Evaluating innate immune cell immunotoxicity of a novel therapeutic delivery system: Assessing immunotoxity of extracellular vesicles in human monocytic cell lines

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1 ABSTRACT

There is an increasing number and array of therapeutics which specifically or non-specifically target immune cells. Current immunotoxicologic evaluations largely focus on immunotoxicity endpoints of the adaptive immune system (i.e. T/B cells). However, changes in innate immune cells (e.g. myelomonocytic) have far-reaching effects on both innate and downstream adaptive immune responses. Monocytes are innate phagocytic cells that can differentiate into macrophages or dendritic cells. Agents affecting monocyte survival or function can dramatically impact the both innate and adaptive immune responses. Extracellular vesicles (EV) are a promising novel therapeutic delivery system that target specific cells to deliver a therapeutic payload. These EV are produced by cultured cells and very little is known about how culture-derived EV interact with cells of the immune system. This study evaluated the potential immunotoxic effects of HED293T-derived EV on the two human monocytic cell lines THP-1 and U937 by evaluating the cytotoxicity (apoptosis versus necrosis) and cell function (phagocytosis) by flow cytometry. Results: Human monocytes are highly permissive to EV entry in a dose-dependent manner but exposure to EV did not affect the homeostatic levels of apoptosis or necrosis. Although lower doses of EV increased phagocytic capacity in both cell lines, phagocytic efficiency of individual cells was not affected by EV exposure at any of the doses evaluated. The study demonstrates the application of *in vitro* immunotoxicity assessment of innate immune cells (human monocytes) of a novel therapeutic delivery system. Evaluating innate immune endpoints are an addition to traditional adaptive immune cell immunotoxicity assessments.

2 INTRODUCTION

Extracellular vesicles (EV) are naturally occurring nanosized lipid vesicles are involved in many biological processes. EV are of increasing and significant therapeutic interest due to their endogenous composition, ability to protect potential therapeutically loaded cargo and ability to attach to target cells (Batrakova 2015 and Gyorgy 2015). EV can be classified into exosomes and microvesicles (MVs) based on size. Exosomes are 30-100 nm, of endosomal origin and contain cytoplasmic contents (e.g. lipids, mRNA, miRNA, proteins, etc.) while MVs are 150-1000 nm and formed from budding of the plasma membrane (Gyorgny 2015, Colombo 2014).

As with any new therapies, evaluating the toxicologic potential is critical. Increasingly, new therapeutic entities, such as EV, are being developed that impact the function, cell populations and signaling pathways of the immune system. Evaluating the immunotoxicity both *in vitro* and *in vivo* of these newly developed therapies is critical and of increasing interest to regulatory agencies (ICH 2006).

Innate immune cells, particularly those of the myelomonocytic cell origin, are critical environmental sensors and key players in immune defense. Monocytes, macrophages and dendritic cells play critical roles in both early innate immune responses and subsequent modulation of adaptive immune response. Cellular viability, phagocytosis, cytokine secretion, respiratory burst, chemotaxis, phenotypic and activation marker expression are some of the *in vitro* assays that are useful to study phagocyte function (Frohlich 2015, Barnett 2010).

In this study, we evaluated the impact of EV on two human myelomonocytic cell lines to determine whether EV influenced cellular viability or phagocytic abilities. At the time of these studies, no immunotoxicologic evaluation of EV had been performed. For these initial studies, we chose to evaluate cellular viability and phagocytic capability.

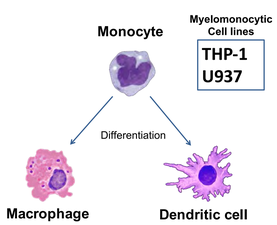

Figure 1. Myelomonocytic cell populations are innate phagocytic immune cells important that are key players in immune defense. Monocytes, macrophages and dendritic cells are phagocytic cells that are environmental sensors. Targeting myelomonocytic cells impact not only innate immune responses but also subsequent adaptive immune responses. THP-1 and U937 are cell lines used to evaluate the immunotoxicity of potential therapies on monocytes or cells (i.e. macrophages and dendritic cells) that can differentiate from monocytes.

3 MATERIALS AND METHODS

MYELOMONOCYTIC CELL LINES:

Human monocyte cell lines THP-1 and U937 were cultured in supplemented RPMI 1640 medium. To differentiate monocyte cells towards macrophage-like adherent cells, cells were incubated for 24 hours in 50 ng/ml PMN followed by 48 hours untreated culture media prior to experimental assays. EV preparations were added to the cells and incubated for 16–24 hours according to each assay protocol. When required, cytochalasin D at a final concentration of 10 uM was added to cells 1 hour prior to the addition of EV to the culture.

EV PREPARATION, CHARACTERIZATION AND CONFIRMATION:

EV were generated in the HEK293T cell line using standard and spinner flask culture. EV were isolated by standard ultracentrifugation protocol as described (Rosas 2015, Thery 2006). Western blotting was performed using manufacturer's protocols and standard conditions and Cryto-TEM was performed as described (Rosas 2016).

FLOW CYTOMETRY AND CONFOCAL OF UPTAKE OF EV:

Cells were harvested after 16 hours of incubation with PKH67-fluorescently labeled EV. A quenching assay with 0.05% trypan blue was used to discriminate intracytoplasmic EV uptake from membrane-attached EV. Permeabilization of cells was achieved using BD Cytofix/Cytoperm and cells were washed, resuspended in FACs buffer and evaluated using the BD Accuri™ C6 flow cytometer. Confocal microscopy was perofrmed on cells adherent to glass coverslips as described and images were captured using an FV1000-Spectral confocal system (Olympus) using a 40x oil objective (Rosas 2015).

APOPTOSIS/NECROSIS:

After 24 hours co-incubation with EV, cells were harvested and stained with FITC Annexin V and 7-amino-actinomycin (7-AAD) to determine apoptosis and necrosis. Cells were run on BD Accuri C6 flow cytometer. Indexes of apoptosis and necrosis were determined as described (Rosas 2016, Roy 2006, Li 2011).

PHAGOCYTOSIS OF BEADS:

Cells and EV were co-incubated for 21 hours at which time 1 um diameter FluoSpheres® fluorescent microsheres were added (ratio of 50 microspheres/plated cells) and incubated for 3 hours. Cells were washed and evaluated by flow cytometry. Phagocytic capacity was determined by the number of positive cells while phagocytic efficiency was determined by the mean fluorensce.

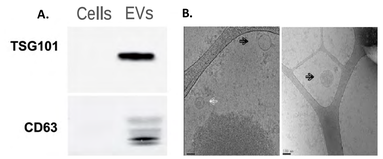

Figure 2. Characterization of extracellular vesicles (EV). **A)** EV were enriched for multi-vesicular bodies (MVBs) and exosomal markers TSG101 and CD63 by Western blot and can be seen by **B)** Cryo-TEM having both double (white arrow) and single (black arrow) membranes.

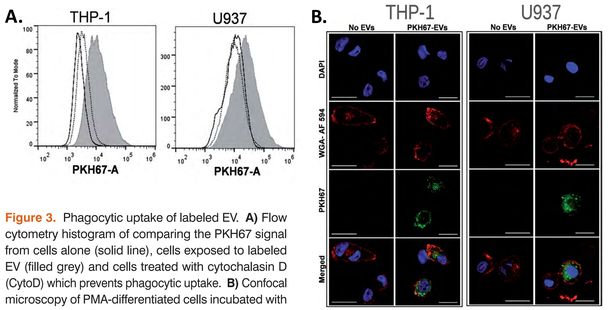

Figure 3. Phagocytic uptake of labeled EV. **A)** Flow cytometry histogram of comparing the PKH67 signal from cells alone (solid line), cells exposed to labeled EV (filled grey) and cells treated with cytochalasin D (CytoD) which prevents phagocytic uptake. **B)** Confocal microscopy of PMA-differentiated cells incubated with EVwhere; PKH67-EV (green), nuclear stain DAPI (blue) and cell membrane WGA-Alexa Fluor® 594 (red).

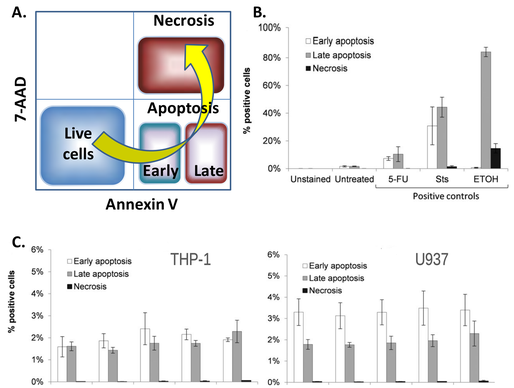

Figure 4. Determining apoptosis and necrosis using Actinomycin and 7-AAD. **A)** Schematic showing dual staining methodology to identify apoptotic versus necrotic cell death and a representative histogram (in THP-1 cells) showing positive and negative control staining. **C)** There is no difference in apoptosis versus necrosis following exposure to increasing concentrations of EV in either THP-1 or U937 cells.

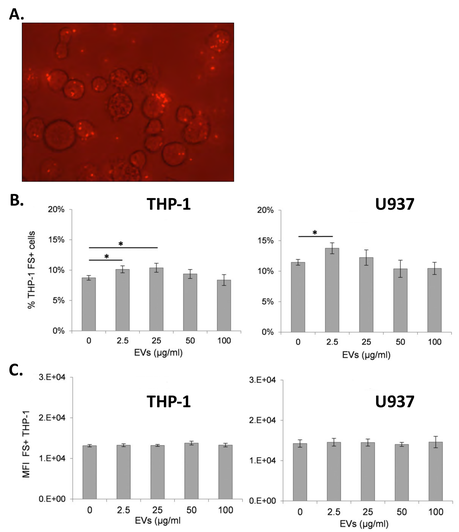

Figure 5. Phagocytosis of 1 um diameter FluoSpheres fluorescent microspheres. **A)** Fluorescence of microspheres in cultured monocytic (canine) cell line. **B)** Upon EV exposure, a slight increase in phagocytic capacity (as indicated by the percentage of positive cells were noted in both human monocytic cell with no differences noted in **C)** phagocytic efficiency (as measured by mean fluorescence intensity (MFI)). Error bars show SEM. Data representative of at least three experiments. *p <0.05

4 CONCLUSIONS AND FUTURE DIRECTIONS

Increasingly, new therapeutics which directly or indirectly target immune responses or cells are being developed. Assessing the safety, particularly toxicity on the immune system, is particularly important. No longer is assessing overall cell numbers, overt immunosuppression, or immunostimulation sufficient in evaluating more subtle effects on the immune system. More advanced phenotypic and functional assays are necessary to understand the complex impact that novel therapeutics have on immune responses. While assessing immunotoxicity of adaptive immune responses (e.g. T cell dependent antigen response; TDAR) is commonly employed, evaluating innate immune endpoints, particularly of myelomonocytic cells, are often less commonly evaluated.

Myelomonocytic cells are key players which impact both innate and adaptive immune responses. Thus, newly developed therapies which have the potential to target or alter their function can have dramatic impacts on multiple aspects of the immune response. In this study, we evaluated the immunotoxicologic effects of extracellular vesicles, a novel therapeutic delivery system, on human monocytic cell lines. We found that human monocytes are highly permissive to EV entry in a dose-dependent manner but exposure to EV did not affect the homeostatic levels of apoptosis or necrosis. Although lower doses of EV increased phagocytic capacity, phagocytic efficiency of individual cells was not affected by EV exposure at any of the doses evaluated. The study demonstrates the application of *in vitro* immunotoxicity assessment of innate immune cells (human monocytes) of a novel therapeutic delivery system.

5 REFERENCES

Barnett JB and Brundage KM. 2010 Methods Mol Biol. 598:75–94.

Batrakova EV and Kim MS. 2015 J Contr Rel. 219:396–405.

Frohlich E. 2015 Int J Nanomedicine 10:3761–78.

Gyorgy B *et al*. 2015 Annu Rev Pharmacol Toxicol. 55:439–464.

ICH (I.C.O.H). 2011 [Internet]. Pre-clinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals. Available from: http://www.ich.org/products/guidelines/safety/safety-single/article/preclinical-safety-evaluation-of-biotechnology derived-pharmaceuticals.html

Leclerc *et al*. 2010 Inhal Toxicol. 22:1091–1100

Li, *et al*. 2011 Arch Toxicol. 85:355–361.

Rosas LE *et al*. 2016 J. Immunotoxicol April 14:1–14 [Epub ahead of print]

Roy *et al*. 2006 Pharmacol Res 53:293-302