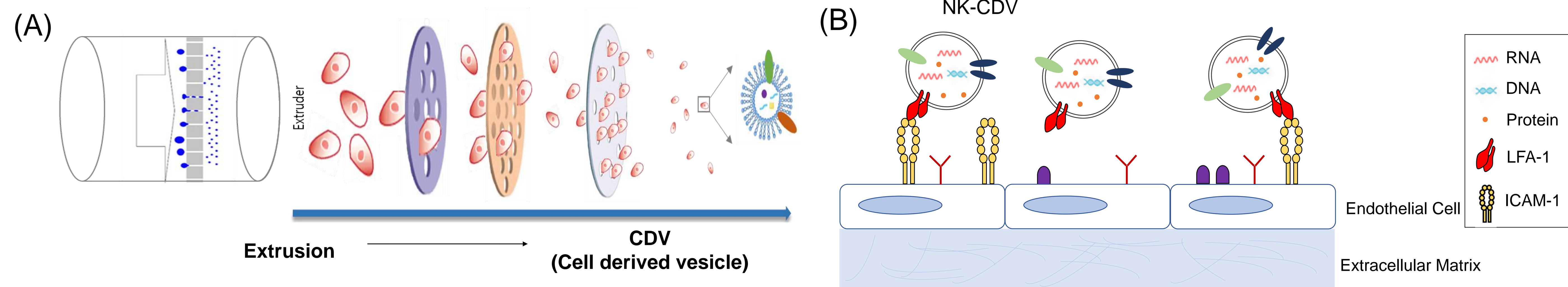


**Figure 1:** (A) Manufacturing-scale production of CDV using syringe extruder. Serial extrusion with different membrane pore sizes produces CDV of the size of interest. (B) Adhesion of lymphocyte function-associated antigen (LFA-1) on NK-CDV to intercellular adhesion molecules-1 (ICAM-1) expressed on endothelial cells such as human umbilical vein endothelial cells (HUVECs).

## METHODS

### NK-CDV labeling

NK-CDVs were labeled with CFSE, a cell labelling dye for *in vitro* uptake study. In brief, NK-CDVs at the concentration of 2E+11 particles/mL was mixed with CFSE at final concentration of 4 µM. The NK-CDV suspension was then incubated for 2 hr at 37°C. Excessive CSFE was removed with 10KDa dialysis Slide-A-Lyzer dialysis cassette against 500 mL of PBS with constant stirring at room temperature. After 1 h, new PBS was added and dialyzed overnight at 4°C.

### Cellular uptake assay

HUVECs purchased from ATCC were seeded overnight on a 24–well plate at a density of 4E+4 cells/well. Cells was then treated with 50 ng/mL of TNF-α for 16 h to induce expression of ICAM-1. ICAM-1 expression was examined with both western blot and FACS. Then, CFSE labelled NK-CDV at different particle number, 1E+3-1E+5 particles/cell were added to the culture media of the recipient cells and incubated for 15, 30, 60, 120, 180 mins respectively at 37 °C After respective incubation period, the degree of NK-CDV uptake into HUVECs were examined using FACS (Sony, 3800). HUVECs treated with 1E+5 particles/cell were further visualized with microscope. These cells were washed twice with PBS, fixed by 4% paraformaldehyde and incubated with DAPI staining and then imaged by confocal microscopy.

### Uptake mechanism study

HUVECs were first seeded overnight in a 24-well plate at 4 E+4 cells/well and then preincubated with 0.8-80 µM of Dynasore (dynamin-dependent endocytosis inhibitor), 0.1-10 µM cytochalasin (actin-dependent phagocytosis or micropinocytosis inhibitor), or 0.1-10 µg/mL heparin (receptor mediated endocytosis inhibitor) before addition of NK-CDVs. Then, CFSE labelled NK-CDV at 1E+5 particles/cell were added to the culture media of the HUVECs and incubated for 15 mins at 37 °C. The degree of uptake into HUVECs were examined using FACS (Sony, 3800).

## NK-CDV IN VITRO UPTAKE STUDY

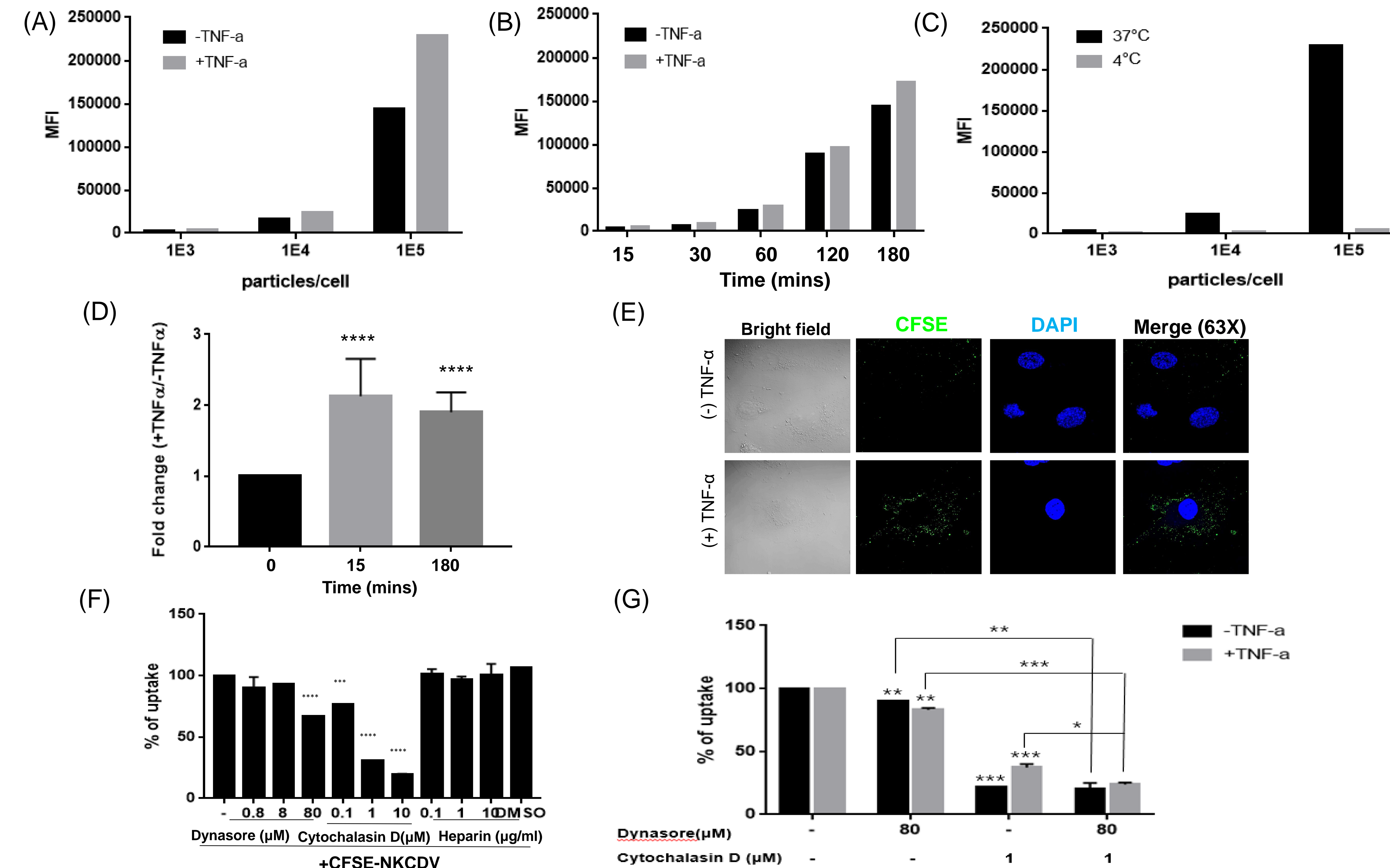


**Figure 2**: Cellular uptake of NK-CDVs into HUVECs. As the particle number (A) and incubation time (B) increase, the uptake of NK-CDV was found to increase gradually. This suggests that the uptake of NK-CDVs into HUVECs is dose- and time-dependent. (C) Incubation at 4°C attenuated the uptake significantly compared to 37°C, suggesting an energy-dependent process rather than passive membrane passage. (D) Increased uptake was observed in TNF-α induced HUVECs compare to non-treated HUVECs. (E) Representative confocal microscopic images of CFSE labelled NK-CDVs taken up by TNF-α treated HUVECs at 180 mins of treatment. (F). Uptake of NK-CDV by TNF- α treated HUVECs occurs via dynamin-dependent endocytosis (dynasore) and actin dependent phagocytosis and micropinocytosis (cytochalasin) but not heparin (receptor or integrin mediated endocytosis). (G) Co-incubation with two different inhibitors to examine the combinatory suppression of NK-CDV uptake. Uptake of NK-CDV was not further enhanced by combination of two inhibitors. There was no significant difference in NK-CDV uptake with or without the TNF- α treatment.

## CONCLUSIONS

- Uptake in CDVs increases as the time of incubation and concentration of NK-CDVs increase.
- NK-CDVs uptake in HUVECs is LFA-1/ICAM-1 dependent.
- The main entry route of NK-CDVs into cells is via an actin dependent phagocytosis and micropinocytosis pathway.
- Our current effort focuses on the *in vivo* distribution of NK-CDV on cancer model, especially the tumor targeting of CDV via ICAM-1 and LFA-1 interaction. Additionally, the investigation of CDV's potential to cross the blood brain barrier (BBB) is in progress.