Differential Uptake of Murine and Human Exosomes by Normal and Inflamed Peripheral Tissues and Brain

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Abstract

Background: Exosomes function as an intercellular communication system conveying messages from donor to target cells in nearby or distant tissues. Many aspects of exosome trafficking remain unresolved, however. Here, we investigated uptake of ten radiolabeled murine or human exosomes of various cellular origins by the liver, kidney, spleen, and lung of male CD-1 mice.

Methods: We radioactively labeled 10 exosomes from mouse or human cancerous or non-cancerous lines, injected them intravenously into male CD-1 mice, and studied their tissue uptake. We examined the ability of wheatgerm agglutinin (WGA), mannose-6 phosphate (M6P), and inflammation induced by lipopolysaccharide (LPS) to modulate uptake. We measured uptake rate using multiple-time regression analysis and used heat mapping and path analysis to correlate tissue and exosomal influences on uptake.

Results: Except for the uptake of SCCVII exosomes by kidney, all exosomes were taken up by all tissues, although the uptake levels varied broadly among exosomes and tissues. The liver/serum uptake ratio for exosomes from primary human T-cells was the highest at 4,500 mL/g. Species of origin (mouse vs human) or source (cancerous vs noncancerous cells) did not influence tissue uptake. The uptake of some exosomes was altered by WGA and LPS but not by M6P, except for uptake inhibition of J774A.1 exosomes by liver, suggesting use of the M6P receptor. WGA or LPS treatments enhanced uptake of exosomes by brain and lung but inhibited uptake by liver and spleen. Response to LPS was not, however, predictive of response to WGA. No evidence for a universal binding site controlling exosome uptake was obtained. Applying path analysis and heat map analysis to the data, including our published results for brain, we found that exosome uptake patterns for lung and brain responded similarly to WGA or to LPS, whereas those for liver and spleen clustered together. In path analysis, the 10 exosomes clustered into distinct groups, suggesting that their bindings sites are similarly clustered.

Conclusions: Uptake of exosomes by peripheral tissues is differentially regulated by both exosomes and target tissues and is dependent on the number and types of mutually interactive binding sites.

Background

Exosomes, a subset of small extracellular vesicles (EVs), ranging in diameter from 30-150nm, are produced by normal and malignant cells and are found in all body fluids (1-3). Exosomes are formed as inverse membrane invaginations inside multivesicular bodies (MVBs) and thus originate from the endosomal cell compartment (4). The topography of exosome surface proteins and their molecular content resemble that of the parent cells (3) which, in part, explains why they can serve as surrogates of the producer cell.

Exosomes circulate freely and are taken up by a variety of tissue cells, resulting in the phenotypic and functional reprogramming of recipient cells (4, 5). The capability of exosomes to convey peptides, proteins, and genetic materials from the parental to recipient cells without a loss of function qualifies them as a highly effective intercellular communication system (4-7). Exosomes exert profound and varied biological effects on cells in the circulation and in tissues, reprogramming their functions (5). They convey signals to immune cells that a tissue injury has occurred, stimulating trafficking of immune cells into tissues (8). In cancer, they can prepare the tissue's endothelial bed for metastasis (9) The role of exosomes as disease biomarkers or biomarkers of response to therapy in cancer, infections or autoimmune diseases is under intense investigation (10-12).

While the reprogramming functions of exosomes are well described, there is a limited understanding of processes used by various cells to sort, package and secrete exosome cargos (4). Similarly, questions relating to exosome trafficking remain incompletely explored, although general mechanisms of exosomes uptake by tissues have been described (13). Vesicular internalization follows the exosome attachment to binding sites on the cell membrane of the target tissue. The binding sites on exosomes and on recipient cells of the target tissues are mainly glycoproteins and glycolipids, with integrins

located on exosomes playing a particularly important role as binding sites. To enter into the tissue such as brain, exosomes must first cross the blood-brain barrier (BBB) and so they first bind to brain endothelial cells, inducing endocytosis and ranscytosis (14). Given the diameter of exosomes, it is likely that induction of vesiculation at the capillary bed is an important mechanism for exosome uptake by most tissues.

A number of unanswered questions regarding exosome uptake by tissues remain, including the preferential or selective uptake of exosome subsets by various target cells and tissues; the role specific cellular receptors might play in exosome uptake; and the apparent ready acceptance of human and murine exosomes injected into mice (15). In addition, it remains unclear whether uptake of exosomes produced by cancer cells is favored over exosomes from nonmalignant cells or whether uptake of immune cell-derived exosomes by immune cells is favored over non-immune tissue cells. An important consideration is whether exosome uptake can be modulated or manipulated under different environmental stimuli and, if so, would uptake be equally affected for all exosomes and for all tissues. In our previous report (15), we addressed some of these questions in the study of in vivo uptake of exosomes by mouse brain. Here, we examine the in vivo uptake of radioactively labeled various human and mouse exosomes by murine kidney, lung, liver, and spleen with an objective to further clarify these outstanding issues related to exosome trafficking.

Methods

Cell lines: Human and murine cell lines used for exosome production are listed in **Table 1**, as previously published (15). Tissue origins of the malignant/non-malignant cell lines are indicated. The growth conditions used to obtain cellular supernatants were previously described (16). Briefly, RPMI media were supplemented with fetal bovine serum (FBS) which was centrifuged at 100,000x g overnight to deplete exosomes. Each lot of medium was filtered using 0.22µm filter (Millipore) prior to cell culture. Cellular supernatants were harvested, concentrated and processed as described by us previously (16, 17) in preparation for exosome isolation by size exclusion chromatography (SEC).

Table 1. Exosome Sources and Producer Cell Lines Characteristics*

Designation	Species	Non-Cancerous/Cancer	Tissue
J774A.1	Mouse	Non-Cancerous	Macrophage
NIH-3T3	Mouse	Non-Cancerous	Fibroblast
SCCVII	Mouse	Cancer	Oral Squamous
Primary T Cell	Human	Non-Cancerous	T Cell
HaCaT	Human	Non-Cancerous	Keratinocyte
MEL526	Human	Cancer	Melanoma
MDA-MB-231	Human	Cancer	Breast
PCI-30	Human	Cancer	Head & Neck
SCC-90	Human	Cancer	Head & Neck
Kasumi	Human	Cancer	Leukemia

*Exosomes were isolated as described in Methods by SEC from the supernatants of the above listed cell lines.

Culture of primary human T cells: Peripheral blood was obtained from healthy volunteers who signed a consent form approved by the Institutional Review Board (IRB no. 0403105). Venous blood was collected in heparin tubes and was processed immediately for recovery of peripheral blood mononuclear cells (PBMC) by centrifugation on Ficoll-Paque Plus gradients (GE Healthcare Bioscience, Pittsburgh, PA, USA). The recovered cells were washed in RPMI medium (Lonza, Basel,

CH) supplemented with 10% (v/v) exosome depleted FBS (Gibco, Thermo Scientific, Pittsburgh, PA, USA) and T cells were isolated by negative selection on AutoMACS (Miltenyi Biotec, San Diego, CA, USA) using an isolation kit from Miltenyi Biotec. T cells were activated using CD3/CD28 T-cell activator (25 μ l/ml, Stemcell, Vancouver, BC, CA) and IL-2 (150IU/ml, PeproTech, Bionity, Rocky Hill, CT, USA) in freshly prepared RPMI for 48-72h prior to T cell culture for exosome production.

Exosome Isolation: Exosome isolation by SEC was previously described (18). Briefly, aliquots of concentrated supernatants (1mL) were loaded on a 10 cm-long Sepharose 2-B column and individual 1mL fractions were eluted with PBS. Fraction #4 containing non-aggregated exosomes was harvested, concentrated, and used for exosome characterization (15, 18) and radioactively labeled as described below.

Radioactive labeling and purification: Exosomes isolated from supernatants of the cell lines listed in Table 1 or primary human T cell-derived exosomes were radioactively labeled with 0.5mCi 125 I (Perkin Elmer, Waltman, MA) using the chloramine-T method and purified on an Illustra NAP-5 (GE Healthcare, Piscataway, NJ) column eluting with PBS. Bovine serum albumin (Sigma, St. Louis, MO) was labeled with 131 I using the chloramine-T method or 99m Tc (GE Healthcare) using the stannous tartrate method. Both I-Alb and Tc-Alb were purified on a column of G-10 Sephadex (GE Healthcare).

Mice: 8-week-old CD-1 male mice (Charles River) were kept in a 12/12-hour light/dark cycle and given ad lib water and food. All studies were performed under protocols approved by the Veterans Affairs Puget Sound Health Care System's IACUC, a facility accredited by the Association for Assessment and Accreditation Laboratory Animal Care International (AAALAC).

IV time curve: Mice were anesthetized by giving an intraperitoneal (ip) injection of 0.15-0.2 mL 40% urethane. The left jugular vein was exposed and an injection of 0.2 ml lactated Ringer's solution containing 1×10^6 cpm of a radioactively labeled exosome and 1×10^6 cpm of a radioactively labeled albumin was into the left jugular vein. At time points between 1 and 60 minutes, blood was collected from the carotid artery. Blood was centrifuged at 5400g for 10 minutes and 50 μ l serum collected. The brain, liver, lung, kidney and spleen were removed and weighed. All tissues and serum were placed into a gamma counter and the levels of radioactivity were measured. Results for serum were expressed as the percent of the injected dose per ml of blood (%Inj/ml). Results for tissues were expressed as the tissue/serum ratio in units of ml/g. For each individual tissue, its ratio for radioactive albumin were subtracted from its ratio for the radioactive exosome, yielding the "delta" value which reflected extravascular or tissue uptake. Subtracting albumin removes the vascular and leakage contributions from the exosome tissue/serum ratios, yielding a better estimate of the selective uptake of the exosome.

Lipopolysaccharide (LPS) preparation and treatment: Mice were given an ip injection of 0.2 ml of 0.9% NaCl solution with half the injections containing 3 mg/kg LPS (Sigma, St. Louis, MO). Mice received 3 such injections at t=0, 6, 24 h. At 28 h after the first injection, mice were anesthetized with urethane and studied as outlined above, except time points after radioactive injection ranged from 1-20 min. Whole brain did not include the olfactory bulb.

Effects of mannose-6-phosphate and wheatgerm agglutinin on tissue uptake: Mice were given an ip injection of 0.15-0.2 mL 40% urethane. The left jugular vein and the right carotid artery were exposed. An injection of 0.2 mL lactated Ringer's solution containing 1×10^6 cpm of the radioactive exosome and 1×10^6 albumin was given into the jugular vein at t = 0. In some mice, this injection contained either 10 μ g wheatgerm agglutinin (WGA; Sigma) or 80 μ g mannose-6-phosphate (M6P; Sigma). At t=20 min, carotid artery blood and tissues were collected, and the results expressed as described above.

Statistical Analysis: To calculate clearance from blood, the %Inj/ml values were transformed to log values and regressed against time. We compared linear and nonlinear (one phase decay) models using the Prism statistical package (GraphPad Inc, San Diego, CA) and report the best fit. Mice were studied over a 60 min period. Blood-to-tissue unidirectional influx rates (K_i 's; units of μ L/g-min) were calculated by regressing the delta values (tissue/serum ratios corrected for the albumin space) against exposure time with multiple-time regression analysis (19, 20). This method yields the K_i and also the initial volume of distribution in the tissue (V_i ; units of μ L/g). Each regression is reported with its n, r^2 , and p value. Means are

reported with their standard error terms and n's. Study period was 60min. Means are reported with their standard errors and n/group.

Path analysis based on methods as outlined by Baron and Kenny (21) was used to further explore relations among uptake of exosomes by tissues. Such analysis produces a graph with pairs of tissues connected, with those pairs with the strongest statistical connection taken to be more closely related. No directionality or dependency among the tissues was assumed. To probe for relations among the tissues, the delta tissue/serum values from the controls of the WGA study for each tissue was regressed against each of the other tissues using Spearman correlation and the values for r^2 ranked from high to low. Starting with the pairs with the highest r^2 values, pairs were graphed unless such graphing would produce a closed loop. Pairs in the resulting graph with higher r^2 values are taken to be more closely related than those with lower r^2 values or that are connected indirectly through other tissues. To probe for relations among exosomes, delta tissue/serum values from the controls of the WGA study for each exosome was regressed against each of the other exosomes using Spearman correlations.

Results

Exosome characteristics

Human or mouse exosomes were obtained from supernatants of cultured malignant or non-malignant cells (**Table 1**). To isolate exosomes, pre-cleared, concentrated supernatants were separated by size exclusion chromatography (SEC) as previously described (16, 18). Vesicles collected in fraction #4 were characterized as exosomes based on their average diameter (~100 nm), the presence of endocytic markers (TSG101, ALIX), absence of cytoplasmic proteins such as Grp94 or calnexin, and the enrichment in tetraspanins (CD63, CD81). According to the recently adopted nomenclature, these vesicles are "small extracellular vesicles"(22), here referred to as "exosomes" that range in size from 30-150nm and display a characteristic vesicular morphology by TEM. All harvested exosome preparations were evaluated for the protein content and functional attributes as measured in vitro by their ability to modulate immune cell functions prior to radioactive labeling.

Uptake of radiolabeled exosomes by tissues

Liver

Figure 1 shows representative results for uptake by liver of exosomes produced by human keratinocytes (HaCaT), human tumor cells (PCI-30), mouse macrophages (J774A.1), and primary human T-cells. For all ten exosomes tested, a sharp increase in the liver/serum ratios peaking at 10-20 min was followed by a decline, except for exosomes produced by HaCaT, where the ratio was flat over time (**Figure 1A**). The largest uptake was for primary human T-cell exosomes (**Figure 1B**). Uptake by PCI-30 (**Figure 1A**), MDA-MB-231, SCC-90 and SCCVII increased to the 1200-1500 $\mu\text{L/g}$ range, whereas uptake by J774A.1, Kasumi, and NIH-3T3 peaked at 800-900 $\mu\text{L/g}$; MEL526 peaked at about 500 $\mu\text{L/g}$. Thus, while 9/10 exosomes showed similar patterns for liver uptake, the degree of uptake varied almost 10 fold.

LPS had no statistically significant effect on liver uptake of exosomes from HaCaT, Kasumi, MDA-MB-231, primary human T-cell, or SCC-90. LPS treatment decreased liver uptake of exosomes from J774A.1 (**Figure 1C**), MEL526, NIH-3T3, and SCCVII, and there was a trend for a decrease for exosomes from PCI-30. For NIH-3T3 and SCCVII, the influx rate was significantly decreased, whereas for MEL526, the intercept was shifted downward. J774A.1 showed a hybrid pattern with the intercept shifted downward and the slope decreased, although there was a statistical effect only for the decrease in intercept (**Figure 1C**). M6P only affected J774A.1 exosome uptake, inducing a small increase (**Figure 1D**), while WGA caused a significant decrease in uptake of exosomes from J774A.1, Kasumi, MDA-MB-23, NIH-3T3, PCI-30, and SCC-90 by the liver.

Kidney

For exosome uptake by kidney (**Figure 2**), MDA-MB-231 showed a rapid increase and subsequent decline, while a hyperbolic relation was seen for J774A.1 (**Figure 2A**). Uptake of SCC-90 exosomes (**Figure 2B**) and primary human T-cell exosomes (**Figure 2C**) was linear, whereas SCCVII showed an inverse relation with exposure time and negative kidney/serum ratios (**Figure 2B**). The negative values for SCCVII indicate that it is taken up less well by the kidney than serum albumin. There was no correlation between kidney/serum ratios and exposure time for HaCaT, Kasumi, MEL526, NIH-3T3, or PCI-30 exosomes, although the ratios were 20-100 $\mu\text{L/g}$ higher than the ratios for albumin (data not shown).

LPS had an effect on kidney uptake for only 3 exosomes derived from primary human T-cell (**Figure 2C**), SCC-90, and SCCVII. For SCCVII, kidney/serum ratios remained negative in LPS-treated mice, but at values closer to zero than in the controls. WGA increased uptake of exosomes from HaCaT, J774A.1, NIH-3T3, and primary human T-cells (**Figure 2D**). M6P had no effect on any of the 10 exosomes.

Lung

Figure 3 illustrates uptake of various exosomes by lung. Only SCCVII and MDA-MB-231 accumulated in lung as a function of time (**Figure 3A, B**). Other exosomes had low lung/serum ratios of 10-100, except for primary human T-cell exosomes, which had ratios of about 500 $\mu\text{L/g}$.

LPS increased lung uptake of exosomes to a modest, but statistically significant degree for Kasumi, MDA-MB-231, primary human T-cell, SCC-90 and SCCVII exosomes, but not for the other exosomes (see examples in **Figure 3C, D**). LPS decreased exosome uptake for J774A.1 (data not shown). M6P had no effect on lung uptake of exosomes, but WGA dramatically increased uptake for all exosomes (**Figure 3E**).

Spleen

All of the exosomes accumulated as a function of time by the spleen, except for MEL526 and SCC-90, which nevertheless had spleen/serum ratios averaging about 350 $\mu\text{L/g}$ and 500 $\mu\text{L/g}$ above those for radioactive albumin. **Figure 4A and B** show the relationships between the spleen/serum ratios and time for all of the exosomes that were taken up over time except MDA-MB-231, which closely resembled the pattern for Kasumi. In general, a plateau or a fall followed after about 20-30 min of uptake.

LPS caused a significant decrease in the uptake by spleen for exosomes produced by J774A.1, MDA-MB-231, MEL526, NIH-3T3, PCI-30, primary human T-cells, and SCCVII, but not for HaCaT, Kasumi, or SCC-90 exosomes (see **Figure 4C** for representative data with PCI-30 exosomes). M6P had no effect on uptake of any exosomes, whereas WGA induced a statistically significant decrease in the uptake of 6 exosomes (**Figure 4D**).

Single time-point analysis of uptake

Figure 5 and **Table 2** show the control data from the WGA experiment. **Figure 5A** shows all tissues and exosomes with tissue/serum values plotted against a logarithmic axis. The kidney values for NIH-3T3, HaCaT, and SCCVII were negative, but appear in **Figure 5A** as values = 1. Spleen and liver show the highest ratio of uptake, i.e., have the highest tissue/serum values, for all exosomes. Lung and kidney have a moderate uptake ratio, while brain has the lowest ratio of uptake for all exosomes. **Figures 5B, C, and D** show the tissue/serum ratios for each of five tissues for uptake of all exosomes plotted against an arithmetic scale to emphasize differences in tissue uptake values. **Figure 5E** is a heat map of uptake.

Table 2. Means ($\bar{x}$), standard deviations (SD) and number per cell (n) for each exosome and tissue. Results are from *control* mice used in the WGA experiment

	Brain			Liver			Lung			Kidney			Spleen		
	X	SD	n	X	SD	n	X	SD	n	X	SD	n	X	SD	X
J774.A1	5.82	0.47	10	537	16.9	10	121	9.8	10	75.3	6.3	10	599	39.6	10
NIH-3T3	2.35	0.55	9	507	92.5	9	21.6	13.9	9	-34	10.3	9	532	76.9	9
Primary T Cell	12.1	1.6	8	2157	255	8	438	105	8	466	123	8	1018	208	8
HaCaT	1.79	0.33	10	282	14.2	10	13.9	5.7	10	-28.3	8.8	10	302	30.1	10
SCCVII	5.38	0.37	8	718	133	8	93.8	13.1	8	-48.8	14.8	8	1097	71.5	8
MEL526	5.18	2.32	8	419	62	8	76.5	38.8	8	55.8	18.5	8	514	44.2	8
MDA-MB-231	8.6	3.7	9	452	50	9	119	51.5	9	79.9	33.8	9	402	64	9
PCI-30	8.41	0.77	10	674	72.1	10	114.9	9.7	10	62.6	10	10	829	90.9	10
SCC-90	6.54	0.47	10	981	50.2	10	99.1	0.3	10	104	9	10	751	73	10
Kasumi	4.82	0.21	8	518	63	8	72.2	36.7	8	73.8	24.3	8	678	70.8	8

Results of path analysis

The observed differences in exosome uptake ratios between tissues were further evaluated by path analysis. The analysis was performed for the four tissues as well as the previously published data for brain, which were from the same animals (15). The first step in path analysis was to determine the correlations among all tissue pairs using Spearman correlations. **Figure 6A** shows the pair with the strongest Spearman correlations (lung/serum ratios vs brain/serum ratios) and **Figure 6B** the weakest Spearman correlation (spleen/serum ratios vs kidney/serum ratios). **Figure 6C** shows the tabulated r^2 values for each of the possible tissue comparisons with those having a statistically significant correlation indicated by an “*”. **Figure 6D** is a graphic presentation of the path analysis showing the r^2 values, where solid lines represent r^2 values that were statistically significant and dashed lines are those that were not significant. A higher r^2 value results from a greater statistical correlation between the pair of tissues. The results show two significant clusters of relationships. Brain and lung are highly correlated (that is, the degree that an exosome, taken up by one tissue is predictive of the degree to which it is taken up by the other tissue), and lung and brain are also correlated with kidney, although less so. Liver and spleen form another highly correlated cluster. The least related pair is that of kidney and spleen with an $r^2 = 0.016$.

Path analysis based on exosome pairs is shown in **Figure 7**. For exosome pairs, there were only 5 tissue/serum ratios (one for each of the five tissues) for constructing the regression lines, giving only 3 degrees of freedom. In contrast, path analysis for the tissue had 10 tissue/serum ratios (one for each exosome) and so 8 degrees of freedom. Therefore, path analysis for exosome clustering is much less detailed. Three sets of exosomes were perfectly correlated ($r^2 = 1.0$): (a) NIH-3T3, HaCaT, and SCCVII; (b) PCI-30, J774A.1, and MEL526; (c) Primary T cell and SCC-90. Statistically significant relations with Kasumi and MDA-MB-231 are also shown. The table in **Figure 7** shows the r^2 values among the exosome pairs that were not statistically significant.

The heat map in **Figure 8** shows the effects on LPS, WGA, and M6P treatments on the uptake of exosomes by various tissues, with gray indicating no effect of treatment on exosome uptake; blue indicating that treatment decreased the exosome tissue uptake; and red indicating that treatment increased the exosome tissue uptake. The uptake data obtained for brain and peripheral tissues are derived from the same mice, although the data for brain have been previously published (15). The heat map shows two main clusters, with liver and spleen responding similarly to LPS or WGA treatment by

decreased exosome uptake, kidney and lung responding to LPS or WGA treatment by increased exosome uptake, and brain responses being more similar to those of kidney and lung.

Discussion

This study examined the ability of four peripheral murine tissues: lung, kidney, liver, and spleen to take up different types of exosomes (n=10) produced by human and mouse cancerous or noncancerous cells. We examined uptake when the innate immune system was stimulated with LPS and also after WGA treatment. M6P was used to block exosome uptake via the M6P receptor. We compared these uptake results with the previously published data for the uptake by brain of the same ten exosome types injected in the same mice (15).

We reasoned that exosome uptake by any tissue is dependent on an interaction between the glycoproteins/glycolipids on the exosome surface and their counterparts expressed on cell surfaces of the target tissue. Binding of exosomes to glycoproteins/glycolipids on tissue cells is sufficient for internalization. The abundance of the available binding sites on the exosome surface is expected to control the rate of exosome uptake by tissues. Similarly, the abundance of these binding sites in the tissue or its vascular bed should control the rate of exosome uptake by this tissue. Spleen and liver showed the most abundant uptake of exosomes, and in 7/10 cases brain took up the least amount of exosomes (**Figure 5**). This observation is consistent with the barrier functions of the brain's vascular bed (23). The three cases (NIH-3T3, HaCaT, SCCVII) where values for kidney uptake were negative (that is, uptake of albumin was greater than uptake of exosomes) can be explained by an absence or low level of binding sites for those exosomes in the kidney. Thus, uptake would depend on leakage and, since exosomes are about 7 times the diameter of an albumin molecule, the rate of leakage for exosomes would be less than that for albumin. The primary T cell exosomes usually showed the greatest tissue uptake, and HaCaT exosomes tended to show the lowest uptake.

A comparison of various tissues for the uptake of exosomes in the presence/absence of LPS, WGA and M6P provides additional key information about exosome-tissue interactions. For example, if exosome uptake relied on a single, universal protein that determined uptake, then all exosomes would be taken up by all cells in proportion to the expression of that protein. To the extent that the uptake of proteins differ among exosomes, target tissues, or both, a greater variety among the uptake patterns would emerge. WGA affects binding to glycoproteins expressing sialic acid and N-acetyl-D-glucosamine and M6P binds to the mannose-6-phosphate receptor (24-27). Thus, an effect of WGA or M6P indicates the involvement in uptake of those respective binding sites. LPS stimulates the innate immune system, which alters many cell membrane receptors and transporters, including expression of the selectins (28, 29). Thus, an effect of LPS indicates a binding site in the target tissue responsive to the innate immune system.

As a rule, a given exosome type showed very few consistent patterns across tissues or conditions. While exosomes from human primary T cells consistently had high uptakes in most tissues, rates and responses varied among other exosomes, clustering into the lung-brain and the liver-spleen groups, as noted above. WGA had an effect on 6 exosomes and LPS an effect on 4, but only two exosomes were affected by both WGA and LPS. This illustrates the variability of responses that exosomes display. In contrast to exosomes, the tissues did show some consistency in that liver and spleen responding to LPS or WGA had decreased exosome uptake, whereas lung and brain had increased uptakes. For most exosomes, liver had the greatest uptake that peaked 10-20 min after injection and then declined. This is similar to the findings reported by Charoenviriyakul et al (30). The high uptake of exosomes suggests the liver is the major clearance site for exosomes, and a subsequent decline in uptake indicates that the liver releases exosomes after their sequestration, although it is unclear if those were intact exosomes, exosomes modified by the liver, or degradation products.

With the exception of kidney uptake of SCCVII, NIH-3T3, and HaCaT, all exosomes under all conditions entered each tissue to an extent exceeding the uptake of albumin, indicating *tissue sequestration*. However, uptake of exosomes was characterized by variety rather than uniformity. It ranged from a high uptake of 4,500 mg/mL for primary human T-cell

exosomes by liver to the nondetectable uptake levels for SCCVII exosomes in kidney. There was no discernable effect of species (i.e., mouse vs human) or source (i.e., cancerous vs noncancerous cell line type) on the degree of uptake, uptake patterns, or responses to LPS, WGA, or M6P. For example, WGA decreased liver uptake for 6 exosomes, but increased uptake by lung for all exosomes. A response of exosomes to WGA was independent of their response to LPS. These findings show *there is no universal receptor or binding site that controls exosome uptake*. Our results favor a view that binding sites vary among exosomes and among tissues, producing novel patterns of exosome/tissue interactions.

Why LPS and WGA would have opposite effects on exosome uptake by lung-brain vs liver-spleen is unclear. LPS could have a differential effect on expression of receptors key to exosome uptake. By analogy, LPS treatment increases immune cell trafficking into brain, but decreases immune cell uptake by spleen (31). In the current experiments, mice but not exosomes were treated with LPS prior to injection, and so the LPS effects here are mediated at the tissue level. WGA affects uptake through the process of adsorptive endocytosis, which has the hallmark of increasing rather than inhibiting uptake of its ligands through stochastic binding mechanisms (32). Because WGA was included in the injection along with the exosomes, WGA could be exerting its effects at either tissue or exosome receptors.

There were, however, some interesting broader patterns. As already noted, both path analysis (**Figure 6**) and heat map analysis (**Figure 8**) showed clustering of lung and brain responses and another clustering of liver and spleen. Path analysis indicated that the ranking of exosomes from low to high correlated well between lung and brain and also between liver and spleen. As an example, if an exosome was robustly taken up by brain, its uptake by lung would also likely be robust. Heat map analysis showed that WGA or LPS treatments tended to increase exosome uptake by lung and brain, but decreased exosome uptake by liver and spleen. The strength of this correlation is highlighted by path analysis, which is based on baseline uptake rates, whereas the heat map in **Figure 8** is based on responses to WGA and LPS. Thus, we conclude that the tissue-exosome interacting receptor combinations may be similar between lung and brain and also between liver and spleen. The only exosome-tissue combination affected by M6P was J774A.1 and liver; and in the previous study of exosome uptake by brain, only J774A.1 was affected by M6P. This suggests that a mannose-6 phosphate receptor is important in the uptake of J774A.1 exosomes by both brain and liver. Future analysis will likely show other receptors shared by lung and brain as well as liver and spleen. Similarly, path analysis showed exosome clustering (**Figure 7**). Because statistical analysis was less robust for exosome clustering than for tissue clustering, results are less finely grained. Nevertheless, the results indicate that groups of exosomes are likely to have similar binding sites. This is consistent with the work showing that distinct integrin expression patterns differentiated exosomes metastasizing to lung from those metastasizing to liver (9).

In comparison to liver, kidney had low levels of uptake, suggesting it is not a major site of clearance for circulating exosomes. Kidney showed the greatest number of uptake patterns, including a peak and decline (like liver), linear, flat or hyperbolic, and the only exosome with uptake lower than that of albumin (SCCVII). The three exosomes for which kidney uptake was less than that of albumin (NIH-3T3, HaCaT, SCCVII) were also shown by path analysis in **Figure 7** to cluster. Kidney had the fewest exosomes whose uptake was altered by LPS (n=3) or WGA (n=4). Lung also had low uptake and most exosomes had uptake patterns that were flat, indicating that the exosomes had rapidly reached equilibrium. Lung showed the greatest difference in response to WGA vs LPS, as all exosomes responded dramatically to WGA increasing their uptake by 30-50 fold. Spleen had levels of uptake intermediate between the high liver uptake and the low kidney and lung uptake. Uptake of the immune cell derived exosomes (J774A.1, human primary T cells, and Kasumi) was not favored by the spleen.

Conclusion

Exosomes were readily taken up by liver, kidney, spleen, and lung, and uptake by these tissues of various exosomes was modified by LPS and WGA. However, patterns of uptake and responses to LPS and WGA varied greatly among the exosomes. No evidence for a universal binding site controlling exosome uptake was obtained. Both tissues and exosomes

differed in uptake patterns, but they clustered reflecting similar binding and uptake patterns. The exosome-tissue binding sites are likely to be highly similar between brain and lung and between spleen and liver. Likewise, exosomes not related by source of origin (i.e., mouse vs human; cancer vs non-cancer) likely cluster by using a common set of binding sites for tissue uptake.

Abbreviations

%Inj/g: The percent of the injected material present in a gram of the tissue

%Inj/ml: The percent of the injected material present in a ml of serum

BBB: Blood-brain barrier

EVs: Extracellular vesicles

I-Alb: Albumin radioactively labeled with ^{125}I

LPS: Lipopolysaccharide

M6P: Mannose-6 phosphate

MVBs: multivesicular bodies

Tc-Alb: (Albumin radioactively labeled with $^{99\text{m}}\text{Tc}$)

WGA: Wheatgerm agglutinin

Declarations

Ethical Approval and Consent to Participate

All authors approve of the ethics and consent to participate

Consent for Publication

All authors consent to the publication of this manuscript.

Availability of Supporting Data

All data generated or analyzed are included in this article. Raw data is available upon reasonable request.

Competing Interests

No author has competing interests and no conflicts of interest

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Author contributions

All authors contributed to experimental design discussions and manuscript preparation.

William A Banks: Experimental design, advanced statistics, manuscript preparation and editing

Priyanka Sharma: Experimental design, exosome preparation, manuscript editing

K.M. Hansen: Experimental design, manuscript preparation, animal work,

initial statistical evaluation

Nils Ludwig: Experimental design, manuscript editing, exosome preparation,

T.L. Whiteside: Experimental design, manuscript editing and preparation, exosomal preparation

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Figures

Figure 1

Liver uptake of exosomes. In **A**, the uptake pattern as exemplified by PCI-30 for those exosomes that were taken up by liver in a time-dependent manner. That panel also illustrates HaCaT as an example of exosomes whose uptake levels did not vary with time. In **B**, the most robustly sequestered exosome was derived from primary human T-cells. In **C**, the typical uptake pattern as exemplified by J774A.1 for exosomes whose liver uptake was inhibited by LPS treatment. In **D**, the six exosomes whose uptake was increased by WGA, and the one exosome affected by M6P. * $P<0.05$; ** $P<0.01$; **** $P<0.001$.

Figure 2

Kidney uptake of exosomes. In **A**, the two patterns of exosome uptake for kidney: a hyperbolic uptake as exemplified by J774A.1 and an increase followed by decrease as exemplified by MDA-Mb-231. In **B**, the uptake pattern for SCC-90 and the uptake pattern with negative values for SCCVII. In **C**, the increased uptake of exosomes with LPS treatment for primary human T-cell exosomes. In **D**, the four exosomes whose uptake was increased by WGA. *** $P < 0.001$; **** $P < 0.001$.

Figure 3

Lung uptake of exosomes. In lung, only SCCVII and MDA-MB-231 showed time-dependent uptakes. SCCVII showed a hyperbolic pattern (A) and MDA-MB-231 showed a decline following its initial uptake. In C and D, modest increases in uptake induced by LPS for exosomes whose uptake either increased (left) or was stable (right) over time. In E, WGA dramatically increased uptake of all of the exosomes; $P<0.001$ for each comparison.

Figure 4

Spleen uptake of exosomes. In A and B, the uptake patterns for spleen for different exosome types. In C, a decrease in uptake with LPS as exemplified by PCI-30. In D, the six exosome types whose uptake was suppressed by WGA. * $P<0.05$; ** $P<0.01$.

Figure 5

Comparison of exosome uptake by various tissues. In **A**, the tissue/serum ratios for all tissues and all exosomes are shown in a logarithmic scale. NIH3T3, HaCaT, and SCCVII for kidney all had negative values but are plotted as a value of one. In **B**, **C**, and **D**, exosome uptake by the five tissues is plotted in an arithmetic scale. In **E**, the heat map for all the data is presented. The data are means with SD and were obtained from mice used as controls for the WGA experiments.

Figure 6

Page 16/18

Path analysis for tissues. In **A** and **B**, examples of correlations of the ten exosomes among paired tissues. In **C**, a list of all r^2 values for various combinations of tissues. *indicates those paired tissues that showed a statistically significant correlation. In **D**, the path analysis for the five tissues studied. Liver-spleen form one strongly-related cluster, whereas lung-brain show a second strongly-related cluster, with kidney also correlated to lung and brain.

Figure 7

Path analysis for exosomes. Exosomes listed in boxes correlated perfectly ($r^2 = 1.0$) with other graphed relations found to be statistically significant. Values below the figure are remaining possible correlations and their r^2 values, none of which were significant.

Figure 8

Heat map correlating changes in exosome uptake following LPS, WGA or M6P treatments. Blue color indicates that LPS, WGA, or M6P induced a statistically significant decrease in uptake of the exosomes, and red color indicates that LPS, WGA, or M6P induced a significant increase in uptake of the exosomes. Grey indicates that LPS, WGA, or M6P did not produce a statistically significant alteration in the exosome uptake. Liver-spleen show strong similarities to one another, whereas lung, brain, and kidney form another group.