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Key Leaders' Opinion on Immune and Exosome

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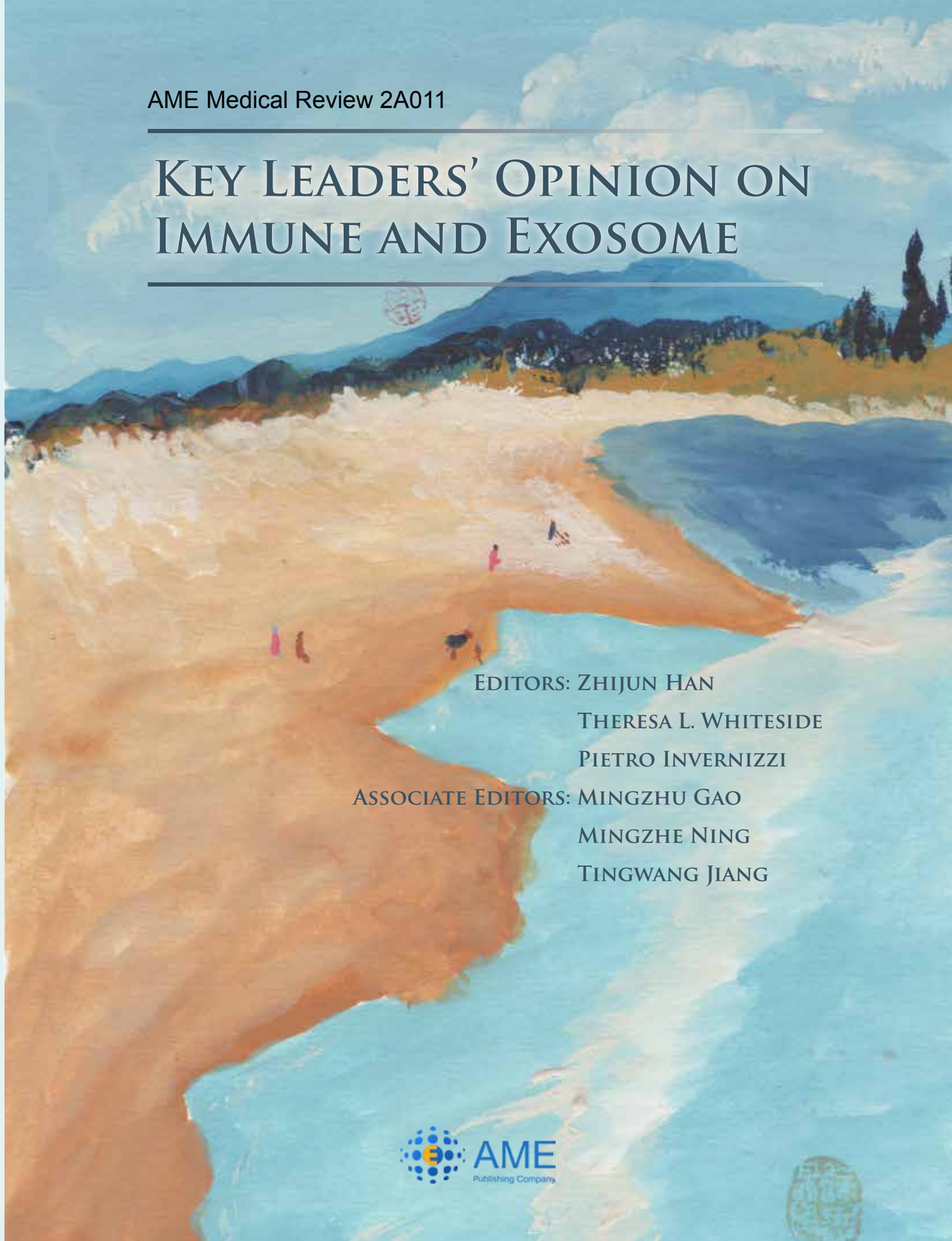

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Key Leaders' Opinion on Immune and Exosome (FIRST EDITION)

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Will scholarly journals perish?

Will scholarly journals perish? This is a question that has puzzled me for years.

The introduction of online journals results in the inevitable recession of print journals. The uprise of the open access journals has been changing the structure of scholarly journals ceaselessly. What keeps me thinking is the open access of clinical trials data. What would be the bigger picture if open access to clinical trials data becomes the mainstream?

It is interesting that with the primary bottleneck lying in the availability of open data, the Big-data Clinical Trial (BCT) seems to stay where it was in spite of the increasingly popularity of "Big Data" among scientists. It has to be the fact that without open data, a statistical analysis is restricted to a particular area (or several areas). Even with big enough data, the study can only be termed as "research with big data sets" rather than "big data research", which are totally different concepts. Big Data is constituted by a plurality of dimensions. On one hand, for an individual (e.g., a patient), the relevant data covering his/her disease course is big enough; on the other hand, for the entire population, as more as individuals (e.g., patients) are expected to be included, to contains all the elements just like the "universe set" in set theory; by doing so, scientists expect to carry out the so-called clinical studies in real-world settings.

Why do the real-world-based clinical trials so appealing? It is understandable that the results and conclusions are likely to be altered in studies targeting the same issue using the same research method with sample size changed. In addition, the probability of such a "likely" is quite high. In many top journals, it is a common phenomenon that some authors tend to validate the results of one study in another population using the same research method. However, if the results are "validated" in one population, it only means that they are "repeatable". Will the results also be repeatable in the second, third, and more populations? If the attempts are not continuing, which should be, the "validation" is equivalent to "self-deception" in a sense.

When clinical research data is open accessed, we can easily integrate data from multiple centers for statistical analysis and meanwhile "validate" the results in multiple populations. If this is the case, then another question arise: can everyone easily publish his/her results/papers in high-profile journals such as the *New England Journal of Medicine*? My answer is NO.

When the open access to clinical research data becomes mainstream, we can easily find the constant update of database on the Internet. Simply by clicking on a button, we obtain the statistical results of the most current data. A further button click would display the validation results based on a specific population. The database would be updated at a certain period of time (e.g., 1 month or 1 day), and the statistical results would "likely" also be changed accordingly. At that time, the questions may change to "would any researchers publish their findings in a journal?" Well, even if someone is still keen to write such articles, journals may be reluctant to publish them because of the indefiniteness of the findings with the risk of being overturned at anytime.

Eventually here it comes the serious question: will scholarly journals perish? My answer is still NO. Then in what way the scholarly journals would probably lead to?

During my Business Administration course, my teacher distributed to us an article from the Case Study column of the *Harvard Business Review*. In this highly respected journal, articles in this column often present one case first, followed by the comments from two experts. These comments could either support or oppose each other. My teacher asked us to study the case, read through the comments and then form our own point of views on the case. He encouraged us to interpret the case from different perspectives independently in what form that I found pretty practical.

The course brought a possible answer to me. When the open access to clinical research data becomes mainstream, the entire publishing industry, especially the publication of "scholarly journals", would eventually experience revolutionary change. It may no longer focus on the rigid and cold outcomes but it would definitely cares more about the reflection on the problems, update of insights, and integration of science and arts.

AME Medical Review Series is a production of the above thinking. As an attempt, we decided to invite experts internationally to provide their views on a specific topic to share their insights with more clinicians and thus benefit more patients. The first chosen topic for the series is the currently controversial one: conventional surgery versus stereotactic body radiotherapy for

the early stage lung cancer. As the first book to the series, we hope it would give you a glance at the coming changes.

The book series will be written by a group of individual experts who are willing to contribute medical reviews and comments to individuals who are interested in clinical research and medical reviews specifically. The book in your hand may possibly be on a heavy subject but we do hope it is presented in an easier way. It will be more than great if it brings you some thoughts and inspire you in some way.

Stephen D. Wang
Founder and CEO,
AME Publishing Company

The field of extracellular vesicle (EV) biology has undergone a rapid and extensive expansion in the last few years. What began as a story of cellular "garbage cans" has in the course of 30 years or so developed into a saga of a nanoparticle-driven universal communication system that appears to be operating in all multicellular as well as unicellular organisms (1,2). It is clear that this communication system is preserved by the evolution and is necessary for the survival of the species.

The number of publications featuring EVs and their participation in biological interactions between cells in health and disease has increased exponentially in the last 10 years, reflecting an enormous interest in the molecular and genetic reprogramming that EVs mediate. The collection of articles presented in this volume serves as an example of the width and breath of research in the EV field that reaches into diverse aspects of physiological and pathological changes induced by EVs in tissues, organs and organisms. The broad coverage of the impact EVs exert in health and disease is intentional, and it serves to emphasize functional versatility of EVs as well as their potential to serve as future biomarkers for many different human diseases.

The remarkable progress in nano-biology has enabled us to recognize, identify and begin to evaluate EVs not only in the supernatants of various human cell lines but also in human body fluids (3). This has led to a better, although still fragmentary, understanding of the complexity of this communication system. As the articles included in the present volume indicate, EVs appear to be involved in the development, maturation and homeostasis of all major signaling pathways and to actively participate in many disease states. An interesting caviat of EV interactions with recipient cells is their dual capability to activate or inhibit responses. The EV cargos contain both stimulatory and inhibitory messages that are delivered as an array or a "bundle" to recipient cells. This implies that the recipient cell has a choice to accept or reject the message, depending on the environmental cues it receives. Interactions of EVs with their cellular targets are thus contextual and environmentally regulated.

As much as the EV functions engage the interest of the scientific community, a number of unanswered questions exist. One commonly voiced criticism is that EVs "are involved in everything," defying the concept of regulated biological processes that are expected to be cell-, tissue- or organ-specific. While it is not known whether or how the EV release by parent cells is regulated, their excessive production by stressed or diseased cells suggests that in case of pathologic situations, EVs are especially needed. The fact that EV mediate autocrine signaling provides additional evidence for the important role of EVs as guardians of the parent cell wellbeing. EV-mediated long-range paracrine-type communication depends on their ability to safely deliver precisely-designed messages from the parent to recipient cells. These messages, in the form of nucleic acids, are safe and protected inside the EV lumen, a distinct advantage for effective communication. Specificity of the EV network might rest on the unique identity of the parent and recipient cells. The address imprinted into EVs by the parent cell might be recognized only by a designated recipient cell. Cross-talk is maintained by the biological mechanisms normally used by a recipient cell that may include endocytosis, phagocytosis or receptor/ligand-type signaling. Thus, messages delivered by EVs are not haphazard but are directed to where they are needed most. While this view of the EV communication network is not yet backed by experimental evidence and might be entirely ficticious, it makes biological sense. A communication network has to operate efficiently and precisely, otherwise it becomes useless. EVs have a potential to meet the required specifications for representing such a network.

EVs are a heterogeneous population of vesicles with different sizes and different cellular origins. The current lack of the nomenclature for various EV subsets reflects this heterogeneity (4). It may be that different EV subsets have distinct phenotypic and functional characteristics and that a division of labor in distributing the messages exists. Technologies for separation and characterization of different EV subsets are rapidly emerging, and it is likely that it will be possible to differentiate among these subsets in the near future. There is great interest in the identification of the cargo EV subsets, such as, e.g., exosomes, carry. This interest is fueled by the likely possibility that EVs will prove to be useful in the near future as non-invasive biomarkers of various pathological conditions. There is also interest in the use of *in vitro* engineered EVs for therapy based on their potential to deliver drugs to disease sites.

The attributes of EVs discussed in this book provide for an interesting overview of the multiple roles EV subsets assume in several different human diseases. The book consists of a series of short and focused chapters grouped by a common theme and covering EV-mediated contributions to cancer, hematopoiesis, inflammatory conditions, tissue repair, infections or immune therapies. This selected series of short and focused presentations is an easy read. At the same time, it provides a comprehensive appraisal of the recent progress in the EV biology.

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On the function and heterogeneity of extracellular vesicles

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Provenance: This is a Guest Editorial commissioned by section editor Dr. Mingzhu Gao (Department of Laboratory Medicine, Wuxi Second Hospital, Nanjing Medical University, Wuxi, China).

Comment on: Fonseca P, V ardaki I, Occhionero A, *et al.* Metabolic and Signaling Functions of Cancer Cell-Derived Extracellular Vesicles. Int Rev Cell Mol Biol 2016;326:175-99.

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Both, prokaryotic and eukaryotic cells contain the ability to communicate with cells in their environment. Classically, intercellular cell communication was thought to be mainly mediated by direct cell-cell contacts and by soluble factors including cytokines, growth factors and hormones. In 1996 Raposo and colleagues showed that intercellular communication can also be mediated by vesicles; specifically they reported that B lymphoblastoid cells release MHC class II carrying vesicles which are able to induce antigen-specific MHC class II-restricted T cell responses (1). Since then, an exponentially increasing amount of studies demonstrates the importance of extracellular vesicle (EV) mediated intercellular signaling in a huge variety of different cellular systems and organisms ranging from bacteria and yeast to plants and humans. It became evident that EV-mediated intercellular communication is essentially involved in many physiological and pathophysiological processes (2). Thus, EV-mediated intercellular communication provides a third, very complex mode of intercellular communication. Unraveling the basic principles of this novel intercellular communication system and gaining insight in its complexity will for sure have large impacts on the overall understanding of biological processes and certainly will pave the way for novel diagnostic and therapeutic approaches (2-4).

EVs carry a number of different molecules. Initially, together with lipids several proteins were identified being expressed on EVs like major histocompatibility complex (MHC) class II molecules and tetraspanins (1,5,6). In 2001 the first proteomic approach was reported by Théry and colleagues, and a number of different proteins were discovered, several of them being connected to the EVs' functional properties (7). In 2006 RNAs have been detected in EV fractions, and it has been shown that EVs shuttle functional RNA molecules between cells (8,9). These findings increased the interest in EV research and massively boosted the field. Although some publications may raise the impression that EVs mainly act as vehicles for miRNAs, they should rather be seen as extracellular cell organelles, which composed by a collection of specific lipids, proteins and RNAs specifically transmit a special combination of signals from signal sending to the signal receiving cells (10). More recently, single stranded and double stranded DNA have also been identified in EV fractions (11-13). However, although EV-associated DNAs have been associated with the progression of certain diseases, e.g., cancer, their function remains elusive, yet.

In addition and as nicely reviewed by Fonseca and colleagues, recently, a collection of metabolic proteins have been identified in EV fractions which are able to control metabolic activities of target cells and tissues (14). Affecting metabolic processes provides another level of complexity to the EV mediated intercellular signaling. Learning more and more about the processes of how EVs can influence their target cells, we have also to bear in mind that different EV entities exist. It is important to consider that eukaryotic cells release a variety of different vesicle types. The most prominent EV types are the exosomes, microvesicles and apoptotic bodies (15). Exosomes are defined as derivatives of the endosomal system and correspond to intraluminal vesicles formed during endosome maturation by inward bodying of

the outer endosomal membrane, the limiting membrane. Previously, it was assumed that the intraluminal vesicles and all their cargo molecules are destined for the degradation in lysosomes. However upon studying transferrin trafficking in reticulocytes it was discovered that a proportion of the endosomes containing intraluminal vesicles, the so called multivesicular bodies or multivesicular endosomes, can fuse with the plasma membrane to release their intracellular vesicles, commonly having sizes between 70 and 150 nm, into their extracellular environment (15-17). In contrast, microvesicles are formed by the outward bodying of the plasma membrane. Microvesicles are reported to have sizes between 100 nm and 1 µm (15). Apoptotic bodies arise by the fragmentation of apoptotic cells, their reported sizes range from 500 nm to several µm (15). However, upon analyzing apoptotic cells, the formation of vesicles in the same size range than exosomes can be observed. Up to now, neither markers have been qualified allowing a clear discrimination between exosomes, microvesicles, apoptotic bodies and other EV types, nor are well established technologies available allowing purification of certain EV subtypes, yet. Conventionally, different vesicle types are enriched in parallel resulting in very heterogeneous vesicle fractions. Accordingly, to use an appropriate terminology, members of the International Society of Extracellular Vesicles (ISEV) recommended to decipher vesicles in experimental enriched vesicle fractions as EVs rather than as exosomes, microvesicles or apoptotic bodies etc. (15,18). The issue of heterogeneity is further complicated by the finding that different pathways have been identified controlling the intraluminal vesicle formation in the endosomal compartment. Specifically, intraluminal vesicles can be formed in an ESCRT-dependent or an ESCRT-independent manner (19). Regarding the ESCRT-dependent intraluminal vesicle formation, different pathways have been defined as well. For now, ubiquitin- and sumoylation-dependent pathways can be discriminated (20). The complexity is further multiplied due to the fact that EVs always contain a set of molecules reflecting their cellular origin. Thus, different cell types release different variants of given EV subtypes. Furthermore, the alteration of environmental parameters regularly results in changes in the EVs' molecular signatures.

So far, the question of heterogeneity in given EV samples is rarely addressed at the experimental level, as mentioned before, mainly due to the lack of qualified technical platforms. Very recently, two studies made use of bead-capturing methods to separate and characterize different EV subtypes (21,22). Despite characterizing the molecular content of the different EV subpopulations, it will be important to analyze their biological functions independently. In the future we need to address the issue of heterogeneity in more detail. It will be important to learn whether all EVs taking part in intercellular communication contain RNAs/DNAs or whether nucleic acids are transported by specific EV subtypes. If it comes to the EVs' function it will also be essential to learn whether there are subtypes specialized on certain functions, e.g., to mediate metabolic activities, to mediate intercellular signaling pathways or to transport waste out of given cells, or whether all functional properties are unified in identifiable subtypes. For sure, novel technological platforms allowing single EV analysis and novel purification techniques enabling enrichment of distinct EV subpopulations that can be analyzed at a functional level will help to increase our understanding of EV mediated intercellular signaling massively. I am convinced that an improved biological understanding of EVs will affect the field of life sciences tremendously.

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Footnote

Conflicts of Interest: The author has no conflicts of interest to declare.

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Extracellular vesicles isolation and their biomarker potential: are we ready for testing?

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Provenance: This is a Guest Editorial commissioned by section Editor Mingzhu Gao, MD (Department of Laboratory Medicine, Wuxi Second Hospital, Nanjing Medical University, Wuxi, China).

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In a recently published paper (Sci Rep 2016;6:33935), a group of Japanese investigators reported on a novel affinity-based method for the isolation of extracellular vesicles (EVs) from supernatants of cell lines or cells isolated from murine tissues (1). The paper calls attention to the growing interest of scientific and medical communities in EVs, their molecular and genetic characterization, their biomarker status and the role they play in human health and disease.

EVs are rapidly emerging as mediators of communication between cells (2). In multicellular organisms, the maintenance of cellular contact within tissues or between distant tissues is of critical importance for the development, organization and functional integrity of the whole organism. The presence in tissues and body fluids of soluble factors, including cytokines and chemokines, has been generally viewed as evidence for the existence of active cross-talk between cells communicating via cognate receptors expressed on their surface. More recently, EVs which are released by all cells and are ubiquitous in all body fluids, have assumed a predominant place as the highly efficient and biologically significant intercellular communication system (2). Cells release EVs of different types, and so, EVs found in body fluids are heterogeneous mixtures of membrane-bound vesicles originating from various cells and ranging in size from 30nm to >5,000 nm (3). The current nomenclature of EVs is based on size, and they have been arbitrarily divided into the smallest, exosomes, which are 30–150 nm in diameter, somewhat larger microvesicles (MVs, 200–1,000 nm) or large apoptotic bodies (1,000 to >5,000 nm). Each EV is bound by a lipid bilayer membrane containing numerous biologically-active transmembrane proteins. The vesicle lumen is filled with cytosolic proteins and nucleic acids derived from the EV-producing cell (4). EVs differ from one another not only by size but also by cellular mechanisms used for their secretion, the molecular content and functional properties (5). MVs are formed by "blebbing" or "pinching off" from the cellular membrane of the parent cell and contain parts of the cytosol more or less randomly enclosed in vesicular "blebs". Apoptotic bodies are remnants of dead parental cells. In contrast, the biogenesis of exosomes is unique: they originate from the endocytic compartment and their molecular content reflects, at least in part, that of the parental cell (5). For this reason, exosomes, serving as surrogates of their cells of origin, have been of the greatest interest among EVs as potential biomarkers and "liquid biopsies" (6). As communication vehicles, EVs transfer proteins, lipids and nucleic acids (mRNA, miRNA and DNA) from the parent to recipient cells, and this transfer of the molecular/genetic cargo is accompanied by re-programming of the recipient cell functions (6). Because the EV cargo determines cellular re-programming, efforts to isolate EVs from human body fluids and to characterize their molecular and genetic content have been intensively pursued.

The methodology for EV isolation was initially developed and used for their recovery from supernatants of cell lines. It involved a series of sequential differential centrifugation steps at increasing speeds (300 ×g,

2,000 ×g, 10,000 ×g) to remove cell debris and large EVs followed by ultrafiltration using 22 nm-pore filters and ultracentrifugation (UC) at 100,000 ×g for 2–3 h (7). The recovered pellets of small EVs or exosomes were then re-suspended in buffer and placed on a continuous sucrose density gradient for further exosome enrichment, taking advantage of the unique ability of exosomes to float at the density of ~1.15 g/mL of sucrose. This method for small EV isolation has been widely adopted as the prototype and is being used as the gold standard despite the fact that UC tends to aggregate EVs, is time consuming, requires special equipment and is not suitable for a high sample throughput. Purification of vesicles on sucrose gradients leads to a loss of aggregated vesicles. Thus, neither the EV morphologic integrity nor their recovery may be optimal with this procedure. Numerous other isolation methods utilizing various technologies such as polymer-based precipitation (e.g., total exosome isolation or TEI), microfluidic separation, affinity capture with antibodies coated on latex beads or size-exclusion chromatography have been introduced and are in use for EV isolation (8). Needless to say, the recovery, quality and molecular content of EVs obtained by these different methods vary. Many of the methods are commercially available. Often, these methods aim only at the isolation of nucleic acids, usually of miRNA or DNA, from EVs. Some methods do not discriminate small from large EVs, and few are concerned with EV integrity, purity and biological functions. EVs have the propensity for binding of exogenous molecules. Thus, EVs obtained from biological fluids such as plasma are always liberally coated with immunoglobulins (Igs) and albumin. The presence of these "contaminants", which stick to the surface of EV membranes but are not *bona fide* parts of the EV molecular content complicates subsequent molecular profiling and may interfere with biologic activities. To date, despite a wide choice of methods available for the isolation of EVs from various fluids, no single method guarantees their recovery for reliable qualitative and quantitative analyzes.

Since isolation of EVs based on size alone is not optimal, affinity-based capture has been considered as an option for improving recovery and purity of EVs. The success of this approach depends on the stable presence on the EV surface of a marker or markers that can strongly bind a detection reagent such as the marker-specific antibody (Ab). For example, Abs specific for the three tetraspanins classically used as exosome markers (CD9, CD63, CD81) have been used for immuno-affinity isolation of small EVs (9). The problems may arise, however, when levels of a given tetraspanin in the exosome cargo vary, as they often do, leading to a partial EV capture. Also, capturing Abs might interfere with downstream EV analyses, specifically mass spectrometry, and their removal following capture could impair vesicular integrity or functionality of EVs. Hence, utmost care has to be taken when selecting reagents for affinity-based EV isolation.

The paper by Nakai and colleagues describes a novel affinity-based isolation method for EVs which, in combination with initial differential centrifugation (the pre-clearing steps), yields highly purified small EVs and avoids some of the above mentioned pitfalls (1). The method uses the T cell immunoglobulin and mucin domain protein 4, Tim4, for EV capture. Tim4 is a transmembrane protein expressed on macrophages. It is a receptor for phosphatidylserine, and the receptor-ligand binding is Ca^{2+}-dependent (10). Phosphatidylserine decorates apoptotic bodies as well as MVs and exosomes. The authors used the extracellular domain of murine Tim4 fused to the Fc fragment of human IgG and immobilized Tim4-Fc protein on magnetic beads for EV capture in the presence of Ca^{2+}. The captured EVs were readily released from the beads by adding buffer containing a Ca^{2+} chelating agent (e.g., EDTA). The yield and purity of EVs isolated by this Tim4-Fc affinity capture were compared with those of EVs obtained by UC or TEI-based precipitation and were shown to be superior. Tim4-Fc affinity capture isolated small EVs or large EVs (if the pre-clearing steps were omitted), were largely free of protein contaminations, enriched in exosomal proteins and morphologically intact after their release from magnetic beads. *Ex vivo* functions of isolated EVs were not tested. The most important advantage of this isolation method was the relative absence of contaminating non-exosomal proteins, which enabled proteomics analysis of the "purified" EVs. The presence of contaminants in EVs isolated by the conventional methods listed above interferes with mass spectrometry and is a major disadvantage in exosome molecular profiling and biomarker studies.

While the new isolation method appears to be advantageous, it has been largely tested with supernatants of cultured cells and not with human plasma (although one example of human urine is presented). This is a significant problem that has plagued the exosome field from the start. Supernatants of cultured cells are a good source of EVs produced in large quantities by one cell type. In contrast, human plasma contains mixtures of exosome subsets derived from many different normal or abnormal cells. Isolation of "contamination-free" EVs from human plasma, especially

with specimens of patients with chronic diseases such as cancer, is proving to be a major challenge. So far, size exclusion chromatography of pre-cleared plasma which removes a bulk of exosome-associated plasma proteins, as recently described (11) appears to come the closest to yielding exosomes that have a low (but not absent) contaminating protein content, are morphologically intact and are functionally competent (11). It may be that the receptor-ligand-based isolation of EV, such as described by Nakai *et al*., will be successful in isolating "pure" EVs from human specimens. However, given the enormous heterogeneity of plasma EVs which likely have a broadly variable content of phosphatidylserine, the recovery as well as EV purity might be compromised. Also, there is no rational explanation for removal of contaminants by this type of capture, unless the step of EV release from beads using Ca^{2+} chelators contributes to overall final "purity".

Yet another aspect of EVs isolation from human body fluids that is in part related to EV purity and to their molecular profiling concerns separation of different EV subsets, specifically EV subsets produced by diseased cells, e.g., cancer cells. Affinity-based EV capture with Abs specific for a component of exosome cargo represents one way for exosome isolation and separation of from human body fluids. The best example of this approach was provided by Melo and colleagues, who used immune capture with Abs specific for glypican-1 to isolate pancreatic cancer-derived exosomes from patients' plasma and to study them as predictive biomarkers of the disease progression and outcome (12).

As disease biomarkers or "liquid biopsies", EV subsets in plasma that are derived from diseased cells and carrying the cargo partly derived from these cells are likely to be more specific and more reliable than total plasma EVs. The major barrier to isolation of such "disease specific" EVs has been the lack of capture reagents with sufficiently high avidity and specificity for unique markers that such EVs are expected to carry. The requirement for a two-step isolation method, first to isolate "pure" total plasma EVs and then to selectively capture the desired EV subset, further complicates the process. Using affinity capture directly from body fluids or after the preclearing steps but without removing contaminating proteins is less effective. Finally, the use of affinity capture for EV isolation means that the capturing agent will have to be removed and EVs safely recovered for downstream analyses, as done by Nakai *et al*. using Ca^{2+} chelation in their Ca^{2+}-dependent capture method. The upscaling of the procedure for high throughput EV capture necessary for human biomarker studies will require additional efforts. To date, selective isolation of "disease specific" EVs from body fluids remains an elusive goal. There are indications, however, that with a better understanding of the EV biology, it will be possible to achieve this goal perhaps by a combination of large-scale size-based fluidics with specific capture of desired EVs. In the context of this effort ongoing worldwide, methods for reliable, effective and uncomplicated isolation of "pure" total EVs such as described by Nakai *et al*. represent an important addition to the existing EV isolation repertoire.

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Footnote

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Extracellular vesicle isolation: present and future

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In recent years, extracellular vesicle (EV)-based biomarker discovery has received significant interest amongst scientist studying diverse disease conditions. Extracellular vesicles are released by variety of cells into the cellular microenvironment and have the natural ability of delivering different cargos and carry bioactive molecules such as non-codding RNA, genomic DNA, lipids, growth factors, and signaling molecules. EVs have been isolated from most biofluids including serum, plasma, serum, and saliva. It has been shown that EVs play substantial roles not only in the regulation of normal physiological processes but also in disease pathogenesis and their cargo reflects the status of parental cells at the time of secretion (1,2). Multiple studies found EVs enriched in lipid raft molecules (Flotillin) (3), membrane trafficking molecules (Annexins) and heat-shock proteins (HSP70, HSP90) (3,4). Based on mode of biogenesis, EVs can be divided into exosomes (30–100 nm), microvesicles (100–1,000 nm) and apoptotic bodies (>1,000 nm). The biogenesis and cellular pathways for generation of these different vesicle types, as well as their cargo, membrane composition and surface molecules are distinct with some overlapping features (5,6).

Although the field of EVs is rapidly growing, it has been hampered by challenges in EV isolation and characterization methods. Employing an efficient, rapid and reproducible isolation method is fundamental to analytical reproducibility.

Different studies employed various EV isolation techniques that can be broadly grouped into five distinct isolation methods: ultracentrifugation, density-gradient separation, polymer-based precipitation, immuneselection, and microfluidic isolation methods (7).

These different methods can be used individually or combined for the isolation of extracellular vesicles from diverse biological sources. It is worth noting that each of these methods, while suitable for extracellular vesicle isolation, have some limitations. In this report we briefly describe some of the positive and negative attributes of each of these methods for EV isolation. We will also discuss a recently described new method of EV isolation published by Nakai *et al*. in *Scientific Reports* (8) by focusing on its novelty and how this method can complement or enhance the reliability of previously described methods for EV isolation.

Ultracentrifugation is generally regarded as the most commonly used method for isolating EVs and exosomes; however, different results from the literature demonstrate inconsistencies in reproducibility of this isolation technique. Such inconsistencies could be a result of different centrifugation time, speed, type of rotor or other technical factors (e.g., temperature) (9). Ultracentrifugation is time consuming, requires an ultracentrifuge and results in relatively low recovery of EVs (1). Another limiting factor is that isolation by ultracentrifugation causes non-vesicular macromolecule contamination (10) and aggregation of EVs that can lead to masking of surface antigens (11).

One of the limitations in using ultracentrifugation for isolation of EVs is coprecipitation of protein aggregates and nucleosome fragments which can lead to decreased sample purity and may affect downstream analysis. The density-

gradient separation, which employs ultracentrifugation combined with a sucrose gradient, can lead to increased sample purity as protein and protein-RNA aggregates can be efficiently separated from the exosomes. This method allows the separation of EVs based on their densities from low density exosomes to higher density microvesicles (12,13).

Polymer-based isolation is a widely used techniques based on poly-ethylene glycol based separation. This method allows for greater yield of the extracellular vesicles (14), but with a high portion of other contaminants (lipoproteins), especially from serum samples (15,16). Microfluidic techniques are based on trapping EVs in micro channels are a good option for low volume input of biofluids. There is lack of evidence about their efficacy and downstream clinical utility in the comparative settings (17).

Immuno-selection techniques use antibody-based separation methods targeting known surface markers on extracellular vesicles. Some of these markers include the well characterized tetraspanins (CD9, CD63, CD81) or immune-regulator molecules (MHC I&II) on the surface of the vesicles (18,19). This method is costly and is not suited for large sample volumes. Captured EVs may not retain desired functionality even if successfully eluted from the bead surface (13). However, the main limitation of this method, if is that not all microparticles can be isolated successfully (in a contrast with, e.g., polymer based techniques) since the isolation relies on surface markers and some of those might not be expressed on all types of extracellular vesicles. However, in specific designs in which enrichment of a subpopulation of EVs or isolation of viral particles from EVs is desired, this selectivity can serve as a distinct advantage (4).

Given the potential limitations with the above described methods, Nakai *et al*. published a novel affinity-based technique for the isolation of EVs, which uses Tim4 protein binding to phosphatidylserine, an enriched component of the EVs surface (8). The authors compared this new isolation method to conventional ultracentrifugation and polymer-based precipitation methods for powerful isolation of both small and large EVs. In their experiments, they used K562 cells and peritoneal macrophages (pMac). With Tim4 affinity-based isolation they demonstrate a robust enrichment of CD63, Flotillin2, CD9 and CD81 markers in the small EVs (sEVs) compared to ultracentrifugation and polymer-based precipitation. They provide Western blot analysis of the large EVs, demonstrating efficient isolation of LAMP2 positive microvesicles from the 10k pellet.

In further studies, they performed a proteomic analysis of sEVs, where they found an excellent recovery in the protein profile of sEVs (78.1%), especially compared to the sEVs isolated by polymer based precipitation (21.8%). They also provided a FACS-based quantification, where they found that exosome isolation by Tim4-conjugated beads was more efficient than that by CD63-cojugated beads, leading to 2 fold stronger signal-to-noise ratio.

While this new method described by Nakai *et al*. (8) provides a novel approach for EV isolation, the major limitation of their method is its inability to distinguish between microvesicles and exosomes since phosphatidylserine is expressed in different subpopulation of EVs including exosomes, microvesicles and apoptotic bodies. Therefore, Tim4 affinity-based isolation should not be used in studies assessing different subpopulations of EVs. Additional limitation of the Tim4-based isolation is that phosphatidylserine is also expressed on the apoptotic micro particles (EVs) and apoptotic bodies. Thus, a Tim4 based isolation can lead isolation of apoptotic bodies and not only EVs, which can significantly affect experimental conclusions and mislead EV focused investigations (1,20).

Moreover, the Nakai *et al*.'s (8) study is limited to the pMacs and K562 human erythromyeloblastoid leukemia cell line model and the result should be replicated in other cells lines and different biofluids. Specifically, the effect of stimulation of cells with monensin which was used in this study should be corroborated. Monensin stimulation increases the cytosolic free Ca^{2+} and increases the release of EVs that harbor phosphatidylserine/Tim4. It is not clear that the EVs released in physiologic states or other pathologic/stress conditions harbor comparable amounts of phosphatidylserine.

Taken together, the Tim4-based isolation method is a novel and a potential powerful EV isolation method with excellent recovery. However, the inability of this method to distinguish between different populations of EVs based on Tim4 (namely exosomes), microvesicles and apoptotic bodies greatly limits its utility. The utility of Tim4 for affinity methods should be reproduced in other cell types and biofluids in physiological and pathological states. Despite these limitations, the newly discovered Tim4-based EV isolation method offers a promising new approach for future EV research.

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Footnote

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Myeloid-derived suppressor cell, arginase-1, IL-17 and cl-CD95L: an explosive cocktail in lupus?

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Myeloid-derived suppressor cells (MDSCs) ($CD33^+CD11b^+$) are a heterogenous population derived from immature myeloid cells consisting of two main subsets: $CD15^+CD66b^+$ granulocytic (G-MDSCs) and $CD14^+HLA\text{-}DR^-$ monocytic (M-MDSCs) cells (1). G-MDSCs consist of relatively immature and pathologically activated neutrophils, whereas M-MDSCs are inflammatory monocytes (2). While MDSCs are associated with a poor clinical outcome in cancer, their role in chronic inflammatory diseases remains unclear probably because, among other explanations, the heterogeneity of this cellular population.

Systemic lupus erythematosus (SLE) is a disorder of largely unknown etiology whose pathogenesis can affect almost all organs and tissues. SLE is characterized by an autoimmune response with circulating autoantibodies secreted by B cells, which are aided by the $CD4^+$ T cell subset, follicular helper T (Tfh) cells. A growing body of evidence in both human and murine studies confirm a pivotal role for IL17-secreting T-cells (Th17) cells in the progression of SLE disease [see (3) for a review] (4). Indeed, lupus-prone mice are partially protected from immunopathology by a reduction in renal Th17 cell accumulation (5). Therefore, modulation of Th17 cell migration and trafficking to inflamed organs is an attractive therapeutic option for reducing disease-related inflammation. However, the precise mechanism of Th17 cell accumulation in damaged SLE organs remains unclear.

Yang and colleagues team demonstrated that the percentage of blood MDSCs is positively correlated with the SLE disease activity index (SLEDAI) in patients. Moreover, this team observed that IL-6 accumulation in SLE patients promotes the expression of arginase-1 (*Arg-1*) by G-MDSCs. Arg-1 enzymatically hydrolyzes the amino acid L-arginine (L-Arg) to ornithine and urea. Unexpectedly, while L-Arg depletion by MDSCs in tumors is responsible for the suppression of the immune response (1), this depletion in SLE patients causes inflammation by activating two kinases, general control nondepressible 2 (GCN2) and mTOR, through a molecular mechanism that depends on the increase in uncharged tRNA (6) and that triggers Th17 differentiation. Indeed, MDSCs promote Th17 development *in vitro* when co-cultured with naive healthy autologous CD4 T-cells. This effect is abrogated in the presence of the Arg-1 inhibitor, nor-NOHA. Using the same experimental set-up, Wu *et al*. demonstrated that MDSCs derived from SLE patients amplify the positive effect of co-culture on Th17 differentiation, again in an Arg-1-dependent manner. Using a humanized mouse model, in which injection of PBMCs from SLE patients into immunodeficient mice (NOD/SCID) gives rise to SLE-like disease, they showed an *in vivo* role for MDSC and Arg-1. Indeed, prior depletion of MDSCs from PBMCs or nor-NOHA-mediated inhibition of Arg-1 halts the development of disease and expression of *il-17a* in both the spleen and kidney. Strikingly, in their hands, MDSCs would appear to exacerbate the pathological role of Th17 cells in

SLE patients. This study emphasizes that although MDSCs block T-cell proliferation in lupus and cancer, these cells can also promote the differentiation of IL17-expressing $CD4^+$ T-cells, fueling inflammation in lupus patients. Taking into account that IL-17 is known to promote the production of IL-6 and directly recruits MDSC in tumor environment (7), one can envision that Wu *et al*. highlighted an amplification loop that might cause acceleration of the pathogenesis in SLE patients.

A mechanistic link between MDSC, Arg-1 and Th17 cells has recently been reported (8). Indeed, Iwata *et al*. observed an increased number of MDSCs ($CD11b^+GR\text{-}1^{low}$) in SLE patients and the percentage of these cells was correlated with the progression of the disease. Nonetheless, they did not evaluate the impact of MDSCs on T-cell differentiation but instead showed that the increased Arg-1 activity in these myeloid cells was responsible for the inhibition of T-cell proliferation in lupus-prone mice MRL^{Lpr}. These differences in the effects of Arg-1 on SLE outcome and progression could be due to the lupus-prone mouse models used in these studies—adoptive cell transfer in Wu *et al*., versus genomic mutation in the *CD95* loci in Iwata *et al*.—or could be reconciled because disparate degrees of proliferation account for Th cell differentiation (9). A more established role for Arg-1 in suppression of Th2 function *in vivo* (10) and in promoting intracellular T. gondii survival have already been described (11).

Our group recently demonstrated that the tumour necrosis factor (TNF) member, CD95L (also known as FasL or CD178) aggravates the inflammatory process in SLE patients by recruiting Th17 cells in inflamed organs (12). The CD95L receptor, namely CD95 (also known as Fas), belongs to the death domain (DD)—containing members of TNF receptor superfamily (13). Regardless of accumulating evidence indicating that this plasma membrane receptor possesses non-apoptotic functions, CD95 is still considered as a death receptor (14). CD95L is expressed as a transmembrane "cytokine" (m-CD95L), whose extracellular domain is composed of a juxtamembrane stalk region and a TNF homology domain. This stalk region can be cleaved by metalloproteases (15) releasing a soluble CD95L into the bloodstream. In presence of m-CD95L, CD95 aggregates and forms a molecular complex consisting of FADD and caspase-8/10 that is called death-inducing signaling complex (DISC) (16). Metalloprotease-cleaved CD95L [cl-CD95L for cleaved CD95L to distinguish this ligand from its soluble, exosome-bound counterpart (17,18)] fails to form DISC but triggers non-apoptotic signaling pathways such as NF-κB (19) and PLCγ1-driven Ca^{2+} response (20). In a pathophysiological standpoint, we showed that this soluble ligand, cl-CD95L, is accumulated in sera of lupus patients and promotes endothelial transmigration of activated T lymphocytes through the formation of a molecular complex designated MISC for motility-inducing signaling complex (20). Binding of cl-CD95L to CD95 triggers the recruitment of PLCγ1 and the implementation of a Ca^{2+} signal promoting the endothelial transmigration of Th17 cells (12). PLCγ1 recruitment occurs at the level of the juxtamembrane region of CD95, designated calcium-inducing domain (CID). A selective inhibitor consisting of the cell-penetrating domain of TAT fused to CID, namely TAT-CID, interacts with PLCγ1 and prevents its recruitment to endogenous CD95. Moreover, injection of TAT-CID in lupus-prone mice ($MRL^{Lpr/+}$) blocks endothelial transmigration of Th17 cells and alleviates clinical symptoms. In this study, we did not investigate the effect of TAT-CID on the recruitment of MDSC cells in spleen and kidney of the lupus-prone mice $MRL^{Lpr/-}$. According to the study of Wu *et al*., it would be interesting to address whether TAT-CID by preventing endothelial transmigration of Th17, could also inhibit the accumulation of MDSCs in damaged organs of lupus-prone mice indicating that either Th17 recruitment precedes MDSC accumulation or cl-CD95L exacerbates inflammation in SLE patients by recruiting simultaneously Th17 and MDSCs initiating the local amplification loop of the inflammatory process in these organs. Like IL-6 and IL-17, we cannot rule out that cl-CD95L, which is also increased in these patients (20), contributes to MDSC and/or Th17 differentiation in organs of SLE patients. Overall these findings raise the question whether MDSCs and cl-CD95L are biomarkers of lupus flare by promoting Th17 differentiation and trafficking, respectively. This question remains to be addressed to better understand the lupus etiology and propose novel and targeted treatments for clinicians to more efficiently treat this pathology.

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Footnote

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Protection against lupus-like inflammatory disease is in the LAP of non-canonical autophagy

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Introduction

Immunological diseases have been proposed to exist as a continuum, with innate immune-mediated autoinflammatory disease at one end of the spectrum and adaptive immune-mediated autoimmune disease at the other (1). Systemic lupus erythematosus (SLE) is often described as the quintessential autoimmune disease, with disease manifestations existing in multiple tissues throughout the body, particularly the skin, kidneys and brain. Phenotype variability among individual sufferers, as well as differences in disease severity also lead to specific diagnostic and therapeutic dilemmas. Symptoms associated with SLE include fever, chest pain, swollen lymph nodes, a red rash most commonly found on the face, as well as painful Raynaud's phenomenon and swollen joints, with many patients also developing arthritis. This diversity in clinical features is due to a loss of control of self-tolerance of both the innate and adaptive immune systems (2). This array of clinical features is mirrored by the complexity of the many factors which may cause the disease, including genetic, environmental and hormonal. It is widely postulated that the root cause of SLE may involve an unknown environmental agent, triggering one's existing genetic susceptibility and underlying immune system defects, with vitamin D deficiency as one such example (3). One certainty is that the adaptive immune response orchestrates the vast majority of SLE manifestations, by the presence of activated autoimmune T- and B- lymphocytes. However, the root cause of the disease remains elusive, and a diagnosis of SLE is typically confirmed once an individual is experiencing at least four of the eleven common signs of the disease (4,5). Of these eleven, a positive test for antinuclear antibody (ANA) is typically the most shared feature among patients.

Abnormalities in apoptosis, a form of programmed cell death in which damaged or ageing cells are intricately disposed of, are leading causes of SLE susceptibility. Many adverse stimuli can initiate a break in immune tolerance, by which healthy cells are destroyed and their nuclear contents, such as DNA and histones, become exposed and proceed to act as autoantigens. Moreover, certain abnormal immunological functions may (potentially) allow adaptive immune cells, such as T- and B- lymphocytes, to avoid cell death and remain in the circulation (6). Sensitised B lymphocytes produce antibodies against exposed autoantigens, such as double-stranded DNA (dsDNA), resulting in the formation of protein-antibody complexes, with the capacity to aggregate and damage blood vessels (7).

Dysfunctional autophagy in lupus

It is perhaps no surprise that certain shortcomings in our body's capacity to clear these 'dying' cells are continually being proposed to be at the core of SLE pathogenesis (8).

In a disease characterised by dysfunctional apoptosis (9) and immune activity against intracellular components (10-12), one process stands out as potentially providing a link between exposure of autoantigens and a break in immune tolerance. Autophagy is a term first coined by Christian de Duve, to describe an intracellular degradation system which seeks to maintain homeostasis by sequestering cytoplasmic constituents and delivering them to lysosomes (13). By achieving this, the autophagic process acts as a cell survival pathway to maintain healthy cells, by degrading damaged organelles and eliminating invading pathogens. Further roles of autophagy include cellular housekeeping, and operating as a component of cellular integrated stress responses. Therefore, the hypothesis that autophagy is dysfunctional or impaired in SLE seems very plausible, when autophagy's role is to clear dying or dead cells and prevent potential intracellular autoantigens from being exposed to the immune system. And indeed, single-nucleotide polymorphisms (SNPs) have been associated with genetic susceptibility to SLE (14-16).

Autophagy was first identified in yeast, with homologues of autophagy-associated genes (ATGs) later being identified in humans (17). In terms of signalling regulation, the autophagy pathway is vast and complex, but mammalian target of rapamycin (mTOR) is often identified as the key player in balancing protein translation and autophagy. mTOR complex 1 (mTORC1) achieves this by operating as a negative regulator of the pathway. Further upstream, other key players in nutrient and energy detection, such as adenosine monophosphate activated kinase (AMPK), also operate as regulators of the pathway (18). When mTORC1 activity is inhibited, due to amino acid depletion for example, the autophagy pathway is activated. This process relies on a series of ubiquitin-like conjugation systems to maintain cellular homeostasis. The processing of targeted material for degradation relies on the formation of a double membrane vesicle, which emerges at a site known as the phagophore assembly site (PAS) in yeast. However, a similar site has yet to be identified in humans, with many investigators believing the site to be endoplasmic reticulum (ER) associated (19).

In humans, an initiation complex with UNC-like kinase 1 and 2 (Ulk1/2) at its core, is responsible for phagophore initiation, followed by a conjugation system stemming from Beclin-1, as the double membrane vesicle begins to elongate. As the phagophore transitions into an autophagosome, the membrane is enriched with microtubule-associated protein light chain 3 II (LC3-II), which is the central component of the next conjugation system. LC3-II is the lipidated form of LC3-I, with the conversion between these two forms often being questionably employed, via immunoblot, as a measure of complete autophagic flux (20). Finally, once sequestered components are ready to be delivered to the lysosome, fusion between the two membranes forms an autophagolysosome, with the final conjugation system utilising Atg12 and Atg5 to achieve this fusion (21) (*Figure 1*).

Canonical *vs.* non-canonical autophagy

Up until recently, there were considered to be three main forms of autophagy: micro, macro, and chaperone mediated. However, in 2011 a novel and alternate, or non-canonical, autophagy pathway was described (22,23). This distinct pathway and its variations, share many characteristics with the canonical pathway, but, its unique in that it operates independently of some of the complexes, which are integral to phagophore formation in normal autophagy. This alternative route is known as LC3-associated phagocytosis (LAP) and it is characterised by a reduced requirement for hierarchical influence of the Atg proteins, a double membrane that does not have to emerge from a single source, and the fact that Atg proteins can be recruited to a distinct membrane, other than the phagophore (24). Although some integral canonical components are not required for LAP, this phagocytic process has been shown to rely on certain components, which are conversely known to be dispensable for canonical autophagy (*Figure 1*). Two such examples include Rubicon and, to a lesser extent, an isoform of NADPH oxidase, NOX2. Recruitment of Rubicon to LAPosomes is integral to the incorporation of downstream proteins in the system, while it also stabilises a NADPH oxidase 2 (NOX2)-containing complex, thereby allowing reactive oxygen species (ROS) to be produced (25). Critically for LAP, phosphatidylinositol 3-phosphate (PtdIns3P) and ROS are both necessary if LC3 is to be conjugated to LAPosomes, as well as also being crucial for subsequent fusion with LAMP1(+) lysosomes (25). It is important to state that both canonical and non-canonical autophagy occur downstream of phagocytosis of cellular debris or dying cells; autophagy is responsible for degradation of this engulfed material. The principle difference between canonical and non-canonical autophagy is the fact that non-canonical autophagy acts independently of the RI1CC1 pre-initiation complex.

When one considers some of the immunological processes that are dependent on autophagy, such as antigen

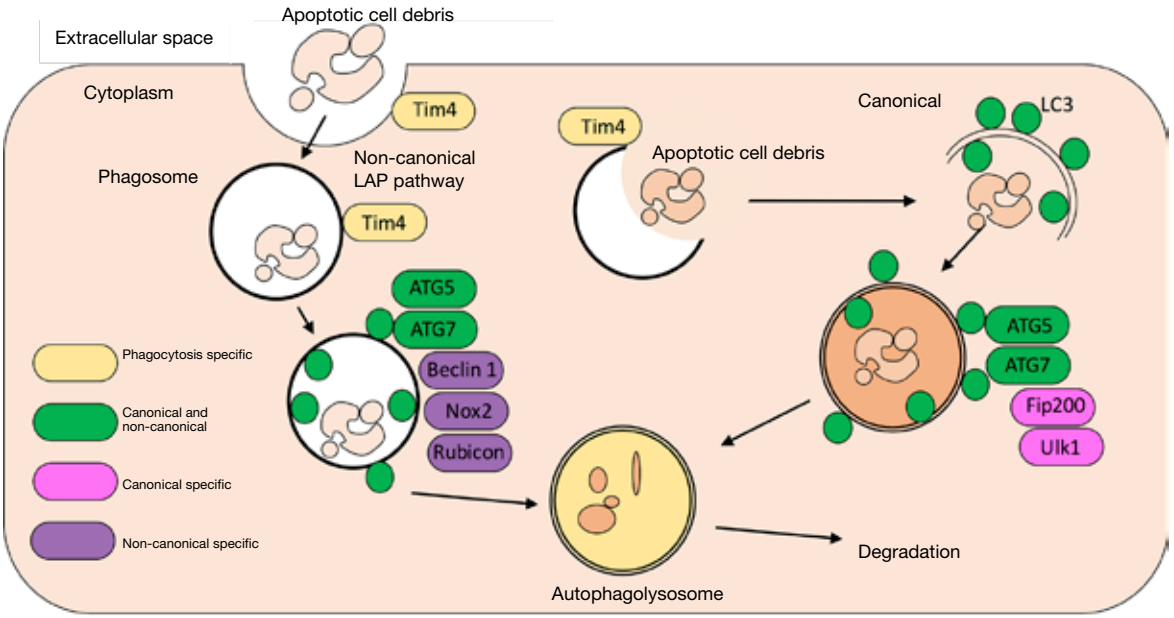

Figure 1 The canonical and non-canonical autophagy pathways. Apoptotic cell debris is phagocytosed and directed to the LAP pathway of autophagy. When the contents of phagosomes escape into the cytoplasm, canonical autophagy is the preferred pathway. Both pathways share certain proteins (green), whereas some are canonical specific (pink) and some are specific to the LAP pathway (purple). LAP, LC3-associated phagocytosis; Tim4, T cell immunoglobulin- and mucin domain-containing molecule-4; ATG, autophagy-related; Nox2, NADPH oxidase; Fip200, FAK family-interacting protein of 200 kDa; Ulk1, uncoordinated (Unc)-51-like kinase 1; LC3, Microtubule-associated protein 1A/1B-light chain 3.

presentation (26), lymphocyte development (27) and regulation of inflammation (28), in the setting of both autoimmune and autoinflammatory disorders, it becomes clear how re-establishing functional autophagy could be beneficial, in terms of suppression, or even full correction of the disease phenotype.

In the 05 May 2016 issue of Nature, Martinez *et al.* published a letter describing the effects of defective non-canonical autophagy *in vivo*. In this article, and with regard to SLE specifically, Martinez *et al.* showed that adverse consequences may arise when non-canonical autophagy is not fully operational, and that disease phenotypes may ensue. Ultimately, both canonical and non-canonical autophagy strive for the same end result in much the same manner, but with intricate variations at key branches of the canonical pathway. Thus, it is likely that LAP has evolved to operate as a cellular safety net for whenever canonical autophagy is bypassed, or fails. This LAP-driven process drives cells towards the desired healthy phenotype that cellular homeostasis requires, and away from unnecessary, and often detrimental, apoptotic phenotypes.

LAP deficiency causes a lupus-like disease

The experimental animals used in Martinez *et al.* were conditionally ablated for key components of the LAP pathway, including components of canonical autophagy or T-cell immunoglobulin mucin protein 4 (TIM4), which is involved in macrophage engulfment of dying cells. This conditional ablation used lysozyme-M to target the knockdown to macrophages, monocytes, neutrophils and conventional dendritic cells. LAP deficiency created an autoinflammatory lupus-like syndrome in mice defective in the LAP specific proteins Becn1, Nox2 and Rubcn, as well as proteins that are common between canonical and non-canonical autophagy such as Atg7 and Atg5. However, proteins exclusive to the canonical autophagy pathway, Fip200 and Ulk1, did not produce the said phenotype; Tim4 ablation has been previously shown to induce a lupus-like phenotype in mice (*Figure 1*). The autoinflammatory lupus-like phenotype was induced by injection of irradiated thymocytes undergoing apoptosis, and was characterised by low body weight, the presence of anti-dsDNA antibodies and anti-nuclear antigens, in addition to increased levels of autoantibodies associated with autoimmune

and autoinflammatory disorders. A key manifestation of SLE is kidney pathology and the mice with LAP deficiency presented with renal damage, as measured by increased serum creatine, blood urea nitrogen and proteinuria. LAP-deficient mice also had a strong interferon signature, a classical feature of lupus.

LAP deficiency causes dysfunctional degradation, not engulfment of dying cells

The engulfment or phagocytosis of cellular debris and dying cells occurs upstream of the autophagy pathways. Martinez and colleagues have previously published *in vitro* data (22) describing how the LAP pathway orchestrates the degradation of phagocytosed material. In their 2011 article, bone marrow derived macrophages (BMDMs) from GFP-LC3$^+$ mice were used in a phagocytosis assay to study activation of the LAP pathway and how disruption of LAP induces a pro-inflammatory response from BMDMs. Not only did a deficient LAP pathway induce a pro-inflammatory cytokine secretion profile in BMDMs, but it also downregulated anti-inflammatory cytokine secretion, contributing to a loss of immune tolerance. In the present study, Martinez and colleagues continued this work by observing the ability of *in vivo* monocytes, macrophages, granulocytes and dendritic cells to not only engulf dying cells but also to degrade them and produce, in response, an appropriate cytokine secretion profile. In order to measure the extent of engulfment and degradation, apoptotic ultraviolet-irradiated thymocytes, labelled with a red fluorescent marker (PKH26), were injected into wild-type (WT) mice as well as Cre$^+$ *Atg7*$^{f/f}$, *Fip200*$^{f/f}$, *Rubcn*$^{f/f}$ and *Tim4*$^{f/f}$ mice. Engulfment was observed using immunofluorescence of labelled thymocytes within the phagocytes. Engulfment of dying cells was equivalent in all canonical and non-canonical autophagy-deficient mice and WT mice, except for the phagocytosis-deficient Cre$^+$ *Tim4*$^{f/f}$ mice, which were unable to engulf dying thymocytes. To measure the degree of degradation of engulfed cells, GFP-tagged LC3 was used as a marker of lysosomal fusion and cargo degradation. The Cre$^+$ *Atg7*$^{f/f}$ and Cre$^+$*RUBCN*$^{f/}$ mice were each unable to convert LC3 to LC3-II and, so, were unable to clear dying thymocytes, as a result of these mice being LAP deficient. WT mice and Cre$^+$*Fip200*$^{f/f}$ mice successfully degraded the engulfed cargo, which is evidence in support of non-canonical, LAP-dependent autophagy being largely responsible for degradation of engulfed cells. When LAP-mediated autophagy is deficient, engulfed cells may remain in the phagocyte without being cleared, despite maintaining a functional canonical autophagy pathway.

In view of the fact that phagocytes which are deficient in the LAP pathway can successfully engulf dying cells but cannot degrade them because of their dysfunctional non-canonical autophagy mechanism, Martinez and colleagues investigated whether *in vivo* LAP-deficient macrophages were more pro-inflammatory than their LAP-sufficient counterparts. LAP-deficient macrophages produced pro-inflammatory IL-1β, IL-6 and CXCL10 after thymocyte engulfment, whereas, in contrast, LAP-sufficient macrophages produced anti-inflammatory IL-10. This was mirrored in serum levels of IL-1β, IL-6 and CCL4 observed in LAP-deficient animals but not in the LAP-sufficient animals, which produced increased serum IL-10 levels.

As LAP-deficient mice were shown to produce a lupus-like phenotype, with defective clearance of dying, engulfed thymocytes, Martinez and colleagues set out to investigate whether persistent administration of dying, engulfed thymocytes in LAP-deficient mice would aggravate this phenotype. And indeed it did, with the development of an SLE-like disease, characterised by increased ANAs, anti-dsDNA autoantibodies, glomerular deposits of IgG and C1q as well as increased alanine aminotransferase (ALT) levels. The increased serum pro-inflammatory cytokine profiles seen in LAP-deficient mice, administered with persistent dying, engulfed thymocytes, corroborated previous observations.

Conclusions

By studying non-canonical autophagy, Martinez and colleagues have uncovered a pivotal pathway, capable of not only general cellular housekeeping and homeostasis, but also protecting phagocytes from generating an autoinflammatory disease phenotype. The LAP pathway is the bedrock of both canonical and non-canonical autophagy and, without it, these processes are unable to achieve their ultimate purpose, namely the degradation of dying cells. LAP deficiency causes phagocytes to enter a severely pro-inflammatory phenotype, when engulfed cargo is resistant to disposal via autophagy. This is in stark contrast to the anti-inflammatory phenotypes of LAP-sufficient cells, which are capable of successful degradation of engulfed cargo. This innate immune dysfunction exacerbates systemic inflammation, by precipitating a vicious circle of apoptosis, failure to clear engulfed dying cells and development of further apoptosis. The resultant exposure

of intracellular autoantigens, associated with lupus, then ensues and contributes to the autoinflammatory lupus-like disease that the authors describe.

Going forward, the link between the LAP pathway and IL-10 production is an intriguing line of enquiry (investigation), with some reports of dysregulated IL-10 in SLE (29). If the autophagy process is successful it then skews phagocytes towards an anti-inflammatory, IL-10 producing cell capable of protecting an individual from lupus, which open up avenues for therapeutic intervention. However, IL-10 levels are often normal or even elevated in lupus, perhaps being sourced from adaptive immune cells (30).

Autophagy is thought to degrade and regulate some essential intracellular pro-inflammatory protein complexes, the inflammasomes. These ancient innate immune cytokine-processing complexes are not often associated with the development of autoimmunity, but are rather characteristic of autoinflammatory conditions at the other end of the immunological disease spectrum, such as hereditary periodic fevers (31). However, cellular stress (32), changes in cell metabolism (33) and neutrophil extracellular traps (NETs) have all been described as priming NLRP3 inflammasome activity, and failure to clear engulfed cellular material certainly fits into this criterion (34,35).

The discovery of the function of the LAP pathway and the organism's reliance on it to prevent the development of lupus is a major step forward in our understanding of the initiation of lupus, as well diseases from the autoinflammatory end of the immunological disease spectrum. Certain autoinflammatory diseases have arisen due to defects in various genes independent of autophagy, which may cause indirect malfunction of non-canonical autophagy, for example cystic fibrosis (CF) or hyperimmunoglobulinemia D with periodic fever syndrome (HIDS). CF is a monogenic disease in which a chloride ion channel is mutated and defective. Autophagy has long been described as being dysfunctional in CF, but as of yet it is not understood how the CF mutation causes dysfunctional autophagy and how this relates to disease phenotype. HIDS is characterised by altered cellular metabolism and activation of the NLRP3 inflammasome (36). However, it has been shown that autophagy is dysfunctional in phagocytes from these patients and this demonstration of the importance of the LAP pathway may explain the severe pro-inflammatory phenotype associated with HIDS (37). The LAP pathway is clearly crucial for maintaining cellular homeostasis, and may prove to be an underlying disease protagonist spanning right across the immunological disease continuum.

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Footnote

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Immune-Related Disease

Vascular changes and perifascicular muscle fiber damage in dermatomyositis: another question of the chicken or the egg that is on our mind

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Dermatomyositis (DM) is a multisystem disease with symptoms spanning diverse body functions including the skin, the joints, the heart and the lungs. The skin manifestations involve a sun-sensitive rash on the face, neck and upper trunk, and erythema of knees, elbows and knuckles. In classical cases, where these typical skin manifestations combine with muscle weakness, diagnosis can be set on clinical grounds. In other cases, diagnosis is confirmed by assessing the level of serum muscle enzymes, electromyographic findings, and a muscle biopsy (1). In the skeletal muscle tissue of DM, the primary target of the immune response is the vascular endothelium of perimysial and perifascicular blood vessels, and inflammatory infiltrates often occur at perivascular sites. Complement deposition of the terminal C5b-9 membrane attack complex (MAC) leads to blood vessel necrosis (2). The trigger that initiates complement activation remains however unclear.

In a recently published article in Brain, Lahoria and colleagues (3) touch upon two of the main research questions remaining in inflammatory muscle disease. Firstly, why does DM display this unique feature of endothelial damage, which is conspicuously in contrast with other subtypes of inflammatory myopathy? In two other main subgroups, polymyositis and sporadic inclusion body myositis, the primary target of the immune response are the muscle fibers themselves, as illustrated by active invasion of nonnecrotic muscle fibers by auto aggressive cytotoxic T-cells and macrophages (4). Secondly, does this endotheliopathy precede the perifascicular muscle atrophy (*Figure 1*) that characterizes the disease, or are these myopathological features simultaneous or more or less unrelated events?

The authors investigated in detail the microvascular changes in 50 patients diagnosed with DM, both of the juvenile (n=15) and the adult form (n=35). Capillary density was found to be reduced 2-fold and transverse vessel density reduced 3-fold in regions with perifascicular atrophy. Material from patients at different disease stages was evaluated, as were patients that had received treatment prior to the biopsy. In a patient in acute phase of the disease, severe capillary depletion was present in the biopsy even in fascicles without perifascicular atrophy. In contrast, a patient treated for 5 months with 20 mg pro day of prednisone displayed a capillary density that was least decreased, and the subject's muscle weakness was mild. The authors found perifascicular inflammatory cell infiltration to be a rare event, and much less frequent than MAC positive perifascicular capillaries. The latter were encountered in the majority of DM patients and could be regarded as one of the most important diagnostic features. The article presents several important observations in favor of DM being an antibody-dependent, complement-mediated disease characterized by capillary injury that results in perifascicular muscle fiber atrophy. An early study in favor of a sequence of events starting with blood vessel dropout was published way back in 1990 (5), and DM biopsies show evidence of

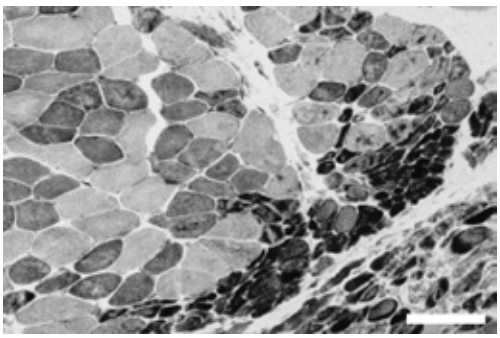

Figure 1 Histochemical fiber-type-staining detecting the reduced form of nicotinamide adenine dinucleotide shows the perifascicular muscle fiber atrophy characteristic of muscle biopsies from dermatomyositis patients. Scale bar =100 μM.

focal ischemia by way of strong expression of markers of oxidative stress and lipid peroxidation (6).

The pro-inflammatory environment in DM is starting to become more and more clear, and many indications point to dysregulated innate immunity as an important pathological factor. Perifascicular pathology is associated with interferon (IFN) type 1-induced alterations, though it needs to be said that inflammatory myopathies are generally associated with increased IFN type 1 levels (7), as are indeed also other rheumatic diseases. However, it appears that the trigger that initiates this IFN signature seems specific for the individual disease entities, leading to the characteristic inflammatory patterns in the different diseases. Sera from DM patients contains higher levels of IFNα and expression is inversely correlated with duration of disease, suggesting that IFNα plays a role in disease initiation (8). The plasmacytoid dendritic cells inside the muscle tissue are thought to be the main local source of IFN type 1 (9), but research results suggest important additional systemic production also exists in patients. Several IFN type 1 signature genes are overexpressed specifically in the perifascicular areas of DM tissues, as is the associated factor *retinoic acid-inducible gene-1* (*RIG-1*). Interestingly, RIG-1 was found absent from the affected muscle fibers of polymyositis and sporadic inclusion body myositis tissues (10). In a subgroup of DM patients, usually presenting an amyopathic phenotype with interstitial lung disease, autoantibodies can be detected that recognize IFN-induced with helicase C domain protein 1 (IFIH1), one of the RIG-I-like receptors (11,12). RIG-I and IFIH1 can interact with viral RNA, activating IFN type 1-inducible cytokine gene expression (13). Based upon these observations, it has been put forward that upregulated IFIH1 might liberate otherwise cryptic epitopes, causing breakage of self-tolerance and consequently autoimmune tissue damage. We are conscious that the affected muscle fibers need to be regarded as active contributors to the inflammatory process, initiating and/or perpetuating a persistent local immune response, and contributing to the associated dysregulation of innate immunity. The vast potential of muscle fibers to produce immune stimulators and the differential expression of cytokines between the subtypes of inflammatory myopathies has been described extensively (14). Of interest in this respect is that Lahoria *et al*. (3) describe the expression of MxA, another type 1 IFN-inducible antiviral protein, on both perifascicular capillaries and muscle fibers. This raises the possibility that a common mechanism could produce these abnormalities rather than one leading to the other, adding nuance to the story of muscle fiber atrophy as merely the result of blood vessel dropout.

Non-immune mechanisms equally are contributors to the muscle tissue damage in DM. Viperin, another IFN-induced anti-viral protein and a modulator of mitochondrial and endoplasmic reticulum (ER) stress, is upregulated in DM (10). Viperin thus represents a link between inflammatory and ER stress response pathways, the latter being significantly elevated in muscle tissue of patients with polymyositis and DM alike (15). Chaperoning heat shock proteins (HSP) are also known to regulate skeletal muscle physiology and adaptation to stress, and are involved in clearing the tissues of damaged, misfolded and aggregated proteins. Expression of HSP70 and 90 families was found strongly increased in perifascicular atrophic fibers and regenerating fibers in DM, a feature shared with the affected fibers in polymyositis, sporadic inclusion body myositis and muscular dystrophy biopsies (16). Most of these non-immune mechanisms could therefore be regarded as universal (failing) tissue protection programs in muscle disease.

The jury is still out on the precise relationship between vascular and fiber damage in DM, but the article by Lahoria *et al*. (3) offers valuable data and insight, and is yet another illustration of how descriptive myopathological analysis is able to aid our understanding of disease mechanisms. Though this particular type of research delivers static data, it offers indirect evidence into the sequence of events concerned.

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Footnote

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The "gut microbiota" hypothesis in primary sclerosing cholangitis

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Primary sclerosing cholangitis (PSC) is a rare, immune-related disease characterized by chronic biliary inflammation and fibrosis of the intra and/or extra-hepatic biliary tree, leading to end-stage liver disease and biliary cancer. No medical therapies have demonstrated impact on the overall survival and liver transplantation (LT) is the only curative therapy (1). PSC is a complex condition characterized by the interplay between genetic predisposition and still unknown environmental factors. A better understanding of the disease etiopathogenesis would allow the development of specific therapies. The gut microbiota has recently evolved as a new important player in the pathophysiology of many intestinal and extraintestinal diseases, such as inflammatory bowel diseases (IBD), diabetes, obesity. It is to note that trials in PSC engaged antibiotics, both metronidazole and vancomycin, have shown a reduction in the alkaline phosphatase (ALP) (2,3). These data might suggest that manipulation of the gut microbiota could potentially influence the disease process in PSC.

In a recently published paper on *Gut*, Sabino *et al*. have investigated the intestinal microbiota composition in patients with PSC and have proposed that PSC has a characteristic microbial signature which is independent from IBD (4). In their study, a total of 175 individuals, divided in four different cohorts of patients were evaluated: patients with PSC only (n=18), PSC and IBD (n=48), IBD only (n=43), and healthy controls (HC) (n=66). Data about therapy in the last 30 days [in particular the use of antibiotics, probiotics, ursodeoxycholic acid (UDCA), immunosuppressors such as corticosteroids and anti-TNF], diet, living style, and disease conditions (stable, cirrhotic and liver transplanted patients) were collected. From each subject, fecal samples were collected, DNA were extracted and quantified, and microbiota analysis were performed in order to assignment a taxonomic sequencing 16S RNA genes (4). First of all, this study showed that the overall composition of the fecal microbiota was significantly different in patients compared to HC, with that from patients affected by PSC and PSC with concomitant IBD were different compared to that from the patients with only IBD. In particular, patients with PSC have a reduced microbiota with *Bacteroides* overrepresented and *Firmicutes* underrepresented compared to HC. In addition, it was found a consistent signature of four genera—*Enterococcus*, *Fusobacterium*, *Lactobacillus* and *Streptococcus*—which were overrepresented in both subgroups of PSC (PSC with and without IBD) compared to HC. Also *Streptococcus* genus was significantly increased in PSC and PSC with IBD patients, but its effect disappeared after taking into account antibiotic use. These results were independent of the treatment with antibiotic or UDCA, sex, smoking and cirrhosis or LT. *Enterococcus* was positively correlated with elevated ALP levels, but this correlation disappeared in the multivariate analysis. In addition, three different operational taxonomic unit (OTU) were assigned to the genus *Enterococcus*; one of these, OTU1, was significantly

associated with PSC, regardless of the subgroups, and correlated with ALP, although not confirmed in the multivariate analysis.

These results are consistent with the recent work of Kummen and colleagues who described a reduced bacterial diversity in PSC, regardless of the presence of IBD (5). In another study performed in a pediatric population of PSC and ulcerative colitis (UC) a lower species richness and abundance of *Enterococcus* (*E. faecalis* especially), *Streptococcus* (with prevalence of *S. parasanguinis*) and *Veillonella* species were found (6).

The big challenge to clarify is still whether these changes represent the trigger of PSC or are only a consequence of this liver disease, for example deriving from an alteration of the bile pool. Also, it is still unclear whether fecal microbiota are entirely representative of communities of mucosa-associated bacteria, which might uniquely interact with immune and epithelial cells.

An imbalanced intestinal microbiota characterized by an increased proportion of pro-inflammatory microorganisms and a decreased proportion of anti-inflammatory microorganisms, has been repeatedly observed in patients with IBD and is now well recognized as a key factor in the gut inflammatory processes. On the contrary, little data is available on the link between cholestasis and microbiota. It has been recently reported that a multidrug resistance 2 knockout ($mdr^{-/-}$) mouse model, a well-established animal model of PSC, shows higher serum markers of cholestasis and more advanced histological damage, such as increased liver fibrosis, ductular reaction, and ductopenia, when raised in a germ-free environment (7). The gut microbiota is essential for bile acid (BA) metabolism and regulates both the levels of primary BA synthesis, through modulation of the nuclear receptor, farnesoid X receptor, as well as production of secondary BA, such as deoxycholic acid, that are absent in germ-free mice (7). The absence of commensal microbial metabolites such as secondary BA with their anti-inflammatory properties might, in part, explains the link between gut microbiota and the liver.

A 'leaky gut' hypothesis has been proposed, suggesting that bowel disease and disruption of bowel permeability may eventually lead to microbial infection of bile, and subsequently causing cholangiocytes to activate a response that leads to inflammation and fibrosis within the liver. Considering that *E. faecalis* has already been associated with impaired intestinal permeability, it is to note that Sabino *et al*. observed an overrepresentation of the genus *Enterococcus* in fecal samples (4). In particular, gelatinase, a metalloprotease produced by *E. faecalis*, has been shown to alter the epithelial barrier, resulting in higher susceptibility to intestinal inflammation. The impairment of the epithelial barrier might allow bacterial translocation and bile colonization. Interestingly, *E. faecalis* and *E. faecium* have been the most frequently isolated species in bile in patients with PSC with dominant strictures (8,9).

High diversity of the intestinal microbiota is known to be a driving force for the evolution of the immune system, allowing the host to accommodate antigens and self-antigens. This ties in well with the 'gut lymphocyte homing' hypothesis in PSC which proposes that T cells are abnormally activated in the gut, with an erroneous recruitment to the liver, and a consequent triggering of hepatobiliary inflammation and fibrosis (10,11).

Most of the studies on microbiota are affects by a number of limits: first, they assess the use of drugs in a short preceding period, but do not consider the long-lasting effect of pharmacological agents on the microbiota. Second, the often neglect the dietary history, although is a key factor in the development of each individual microbiota. Unfortunately, while adjusting for structured diet approaches such as vegetarianism or gluten-free diet is feasible, there are no standardized methods to adjust for a detailed dietary history. Finally, the scenario is even more complex since recent evidences suggest that other factors such as the type of delivery (vaginal versus cesarean) and breast-feeding *vs.* formula-feeding affects the development of the intestinal microbiota, and this has been associated with chronic inflammatory conditions such as IBD (12-14). It is warranted that these factors should therefore be explored in future studies looking at the microbiota in PSC.

The study by Sabino and colleagues (4) provides a rationale for further studies on the microbiota in PSC with the aim to better understand its mechanisms and contribution to the disease, and to develop strategies to modulate the microbiota to treat or prevent disease. Ideally, in the future it may even be possible to use the microbiota to detect gut-related diseases before conventional diagnostics can.

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Extracellular vesicles: important players in immune homeostasis

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Exosomes are nano-sized extracellular vesicles (EVs) released from cells that act as mechanisms of cellular communication both between the surrounding cells that make up the cellular mileu and cells at distant locations in the body (1). Not only do they play many different roles in normal physiology, exosomes have been shown to exacerbate the progression of numerous different pathologies (1), including playing dual roles in immune regulation (2). Exosomes activate immunity by way of transferring antigens and promoting activation of antigen presenting cells. They also have been shown to participate in immunosuppression under different circumstances (2). For example, exosomes released from intestinal epithelial cells have the ability to suppress global immune function and are critical players in immune evasion in tumor models. Additionally, exosomes released by mesenchymal stem cells suppress immune function in various animal models of disease and tissue damage when used for therapeutic cellular regeneration (2).

An activated immune state contributes to the progression of many different types of inflammatory diseases, namely those irreversible degenerative eye diseases such as age-related macular degeneration (AMD). In the case of this disease, retinal pigment epithelium (RPE) cells, which are critical for maintaining integrity of the blood-retinal barrier, become damaged in an inflammatory environment. Unfortunately, regeneration does not occur in RPEs. Under normal conditions, RPE cells suppress the inflammatory response, protecting themselves and the retina from immune mediated damage. However, RPEs release both immunosuppressive and inflammatory cytokines that are important for immune responses, as they have the ability to initiate a rapid defense system to protect the retina from pathogens (3). Although RPEs play a critical role in the protection of the retina by upregulating the inflammatory response, immunoregulation creates a balance in inflammatory states, and is a vital mechanism that protects both RPE cells and the retina from excessive cellular damage potentially caused by an activated immune response.

Korthagen *et al*., demonstrate modified gene expression in RPE cells when stimulated by TNF-α, one of the cytokines involved in ocular inflammation. Other altered gene expressions include those involved in apoptosis, cell motility, immune response, protein transport, and cell signaling, suggesting TNF-α mediates numerous RPE cellular functions (4). Furthermore, it was proposed that vesicular trafficking within the RPE cells may reduce inflammation in the retina (5), however, the precise mechanisms, and the role of EVs in this process, are unknown.

In the current paper, Knickelbein *et al*., explored the notion that EVs, such as exosomes, released from RPE cells play in the immunosuppressive environment required to protect the cells from immune-mediated degeneration. These authors mimicked an inflammatory environment in an *in vivo* setting by stimulating the cells with inflammatory cytokines, including IL-1β, IFN-γ, TNF-α, then they characterized the EVs released by the RPE cells under those conditions and compared to the untreated controls. These

authors found that although the size of the vesicles released by the activated cells did not change (~100 nm in diameter), there was an increase in the amount of the CD81 protein, which, the authors believe, suggests an increase in the quantity of exosomes released from the activated RPE cells under inflammatory conditions. These results propose an increase in the release of EVs from these RPE cells under inflammatory conditions, which is consistent with increased exosome release under oxidative stress conditions shown previously (6).

Although the data suggested an increase in CD81 protein in exosomes under inflammatory conditions, it was important to determine the effect these EVs have on the immune system. The authors specifically investigate T-cell and monocyte response to the EVs released by activated RPEs. T-cell proliferation was reduced when exposed to both stimulated and untreated RPE cell exosomes, consistent with previous data implicating the role of RPEs in the suppression of T-cell activation. However, neither the exosomal samples from stimulated or non-stimulated RPEs resulted in T-cell death, which is a function of RPE cells under normal conditions. Taken together, these findings show that exosomes may play a role in the reduction of T-cell proliferation, however, despite one of the mechanisms of immune suppression by RPE cells being the induction of T-cell apoptosis, it is unlikely to be a result of exosome release.

These authors next explored the effects of RPE EVs have on monocyte population. Monocyte phenotype is based on surface expression of CD14 and CD16, and an increase in the population classified as intermediate phenotype ($CD14^{++}CD16^{+}$) was detected when monocytes were incubated with unstimulated RPE EVs when compared to untreated monocytes. This shift of population phenotype suggests an increase in the immunoregulatory functions in the monocytes. In addition, when monocytes were incubated with cytokine-induced RPEs-derived EVs, an induction in monocyte death correlated with the volume of EVs added to the monocytes. This cell death was not seen with EVs from non-cytokine stimulated RPE cells. Furthermore, a greater amount of TGF-β1 (which has immunosuppression functions) was released by monocytes exposed to the non-stimulated RPE EVs. In contrast to those monocytes exposed to cytokine-stimulated RPE-derived EVs, an increased amount of inflammatory cytokines such as TNF-α, IL-6 and IL-8 was released.

Overall, this manuscript suggests that EVs from RPE cells mediate changes in T-cell proliferation and monocyte phenotype depending on the inflammatory environment of the RPE cells. Importantly, results from the current studies implicate EVs of the RPE cells as a mechanism of immunomodulatory effects of the cells by way of suppressing T-cell proliferation.

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Characterizing the contribution of inflammasome-derived exosomes in the activation of the immune response

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The interaction of cells with the environment is well known and documented in different processes associated with the fate of the diseases. How do different cells from different organs with different and complex environments communicate in a global and integrative manner is a question that researchers from complementary scientific areas are trying to address over the past decades. Extracellular vesicles (EV), small vesicles secreted by most cell types, participate in intercellular communication allowing exchange of proteins, lipids and nucleic acids between the EV-producing and target cells. Among them, exosomes as vesicles with diameters of 30–150 nm intervene in the transfer of proteins, mRNAs, and miRNAs to recipient cells to mediate many biological processes. The impact of this type of intercellular communication and its relevance at the clinical setting is being recognized by the scientific community, and the role of exosomes as mediators of this interaction is becoming a new and exciting field of research with promise to address relevant clinical questions in the next future.

A recent paper approaches the study of exosomes as mediators of the inflammasome, complex multimeric protein complexes formed during innate immune response (1). The intercellular transfer of pathogen- or host-derived RNA, DNA and proteins from infected cells to neighbor cells impacts on host innate immunity (2). Strong evidence also indicates that exosomes-delivered microRNAs undergo a functional transfer between immune cells and constitute a mechanism of regulating the inflammatory response (3). But it is now clear that not only immune cells but probably all cell types are able to secrete exosomes: their range of possible functions expands well beyond immunology to neurobiology, stem cell and tumor biology, and their use in clinical applications as biomarkers or as therapeutic tools is an extensive area of research (4,5).

An effective innate immune response relies on the detection of pathogen associated molecular patterns (PAMPs) by various host pattern recognition receptors (PRRs) that result in the production of pro-inflammatory cytokines and chemokines. Exosomes have been proposed to mediate one of the mechanisms involved in the stimulation of P2X purinoceptor 7 receptor (P2X7R) that rapidly triggers a key step of the inflammatory response: the induction of NLRP3/caspase-1 inflammasome signalling complexes that drive the proteolytic maturation and secretion of the pro-inflammatory cytokines interleukin-1β (IL-1β) and interleukin-18 (IL-18) (6). ATP stimulation through a P2X7R-dependent pathway activates robust vesicle-mediated unconventional protein secretion, including exosome release and membrane shedding, and drives NLRP3 inflammasome activation in a calpain activity dependent manner (7). Also related, central nervous system trauma induces inflammasome activation and secretion of exosomes containing inflammasome protein cargo into

cerebral spinal fluid that fuse with target cells to activate the innate immune response in peripheral tissues (8).

In this new work, the group of Dr. Wang describes a proteomic approach to the inflammasome signaling and exosome secretion by challenging murine macrophages with the agonist of NLRP3 inflammasome signaling LPS/nigericin. Two main issues systematically impact on this type of studies: first, the design of the model system that must address a complex and dynamic scenario involving different cellular systems and a highly interactive environment, and second, an appropriate methodological approach for the purification and characterization of the EV and exosomes. This study includes the generation of bone-marrow-derived macrophages (BMDM) from 8-week-old C57BL/6 mice cultured for 7 days, with 98% isolation efficiency as evaluated by $CD11b^{+}/F4/80^{+}$. Activation of NLRP3 inflammasome signaling pathway has been conducted in two-step stimulation, first by treatment of BMDMs with endotoxin for 6 h, and second challenged with nigericin for 30 min; BMDMs treated with endotoxin and mock were used as controls. In addition to the characterization of the proteome from inflammasome-associated exosomes, their functional characterization includes microscopy visualization of fluorescently-labelled exosomes internalization by BMDMs, the triggering of pyroptosis, a Caspase-1 dependent pro-inflammatory form of programmed cell death, and the secretion of cytokines.

Exosomes from culture supernatants [fetal bovine serum (FBS) used in culture was previously depleted of endogenous exosomes by overnight centrifugation] were isolated by differential centrifugation. Proteomic analysis was performed by LC-MS/MS, comparing three biological replicates from each condition. The presence of exosomal proteins confirmed the origin of exosomes, with a 50% overlap of the total identified proteins from the different stimulus and a 20% of specific proteins associated with the inflammasome activation by LPS/nigericin, mainly up-regulated. These proteins were involved in immune system process, innate response and inflammatory response; together with proteins of the inflammasome signaling pathway, toll-like receptors and members of the tumor necrosis factor (TNF) and nuclear factor-κB (NF-κB) families were highly enriched in exosomes with treatment of LPS/nigericin, further reinforcing the relationship between inflammasome-derived exosomes to the immune response and infection. In addition to the proteomic characterization, inflammasome-derived exosomes were found to be preferentially uptaken by macrophages leading to the up-regulation of NLRP3 and IL-1β, processing of Caspase-1 and triggering of pyroptosis. Finally, the analysis of secreted cytokines upon treatment with inflammasome-derived exosomes further confirmed the activation of the NF-kB signaling pathway.

This work demonstrates the ability of exosomes derived from the inflammasome to enter macrophages and activate the immune response, amplifying the inflammatory signaling in neighbor cells. The next challenge is to add complexity to the model systems simulating these eventual therapeutic opportunities; exosomes from different cell type origins in a more interactive 3-dimensional co-culture environment and submitted to dynamic processes would ideally result in a more clinically relevant conclusion. Also, the transference of other cargo into exosomes mediating NLRP3-inflammasome activation like microRNA (9,10), or the involvement of other EV during acquired and innate immunity (11), would provide a more complex and complete overview of the interaction of cells with the environment. Finally, the potential modulation of this process may serve to potentiate the immune response in cancer immunotherapy or in the control of autoimmune diseases; innovative approaches like DNA vaccines encoding extracellular vesicle-associated antigens represent promising immunotherapy tools for different diseases (12).

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Exosomes, your body's answer to immune health

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Mesenchymal stromal cells (MSC) have a profound effect on the regulation of the immune system. MSCs show low expression of major histocompatibility complex (MHC)-II and costimulatory surface molecules that include CD40, CD40L, CD80 and CD86, indicating immunomodulatory properties (1). Interestingly, prior research indicated that MSCs are important immune modulators that exert their biological effects in a paracrine manner, involving secretion of exosomes. Exosomes have emerged as an important means for cellular communication through the transfer of proteins and genetic material between cells. Exosomes are a form of extracellular lipid vesicle that are usually 40–100 nm in diameter, have a density of 1.10–1.18 g/mL on sucrose gradients and contain exosome membrane-specific proteins such as CD9, CD63 and CD81 (2).

Many cell types have the ability to form exosomes through the inward budding of a lipid bilayer membrane. It was reported that exosomes can contain factors such as microRNAs (miRNAs) that maintain functionality after cellular transfer (3). These findings strongly support the immunomodulatory role of exosomes that originate from MSCs and their influence on host homeostasis. While the underlying mechanism(s) by which MSCs, exosomes and their secretory factors affect immunity is still lacking, Chen *et al*. (4) examined the immunomodulatory properties that MSC-derived exosomes exert on peripheral blood mononuclear cells (PBMCs), with special emphasis on the T-lymphocyte immune subset.

Exosomes have been reported to originate from many different cells types, thereby contributing to the wide array of biological functions observed (5). It has been theorized that exosomes are the paracrine effectors of MSCs and mimic important activities of their parental cell in a range of different models of disease. Coordinated inflammatory responses require intercellular communication in addition to immune cells such as dendritic cells (DCs) and T-lymphocytes, which are able to absorb and secrete exosomes. Several studies have reported that exosomes contain miRNAs that modulate the function of recipient cells such as those involved in cancer, heart disease (6) and dysregulated inflammatory states such as sepsis (7). MSC-derived exosomes exert various biological functions that include multi-lineage differentiation, cytokine(s) secretion, cellular proliferation and immunomodulation. Therefore MSC-derived exosomes are attractive as potential therapeutic agents.

In their study Chen *et al*., reported on the immunomodulatory effects of MSC-derived exosomes towards PBMCs and especially, T-cells (4). MSCs were validated by their capacity to differentiate into multi-lineage cell types and possessed the typical mesenchymal markers according to flow cytometry. Exosomes generated from MSCs were isolated and identified via transmission electron microscopy, size distribution and content of exosome-specific proteins CD9, CD63 and CD81 by Western blot. MSC-derived exosomes were found to suppress T-cell activation and help maintain immune homeostasis.

PBMCs were stimulated with a range of concentrations of MSC-derived exosomes, and the ratio of CD4+/CD25+/CD127^{low} regulatory T-cells increased along with the expression of cytotoxic T lymphocyte-associated molecule-4 (CTLA-4), a negative regulator of T-cells. Previous studies

had shown that MSC-derived exosomes promoted cellular proliferation (5) and indoleamine 2,3-dioxygenase (IDO) activity, but this study reported that proliferation and IDO activity was unaffected (8). Additionally in this study (4), apoptosis was found to be upregulated in both PBMCs and CD3+ T cells but not in prior studies (8). It should be noted that these discrepancies might be due to the different components that are carried within different types of exosomes or the underlying complexity that differentiates MSCs from MSC-derived exosomes and thus may significantly affect biological activities.

Of interest was the shift in cytokine profile generated by MSC-derived exosomes. Chen *et al*. (4) found significant inhibition of the pro-inflammatory cytokines, IL1β and TNFα but enhancement of the expression of the anti-inflammatory cytokine, TGFβ. This cytokine profile mimics the immunomodulatory effects of MSCs (5,6,9). These findings thus support a secretory mechanism by which MSCs might export exosomes-associated factors to the surrounding environment. The authors also reported that exposing CD4+ T-lymphocytes to MSC-derived exosomes increased the population of Th2 cells while also limiting the Th1 and Th17 T-cell subsets. They proposed that Th1 cells might differentiate into Th2 cells; however since only a single surface marker was used to distinguish these subsets, the heterogeneity and plasticity of T-cell populations cannot be fully addressed (10). Further identification of the T helper cell subsets might provide more conclusive insights.

The key finding of Chen *et al*. (4) was the role that MSC-derived exosomes play in dampening the activation of T-cells, enriching regulatory T-cell populations and their activity. The findings of their study further support the notion that MSC-derived exosomes can promote and maintain a modulated immune state. Although the mechanism by which MSC-derived exosomes function is not completely understood, it would be beneficial to define the internal constituents of exosomes since the origin of the parent cell contributes to the resulting components found within (5). Although this study did not identify the components of exosomes that drove the observed immunomodulatory effects, other published studies have implicated miRNAs as functional effectors.

miRNAs are small non-coding RNAs with the ability to target genes and initiate an immune response. miRNAs are typically short, 18 to 22 nucleotides in length, highly conserved and regulate diverse aspects of development and physiology through RNA silencing and post-transcriptional regulation. Moreover, miRNA expression has been found to correlate with disease states (3,11). It has been widely reported that exosomes can transport miRNAs that are functional in recipient cells, thereby mediating cell-to-cell communication. Therefore, miRNAs might display a protective role in attenuating inflammation or modulating the immune response.

Systemic inflammatory diseases such as sepsis are usually triggered by infection and can lead to organ failure and death. Although the clinical definitions have been updated, there is still a great need for the general agreement of definitions and identification of the factors at play during early sepsis (12). The mortality of sepsis is more than 30%, typically targeting the very young and elderly (13), but there are currently a lack of efficacious treatments. Sepsis was originally considered as the dysregulated response of the host to infection, resulting in elevated and sustained levels of cytokines and chemokines (14). Additional study of this disease state observed much more complexity than initially considered. Rather than a primarily predominant hyper-inflammatory state, host blood cells undergo cellular reprogramming (CR) leading to an inability to respond to danger signals (15,16). The need for improved outcomes in sepsis makes MSC-derived exosomes an attractive therapeutic agent to help regulate immune function. Many studies have reported the importance of exosomal miRNAs and their ability to communicate and regulate cellular functions, including immunity (7,11).

Wang *et al*. found that miR-223 plays a cardio-protective role by dampening the inflammatory expression of immune cells in a polymicrobial murine cecal ligation puncture (CLP) sepsis model. Another study had reported that miR-223 has the ability to limit the expression of inflammatory genes (17) and Wang *et al*. further explored the impact it had on immune cells during sepsis and ultimately how mortality was affected. The study found that exosomes released from wildtype MSCs conferred survival to CLP mice while exosomes from miR-223 knockout MSCs proved detrimental. Thus, the lack of miR-223 within exosomes resulted in sepsis-induced heart failure, inflammation and death, while miR-223-containing exosomes attenuated the inflammatory response and minimized morality. These immune modulatory properties of MSC-derived exosomes are thus consistent with the findings by Chen *et al*. (4).

Altogether, these findings strongly implicate exosomes as a crucial component in modulating the immune system during a variety of different physiological perturbations. Therefore, the potential importance of exosomes and the

significant roles they exercise in cellular communication necessitates further studies to clarify their mechanism(s) of action.

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Tumor exosomal microRNAs thwarting anti-tumor immune responses in nasopharyngeal carcinomas

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The considerable progress achieved in the past 10 years in the field of tumor biology and therapeutics has strengthened the idea that cancer is not only a cellular but also a tissue disease. This concept is likely to apply to nasopharyngeal carcinoma (NPC), characterized by the consistent expression of oncogenic viral proteins in a context of inflammation and immune escape (1). In its typical undifferentiated form, NPC is constantly associated with the Epstein-Barr virus, whose genome is contained in the nuclei of all malignant cells, but not in the surrounding tissue. Latency is the predominant mode of virus-cell interactions, meaning that most viral genes are silent in the vast majority of malignant cells. However, a small fraction of them are consistently expressed coding a handful of viral products, both viral proteins and untranslated RNAs, most of them with proven oncogenic properties. The inflammatory context of NPC is obvious for pathologists: almost all NPC primary tumors are heavily infiltrated by non-malignant leucocytes, mainly T-lymphocytes but also B lymphocytes, macrophages, dendritic cells and neutrophils. This inflammatory infiltration often disappears in metastatic lesions. The immune escape is also obvious because of the rapid proliferation of malignant cells despite the consistent expression of EBNA1, LMP1 and LMP2 which are known to be the targets of CD4+ and CD8+ cytotoxic T-cells in EBV-carriers. One interpretation of the paradox of tumor inflammation combined with tumor immune escape is that malignant cells in the primary tumor benefit to some extent from the proximity of leucocytes while developing mechanisms of immune escape.

High-scale genomic studies have brought evidence that the immune escape mechanisms in NPC can be cellular intrinsic alterations, for example defects in the expression of HLA class I molecules (Lo KW, 17th International Symposium on Epstein-Barr virus and associated diseases, Zurich, August 2016, abstract EBV2016-1040). These alterations are probably the most difficult to deal with for the oncologist. However, there is also evidence of a major contribution of extra-cellular "micro-environmental" immunosuppressive factors. Data from previous studies support the role of immunosuppressive proteins either secreted in a soluble form or carried by tumor exosomes, for example CCL20, galectin-9 or IDO (indoleamine 2, 3-dioxygenase) (2-4). One recent elegant publication from Jiang Li's group in Guangzhou provides new insight on the role of tumor exosomes carrying immunosuppressive microRNAs (5) (*Figure 1*). For the sake of brevity, one can distinguish two types of results in this study. Most data are based on *in vitro* experiments. They demonstrate that malignant cells mixed with T-cells from healthy donors can deliver miR-24-3p to these T-cells using exosomes as intercellular carriers. Then it is shown that miR-24-3p decreases the proliferation of target T-cells by down-regulation of FGF11 and subsequent modifications of ERK and STAT protein phosphorylation. Simultaneously, there is a decrease in the expression of interferon-γ and

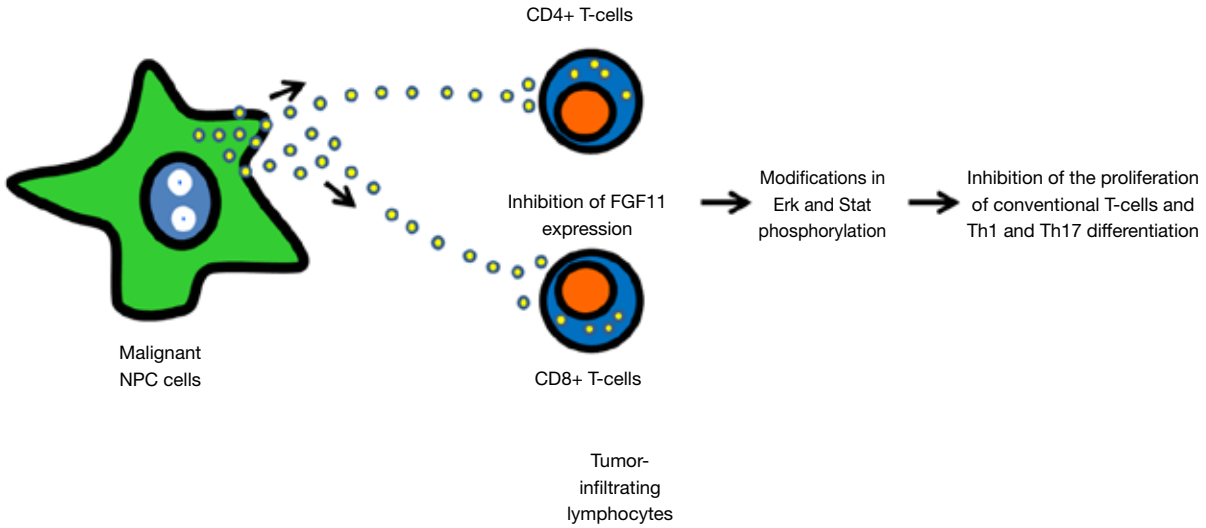

Figure 1 Main steps of the scenario linking the inhibition of the proliferation and differentiation of tumor-infiltrating T-lymphocytes to the release of tumor exosomes carrying miR-24-3p in the microenvironment of nasopharyngeal carcinomas. Exosomes carrying miR-24-3p are released by malignant NPC cells and up-taken by tumor-infiltrating lymphocytes. The internalization of miR-24-3p in target cells results in the downregulation of FGF11 with subsequent modifications in the phosphorylation of Erk and Stat proteins. These signaling events result in the inhibition of the proliferation of conventional T-cells (CD4+ and CD8+) and probably impair Th1 and Th17 differentiation. This scenario is based on *in vitro* and *in vivo* investigations reported by Ye *et al.* (5).

IL-17 expression in CD4 T-cells suggesting the impairment of Th1 and Th17 differentiation. From these data, the authors infer that similar interactions are likely to occur in the tumor microenvironment where malignant cells are in close contact with tumor infiltrating lymphocytes (TILs). Other data, based on investigations of serum samples or tumor tissue sections, support this inference. First, the authors show that the abundance of FGF11 in TILs is inversely correlated with the serum concentration of miR-24-3p. Later, they show on tumor sections that a low abundance of CD4+ and CD8+ TILs correlates with a low abundance of FGF11 in TILS (and in malignant cells as well). Moreover, a high concentration of exosomal miR-24-3p in the serum and a low amount of FGF11 in TILs and malignant cells were associated with a shorter disease-free survival. A few more experiments demonstrated that, at least *in vitro*, hypoxia enhances the concentration of miR-24-3p in tumor exosomes. The data are less consistent with regard to regulatory T-cells (T-regs). Indeed, *in vitro* T-regs' expansion and Fox-P3 expression were enhanced by the uptake of miR-24-3p and FGF11 down-regulation. However, *in vivo*, there was no significant correlation between the abundance of Fox-P3-positive cells among stromal cells and the depletion of FGF11 in TILs and malignant cells. This reminds us that the role of T-regs in NPC remains controversial (3,6).

In terms of methodology, it is important to note that almost all *in vitro* investigations were done using an EBV-negative malignant epithelial cell line, TW03. This approach is likely to have both positive and negative consequences. TW03 cells are easier to handle *in vitro* that genuine NPC cells carrying an endogenous EBV genome. The authors have largely taken advantage of the ease of DNA transfection into these cells to make an intense use of microRNA mimics, microRNA sponges and reporter assays. However, in many respects, TW03 cells lack several major characteristics of NPC cells. For example, NPC cells carry on their surface an array of inflammatory molecules like HLA class II molecules, CD54 and CD70 which are not found on TW03 (1). Moreover, a huge fraction of the total microRNAs produced by NPC cells—often as much as one sixth or even one third of them—are EBV—encoded microRNAs of which some might have an impact on T-cell functions (7). On the other hand, to a large extent, TW03 cells have a phenotype which is reminiscent of the phenotype of malignant cells from squamous cell carcinomas of the upper aero-digestive tract. Therefore, the findings reported by Ye *et al.* may have applications for

non-NPC epithelial malignancies, for example squamous carcinomas of the upper aero-digestive tract where hypoxia is often highly prevalent.

Tumor immunosuppression is usually a multifactorial process. As mentioned previously, other immunosuppressive factors, especially proteins are known to be released by NPC cells either in a soluble form or conveyed by exosomes. Therefore an integrated approach will be required to assess the respective contributions to the immune evasion of NPCs of the various immunosuppressive agents, regardless of their chemical nature, proteins, nucleic acids and probably lipids like prostaglandins. It is noteworthy that in virtually all experiments reported by Ye *et al.*, the reduction of the T-cell proliferation induced by exosomes carrying the miR-24-3p did not exceed 20%. Thus, there is ample room for potential synchronic or non-synchronic cooperation of miR-24-3p with other immunosuppressive factors. In future research, one major challenge will be to identify the predominant mechanisms of immune suppression for each clinical and molecular subtype of NPC or even for a given patient, at each stage of his treatment and surveillance. NPC clearly is a heterogeneous disease, in terms of growth pattern (with either early metastases or predominantly local growth), in terms of malignant cell phenotypes (with more or less epithelio-mesenchymal transition) or in terms of immune "contexture" (variable relative abundance of various types of T-lymphocytes, macrophages, NK cells and dendritic cells) (1,6,8). Although currently there is no consensus on molecular subcategories, it is obvious that the NPC malignant phenotypes can be supported by different genetic and epigenetic alterations as well as different modes of virus-cell interactions, for example a high, low or very low level of LMP1 expression (Lo KW, 17th International Symposium on Epstein-Barr virus and associated diseases, Zurich, august 2016, abstract EBV2016-1040). The work published by Ye *et al.* is also quite exemplary insofar as it shows the importance of combining the investigations on the tumor tissue with investigations on serum or plasma samples (5). One can presume that, in the future, the diagnosis of the immune "contexture" and immune suppressive mechanisms will rely on tumor tissue analysis combined with assays performed on serum or plasma factors including microRNAs and proteins like CCL20 and galectin-9 (8).

What are the consequences of the findings reported by Ye *et al.*, in terms of therapeutics? One option seems to be the use of miR antagonists (antagomiRs or anti-miRs) to neutralize plasma miR-24-3p. Vectorization of these antagonists to the malignant cells or the tumor-infiltrating leucocytes remains a major challenge. Another option is to attempt a capture and depletion of tumor exosomes using systemic injections of therapeutic antibodies. Because exosomes released by endothelial cells or leukocytes are very abundant in plasma and many interstitial fluids, it will be necessary to use antibodies reacting with molecules expressed selectively on the surface of NPC exosomes. Some years ago, we made the empirical and surprising observation that selective capture of tumor exosomes from plasma samples from NPC patients was facilitated by the use of anti-HLA class II antibodies (2). Galectin-9 is another protein present on the surface of NPC tumor exosomes (ibid.). Thus, the use of anti-galectin-9 or anti-HLA II antibodies might play a role in a future strategy of NPC exosome capture and depletion.

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Footnote

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Future directions of extracellular vesicle-associated miRNAs in metastasis

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Abstract: Numerous studies have demonstrated the dynamic cell-to-cell communication mediated by extracellular vesicles (EV) in cancer cell survival and metastasis development. EV content includes proteins, lipids, DNA, and RNA like microRNAs. Non-protein coding microRNAs play a very active role in almost all cellular processes targeting mRNAs for silencing. Different miRNA profiles have been found in different cancer types, and clarification of miRNAs packed in EV from different types of cancers will allow the understanding of metastasis and the application of miRNAs as biomolecules in diagnostic, prognostic and therapeutic approaches to fight cancer. The profound review of Dhondt *et al*., 2016, provides a wide view of EV miRNAs involved in various steps of the metastasis process to illustrate how the cancer cell interaction with the near and long distance microenvironment allows metastasis. These studies will surely conduce to additional patient studies to prove the relevance of EV miRNAs in metastasis *in vivo*. It remains to be elucidated how the tumoral cell sorts the miRNAs for secretion to send a message, and to well recognize the type of EV performing this message delivering. It will be very useful to identify whether miRNAs are delivered with post-transcriptional modifications since this is an important feature for miRNAs activity and stability.

Keywords: Microvesicles (MV); microvesicles particles; exosomes; extracellular vesicles; microRNAs

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Significant amount of information supporting extracellular vesicle (EV) associated miRNA mediated cell-cell communication has accumulated along the years (1,2). Specific miRNA vesicle loading is an important question to be unveiled due to its implication in tumor cells communication to near and long distance tissue cells. There are three types of EV involved in molecules secretion: microvesicles (MV), exosomes (with different types of biogenesis pathways, implying different types of exosomes) and apoptotic bodies. The variety of EV has emerged the question whether different types of vesicles are related to different miRNA loading and miRNA function in recipient cell. However, from the studies shown in the profound review of Dhondt *et al*., 2016, miRNA loaded exosomes are found either from primary tumors and from metastasis cells, lowering the possibility that this type of EVs are preferentially secreted by one or the other type of cell (3). Nevertheless, more studies discriminating between MV and exosomes and differentiating between exosomes sizes or types would lead to more definitive conclusions. The question of miRNA load selection still remains open. In the same review it is

shown that some anti-oncomirs are discarded in exosomes favoring tumoral cell hallmarks as miR-145 (4), miR-146b, miR-122 (5) and miR-23b (6). It still remains to know whether miRNAs in these exosomes are active or not, since recently it has been shown that post-transcriptional regulation mediates miRNA activity and stability (7). It is known that total miRNA cell profile differs depending on the type of cancer cell (8-10). It is interesting to notice that the majority of studies shown in this review focused in oncomirs and less in anti-oncomirs analyses, whether there is a preference of the type of miRNA secreted by these cancer cells is not known. Therefore, additional miRNA loaded EV studies in different cancer cell types will widen the understanding of this cancer progression mechanism. On the other hand, it is shown in Dhondt *et al.*'s review that oncomirs loaded in exosomes of primary tumors or metastasis cells favor the metastasis process for example preparing other tissues niches like exosomes carrying miR-105 secreted by breast metastatic cancer cells and internalized by endothelial cells (11); favoring angiogenesis like miR-214 found in exosomes of epithelial cells (12); inducing migration by miR-409 from cancer associated fibroblasts in EV (13); stimulating invasion by mir-105 found in breast cancer cell exosomes (11); promoting proliferation like miR-429 in hepatocellular carcinoma (14); potentiating invasive and adhesive capabilities as miR-210 in brain metastasis competent cell-derived exosomes (15); triggering epithelial to mesenchymal transformation by miR-409 from cancer associated fibroblasts in EV (16) and miR-221 in extra-hepatic cholangiocarcinoma (17); or additionally, participating in mesenchymal to epithelial transition (18). These results direct to the question: how do cells tag a type of miRNA to be discarded or used to enhance cancer traits? The study of the mechanisms responsible of vesicles miRNA specific loading will be useful to respond this question. If there are proteins or miRNA post-transcriptional modifications involved in miRNA sorting is a tentative subject of study. There are important studies supporting this question. Recently, Janas *et al.*, 2012, suggested that miRNA sorting could be explained by contacting of the miRNAs and the microvesicular body (MVB) membranes due to the well-known affinity of RNAs to the raft-like membrane regions (19). Besides this mechanism, secretion of miRNAs could additionally be more specific. Makarova *et al.*, 2016 (20) suggested the presence of universal sequence-specific sorting mechanisms for miRNA loading into EVs since miRNA repertoires in EVs derived from different cell types shared a higher similarity than that of EVs and their corresponding parental cells (21), moreover, significant differences in intracellular and extravesicular miRNA profiles reported by several research groups strengthen this hypothesis (22-28).

Extensive studies have shown that intracellular and extracellular miRNAs are mainly found bond to AGO proteins (29-32). However, recent findings suggest miRNA stabilization can be different as well. Makarova *et al.*, 2016, revealed that in HeLa cells, the amount of miRNA is approximately 13 times higher than the amount of AGO proteins (~200,000 and 15,000 molecules per cell, respectively) (20). Moreover, two recent studies have reported the discovery of more than a thousand new human miRNAs (19,33). In addition, a minor fraction of AGO proteins are associated with other classes of short RNAs (33,34) which further reduces the amount of AGOs available for the association with miRNA. All this information leads to speculate that other type of miRNA regulation may play a role in the different processes where miRNAs participate, as it could be miRNA sorting in EV.

In the same review, it is shown by in silico analyses that 50%–70% of animal and plant miRNAs are able to form intrinsic secondary structures (hairpins and homoduplexes) (35,36). Many of these miRNA structures strongly resembled to anti-tenascin C aptamers (37) implying that miRNAs may directly modulate protein activity (36), but also it may imply that miRNAs may have the ability to bind to different proteins and become stabilized or be sorted for EV loading. Exosomes studies have shown that among several pathways of exosome biogenesis, the ceramide-dependent mechanism is a way contributing to circulating miRNA release since export of miRNA outside the cell was impaired upon inhibition of neutral sphingomyelinase 2 (nSMase2) an enzyme mediating ceramide biosynthesis (20,38-40). Another way of miRNAs secretion mediated by ESCRT machinery of exosome formation seems to be more controversial since in HEK293 cells, after transient knock-down of the ESCRT-associated protein ALIX, which regulates ESCRT-dependent intraluminal vesicle (ILV) formation (41) the levels of miRNA in conditioned media remained unaltered (42). However, another study reported that miRNAs activity is an ESCRT MVB dependent mechanism, suggesting this may be another way of miRNA secretion (2). Therefore, additional studies are needed to clarify the types of vesicles mediating

miRNA export. MiRNA sorting into the MV, the other type of EVs, is also unclear and it is subject of study (20). The existence of different ways of secretion may imply the existence of miRNAs sorting mechanisms for their specific secretion and could also be linked to the type of function that miRNAs are performing in recipient cells.

Different protein modifications are implicated in protein function and fate therefore miRNA modifications can probably serve as miRNAs sorting. The global localization of miRNAs in the cell has emerged the idea of multiple miRNA functions beyond that of gene silencing. It was strongly thought that miRNA activity was limited to P bodies (43-45) but it has been recently shown that miRNAs and miRNA machinery are also found in ER, Golgi apparatus, lysosomes and endosomes and that miRNAs are functionally active in ER (46,47). ER is a cell organelle where protein modifications are carried out (48). The fact that miRNAs are found very close to these organelles gives a possibility that post-transcriptional miRNA modifications may be happening in this place. MicroRNAs are modified through a series of processing events after transcription like 5'-end phosphorylation, 3'-end adenylation or uridylation, and terminal nucleotide deletion (49). The study of Salzman *et al*. showed that miR-34, a tumor-suppressor miRNA that is important in cell survival and that is transcriptionally upregulated by p53 in response to DNA damage is found in a pool of mature miRNA in cells that lack a 5'-phosphate and is inactive. Following exposure to a DNA-damaging stimulus, the inactive pool of miR-34 is rapidly activated through 5'-end phosphorylation in an ATM- and Clp1-dependent manner, enabling loading into Ago2. In a different study, it was shown that post-transcriptional addition of nucleotides to the 3' end of miRNAs is a mechanism for regulation of miRNA activity. For example, such modification in plants and C. elegans influence miRNA stability (49,50). In humans, miR-122 was shown to be adenylated by the RNA nucleotidyl transferase GLD-2, which resulted in an increase in the stability of the miRNA (51). On the other hand, uridylation of miR-26a had no effect on miRNA stability, but had an effect on the ability of miR-26a inhibition of its mRNA target (52). It has been shown that 3' modification of miRNA is a physiological and common post-transcriptional event that shows selectivity for specific miRNAs and is observed across species ranging from C. elegans to human (53). Thus, miRNA post-transcriptional modifications are important to this molecule for different purposes. This or other uncovered modifications may be taking place in order to select miRNAs for vesicles sorting. Concerning miRNA EV loading and release, some studies have focused on mechanisms involved in miRNA release from EV to recipient cells. The protein neurophilin 1 has been found implicated in the mechanism of EV and recipient cell interaction. miRNA EV loading on the other hand has been less documented (54).

Given that exosomes can be isolated from almost any cell, are involved in cell-to-cell communication, and participate in both normal and pathobiological mechanisms, there have been extensive studies exploiting their use both as diagnostics and therapeutics (55). For example, exosomes are used to detect tumors in patients with prostate, breast, and ovarian cancers (56-58). The naturality of exosomes to carry nucleic acids, such as DNA, RNA, and miRNAs to targeted cells, inducing genetic modifications in both biological and pathogenic processes, exosomes became a major interest in treatment strategies involving genetic therapy as drug delivery systems (55). The understanding of miRNA loading and sorting in EV will strongly improve the design and efficiency of this potential therapy approach. The recent revelation that miRNAs activity is modulated by phosphorylation (7) should be taken in consideration when designing diagnostic and therapeutic methodologies for more secure and effective methodologies. MiRNAs have been extensively reported to be implicated in the process of drug and radiation resistance, being both miRNA under or over expression important determinants of clinical response after cancer therapy (59-62). For example, Pedroza-Torres *et al*., 2016 (63) identified 101 miRNAs that showed significant differences between non-responders and complete pathological responders. Thus miR-31-3p, miR-3676, miR-125a-5p, miR-100-5p, miR-125b-5p, miR-200a-5p and miR-342 were significantly associated with clinical response. Expression of miRNAs above the median level was a significant predictor of non-response to standard treatment. Interestingly, it has also been reported that miRNA expression is affected after radio and/or chemotherapy (64). MiRNA signatures are currently being used to study miRNA based cancer prognosis after conventional therapy. How may this treatment influence the afflux of EV and miRNA load has not been profoundly investigated and would provide important information to the understanding of cancer relapse and the intervention of EV to this process. Dhondt *et al*.'s review is a very useful collection of sophisticated information that clarifies the important function of EV miRNAs in cell-cell communication in the metastasis process. It provides

researchers with potential candidates for diagnostic and prognostic markers and therapeutic targets to fight cancer. It is a scaffold that will lead researchers to perform new assays to gain additional reliable EV miRNA data as the elucidation of the miRNA sorting mechanism to EV loading that would also clarify the metastasis process and lead to new anticancer approaches.

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Footnote

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Vesicles & Cancer Immunotherapy

Therapeutic targeting of dysregulated cellular communication

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Intercellular communication represents a fundamental phenomenon in multicellular eukaryote organisms, such as in the human beings. Multicellularity has allowed developing cells involved in different works. Within the same organisms, some cells function as nutrient providers, while other cells are involved in defense responses or reproduction. Multiple cell-types join to form tissues, such as muscle, blood, vascular and brain tissue. In this scenario, signaling systems are crucial to ensure the harmonious development of multicellular structures and functions. Cells have to sense information from the outside environment, including the availability of nutrients, changes in temperature, or light levels and then the information has to be transmitted intracellularly. Considering that tissues are constituted by different cell-types which have to collaborate to carry on a particular function, the intercellular communication is a major factor (*Figure 1*), and dysfunctional cellular crosstalk promotes the development of various types of diseases, including atherothrombosis and cancer (1).

Defective intercellular communication in cancer

Within the same cell-type, such as fibroblasts, it has been shown that cell collective responses to the sensory stimulus ATP (adenosine triphosphate), is dictated not only by the stimulus concentration but also by the degree of communication within the cellular population (2). In fact, when cells interact, they form a small channel called gap junction between adjacent cells. These pores allow small molecules and ions to move from one cell to another, thus promoting the transmission of the signal to adjacent cells and increasing the sensitivity to the stimulus (3). Interesting, Potter *et al.* (2) found that the cancer cells are defective in the multicellular network due to reduced gap junction communication. In fact, the coculture of breast cancer cells with fibroblasts is associated with the reduction of the intercellular communicative process (4).

Direct cell-cell interaction and autacoid release in health and disease

The communication between cells also occurs through specific proteins which mediate the adhesion between cells, one important class of adhesive molecules is that of the integrins. They transmit signals to and from cells, thus sensing the environment and controlling cell shape and motility of cells (5). Specific integrins mediate the interaction of platelets with other platelets or other cell types, including endothelial cells and cancer cells. Integrins mediate platelet adhesion and aggregation two phenomena involved in the maintenance of the integrity of the vasculature (5).

Cells also communicate through the release of several soluble molecules of different chemical nature, including lipids and proteins. They are called autacoids and have the characteristic to act near the site of synthesis because

they usually have a very brief lifetime *in vivo* due to a rapid and intense catabolism. Autacoids work through the induction of paracrine and/or autocrine signaling pathways in adjacent cells or in the same cell which has produced them, respectively. Finally, they can also induce an intracrine signaling, by acting on the producing cell before being secreted (6) (*Figure 1A*). Autacoids are involved in physiologic processes and act via the interaction with specific receptors thus inducing a complex network of signaling pathways which participate in a vast number of cellular responses. Interestingly, some cells, such as platelets, uptake various autacoids and store them into specific granules that will be released upon appropriate cellular stimulation. This a mechanism which can prolong the lifespan of the autacoid in the circulation. An important family of autacoids is that produced by the metabolism of arachidonic acid (AA) (7).

It is oxygenated and further transformed into a variety of lipids which mediate or modulate important physiological and pathological functions, including inflammation, cancer, and atherothrombosis. AA (20:4, ω6) is a major component of cellular membranes esterified in the sn-2 position of glycerophospholipids. Upon cell activation, AA is released by the activity of different phospholipases (PLs), including cytosolic PLA_2. Then, various enzymatic pathways transform AA into the large family of eicosanoids. Among them there are: (I) the prostanoids, i.e., prostacyclin [prostaglandin(PG)I_2], thromboxane A_2 (TXA_2), PGE_2, PGD_2 and $PGF_{2\alpha}$, produced by the activity of cyclooxygenases (COX-1 and COX-2) and downstream synthases (7); (II) the leukotrienes (LTs) LTC_4 and LTB_4 formed by the activity of 5-lipoxygenase (5-LOX) coupled to glutathione S-transferase (GST) and LTA_4 hydrolase, respectively (8); (III) epoxyeicosatrienoic acid (EET) and dihydroxy acids by cytochrome p450 epoxygenase (9) (*Figure 2*). The important role of prostanoids in health and disease has been clearly demonstrated by the use of drugs which target specifically COX-1 and/or COX-2, i.e., the nonsteroidal anti-inflammatory drugs (NSAIDs) nonselective for COX-isozymes (named traditional NSAIDs) or selective for COX-2 (called coxibs). The use of these pharmacological tools together with that of gene-modified mice have enlightened a central role of endothelial COX-2-dependent PGI_2 to protect the vascular system from an excessive adhesion of platelets which leads to the recruitment of further platelets and leukocytes (10). Altogether, these events promote the development of a chronic inflammatory response within the vasculature which is a hallmark of atherosclerosis and atherothrombosis. Moreover, the use of the NSAID aspirin, which at low-doses causes a selective inhibition of platelet function via the acetylation of COX-1, has allowed clarifying the crucial role of platelets in the thrombotic occlusion of arteries involved in blood supply to the heart and brain. Finally, the key role of COX-2-dependent PGE_2 in cancer development and progression has been convincingly demonstrated by clinical and experimental evidence (11).

Platelets induce phenotypic changes of cells

Functions of platelets, beyond thrombosis and hemostasis, include the capacity to influence the gene expression program of other cells. This may occur via the biosynthesis and release of prostanoids, including PGE_2 and the secretion of proteins, such as PDGF and TGF-beta which are stored in α-granules. These molecules present in the platelet releasate influence the phenotype of cancer cells, leading to enhanced invasiveness and malignancy (12,13). These effects are mediated by the overexpression of COX-2, which increases survival of cancer cells, and by the induction of epithelial-mesenchymal transition (EMT), which promote migration and invasiveness of cancer cells. To realize these effects, the direct interaction of platelets with cancer cells synergizes with the release of soluble factors by platelets (12,13).

A vast number of results have shown that an anti-metastatic effect is associated with the use of therapeutics which interrupt the crosstalk between cancer cells and platelets. This is strongly supported by the clinical efficacy of the antiplatelet agent low-dose aspirin to reduce the incidence and mortality for cancer (14).

Extracellular vesicle trafficking and signaling in cellular communication

Another way that cells may use to communicate is via the biogenesis of extracellular vesicles. They are classified as exosomes, shedding vesicles (ectosomes, microparticles) or apoptotic bodies (15).

Exosomes have a diameter of 30–100 nm and are intraluminal vesicles (ILVs) formed inside the multivesicular bodies (MVBs). They are released by the exocytosis of MVBs. RAB proteins have been shown to have a role in exosome secretion (16).

Ectosomes are shedding vesicles larger than exosomes

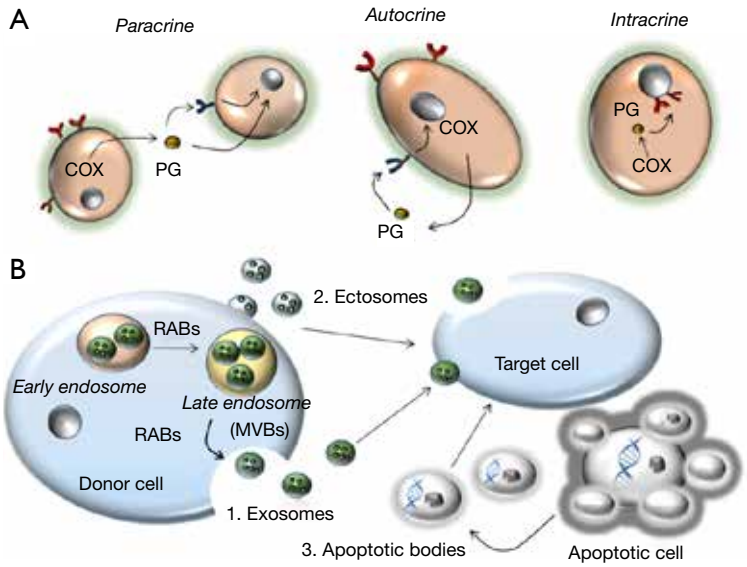

Figure 1 Mechanisms of cell-to-cell communication. (A) Cells can communicate through the release of soluble local mediators called autacoids. Prostanoids (PG) are an important family of autacoids generated from arachidonic acid (AA) by the action of cyclooxygenase (COX) isoenzymes. PG can signal by multiple routes: autocrine, paracrine or intracrine. They usually are secreted and bind to G protein-coupled receptors on the cell surface. In most cases, the target cell is either the cell of origin (autocrine) or a neighboring cell (paracrine). In some cases, prostanoids can bind to nuclear receptors to induce gene expression in the same cell that synthesized them without the occurrence of the release into the pericellular compartment (intracrine); (B) intercellular communication can be mediated by extracellular vesicles (EVs). Indeed, EVs, which are secreted into the extracellular milieu, can deliver the content of a donor cell, such as proteins, messenger RNA (mRNA), noncoding RNA (ncRNA), microRNA (miRNA) and DNA, to target cells. Extracellular vesicles are classified as exosomes, ectosomes or apoptotic bodies: (I) exosomes are formed inside late-endosomes [or multivesicular bodies (MVBs)] that can release them by fusing with the plasma membrane, with the contribute of several RAB proteins; (II) ectosomes are ubiquitous vesicles assembled at and released from the cellular plasma membrane; (III) apoptotic bodies are released as blebs of apoptotic cells and contain fragmented DNA arising from breaking of dying cells.

(with a diameter of 100 nm to 1 μm) and are produced by direct plasma membrane blebbing in regions of lipid rafts (i.e., specialized lipid microdomains constituted by cholesterol associated with sphingomyelin and glycerophospholipids). Tumor cells constitutively shed scores of ectosomes. In contrast, in normal cells this phenomenon is small, but the rate of release can be increased by cell activation. Finally, cells can release apoptotic bodies which are larger than ectosomes or exosomes (>1 μm in diameter) and are released as blebs of apoptotic cells (17) (*Figure 1B*).

Extracellular vesicles contain proteins, mRNAs, and microRNAs (miRNAs) that can be transferred to nearby cells or a distant cell via the blood circulation. However, the mechanisms involved in the uptake and the integration of these molecules into the recipient cell are poor understood (17).

Several lines of evidence suggest that the mRNAs of secreted vesicles derived from different types of cells (including embryonic stem cells or tumor cells) can be delivered to target cells where they are translated into functional proteins. Also, miRNAs can be delivered to target cells (18). Moreover, it has been reported that the phagocytosis of apoptotic bodies leads to the transfer of DNA into fibroblasts and endothelial cells (19).

Colorectal cancer cell-derived exosomes have important roles in tumor progression and the formation of distal metastasis by delivering miRNAs, mRNAs, and proteins.

Recently, it has been shown that hypoxic colorectal cancer cell-derived exosomes promote angiogenesis through the delivery of Wnt/β-catenin signaling in endothelial cells. Interestingly, RAB27a knockdown suppressed exosome secretion by colorectal cancer cells associated with the inhibition of proliferation and migration of endothelial cells (20).

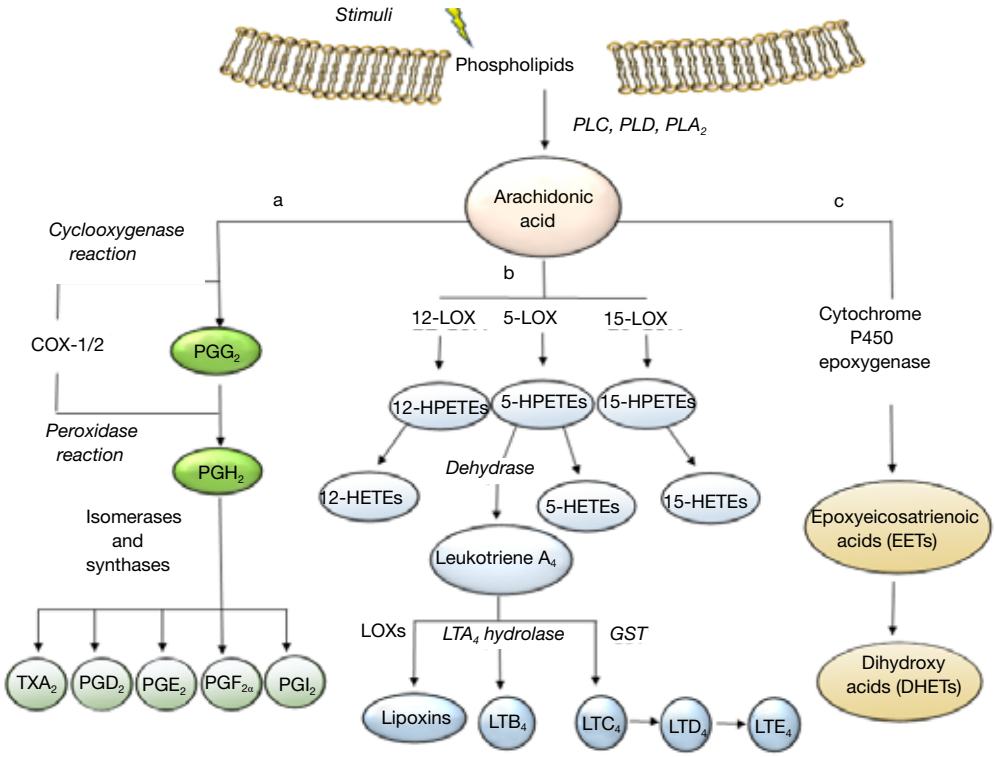

Figure 2 Arachidonate metabolism pathways. Several stimuli, such as thrombin, histamine and platelet-derived growth factor (PDGF), can induce the transformation of membrane phospholipids into arachidonic acid (AA), through three different phospholipases: phospholipase A_2 (PLA_2) phospholipase C (PLC) and phospholipase D (PLD). AA is oxygenated and transformed into several products which mediate or modulate different biological responses by three different major pathways: the cyclooxygenase (COX), the lipoxygenase (LOX) and the cytochrome P450 epoxygenase (CYP-P450) pathways. (a) AA can be transformed to prostaglandin G2 (PGG_2) by the cyclooxygenase reaction and, then, to prostaglandin H_2 (PGH_2) by the peroxidase reaction. PGH_2 is metabolized in prostaglandins (PGD_2, PGE_2, $PGF_{2\alpha}$), prostacyclin (PGI_2) and thromboxane A_2 (TXA_2) by different isomerases or synthases; (b) the second major pathway of AA metabolism involved a class of enzymes called lipoxygenase (12-, 5-, and 15-LOX) which transform the arachidonate to hydroperoxides (12-, 5- and 15-HPETE). The hydroperoxides are converted by the peroxidases into hydroxyeicosatetraenoic acid (12-, 5- and 15-HETE). The 5-HETE can be dehydrated, by the dehydrase enzyme, into the unstable epoxide intermediate leukotriene A_4 (LTA_4) which can be hydrolyzed to form dihydroxy-derivative (LTB_4) or can be linked with glutathione to produce LTC_4. This leukotriene is transformed to LTE_4 by glutamyl transferase and aminopeptidase actions while LTA_4 can also function as a precursor of the lipoxins; (c) cytochrome(CYP) P450 epoxygenases convert AA to epoxyeicosatrienoic acids (EETs). The EETs are short-lived, being rapidly converted to less active or inactive dihydroxy-eicosatrienoic acids (DHETs).

Platelet-dependent cellular communication via the delivery of vesicle cargo

Upon activation, platelets release exosomes and microparticles (MPs). Recently, Dervin *et al.* (21) have characterized the proteome of human platelet exosomes thus showing that a population of these vesicles carries active Wnt glycoproteins on their surface that can modulate Wnt signaling activity in both endothelial and monocytic cells (21).

We have recently shown that incubation of the HT29 colon cancer cell line with platelets is associated with a time-dependent induction of β-catenin translocation into the nucleus of HT29 cells causing the rapid increase in COX-2 mRNA levels (22). This effect was associated with the detection of Wnt3a in the supernatant of platelet-HT29 cell cocultures but not in that of HT29 cells cultured alone. Altogether, these results suggest that Wnt released by activated platelets leads to β-catenin translocation into the nucleus, thus causing the activation of target genes, such as COX-2, of the transcription factor T-cell factor (Tcf)/lymphocyte enhancer (Lef) (22).

miRNAs are short (20–25 bp) RNAs that can regulate gene expression through various mechanisms. Profiling miRNAs (miRNome) of exosomes derived from human platelets has been recently characterized. Platelets contain miRNAs which may regulate platelet function. Moreover, platelet exosomes enriched with miRNAs can be secreted upon activation to influence the behavior of targeted cells (23).

Platelets can uptake different molecules, including genetic material, thus, these cells can change their cargo depending on a pathological environment. It has been shown that tumor-associated blood platelets provide accurate information on the location and molecular composition of the primary tumor. This information can be used for early detection of cancer or its progression (prognostic information), real-time monitoring of treatment (24). However, the different platelet phenotype of cancer patients may also play a significant role in the progression of the disease through the possible delivery of pathological signals to stromal cells and cancer cells.

Conclusions and perspectives

The rapid development of new omic technologies, such as genomics, transcriptomics, proteomics and metabolomics together with that of microscopic imaging platform technology combined with the development of novel optical biosensors and sophisticated image analysis solutions have allowed making a leap forward in understanding how cells communicate with each other. Importantly, it has been clarified that cells can exchange genetic material which can be incorporated into the recipient cells but also different proteins. In this context, it has been enlightened the role of extracellular vesicles in the crosstalk between cells. The release of the various types of vesicles may be altered in pathological conditions thus contributing to the development of different diseases (25). In fact, extracellular vesicles can contain a different cargo depending on the clinical conditions. Vesicles released from nucleated cells contain different molecules derived from the cell itself.

In contrast, the anucleate platelet uptakes various factors present in the environment which are then released as soluble forms or within vesicles. The delivery of the cargo of exosomes to the recipient cell may induce important changes in cellular functions, thus contributing to the development of diseases, including cancer (25). This knowledge opens the avenue to the development of novel therapeutic strategies to prevent cancer and other pathological conditions, including atherothrombosis and tissue fibrosis, by affecting the release of vesicles and their delivery to target cells. To realize this aim, we have to improve our knowledge on the biogenesis of extracellular vesicles and their trafficking. The time is ripe to achieve this goal because we own the appropriate technology and experimental models. The new information should be rapidly translated to humans to confirm the efficacy of these novel therapeutics approaches to prevent and cure different diseases.

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Footnote

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Is the exosome a potential target for cancer immunotherapy?

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Exosomes are nano-sized (30–100 nm in diameter) membrane vesicles of endocytic origin, which interact with other cells by transferring proteins, lipids, DNA, mRNA, and microRNA (miRNA) (1,2). Exosomes have diverse biological functions depending on the cell type of origin, such as tumor cell invasion, intercellular communication, and antigen presentation (3,4). In 2012, Peinado *et al*. (5) revealed that highly metastatic melanomas could reprogram bone marrow progenitor cells through transferring the receptor tyrosine kinase MET via exosomes, and thus increase their metastatic ability. Subsequently, Hoshino *et al*. (6) demonstrated that tumor-derived exosomes uptaken by organ-specific cells prepare the pre-metastatic niche. Specifically, exosomal integrins α6β4 and α6β1 were associated with lung metastasis, while exosomal integrin αvβ5 was linked to liver metastasis. More recently, Nakamura *et al*. (7) reported that ovarian cancer-derived exosomes transfer CD44 to surrounding peritoneal mesothelial cells, thereby facilitating cancer invasion.

Tumor-derived exosomes are also known to exert different actions on the immune system, which affects cancer progression. Several reports have shown that tumor-derived exosomes facilitate immunosuppression and promote tumorigenesis by inhibiting the immune response (8). For instance, Zhou *et al*. (9) demonstrated that pancreatic cancer-derived exosomes down-regulate the expression of Toll-like receptor 4 in dendritic cells (DCs) via miR-203, inducing immune tolerance. Thus, they considered that tumor-derived exosomal miRNAs may down-regulate the anti-tumor activity of DC/cytokine-induced killer cells (CIKs), suggesting that the depletion of exosomal miRNA would enhance the anti-tumor activity. Recently, the same group further demonstrated that exosomal miRNAs can be removed by lysis and ultrafiltration without eliminating immune-regulating proteins, and that treatment with these miRNA-depleted exosomes could enhance the tumor cell-killing capacity of DC/CIKs (10). Thus, they suggested that miRNA depletion from tumor-derived exosomes may be a promising approach for activating DC/CIKs against cancer, opening the door toward development of a novel cancer immunotherapy.

Other researchers have also shown that tumor-derived exosomes can enhance immunostimulation and therefore serve as cancer vaccines. Chen *et al*. (11) evaluated the efficacy of exosomes derived from heat-shocked mouse B lymphoma cells (HS-Exo) in the induction of antitumor immune responses. They demonstrated that the heat-shock proteins HSP60 and HSP90 were more abundant in HS-Exo compared with control exosomes derived from the same cells, and induced significantly increased antitumor immune responses. Rao *et al*. (12) showed that hepatocellular carcinoma cell-derived exosomes (HCC TEX) serve as a carrier of tumor antigens and induce a strong DC-based antitumor immune response. In their study, HCC TEX-pulsed DCs increased the number of $CD8^+$ T lymphocytes and interferon-γ levels, and reduced the levels of immune-inhibitory interleukin-10 and transforming growth factor-beta in orthotopic HCC mice. In another study, vaccination with nanovesicle-bound antigens derived by the homogenization and sonication of primary melanoma

tissues decreased tumor growth and metastasis in mice (13).

The therapeutic benefit of exosome vaccination has not yet been verified in a clinical setting; however, some clinical trials have shown that disease progression was halted in a portion of patients in phase I clinical trials (14,15). Dai *et al*. (15) reported a phase I study in which tumor ascites-derived exosomes (Aex) were isolated and reintroduced in combination with granulocyte-macrophage colony-stimulating factor (GM-CSF) into a total of 40 patients [HLA-A0201(+)CEA(+)] with advanced colorectal cancer. They found that Aex plus GM-CSF but not Aex alone could induce a beneficial tumor-specific antitumor cytotoxic T lymphocyte response with safe and tolerable profiles. The National Institutes of Health funds research related to extracellular RNA (exRNA) encapsulated in extracellular vesicles such as exosomes, including studies on the clinical use of exRNAs for therapy development (16). Collectively, the evidence collected to date indicates that cancer vaccine therapy targeting exosomes has potential as a novel cancer treatment in the near future.

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Footnote

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Vesicles & Cancer Immunotherapy

Cancer exosomes: wanted by many, explored by few, waiting for one

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Hepatocellular carcinoma (HCC) is the second most common cause of cancer-related deaths worldwide and has a five-year survival rate of fifteen percent. It is projected that liver cancer will be one of the top three cancer killers by 2030 (1). HCC is an aggressive tumor type with poor prognosis due to the diverse etiological factors implicated in the development of HCC. In addition, heterogeneity and late diagnosis further complicate survival of HCC patients. Despite many potential therapeutic targets, the overall survival of HCC remains very poor.

Tumor cell-derived exosomes (TEXs), secreted nanovesicles that transfer proteins, DNAs, messenger RNAs, and miRNAs, are emerging as key players in HCC progression. Recent models suggest that exosomes play an important role in cancer progression by mediating immune responses. Exosomes also participate in horizontal intercellular communication (i.e., trafficking among different cell types). A recent review by Wu *et al.* provided insights into the roles of exosomes in HCC and its potential as diagnostic and prognostic tools to improve HCC therapies (2).

Exosomes are extracellular vesicles (EVs) released from cells for meditating intercellular communications. While there are many nomenclatures for exosomes, it should be noted that exosomes are different from ectosomes, which are larger in size, released by the plasma membrane, and are characterized by TyA and C1q markers. Exosomes, however, are smaller in size, released through multivesicular bodies (MVB) at a much slower rate, and are characterized by specific markers such as CD63 and CD61. These subtle differences, while distinct, are difficult to differentiate during purification or isolation. While exosomes and ectosomes presumably function very similarly once released, it remains to be clarified whether they possess different biological effects. The lack of standardized procedures to purify, isolate, or prepare EVs and the inevitable contaminants, such as protein aggregates, lipoproteins, or apoptotic fragments (3), further complicate the exact roles of exosomes in cancer.

As highlighted by Wu *et al.*, the cargoes of TEXs differ greatly and are dependent on the cell types. Hepatocytes, non-parenchymal immune cells, and parenchymal liver cells in liver can release exosomes. Thus far, the majority of exosomal cargoes have been characterized from hepatocytes. These include miR-21 as a biomarker for chronic cirrhosis and HCC from healthy patients (4,5). Members of the *let*-7 miRNA family and the MET oncoprotein are also found (6,7). Further investigations on exosomes released by hepatic stellate cells and Kupffer cells, however, will be equally important to elucidate their roles in tumorigenesis, inflammation, and fibrosis in the liver.

The unusual participation of secreted exosomes in horizontal communication further compounds the sophistication of exosomes in liver pathology. As discussed

by Wu *et al.*, miRNAs, such as miR-21, miR-192, and miR-221, enriched in colorectal cancer cell exosomes could mediate invasion and metastasis of HCC cells. Uptake of pancreatic cancer cell exosomes by Kupffer cells further illustrates previously uncharacterized mechanism of action in establishing pre-metastatic niche required for liver metastasis (6). TEXs have also been demonstrated to transfer its contents to modulate immune functions in T and B lymphocytes, promote angiogenesis *via* endothelial cells, and enhance cell migration or invasion by modulating macrophages in microenvironment (6,8,9). Thus, exploring trafficking of exosomes among different cell/tissue types will also be important to investigate HCC progression.

The regulation on exosome stability and its carried cargoes are also emerging as critical parameters in exosome research. TEXs are encapsulated in a lipid bilayer that is stable, allowing protection of its cargos within. Wu *et al.* discussed that modulation and degradation of exosomal RNA species may lie on RNA binding proteins (RBP), such as high-density lipoproteins, AGO2 and ELAVL1. For example, recent evidence suggests that RBP-RNA complex is important in maintaining the stability and loading of cargoes into exosomes (10). Since RBPs regulate gene expression by modulating the maturation, stability, transport, and translation of its RNA targets, the biological function of exosomes could be altered by changes in RBP-RNA complex. This is demonstrated in a recent study which showed that active KRAS signaling suppresses AGO2 interactions with endosomes and secretion of miRNA *via* exosomes (10). Given that RAS activation has been shown to promote HCC and approximately 5% of HCC harbors a KRAS mutation, it is reasonable to surmise that exosomal dysregulation due to aberrant RAS activation also drives HCC progression (11). In addition to RAS activation, other key cancer drivers, such as β-catenin, may promote tumorigenesis through the deregulation of RBP-RNA complexes (12). It has also been suggested that the alterations of the tumor suppressor p53 would affect overall exosome biogenesis, landscape of EVs, and the secretory profile in tumorigenesis as previously proposed (13).

Besides RNA species, TEXs have been described to carry oncoproteins and tumor suppressors such as PTEN (14). Membrane bound ligands and growth factor signaling receptors are also found in exosomes. The vast difference in protein cargoes expands the diversity and subtypes of exosomes. Investigation of exosomes and its cargoes will be key to illuminate diagnosis and prognosis for HCC and other cancers.

Last but not least, exosomes serve as a great means for delivery. Given that many ideal therapeutic targets are expressed intracellularly, and thus "un-targetable" by conventional therapy, a Trojan horse exosome approach may find utility for cancer drug delivery. Previous studies propose a Trojan horse model for customized exosomes (15). In reference to current trends in artificial nanoparticles, selection of cargoes, destination of specific targeting, and functional consequence upon its uptake/delivery will be critical parameters for the potential therapeutic use of customized exosomes.

In conclusion, exosome research is certainly an exciting field with strong clinical implications and vast basic biology for future investigation. Complexity and confusion of current exosome research, however, should strongly be noted. For example, the lack of standardized protocols for isolating and purifying exosomes make it difficult to distinguish different EVs, their sub-types and trafficking in HCC. We suspect that as the field progresses, new and improved isolation methods including biophysical, molecular and microfluidic methods will significantly augment the current limitations. With these improvements, our understanding of exosome stability and secreted cargoes will evolve, giving hints to the underlying mechanism of exosomal targeting and kinetic profile. Another important highlight in exosome research is our current understanding of how components of exosomes such as proteins, miRNAs and lipids may be effectively used for prognostic and diagnostic purposes. While recent studies demonstrated the potential importance of exosomes for clinical diagnostics, the field is still at its early stage. Future studies will broaden the understanding of exosome biology and improve our ability to exploit exosomes for the treatment of HCC and other cancers.

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Footnote

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Extracellular vesicles: emerging mediators of intercellular communication and tumor angiogenesis

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Rapidly expanding solid tumors demand large quantities of nutrients and oxygen. Growing tumors exploit pre-existing vessels and/or develop new vessels (vascularization) to obtain these nutrients. Vascularization is classified into two main processes, vasculogenesis and angiogenesis. Vasculogenesis is the formation of primitive blood vessels by endothelial progenitors, and angiogenesis encompasses the subsequent remodeling processes, including growth and migration of endothelial cells, sprouting, and stabilization of these sprouts by mural cells (1,2). Tumor cells utilize these processes to satisfy their needs and grow (tumor angiogenesis), especially under hypoxic conditions. One of the pro-angiogenic mechanisms exploited by hypoxic cancers is hypoxia-inducible transcriptional factor (HIF)-mediated signaling. Hypoxia leads to stabilization and nuclear translocation of HIF-1α, and subsequently increases transcription of pro-angiogenic genes, including vascular endothelial growth factor (VEGF) (3). Overexpression of HIF-1α has been reported in various solid tumors including brain, bladder, breast, colorectal, ovarian, pancreatic, and prostate cancers (4,5), and increased vascularity is a hallmark of poor prognosis.

Recent studies have offered new insights into the significant role of extracellular vesicles (EVs) in tumor angiogenesis. EVs are nanometer-sized membranous vesicles that carry genetic information including proteins, mRNAs, and microRNAs. EVs were previously thought to be waste materials; however, recent accumulating evidence has revealed that almost all cell types secrete and use EVs for horizontal exchange of cargo, that is, primitive intercellular communication. Cancer cells in particular are known to secrete large amounts of EVs to expand their niche. A recent article published in *Oncology Research* by Huang and colleagues demonstrated that HIF-1α is overexpressed in hypoxic colorectal cancer (CRC) cells, which increased Wnt4 secretion via EVs, activated Wnt/β-catenin signaling in recipient endothelial cells, and consequently promoted angiogenesis and tumor growth (6). This evidence has highlighted the importance of EVs as a tool for intercellular communication in rapidly expanding tumors. However, as EV biology is still a developing research field, several issues are currently the subject of intensive discussion.

How is cargo sorted and loaded into EVs?

It remains unknown why EVs from hypoxic cancer cells have a stronger pro-angiogenic effect than normoxic cancer cells. Huang *et al*. showed that Wnt4 was enriched in EVs from hypoxic CRC cells compared with EVs from normoxic CRC cells. As shown in *Figure 1A*, it was unclear whether this was due to an increase in the quantity of specific cargo (in their study, Wnt4) loaded into single EVs. The standardized methods for EV isolation and quantification remain controversial (7). Furthermore, it remains unclear how cargoes are sorted and loaded into EVs. No specific

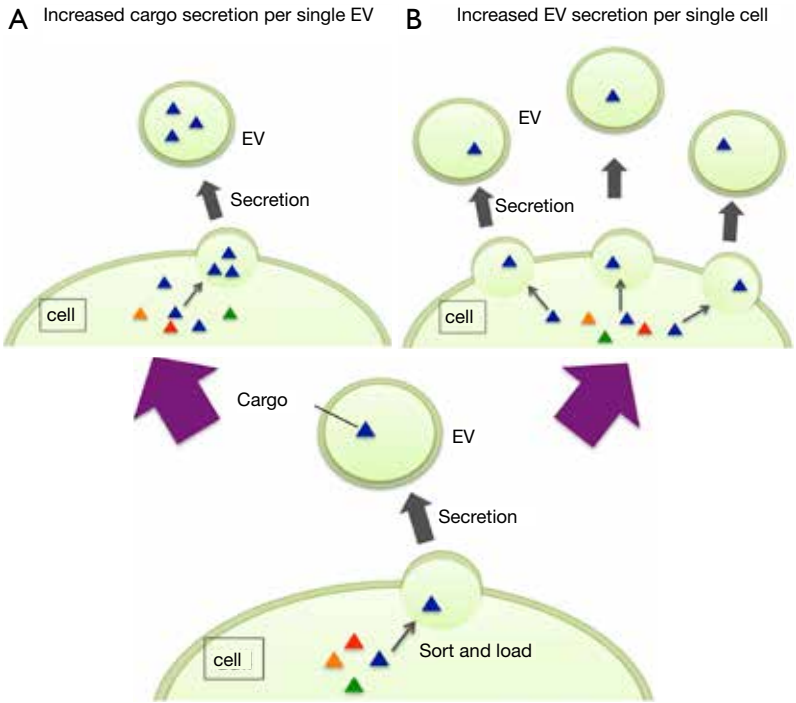

Figure 1 Absolute quantification of EV contents is difficult. Specific molecules are sorted and loaded into EVs. (A) Increased cargo quantity per single EV; (B) increased secretion of EVs per single cell. The total amount of cargo secreted per single cell is the same in (A) and (B), but it remains unclear how they can be distinguished. EV, extracellular vesicle.

molecule has been identified that can be used as an internal control for the quantity of EV contents. To date, researchers have managed to estimate relative quantities using various methods, including nanoparticle tracking analysis (NTA) to estimate the number of EVs per mL (medium) or per cell, and division of a specific cargo by the total quantity of EV proteins or EV RNAs to estimate the quantity of a specific cargo per EV (8-10). However, although these relative quantifications are acceptable for use in single studies, they cannot be used to conduct meta-analysis between independent studies. To promote further understanding of EV biology and the clinical significance of EVs, many challenges in the evaluation of EV contents remain.

Regulation of EV secretion

Again, why do EVs from hypoxic cancer cells have such a strong pro-angiogenic effect? As shown in *Figure 1B*, it might be due to an increase in the quantity of EVs secreted by single cells. However, it is possible that, although cargo quantity differs between EVs in (A) and (B), the total amount of cargo secreted per cell is the same. There is no method to distinguish them. It is also unknown which one we observe as "an increased secretion of cargo". The mechanism of EV secretion is currently being intensively investigated, and the full picture is gradually being revealed.

Anti-angiogenic therapy has been studied as a powerful tool to prevent cancer progression by reducing the supply of nutrients and oxygen. The first anti-angiogenic molecular targeting agent, bevacizumab, which is an anti-VEGF-A monoclonal antibody, is now included in various chemotherapy regimens. There is a possibility that anti-EV secretion therapy will be a new strategy to starve tumors, by cutting off intercellular communication between cancer cells and stromal cells. Liquid biopsy for EVs is also considered to be a promising non-invasive method for cancer diagnosis and monitoring. Although many researchers tend to concentrate on the clinical utility of EV contents, basic knowledge of EV biology remains limited. I hope that furthering our understanding of EV biology will reveal the complex pathways of communication between the human body and tumors and contribute to the development of new cancer therapies.

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Footnote

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Vesicles & Cancer Immunotherapy

Exosomes as delivery vehicles: a commentary on "Amoxicillin haptenates intracellular proteins that can be transported in exosomes to target cells"

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Exosomes are lipoproteic nanosize vesicles of endocytic origin that are released in the extracellular space by several cell types when a multivesicular body fuses with the plasma membrane (1). To date, accumulating evidences highlight the potential of exosomes as messenger in intercellular communication. Indeed, several studies reported the presence of lipids, proteins, metabolites and nucleic acid (DNA, mRNA, microRNA, lncRNA) in exosomes and highlight their functional role in physiological and pathological processes (2).

These specialized nanovesicles behave as shuttles among cells; it is now largely accepted that exosomes influence tumor formation, progression as well as its dissemination by spreading cancer cell properties (3). In addition, exosomes are involved in several neurological diseases, by affecting neuronal communication (4), and are able to induce immunosuppressive or immune-activating effects on different steps of the immune response (5). Furthermore, accumulating evidences highlight the possibility that exosomes can modulate the response to therapy, inducing drug resistance, by exporting chemotherapeutic agents (6).

In addition to animal cells, exosome-like vesicles are released by plant (7) and bacteria (8). It has been observed that bacteria-derived extracellular vesicles have a role in the development of antibiotic resistance; indeed, Ciofu and colleagues showed that β-lactamase, the enzyme responsible for β-lactam resistance, is a constituent of *P. aeruginosa* derived membrane vesicles (9).

Recent studies provided evidences of the use of exosomes for therapeutical applications. Several groups demonstrated that, based on the molecules expressed on their surface, exosomes can be used as drug delivery systems to target specific cells. It has been shown, for example, that exosomes can deliver drugs or small RNA molecules, inhibiting cancer cells proliferation *in vitro* and *in vivo* animal models. Furthermore, exosomes have been considered in the development of vesicle-based vaccines in particular to induce an immune response against tumors or in some infectious diseases (10,11).

However, further studies are needed for the clinical translations of exosome-based therapeutics.

In addition, due to their content, that reflects that one of the cell of origin, exosomes represent a rich source of information. Exosome lipid bilayer confers stability to the vesicle content in biological fluids; thanks to the advances in exosome detection and purification, as well as to the sensitive technologies applied to the analysis of their content, exosomes are now considered as biomarker transporters in several pathological conditions.

Several drugs, and in particular β-lactams, have been shown to stimulate allergic reactions. Among the causes of the allergic response observed in β-lactams treated patients, there is the hapten hypothesis. Specifically, β-lactams are responsible for the formation of drug-protein adducts, a

protein complex that is generated when electrophilic drugs or reactive drug metabolites covalently bind to protein molecules. Indeed, usually, drugs bind to plasma or tissue proteins in a reversible way, when this binding is covalent, an immune response may be induced.

β-lactams have high capacity to form covalent adducts with proteins through the opening of the β-lactam ring by the free amino groups of the protein; in particular, amoxicillin mainly forms adducts with albumin, transferrin, and with heavy and light chains of immunoglobulin.

Among β-lactams, amoxicillin is a wide spectrum antibiotic largely used for several bacterial infections and is among the drugs that most frequently induces allergic reactions (12).

To date haptenated proteins have been found in serum and several methods have been developed to detect and quantify them, aiming to a better understanding of drug toxicity. Identification approaches can be divided in global analysis and targeted analysis. The global approach is based on the use of radiolabeled drugs combined with 2D-gel electrophoresis and mass spectrometry in order to identify protein target of drug metabolites in *in vitro* experiments or *in vivo* animal models. A targeted method is a more sensitive approach and can be applied to patient samples; specifically it is based on the identification of a single protein target, giving specific information on how drugs modify the target, altering its function.

However, monitoring drug-protein adducts still remain one of the challenge in the field, and in particular for pharmaceutical industries. One of the major problems associated with adducts detection is its half-live and the *in vivo* concentration which is generally very low; indeed, around 1% of the protein is generally adducted. Therefore, efforts have been made for the development of more sensitive methods for haptenated proteins identification with a consequent improvement of the diagnostic procedures applied to avoid drug allergic reactions.

In the past, the most used approaches to identify β-lactam-protein adducts were based on immunological methods. Carey *et al*. reported that the treatment with flucloxacillin results in the formation of hepatic protein adducts (13), while Magi showed that ampicillin treatment induced the formation of adducts with albumin and transferrin (14).

Recent advances in proteomic techniques allowed to apply mass spectrometry analysis in order to study β-lactams adducts with human serum albumin (15).

In 2012 Ariza *et al*. used for the first time high resolution MS analyzer (orbitrap) coupled to nanoscale capillary liquid chromatography to an in-depth identification of amoxicillin-protein adducts. In addition to human serum albumin, authors identified other proteins as amoxicillin target; among these proteins, transferrin appeared highly modified by the drug (16).

More recently, the same research group developed a new sensitive tool for the study of protein haptenation by amoxicillin, by creating a biotinylated amoxicillin (AX-B) analog (17). Authors found that AX-B is able to form protein adducts similarly to the drug alone; in particular the two forms of the drug interacts with the same serum targets. By using the same approach, authors recently observed that amoxicillin binds to proteins present in exosome and that amoxicillin-protein adducts are transported by the vesicular carriers (18).

In their recent paper "Amoxicillin haptenates intracellular proteins that can be transported in exosomes to target cells" (18), Sánchez-Gómez and coworkers provided the first evidence of the presence of drug-adducts in circulating exosomes. In particular they showed that several intracellular proteins are haptenated by amoxicillin (AX) and released by cells in the soluble fractions as well as in exosomes.

Specifically, authors treated a lymphocytic B cell line with penicillin, amoxicillin and AX-B analog (17); they found that the treatment with the drugs induced the haptenation of several proteins that were present in the cell lysate as well as in the three fractions of the conditioned medium corresponding to membrane, extracellular vesicles and soluble proteins. These data suggested that modified proteins could be secreted from cells both in soluble and exosomal fractions. Mass spectrometry analysis allowed to identify proteins haptenated by amoxicillin; among these, authors found HSP70, EF-2, actin and α-enolase. In addition they found that haptenated proteins present in the exosome fraction could be delivered among cells and internalized by endothelial cells, contributing to the allergic response observed after amoxicillin treatment (18).

Overall, the presence of drug-protein adducts in exosomes could represent a new way to detect haptenated proteins in the circulation.

At the same time this study strongly highlights, as noted by the authors, a new mechanism for drug-protein adduct transfer among cells, thus providing the evidences of a vesicular sorting (*Figure 1*).

However, further studies are required in order to better understand the role of exosome-enclosed drug-protein

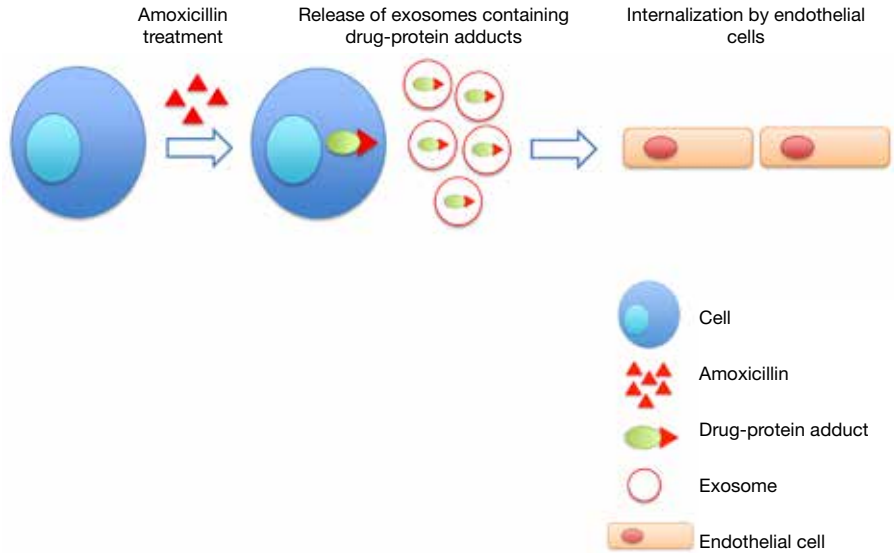

Figure 1 Schematic representation of exosome delivery of drug-protein adducts. Amoxicillin treatment induces the intracellular formation of drug-protein adducts that are released in the extracellular space by exosomes. Exosomes, containing proteins haptenated by amoxicillin, influence target cells phenotype; the internalization of drug-protein adducts by endothelial cells could be responsible for the development of the allergic response.

adducts in contributing to the *in vivo* allergic responses observed in amoxicillin-treated patients.

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Footnote

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Dendritic cell-derived exosomes for cancer immunotherapy: hope and challenges

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Dendritic cells (DCs) are the most potent antigen-presenting cells in the human body. When primed with antigens, DCs activate both helper and killer T cells and B cells. In the past decade, DC-based vaccine has emerged as an important strategy for cancer immunotherapy (1). Several phase I or II clinical trials of DC-based immunotherapy to treat different cancers have been reported with increased survival and mild vaccination-related side effects (2,3). However, DC-based immunotherapy has several limitations. For example, molecular composition of DCs may change and is difficult to be defined (4). Tumor cells may secret soluble immunosuppressive cytokines that could convert immature DCs into tolerogenic DCs, which may activate Treg cells (5). Live DCs are inconvenient for storage (4) and have to be produced locally with quality control issues.

To address these challenges, DC-derived exosomes (dexosomes or Dex) have been developed as an alternative approach of cancer vaccines (4). Exosomes are cell-derived vesicles of endosomal and plasma membrane origin, which are released into extracellular environment. These extracellular vesicles were first discovered in the immature red blood cells in 1987 (6) and represent an important means for intercellular communication. B cells were reported to secrete antigen-presenting exosomes capable of inducing antigen-specific MHC class II-restricted T cell response in 1996 (7). Subsequently, Zitvogel *et al*. described that DCs also secrete antigen-presenting exosomes (8). Both immature and mature DCs can release exosomes. Dex possess many molecules necessary for antigen presentation, such as MHC class I, MHC class II, and costimulatory and adhesion molecules (9). Tumor peptide-pulsed Dex activate antigen-specific cytotoxic T lymphocytes (CTLs) *in vivo* to eradicate or suppress growth of established murine tumors in an MHC- and $CD8^+$ T cell-dependent manner (8). Thus, exosome-based cell-free vaccines represent an important strategy to suppress tumor growth.

In a recent phase II clinical trial published in *Oncoimmunology* (10), Besse *et al*. attempted to use Dex as maintenance immunotherapy after chemotherapy cessation for patients with non-small cell lung cancer (NSCLC). They reported that Dex boosted antitumor activity of natural killer (NK) cells. In this study, they prepared second-generation Dex by differentiating patient's monocytes into immature DCs with GM-CSF and IL-4, followed by induction of mature DCs with INF-γ and loading of tumor-associated antigenic peptides. IFN-γ was reported to induce the expression of costimulatory molecules and ICAMs in DCs (11). Compared to the first-generation INF-γ-free Dex for phase I trial (12), INF-γ-Dex prepared from peptide-loaded mature DCs exhibited a mature phenotype with upregulated expression MHC class II molecules, tetraspanins, CD40, CD86 and ICAM-1/CD54. These data suggest that INF-γ treatment has the potential to improve the capacity of Dex for antigen presentation and T cell activation, as previously reported (11).

The phase II trial was to investigate whether INF-γ-Dex as maintenance immunotherapy can improve the rate of progression-free survival (PFS) at 4 months after chemotherapy (10). This study enrolled 22 patients with advanced NSCLC, all of which had received four cycles of platinum-based chemotherapy. Before the Dex trial, 14 patients showed stabilization, while eight patients experienced a partial response. Patients were administered metronomic oral cyclophosphamide to reduce Treg cell function and stimulate dual INF-γ/IL-7-producing T cells before intradermal injection of INF-γ-Dex. Presumably, this may facilitate Dex-mediated T cell priming and restore T cell and NK cell functions.

The results showed that seven patients (32%) remained stable after nine injections. The PFS rate at 4 months was 32%, and the median PFS for all 22 patients was 2.2 months. There was no objective tumor response according to the Response Evaluation Criteria in Solid Tumors (RECIST) criteria. The median overall survival (OS) for all patients was 15 months with a survival rate at 6 months of 86%. One patient developed a grade three hepatotoxicity, and there was no treatment-related death. Thus, Dex are well tolerated.

Despite the intention behind the development of the second-generation Dex, the study failed to detect T cell response (10). However, an increase in NK cell functions was observed in a fraction of these NSCLC patients. Importantly, MHC class II expression levels of the final INF-γ-Dex product correlated with expression levels of the NKp30 ligand BAG6 on Dex, and with NK functions. NK cell activation in turn was associated with longer PFS. Interestingly, a potential increase in NK cell lysis ability was also observed in a phase I clinical trial of INF-γ-free Dex (13).

This phase II trial showed only limited efficacy for Dex immunotherapy. The primary endpoint of the trial was to observe at least 50% of patients with PFS at 4 months after chemotherapy cessation (10). However, this objective was not achieved. Several reasons may explain why Dex therapy had limited efficacy. First, cancer antigens loaded onto exosomes might not be clinically relevant due to the heterogeneity of the patient cohorts. Second, INF-γ used in the process of Dex production may upregulate programmed death ligand-1 (PD-L1) on DCs and Dex (14). Finally, the limited efficacy may be attributed to the lack of adaptive immune response, particularly antitumor $CD8^+$ T cell response.

Several strategies may improve the efficacy of Dex immunotherapy for future clinical trials. A previous study indicated that protein-loaded Dex, but not peptide-loaded Dex, induced CTL response and inhibited tumor growth, both of which require the activation of $CD4^+$ and B cells (15). Therefore, full-length tumor-associated antigens (TAAs) may be used to replace their peptides to promote the activation of helper T and B cells, which may in turn boost $CD8^+$ T cell response. Dex loaded with multiple TAAs may expand the coverage for the heterogeneity of human tumors. Mutated TAAs could be used to improve T and B cell response (16). Dex may be engineered to upregulate the expression of costimulatory molecules. Engineered Dex could also carry mRNAs coding for relevant TAAs or activation molecules. The expression of PD-L1 and PD-L2 on Dex should be quantified and downregulated. Immunotherapies to block immune checkpoint molecules, such as CTLA-4 or PD-1, can enhance lymphocyte response (17) and could be used in combination with Dex to boost antitumor T and B cell response. A previous phase I clinical trial showed that ascites-derived exosomes (Aex) in combination with GM-CSF, but not Aex alone, induced beneficial tumor-specific CTL response (18). Combination therapy of Dex with GM-CSF may further elicit antitumor CTL response. These strategies could improve the outcomes of future clinical trials with Dex.

Despite the drawbacks and challenges, the study by Besse *et al.* is the first phase II clinical trial of Dex. As a new vaccine strategy for cancer immunotherapy, Dex remain promising with potential for improvement. The prospect of Dex therapy is encouraged by the activation of NK cells (10) as well as the promising outcomes of clinical trials with DC-based vaccines (1). Future basic research and clinical trials should focus on how to stimulate CTL response by Dex. The successful development of Dex-based immunotherapy will further expand the weaponry to battle cancer.

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Footnote

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Immunosuppressive role of extracellular vesicles: HLA-G, an important player

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Comment on: Rebmann V, König L, Nardi Fda S, *et al.* The Potential of HLA-G-Bearing Extracellular Vesicles as a Future Element in HLA-G Immune Biology. Front Immunol 2016;7:173.

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Extracellular vesicles (EVs) are bilayer membrane structures composed of proteins, lipids and nucleic acids (DNA and RNA). Their specific cargo is peculiar and derives from the cell of origin. They have been involved in many physiological and pathological processes. Regarding immune biology, EVs play an important role, as they may enhance or suppress the immune system (1).

Therefore, EVs act as regulatory agents in the immune response. They are a heterogeneous population of vesicles originated from cell plasma membrane or from the endosomal compartment. EVs, independently from their origin, may remain in the extracellular space in proximity of their origin or may move into biological fluids reaching distant sites. Indeed, they have been found in plasma, milk, urine, cerebrospinal fluid, amniotic fluid and tumors (2). Cell-released EVs transfer their cargo into receiver cells, transferring information from one cell to another (3). The EVs cargo includes a variety of receptors, soluble proteins, adhesion molecules, enzymes, chemokines, and cytokines; they also contain several nucleic acid species such as mRNAs, microRNAs or long non-coding RNAs (4).

EVs have been proposed as immunotherapeutic agents as they are involved in several aspects of immunity including immune response, immune suppression, immune surveillance and antigen presentation. In particular, EVs isolated from immune cells, for example, dendritic cells (DCs) promote the immune response. At variance, tumor-derived EVs may favor immune escape (5). In this contest, EVs may act in concert with several soluble factors such as PGE^2, IDO and HLA-G (6).

The important role of HLA-G and EVs was well described in a recent review (7). In this paper, the authors clearly describe the function of the secreted form of HLA-G within EVs, compared with the free soluble fraction. The secreted forms of HLA-G are mainly involved in immune tolerance in cancer and pregnancy (7). However, most of the studies do not discriminate the free soluble form from the EV associated HLA-G.

The HLA-G molecule belongs to the family of non-classical human leukocyte antigen (HLA) class I with a low polymorphism and a restricted tissue distribution. HLA-G is present in different molecular structures due to different splicing of the primary transcript. There are seven isoforms. The isoforms HLA-G1, G2, G3 and G4 are transmembrane while the isoforms G5, G6 and G7 lack the transmembrane domains and are soluble (7,8). In addition, all the membrane-expressed isoforms may be cleaved by metalloprotease enzymes and became soluble (9).

Initially, HLA-G was first described to protect the fetus versus the maternal immune system and to be involved in immune-privileged adult tissues. HLA-G has also been shown to regulate immune cells, inhibiting the activation of NKs, T lymphocytes and DCs and to favor tumor immune-escape. In the past, the immune modulatory function of circulating HLA-G was only related to its free soluble form. Riteau *et al.* first described the presence of HLA-G within EVs (HLA-G-EVs) in supernatant of melanoma cell line (HLA-G positive) (10). They found the full-length G1

isoform, usually detected only in cell membrane. It is well known that tumor cells release many factors to orchestrate their progression and EVs play a relevant role in this phenomenon. A few other groups have observed HLA-G within EVs in a tumor microenvironment (11-13). Tumor EVs stimulate angiogenesis, tumor cells growth, favor the metastatization process and induce immune escape (14). In the review of Rebmann *et al.*, the role of HLA-G-EVs in tumor immune escape is well highlighted (7). In renal cell carcinoma cells, EVs carrying HLA-G impair the differentiation process of monocytes in DCs, inhibiting T cell activation and proliferation (7,11). Moreover, the presence of HLA-G-EVs has recently been proposed as negative prognostic factor for neoadjuvant chemotherapy-treated breast cancer patients. Furthermore, increased level of HLA-G-EVs in peripheral blood has been related to circulating stem cell-like tumor cells and poor prognosis. On the contrary, high levels of soluble HLA-G in blood stream suggests a better scenario. This is the first demonstration of a differential role of soluble fractions of HLA-G, depending on encapsulation or not within EVs (7,12).

Then again, it has been known for decades that overexpression of membrane-bound and soluble HLA-G are up regulated or re-expressed in tumors (both solid and hematological) such as renal cell carcinomas, esophageal squamous cell carcinoma, colorectal cancer tissues, breast cancer, melanoma and pancreatic cancer (15). In some cases, the soluble form of HLA-G has been proposed as a diagnostic or prognostic biomarker.

Another fundamental process in which HLA-G is involved is the tolerance of maternal immune system against fetus. In the review of Rebmann *et al.*, it has been underlined that early and term placenta release exosomes containing HLA-G5 (7,16). This observation has important implications in the relationship between trophoblast and maternal immune system (17).

Although the biological activity of HLA-G is well known, the mechanism of action of HLA-G-EVs is still debated. One possibility is the interaction of HLA-G carried by EVs with specific receptors expressed by target cells. Another possibility is the direct transfer of HLA-G to target cells by the EV fusion with the cell plasma membrane. This second mechanism has been reported for T cells which may acquire HLA-G from tumor cells with consequent changes from an activated to a regulatory phenotype. Another option is the internalization of EV-carrying HLA-G with the activation of immune-modulatory intracellular pathways (7).

In conclusion, the HLA-G-bearing EVs is an important element in HLA-G biology. Although many open questions regarding the mechanism of action remain, it is clear that EVs containing HLA-G are biologically active and are potential diagnostic biomarkers in several diseases.

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The discovery of HLA-G-bearing extracellular vesicles: new perspectives in HLA-G biology

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In contrast to classical human histocompatibility antigen (HLA) class I molecules, HLA-G has been discovered quite recently, in 1990. Since its discovery several efforts have been made to define HLA-G biology and its role in regulating immune responses. The main distinctive characteristics of HLA-G are the limited protein variability, alternative mRNA splicing that generates seven different isoforms(both soluble and membrane-bound) with the ability to form multiple structures via disulfide bounds, and restricted expression to certain tissues (1). Moreover, the discovery that HLA-G binds different inhibitory receptors (i.e., ILT2 and ILT4, and KIR2DL4) led to the classification of HLA-G as a tolerogenic molecule (2).

In the past decade much effort has been devoted to define the role of HLA-G in modulating immune responses in transplantation, inflammatory and autoimmune diseases, and cancer. HLA-G and its secreted forms are considered key players in the induction of short- and long-term tolerance. These tolerance-inducing activities render membrane-bound and soluble HLA-G attractive biomarkers for clinical approaches as prognostic factor to monitor disease stage and progression, or efficacy of treatments (3,4).

In different settings of transplantation it was demonstrated that the expression levels of membrane-bound and soluble HLA-G could be a predictive marker for graft stability, suggesting that increased HLA-G levels are associated with down-regulation of immune responses (5). Similarly, several studies performed in solid tumors highlighted the relationship between membrane-bound HLA-G expressed by tumor cells or soluble HLA-G in sera of patients with advanced disease stage, tumor load, or clinical outcome (5). Thus far, membrane-bound HLA-G expression has been proposed as diagnostic tool to stage breast cancer (6). In hematological malignancies, although several studies reported higher plasma levels of HLA-G in patients compared to healthy controls, the correlation of membrane-bound and soluble HLA-G expression with disease staging gave controversial results; therefore, it is still debated whether HLA-G can be used as biomarker of disease progression (5).

The complexity to apply HLA-G as a significant clinical biomarker has been limited by structural diversity of the molecule: HLA-G can be expressed as monomer and dimers in soluble form, and, more recently, it has been shown that it can be expressed associated with extracellular vesicles (EVs) (3). EVs are membrane-limited vesicles released in biological fluids by normal and malignant cells (7). Cells of the innate and adaptive immune system, including T and NK cells and antigen presenting cells, have been reported to release or to acquire informations via EVs. EVs indeed can contain proteins, lipids, and microRNA and, therefore, can provide molecules for immune modulation, modification in gene expression, and induction of apoptosis (8). Tumor cells can release EVs and can contribute to immune escape

by limiting tumor-specific effector T cells and promoting T regulatory cells expansion or expansion (9,10).

Tumor-derived EVs contain HLA-G, as demonstrated by Riteau *et al*. (11), who described for the first time the existence of HLA-G-bearing EVs in culture supernatants of a melanoma cell line expressing HLA-G. Subsequently, the presence of HLA-G-bearing EVs was observed *in vivo* in ascites and pleural exudates from cancer patients (12), and in exosomes released from first trimester and term placental explants (13). Recently, König *et al*. (14) isolated, for the first time, HLA-G-bearing EVs from plasma of breast cancer patients. Through the analysis of the levels of soluble HLA-G free and HLA-G-linked to EVs, König *et al*. demonstrated the prognostic relevance of HLA-G-bearing EVs, since HLA-G-linked to EVs was associated with disease progression, whereas the levels of soluble HLA-G free form were associated with response to treatment (14). This study highlighted the critical importance of defining the presence of HLA-G free or EVs-linked and how these two molecules may have opposite impact on disease progression, as they may promote immune escape or not. Based on this first demonstration, Rebmann *et al*. (15) discussed and proposed in their perspective the importance to discriminate the source of the different forms of soluble HLA-G, free or EV-linked, present in plasma or in serum of patients to better define their contribution in cancer, and, therefore, their use as biomarker of disease stage, progression, or response to therapy.

Rebmann *et al*. (15) also highlighted the consequences of the discovery of HLA-G-bearing EVs in the biology of HLA-G. In this contest, it has to be taken into account that EV-linked HLA-G can be expressed either as membrane-bound or as soluble form within the vesicles. Up to now, it has not been defined whether membrane-bound HLA-G expressed by EVs is structurally and biologically superimposable to the "classical" membrane-bound HLA-G. Thus, it cannot be excluded that EV-linked HLA-G may counteract the activity of "classical" soluble or membrane-bound HLA-G by competing for receptor occupancy. However, the evidence described by König *et al*. (14) suggested that HLA-G-linked to EVs is functional active, since higher levels of HLA-G-bearing EVs are associated with disease progression indicating its involvement in the down-regulation of immune responses. Future investigations are warranted to better define the mode of action of this new form of HLA-G (3). On the other side, the presence of soluble HLA-G within EVs suggests that HLA-G may be released within target cells independently from the expression of its receptors LILRBs and KIR2DL4 on the cell surface. It can indeed be possible that, upon interaction with target cells, EVs release soluble HLA-G directly into the cytoplasm, where it can interact with unknown receptors participating to yet undefined intracellular pathways. Moreover, since EVs have different composition based on the cell type from which they originate, it can also be speculated that the release of different bioactive effector molecules in conjunction with soluble HLA-G may have either positive or negative effects on HLA-G-mediated activity.

Several evidences indicated that HLA-G-expressing EVs mediate a number of immune-modulatory activities: HLA-G-expressing EVs isolated from renal cancer stem cells modulate monocyte-derived maturation and their ability to stimulate T cells *in vitro* (16). Recently, it was shown that mesenchymal stromal cells (MCS) isolated from patients with refractory graft versus host disease released EVs containing high levels of HLA-G, IL-10, and TGF-β (17). Taken together, these studies highlighted that HLA-G-expressing cells may exert tolerogenic functions not only via the expression of membrane-bound or the secretion of soluble HLA-G, but also through the release of HLA-G-expressing EVs. This can be the case also for HLA-G-expressing DC-10, a population of human tolerogenic dendritic cells, present in peripheral blood (18) and enriched in human decidua of pregnant women (19), that play an important role in promoting tolerance via T regulatory cells. Our group showed that the tolerogenic activity of DC-10 is associated with the expression of membrane-bound HLA-G: DC-10-expressing high levels of HLA-G are more potent inducers of T regulatory cells compared to DC-10-expressing low levels of HLA-G (20). DC-10, as other dendritic cells release EVs, and preliminary data indicated that DC-10-derived EVs contain IL-10 and HLA-G, suggesting that they may contribute to DC-10-mediated tolerance.

In conclusion, a more in depth study on the HLA-G-linked EVs will better define the role on HLA-G-mediated tolerance. Results will lead to improve the knowledge on the activity of the different forms of HLA-G and will shed light on the selection of the best-suited HLA-G form to be used as biomarker for disease stage, progression, and response to therapy.

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Footnote

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Exosomes may play a crucial role in HIV dendritic cell immunotherapy

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The human immunodeficiency virus (HIV) is a lentivirus that infects cells of the immune system, resulting in cell death and loss of cell-mediated immunity. When the number of immune cells drops below a critical population, HIV can result in the acquired immunodeficiency syndrome (AIDS), wherein opportunistic infections and cancers flourish in the absence of immune surveillance. Worldwide, there are over 30 million people infected with HIV, resulting in a significant global health burden (1). The current standard of care for HIV is combined antiretroviral therapy (cART), and while this effectively prevents disease progression and death, treatment must be continued for the rest of the patient's life (2). Therefore, new therapies that can eradicate and prevent the disease are a crucial area of research in global health.

HIV enters the body through the mucus membranes. The first cells it encounters are immature dendritic cells (iDCs), which act as sentries and recognize foreign microorganisms. Upon contact with pathogens, iDCs endocytose the microorganism, resulting in iDC activation and migration to the secondary lymphoid organs. In the lymph nodes, the now mature dendritic cells (mDCs) present the epitopes derived from internalized microorganisms to CD4+ and CD8+ T lymphocytes (CD4TL, CD8TL), priming them for the active immune response (3). However, when an iDC presents the HIV particle to CD4TL, rather than activating them for the immune response, it results in the infection of CD4TL through a process called trans-infection (described as the "Trojan horse" hypothesis) (4). Despite this observed mechanism, it has also been demonstrated that the HIV particle is rapidly degraded within the iDC. Therefore, it is likely that CD4TL infection occurs through two different methods, both through trans-infection and through the production and secretion of *de novo* viral particles by infected iDCs (3).

One of the most compelling novel therapeutic strategies for HIV is that of dendritic cell immunotherapy (DC-IT). In this treatment, patients' myeloid iDCs are removed and treated with an inactivated form of the HIV particle *ex vivo*. This stimulates DCs to mature, and upon reintroduction into the body the mDCs stimulate a CD4TL response to HIV, resulting in an effective immune response (1). To date, 13 clinical trials using DC-based immunotherapy have been completed with varying degrees of success (5). While DC-IT has been used to induce a persistent immune response, the heterogeneous factors involved in a patient's HIV infection, including host/viral genome, specific infection characteristics, and level of HIV-mediated immune suppression, result in differing responses to DC-IT (2). Therefore, it is critical that we continue to identify the intricate biomarkers and signaling mechanisms associated with HIV infection and DC function.

One such mechanism is that of exosome secretion. Exosomes are minute extracellular vesicles 30–100 nm

in size that are generated through the budding of the inner endosomal membrane and fusion with the plasma membrane. Exosomes are highly heterogeneous, with diverse biological functions depending on their molecular contents. They can contain most kinds of macromolecules, and have been identified as a mechanism by which cells communicate with each other through the transfer of proteins, mRNAs, and non-coding RNAs (6). In addition to their functional effects, the use of exosomes as pathologic biomarkers is a burgeoning field of study. As exosome contents differ based on cell of origin and cell state, profiling of exosomes isolated from a patient's blood may be a useful method of non-invasively examining a cells' pathologic state (7).

DC derived exosomes have been shown to regulate nearly all aspects of the immune system. Depending on the maturity status of the parent DC, DC-exosomes may contain major histocompatibility II complexes, allowing them to act as antigen-presenting bodies and stimulate the adaptive immune response, including both CD4TL and CD8TL. They have also been shown to induce antigen-specific humoral immunity, activate natural killer cells, promote the allergenic response, and aid in the immune system's response to tumor cells (5).

In a recent issue of *Medical Hypothesis*, Ellwanger *et al.* described their hypothesis based on preliminary data collected by Pontillo *et al.*, and examined exosome secretion and the CD4TL response to DC-IT (8). Using monocytes and monocyte-derived DCs collected from six phenotypically matching patients enrolled in a DC-IT trial, the authors analyzed the expression of eighty-four genes involved in the anti-HIV response, as well as the expression of the *TSG101* gene, an exosomal marker. Pontillo *et al.* separated the patients into two groups based on whether the genes were predominantly downregulated (group A) or predominantly upregulated (group B) compared to control monocytes. While the patients' diseases were phenotypically similar, group A exhibited higher levels of CD4TL than group B, indicating that these patients may be more responsive to DC-IT. They also found that the expression of *TSG101* negatively correlated with anti-HIV response genes (increased in group A, decreased in group B). This may indicate that the production and secretion of exosomes impacts the HIV response (5,8).

These early findings highlight interesting aspects of exosome signaling in HIV. Exosomes are believed to often act in support of the immune response through antigen presentation and activation of the secondary immune response (6). However, the down-regulation of *TSG101* combined with an increase in CD4TL implies that in HIV and DC-IT, exosomes may be having an opposite effect. It is possible that these data support what has been termed the "Trojan exosome hypothesis". First described by Gould *et al.*, this hypothesis proposes that retroviruses utilize the exosomal biogenesis and secretion pathway for replication of their viral particles. This is founded in the observation that exosomes and HIV particles share a similar protein and lipid composition, although similar findings have also been reported using other retroviruses (4).

However, recent data indicate that while HIV and exosomes use similar pathways within the cell, they appear to be separate particles. Subra *et al.* successfully separated HIV particles from exosomes using ultracentrifugation and immunocapture. They found that after pulsing DCs with HIV, DC-exosomes induced apoptosis in CD4TL, and were not capable of spreading the HIV infection. On the other hand, HIV particles secreted by DCs and separated from exosomes were infectious without inducing apoptosis (9). A recent study from the same research group by Mfunyi *et al.* identified the dependence of DC exosome release on dendritic cell immunoreceptor (DCIR), the same receptor which DCs and CD4TL use to bind the HIV virion. Inhibition of this receptor prevented exosome secretion, solidifying the parallels between the cellular mechanisms used by HIV and exosomes (10). Further work demonstrated that exosomes from HIV stimulated DCs contained the pro-apoptotic protein DAP-3, whereas exosomes from non-stimulated DCs did not (10).

While the full parallels between exosomes and HIV particles remain undefined, it is possible that HIV is utilizing exosomal biogenesis as an additional mechanism of trans-infection. Exosomes produced by fully matured antigen presenting mDCs can serve as additional antigen presenting bodies, activating immature iDCs and CD4TL (6). Upon infection, HIV may hijack these processes in DCs, resulting in the production of HIV containing exosomes that can infect other DCs and CD4TL, greatly increasing the spread of HIV infection (3). However, based on the findings of Mfunyi *et al.* and Subra *et al.*, it appears likely that HIV is merely hijacking the exosomal biogenesis process for creation of discrete HIV particles, separate from HIV containing exosomes.

Ellwanger *et al.* drew two potential conclusions from their findings. First, since they observed that group A patients with decreased expression of *TSG101* also had increased numbers of CD4TL, it is possible that

exosomes have a negative effect on HIV immunity. This is likely through HIV-DC exosome-mediated apoptosis of CD4+T cells. Therefore, the DC-IT patients whose DCs produced fewer exosomes resulted in increased numbers of apoptotic CD4TL. However, patients in group A also exhibited down-regulation of anti-HIV response genes, implying that DC-IT is decreasing HIV resistance. On the other hand, group B patients had higher expression of HIV response genes after DC-IT, as well as increased *TSG101* expression. Based on these results, it is possible that the increased secretion of exosomes seen in group B patients may correlate to increased release of the HIV viral particle, thereby activating HIV response genes in additional DCs (5).

While these preliminary results are inconclusive, they highlight the continued need for investigation of exosomal functions in the immune system. Based on the manuscript by Ellwanger *et al.* and the previous findings of Mfunyi *et al.* and Subra *et al.*, it appears likely exosomes are playing a critical role in the DC HIV response. Modulating exosome expression, potentially through DCIR, may be a mechanism of HIV immunity both in conjunction with DC-IT and alone. However, these findings must be further examined. The hypothesis by Ellwanger *et al.* is based on minimal data, with two patient groups containing three members each. Moreover, they did not examine actual exosome release, merely the expression of exosome related gene *TSG101*. Further verification of these findings that build off the data presented by Mfunyi *et al.* will provide important insights into exosome and HIV biogenesis, immunity, and treatment.

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Footnote

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An immunoregulatory role of dendritic cell-derived exosomes versus HIV-1 infection: take it easy but be warned

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Introduction

Dendritic cells (DCs) are professional antigen-presenting cells capable to initiate and then drive T cell responses. Naturally, DCs sense various pathogens and their products in order to present those to immune cells and in turn initiate immune reaction. In a case of wounding, DCs recognize products released by damaged cells and then contribute to the induction of inflammation associated with further clearance of necrotic and apoptotic cells (1). In addition to DC subtypes that initiate inflammatory reaction, there are DC subsets, which exert tolerogenic properties directed to dampen extensive inflammation and promote switching to wound healing (2).

During HIV-1 infection, host DCs (especially mucosal DCs) can sense virus through innate cytosolic immune receptors and then initiate antiviral responses associated with production of type 1 interferon (IFN) and up-regulation of IFN-stimulated genes (3). Type 1 IFN induces death of infected T cells by bystander effect and suppresses viral replication (4). Furthermore, in some individuals, HIV-1-dependent activation of DCs leads to the induction of highly potent antiviral response related to the appearance of virus-specific $CD8^+$ T cells capable to cause natural, drug-free resistance to the infection and support effective HIV-1 control. There is a reciprocal interaction between DCs and HIV-1-specific T cells that involves innate major histocompatibility complex (MHC) class I receptors from the Ig-like receptor family and facilitates HIV-1 control (5).

However, this virus can overturn the protective function of DCs and use these cells for invasion. HIV-1 enters the organism mostly through vaginal and rectal ways whose submucosa contains high numbers of residential DCs. Compared to infected $CD4^+$ $CCR5^+$ T cells where the rapid burst of infection is observed (6), it is difficult to detect HIV-1-infected DCs. Mucosal DCs catch HIV-1 through the endocytic mechanism after binding to C-lectins such as DC-SIGN, langerin, DC immunoreceptor (also known as C-type lectin domain 4A or CLEC4A), etc. (7). Langerhans cells, i.e., resident DCs of skin and mucosa, are more resistant to HIV-1 infection compared with other DC subsets and can efficiently degrade viral particles in Birbeck granules (subdomains of the endosomal recycling compartment) after internalization (8). However, at higher concentrations, virus is able to infect Langerhans cells and then be transferred to $CD4^+$ T cells (9).

DCs transfer virus to $CD4^+$ T cells in secondary lymphoid tissues. Moreover, DCs can serve as a depot for virus and support HIV-1 replication at low levels (10). Viral transmission from infected DCs to T cells employs

formation of infectious synapse (IS) between a DC and a T cell (11,12) or exosome secretion pathway (13,14). Upon IS formation, captured viral particles are switched from the transfer to the endolysosomal pathways towards trafficking to the synapse (15). The viral trafficking to IS uses a pathway of tetraspanin sorting to the immunological synapse between a DC and naive T cell thereby impairing the mechanism of the immunological synapse formation (16). In the exosome-dependent pathway, virus exploits the exosome antigen-dissemination pathway for transmission (14). HIV-1-containing exosomes are released by both immature and mature DCs. Interestingly, on a per-particle basis, exosome-derived viral particles were 10-fold more infectious than cell-free HIV-1 particles (17). In addition, exosome-associated virus can be targeted by virus-specific immune response significantly less efficiently suggesting for an avenue for virus escape.

In DCs, poor HIV-1 replication can be explained by the existence of cell-protective restriction mechanisms. In myeloid cells and DCs, sterile α-motif and HD domain 1 (SAMHD1), a dNTP triphosphohydrolase, decreases the intracellular dNTP pool essential for virus reverse transcription and synthesis of cDNA. This in turn blocks HIV-1 replication after virus entry to a DC (18). Tripartite motif-containing protein 5 (TRIM5) acts as a receptor able to sense retroviral capside lattice and then induce the innate immune response through up-regulation of the intracellular innate immune signaling (19). DNA dC- > dU-editing enzymes belonging to the APOBEC3 family provide retroviral resistance by inducing G-to-A hypermutation in the provirus that initiates further degradation of viral transcripts (20).

Finally, DCs employ a set of receptors capable to recognize viral particles and viral RNA/cDNA. In addition to HIV-1-sensing C-type lectin receptors, there are Toll-like receptors (TLRs). However, virus can use TLR8 and D-SIGN for infection and replication in DCs (21). Cytosolic 3' repair exonuclease (TREX1) can hide virus from cytoplasmic viral DNA sensors by binding to HIV-1 DNA and degrading excessive viral DNA (22). By down-regulation of IFN-stimulated genes, this nuclease suppresses the anti-HIV-1 innate immune response and also limits expansion of the lysosomal compartment (23). In contrast, another cytosolic DNA sensor, cyclic GMP-AMP synthase (cGAS), by binding to viral DNA, synthesizes cyclic GMP-AMP, an intracellular messenger, that triggers production of type 1 IFN and other cytokines through STING/TRAF family member-associated NF-kB activator (TANK-1)-dependent stimulation of transcription factors NF-kB and IFN regulatory factor 3 (IRF3) (24,25).

A great capacity to recognize viral products and then induce specific anti-viral immune response along with a relative resistance of DCs to HIV-1 infection served as a basis for the development of a rationale for use dendritic cells in anti-HIV immunotherapy. Loading of autologous virus-free DCs with a HIV-1-specific antigen with subsequent vaccination of a HIV-1 infected individual with antigen-activated DCs was suggested to boost the restoration of the host anti-viral immune response (26). Basically, any viral product such as a whole autologous heat- or chemically-inactivated virus particles, viral peptides (such as pol and gag) or viral RNA transcripts may serve as an antigen for generation of DC-based vaccines (27).

In this review-comment, we will consider the impact of DC-based immunotherapy on the treatment of HIV-1 infection and a role of exosomes in the control of DC vaccine-induced antiviral immune response.

DC-based immunotherapy of HIV-1 infection

In the context of HIV-1 infection, the main purpose of DC-based vaccines is the activation of cytotoxic $CD8^+$ T cells since $CD4^+$ T cells can be widely infected by the virus and therefore become ineffective or impaired (28). In chronically HIV-1-infected patients, function of $CD8^+$ T cells and DCs are also defective. Gag-specific $CD8^+$ T cells were shown to produce IFN-γ but failed to produce reliable amounts of perforin and IL-2. These cells are unable to expand in co-culture with gag-specific DCs suggesting for the loss of recall memory to HIV-1protein gag. DCs from HIV-1 patients can effectively present antigens to other viruses such as influenza, Epstein Barr virus, and cytomegalovirus but lack the capacity to expand gag-specific $CD8^+$ T cells (29).

The development of DC-based vaccines is a multi-step process. After antigen loading, DCs should be maturated by supplementation of culture medium with cytokines and/or growth factors. For example, in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF), antigen-loaded DCs maturate to conventional (i.e., "classical") $CD4^+$ DCs with propensity to the proinflammatory activation of naïve T cells. When exposed to type I IFN and macrophage colony-stimulating factor (M-CSF), DCs maturate to plasmacytoid-like DCs characterized by a potent production

of type 1 IFN (30). After vaccination, immune response in DC-immunized subjects can be monitored with help of enzyme-linked immunosorbent assay (ELISA), enzyme-linked immunospot (ELISPOT), flow cytometry, and their combinations.

To date, over 15 clinical trials have been performed in order to assess the efficiency of DC vaccines in HIV-1 therapy. In these trials, there were various clinical settings. In some clinical studies, asymptomatic HIV-1-infected untreated subjects were recruited (31-36), while patients on antiretroviral therapy were enrolled in other studies (37-41). The trials were small since they involved only from 4 up to 56 subjects.

Investigators also used various protocols for development of DC-based vaccines. In some studies, DCs were pulsed with a variable mixture of peptides derived from HIV-1 proteins Gag, Env, Pol, Nef, Vif, Vpu, and gp160 (31,34,36,37). Other researchers loaded DCs with a whole inactivated virus (32,33,38). Yet other investigators used viral mRNA encoding Gag, Nef, Ref, Vpr, and Tat for electroporation of DCs (39,40). Macatangay *et al.* (41) reported development of DC-based vaccine loaded with apoptotic bodies released by dying autologous HIV-1-infected T cells. An interesting approach was implicated by Norton *et al.* (42) to obtain DCs constitutively expressed HIV-1 specific epitopes. For transduction of DCs, Norton *et al.* (42) used lentiviral vectors encoding HIV-1 epitopes fused through self-cleaving peptide to CD40L that helps to release peptide to the endoplasmic reticulum after entry. The vectors contain Vpx, a lentiviral protein, which neutralize SAMHD1-dependent inhibition of viral replication and support long-lasting expression of viral epitopes in DCs (42).

The protocols used for the maturation of antigen-loaded DCs were also different. Generally, in most studies, investigators supplemented the culture medium with cytokines as follows: GM-CSF, IFN-α, interleukin-1β (IL-1β), IL-4, IL-6, and tumor necrosis factor (TNF)-α (27). In all studies, vaccines were injected percutaneously in different doses varying from 2×10^6 (33,36) to 1.2×10^7 (40) cell per dose. Vaccination regimens significantly varied. A total of 3 to 6 doses of a vaccine were injected with a periodicity ranging from every 2 weeks to 1 month (i.e., 4 weeks).

Generally, DC vaccination was safe and well-tolerated by patients. Side effects of immunization were minimal or moderate including erythema, local inflammation, or subcutaneous bleeding at the injection site, and asymptomatic enlargement of peripheral lymph nodes. Adverse side effects of vaccination included thrombocytopenia, neutropeni (38), and severe pruritus (42).

The vaccination led to marked decrease of plasmatic HIV-1 virus load up to 4–5 copies per mL of blood (35,37,40) mainly due to the DC-dependent activation of antigen-specific cytotoxic T cells and enhancement of the anti-viral immune reaction mostly mediated by $CD8^+$ T lymphocytes. Induced cytotoxic T cells perform the destruction of infected T cells thereby limiting cell sources for virus replication and storage. DC vaccination also led to increase in numbers of circulating $CD4^+$ T cells and functional $CD4^+$ T cells, reactivation of latent HIV-1 thereby reducing the reservoir of latent virus in affected patients (42). Andrés *et al.* (43) replicated these results suggesting for the clinical value of DC vaccination for inducing virus-specific T cell subsets capable to control HIV-1 replication and intracellular viral reservoir.

The immunization with DCs also resulted in enhanced production of inflammatory cytokines (IFN-γ, IL-17), IL-21 (essential for proliferation of cytotoxic T cells and natural killer cells), and IL-2 (essential for differentiation of naïve T cells to regulatory T cells and differentiation of antigen-activated T cells to effector T cells and memory T cells (36). Notably, after completing vaccination course, patients were able to stay off the antiretroviral therapy for a long time. For example, Allard *et al.* (39) reported a 96-week patients' withdrawal from the antiretroviral treatment for 6 of 17 participants after DC vaccination. Lu *et al.* (32) showed long-term inhibition of virus load by >90% with stable T cell counts for at least 1 year after completing of DC vaccination.

However, not all but only a part of HIV-1 patients responded to vaccination. Monocyte-derived DCs from HIV-1 patients that undergo the antiretroviral therapy were shown to secrete low levels of IL-12 (essential for differentiation of naïve T cells to proinflammatory Th1 cell) after induction of CD40L (44). Reduced IL-12 correlated with the lack of post-vaccination viral load control (41). However, DCs from patients subjected to the antiretroviral therapy after exposure to IFN-γ and CD40L able to produce more IL-12 (44). Accordingly, $CD8^+$ T cells become more potent IFN-γ producer after activation by DCs treated with CD40L and poly (I:C), a synthetic mimic of viral double-stranded RNA and TLR3 ligand (45). Indeed, pre-vaccination conditions are important for the

control of DC function.

There is a significant heterogeneity in the magnitude of DC vaccine-induced immunoproliferative responses in vaccinated HIV-1 patients. Gandhi *et al.* (38) detected no significant immunoproliferative response while other studies showed modest or transient responses and increase in $CD8^+$ T cell counts that correlated with partial virus load control (33-35). However, other researchers observed a prominent increase of $CD8^+$ T cell mediated antiviral response associated with reduced viral load (36,38).

The variability of DC vaccination-induced responses may possibly arise from the difference between experimental design of clinical studies and selection criteria for participants. Indeed, vaccine preparation methods and clinical vaccination protocols (at least key points such as vaccine response criteria) are needed to be standardized. Standardization is also necessary for the assessment of vaccine efficiency since naïve, untreated patients and subjects under the antiretroviral therapy respond to DC vaccine in a different way. Probably, standardization of selection criteria for patients may be also helpful. Asymptomatic subjects with early chronic HIV-1 infection may be more relevant for evaluation of DC-based vaccines since they have a pretty normal immune response, virus is not subjected to strong immunological pressure, and viral reservoir is still small.

Combination of DC-based immunotherapy with other antiviral pharmaceutical agents may be beneficial and result in the reciprocal enhancement of therapeutic effect. For example, combined treatment with Vacc-4x (a four-HIV-1-specific peptide vaccine), recombinant GM-CSF, and romidepsin (a histone deacetylase inhibitor) resulted in decrease of total HIV-1 DNA by nearly 40%, virus reactivation, and significant decrease of the size of latent HIV-1 reservoir (46). Administration of the peptide vaccine and GM-CSF before treatment with romidepsin was directed to recover DC-mediated anti-viral immune response. Therefore, DC vaccination before pharmaceutical medication may be preferential in order to enhance the efficiency of the anti-HIV-1 therapy.

At the distance, DCs were shown to communicate to each other by releasing exosomes that assisted in the propagation of the immune response and enhance responses against pathogens. DC-derived exosomes also serve as a communication tool with other immune cells such as T cells and B cells (47).

DC-derived exosomes: role in immune regulation

Exosomes are extracellular vesicles surrounded by a lipid bilayer and capable to transfer a variety of molecules such as proteins, DNA, mRNA, microRNA (miRNA), lipids, *etc*. There are several types of extracellular vehicles that are differentiated by size and origin. Exosomes (or microvesicles) are released by budding from the cell surface. By contrast, exosomes (size range, 30–150 nm) are generated by indrawn budding of the internal endosomal membrane followed by formation of multivesicular bodies, their fusion the plasma membrane, and liberation of exosomes to the extracellular space (48).

Exosomes are highly enriched with specific proteins like tetraspanins (CD63, CD81, and CD914) and 70 kDa heat shock protein (HSP70), which can be used as exosomal markers. The exosomal membrane also contains receptors and other proteins that are necessary for targeting of recipient cells and assisting in exosome internalization by endocytosis or phagocytosis and further release of the exosomal cargo into the cytoplasm of the acceptor cell (49). Delivery of the exosomal material to recipient cells can influence intracellular signaling and induce changes in cell function and behavior (50). The exosomal composition and exosome-mediated biological effects depend on the exosome-releasing donor cell and local microenvironment. Changes in inflammation, infection or transformation influence the exosomal content. Exosomes can play protective or pathogenic role (51). As mentioned above, DC-derived exosomes can serve as a vehicle for HIV-1 propagation and infection of $CD4^+$ T cells. Exosomes secreted by tumor cells transfer the biomaterial that can induce malignant changes in the normal recipient cells upon delivery (52).

As known, DCs as immune cells, which recognize, process and present antigens, represent a link between the innate and adaptive immunity. DCs present processed antigens bound either to MHC class I or MHC class II molecules to naïve $CD8^+$ T cells or $CD4^+$ T cells respectively, which then transform to effector cells or memory cells. Effector cells are actively mediate the immune response while memory cells are responsible for storage of immunological antigen-specific information to be reactivated in case of a repeat infection of the pathogen that contains this antigen (53).

Exosomes are extensively involved to the transport and presentation of antigen-MHC complexes to $CD8^+$ and

$CD4^+$ T cells. DC-derived exosomes can present antigens directly or through the mechanism of cross-presentation. In direct presentation, T cells directly accept an antigen that leads to their activation. In cross-presentation, DCs accept antigens transferred by exosomes, additionally process antigens and then present to T lymphocytes (54). In addition, DC-derived exosomes promote cytotoxic effects mediated by $CD8^+$ T cells (55). Exosomes also up-regulate antibody production against bacterial pathogens (56) and humoral immunity in overall (57). Finally, exosomes released by DCs stimulate immune responses against neoplastic cells (58).

Exosomal miRNAs are an important component of the immunoregulatory properties of DC-derived chromosomes. The formation of the functional immunological synapse leads to a significantly more intensive exchange by miRNA-containing exosomes between DCs and T cells (59). DCs were also shown to transfer functional miRNAs to each other *via* exosomes (60). MiRNA are involved in post-translational control of gene expression and silencing of a wide range of mRNA targets. However, in HIV-1 infection, miRNAs are likely to play a minimal role in antiviral fight. HIV-1 protein Nef was observed to bind to Ago2, a key factor in miRNA biogenesis that leads to the inhibition of any RNA interference (61). Again, for example, the virus can stimulate overproduction of miR-29b and cause neural complications through exosome-dependent transport of this miRNA to astrocytes and subsequent down-regulation of expression of platelet-derived growth factor-B (PDGF-B), a growth factor of healing and amplification of neural cells (62). Furthermore, the virus can rule from infected cells the exosome-dependent trafficking of trans-activation response element RNA, an essential activating product for HIV-1 replication (63).

DC-derived exosomes and anti-HIV-1 therapy

In fact, HIV-1 is a trick virus that mimics the human nature to survive. A set of viral mutations was detected to explain how HIV-1 can to reorganize the local immune microenvironment to be effective for replication and further invasion. We are quote sceptic on the use of DC-derived exosomes to heal that viral infection. First, three patients only were in that little examination (63).

Exosomes are possibly used as a tool of immune regulation during the dendritic cell-based immune therapy against HIV-I Second, using autologous DCs is a challenge for HIV-1 infection because of an easy adaptation of the virus to novel microenvironment and these particles may be back-infected.

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Footnote

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Donor-derived exosomes: key in lung allograft rejection?

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In a recent publication, Gunasekaran and colleagues conducted a case-control observational study in patients who underwent bilateral lung transplantation (LTx), determining whether exosomes are generated during allograft rejection and define their origin and antigenic phenotype (1). LTx is the sole effective treatment to save the life for patients with end-stage lung disorders (2). One of the major drawbacks for the clinical outcome of LTx is allograft rejection by acute rejection (AR) or bronchiolitis obliterans syndrome (BOS) (2). A link between autoimmunity and immune responses to tissue restricted self-antigens (SAgs) such as K-alpha-1-tubulin (K-α1T) and Collagen V (Col-V) with acute or chronic lung allograft rejection has been proposed (3,4). However, the mechanisms underlying the evolution of acute or chronic lung rejection are poorly understood.

Previous studies have analyzed the intercellular communication profiles in allograft rejection by studying exosomes, the smallest membranous extracellular vesicles (40–100 nm) secreted into body fluids. Donor-derived exosomes induce specific regulatory T cells to suppress immune inflammation in the allograft heart (5) and urinary exosome-specific protein alterations provide a potential unique panel of biomarkers for monitoring AR in kidney transplant (6). In LTx, exosomes have been identified in bronchoalveolar lavage (BAL) fluid and were different in normal and inflammatory states (7,8). A recent study described a differential exosomal shuttle RNA in BAL and suggested this exosome RNA like a biomarker of AR (9). However, before Gunasekaran's study the presence of mismatched donor HLA and SAgs on exosome surfaces such as sensitive biomarkers for monitoring allograft rejection in LTx, serum exosome miRNA profile and their targeted pathways involved in lung transplant rejection have never been settle.

The strong adaptative immune response against organ allografts was attributed to migration of donor-derived professional antigen-presenting cells (APCs) to recipient lymphoid tissues, where donor APCs trigger activation of allospecific T cells against donor MHC molecules (10). Nevertheless, there is indirect evidence that donor DCs mobilized from organ allografts into graft-draining lymphoid tissues in relatively low numbers; although, these low donor DCs are able to elicit the potent anti-donor response that acutely rejects the graft (10,11). These apparently contradictory findings could be explained by the transference of clusters of extracellular vesicles bearing functional donor MHC molecules and APC-activating signals to a higher number of recipient cells (12). In this sense, Gunasekaran *et al*., suggested exosome-mediated immune responses due to the presence of SAgs and donor HLA on exosome surfaces originated from the transplanted lung. Furthermore, these exosomes containing SAgs are detectable in sera before clinical diagnosis of AR and chronic rejection. Many investigators have attempted to identify diagnostic and predictive cellular and biochemical biomarkers, especially in BAL fluid, of acute

and chronic rejection in lung transplantation. Gunasekaran and colleagues performed serial analysis of exosomes in circulation and have demonstrated that exosomes containing Col-V were detectable 1 month after LTx, 2 and 6 months before clinical diagnosis of AR and BOS, respectively. Thus, the quantification of these exosomes could be considered as a noninvasive biomarker of impending rejection.

Moreover, molecular (genetic and epigenetic) alterations by which a normal and abnormal process can be recognized or monitored are also considered like biomarkers. Therefore, identification of single nucleotide polymorphisms, gene expression and miRNA arrays analysis in BAL or serum fluids rise as potential marker candidates of lung allograft rejection in the last years (9,13). The results presented by Gunasekaran's group showed that serum miRNAs were differently expressed in exosomes from BOS and AR LTxRs compared with stable LTxRs, including lower and higher miRNA signals. Microarray analysis is particularly well suited for discovering complex changes in miRNA expression profiles under different conditions. Inherent within these changes is that knowledge of the dynamics of a single miRNA or even a small group of miRNAs may be insufficient to understand the process occurring due to the change in specific conditions. Therefore, the comparative pathway analysis performed in this study by authors suggests that exosomal miRNAs are involved in the B cell, T cell and TGF-β signaling pathways, regulating significant cellular processes involved in auto- and alloimmune responses in pathogenesis of AR and BOS. Consequently, this report points out exosomes such as a key in mediating immune responses that could contribute to acute and chronic pathogenesis, ultimately leading to allograft rejection.

To reach these conclusions, Gunasekaran *et al*. performed western blotting and transmission electron microscopy techniques to determine the presence of SAgs (Col-V) on exosome surfaces in sera and BAL in LTxRs with AR and BOS. Furthermore, flow cytometry analysis have allowed them to demonstrate donor origin (HLA-A2 antigen positive staining, but not recipient HLA-A3) followed by alloimmune responses from sera of LTxRs with BOS. These selected methods have been widely approved and tested to phenotype exosomes in different biofluids such as serum, urine, breast milk and BAL (14,15). Moreover, in this report the global miRNA profile from pooled sera was performed using a GeneChip miRNA array and miRNA-mediated targeted pathways were also generated. However, the small RNA sequencing of the samples instead of miRNA array could be a better approach to identify miRNAs involved in these processes. Since deep sequencing (miRNA-seq) generates millions of reads from a given sample, it allows to establish individual profile miRNAs and discover novel miRNAs that may have eluded by traditional screening and profiling methods (16). Furthermore, after the amount of miRNAs are quantified for each sample, their expression levels can be compared between samples. Perhaps, miRNA sequencing of the individual serum samples from each AR and BOS patient could have provided an important additional information. But, miRNA profiling analysis approached by authors is enough to show a differential expression in LTxRs with AR and BOS and stable LTxRs.

On the other hand, the study have two methodological flaws that undermine their conclusions. One limitation is the relative small sample size analyzed for LTxRs with AR and BOS and stable LTxRs. The number of patients and patient samples in this work needs to be increased greatly to improve statistical validity. This will require the establishment of collaborative study groups that share biological samples from hundreds of subjects. Another potential shortcoming is they do not address the biological role of exosomes in transplanted lungs. The mechanism by which exosomes may participate in augmenting immune responses that lead to rejection is not yet defined.

Overall, although the mechanisms that lead to lung allograft rejection are mixed, well conducted investigation as the one of Gunasekaran and colleagues provides a light that rose in the darkness of the AR and BOS pathogenesis. However, a lot of questions remain unanswered, with the most relevant being the functional role of exosomes and the cross-talk between them and their recipient cells involved in the lung allograft rejection.

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Footnote

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Exosomes derived from stimulated monocytes promote endothelial dysfunction and inflammation *in vitro*

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During the last few years, the scientific community interest on the role of extracellular vesicles (EVs) in physiology and pathophysiology of several human diseases has increased exponentially (1). These vesicles present the capability of transferring different kind of molecules (lipids, RNAs, DNA, protein…) between cells and may exert some effects on the cell phenotype. The content of these vesicles can vary depending on the cell type of origin (2). Although nowadays there is no consensus regarding the appropriate nomenclature, three well-known types of vesicles can be categorized on the basis of size and biogenesis: apoptotic bodies (>1 μm), microvesicles (150 nm–1 μm, budding from plasma membrane) and exosomes (30–150 nm, formed within the endosomal network and released upon fusion of multi-vesicular bodies with the plasma membrane) (1,2). Exosome is the most commonly used term to designate any type of EV; it has become a "catchword" for EV-related science. Despite the fact that the isolated fractions generally studied consist of a mixture of EVs. Unfortunately, the EVs scientific community is not able to propose a list of EV-specific markers to classify subsets of EVs (3).

Even though there are numerous methods for the isolation and purification of EVs, with ultracentrifugation considered as the "gold standard", there is not a single technique which allows the separation of the different EVs populations efficiently, that is why the International Society for Extracellular Vesicles (ISEV) recommends the use of various techniques with the goal of obtaining the purest (i.e., the most exosomes-enriched) fractions (4). Regarding the vesicles characterization, it is also recommended to combine the use of different techniques (three or more) which would allow confirming that actually, this kind of vesicle is present in the sample.

Nowadays, methods based on polymeric precipitation have become very trendy due to their easy and quick usage, but surely these are not adequate techniques for different reasons: (I) it does not avoid the co-precipitation of non-exosomal components (lipoproteins and the culture medium components) that can interfere on the biological properties of the EVs; (II) the loss of an EV-population of interest, due to the limitation of this technique to precipitate the smallest EVs (5). Culture medium components may possibly exert some kind of interaction with the target cells that could alter the biological effect associated with the studied EVs: even on commercially labeled exo-free culture media after routine quality assays, different RNAs have been detected (6,7). Henceforth the standardization of a unique EV isolation technique is necessary that could prevent the presence of non-exosomal components in EV pellets.

Exosomes vesicles have been associated with the progression of different diseases, including cardiovascular

diseases and cancer. Exosome release from tumorous cells contributes to the tumor progression facilitating the angiogenesis and metastasis (8,9). Additionally, pro-inflammatory conditions, such as atherosclerosis, promote the release of EVs derived from vascular endothelial cells, smooth muscle cells, macrophages and other immune cells that have mainly pro-inflammatory properties, which accelerates the development of vascular diseases (10). The capability of these EVs to transport and deliver biomolecules contained therein makes these nanovesicles a powerful tool for the prognosis and diagnosis of different diseases.

On the study carried out by Tang and colleagues entitled "*Monocyte exosomes induce adhesion molecules and cytokines via activation of NFκB in endothelial cells*", the authors analyzed the effects of EVs derived from stimulated monocytes with IFNα, LPS, or a combination of the two of them (I/L). The authors observed a variation of the exosomal cargo regarding the stimuli applied: when EVs from LPS or I/L stimulated monocytes were added to HUVEC cells an increase in pro-inflammatory molecules (ICAM-1, CCL-2 and IL-6) were observed when compared with IFNα stimulated monocytes and non-stimulated monocytes. Thus, the authors conclude that the EVs from LPS or I/L stimulated monocytes could be related to the formation of atherosclerotic plaques by inducing an overexpression of ICAM-1, CCL-2 and IL-6, via NFκB activation, and consequently favoring the adhesion of monocytes and their subsequent migration to the subendothelial space (11).

Gao *et al.* also demonstrated that exosomes from mature dendritic cells (stimulated with LPS) were capable of favoring endothelial inflammation and atherosclerosis via TNFα-mediated NFκB pathway in both *in vivo* and *in vitro* assays (12).

The results of this study together with the results published by Tang *et al.* seem to confirm that in the presence of certain stimuli (e.g., LPS), monocytes and dendritic cells secrete EVs that could promote endothelial inflammation and atherosclerosis progression.

The exosomal features and their potential role in inflammatory modulation suggest a possible clinical use of EVs as biomarkers or therapeutic agents. The fact that EVs have been isolated from various biological fluids (blood, urine, saliva, breast milk) (13) and the increasing knowledge of their cargo makes them useful both in prognosis and in early diagnosis of different diseases, since it is possible to identify expression patterns of different molecules such as miRNAs, specific for different types of diseases (14).

The authors of this manuscript detected differences on two miRNAs expression in exosomes from stimulated monocytes. On the one hand, an overexpression of miRNA-155, involved in inflammation and signaling via TLR-4 was identified; on the other, a downregulation of miRNA-223 was detected, which is involved mainly in cardioprotective and anti-inflammatory processes.

It is necessary to identify the pathways implicated on the exosomes mediated IL-6 induction. Most of the proinflammatory genes, including those codifying IL-6, E-selectin, ICAM-1 y VCAM-1 have been reported in the literature that may be regulated via JNK pathway (15).

Finally, the long term stimulation of immune cells by HIV seems to activate a novel mechanism in which exosomes could play a crucial role in endothelial cell communication, transferring proinflammatory factors, promoting endothelial dysfunction and inflammation; and as a consequence of all this, enhancing atheroma plaque formation and atherosclerosis development in HIV patients. However, it is essential to identify the exosomal molecules responsible for these proinflammatory and proatherosclerotic effects (miRNA, lipids or proteins) and to perform *in vivo* assays to contrast with *in vitro* studies.

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Extracellular vesicles: small bricks for tissue repair/regeneration

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Comment on: Silva AM, Teixeira JH, Almeida MI, *et al.* Extracellular Vesicles: Immunomodulatory messengers in the context of tissue repair/regeneration. Eur J Pharm Sci 2017;98:86-95.

Abstract: Extracellular vesicles (EVs) are nano-sized membrane vesicles involved in intercellular communication. EVs have pleiotropic actions in physiological and pathological conditions. The ability of EVs to transports proteins, drugs and nucleic acid, to target specific cells and to increase the stability of therapeutic cargo, make EVs interesting as new devices for the treatment of human disease. In a recently published issue of European journal of pharmaceutical sciences, Silva and colleagues reviewed the ability of EVs to modulate tissue repair and regeneration, focusing on their roles and therapeutic potential as immunomodulatory messengers. In this perspective, we discussed the open questions regarding the dual role of EVs in immune system, as well as the technical limitation of the procedure for EVs isolation and administration in clinical practices. EV-based therapies require further studies to consider EVs as promising candidate for a novel cell-free therapy in the context of regeneration medicine.

Keywords: Extracellular vesicles (EVs); immune system; tissue repair/regeneration

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In the past decade, the scientific interest in the role played by extracellular vesicles (EVs) in human physiology and in pathogenesis of major human diseases has rapidly increased. Huang-Doran and colleagues considered EVs an "umbrella term" that includes exosomes and different microvesicles, distinguished by their size and biogenesis (1).

EVs are commonly heterogeneous in size, ranging from 20 to 1,000 nm in diameter depending on their origin and mechanism of release, direct shedding or budding from the plasma membrane. Exosomes are vesicles with a diameter of 20–100 nm formed by the inward budding of endosomal membranes to form large multivesicular bodies and released extracellularly when MVBs fuse with the plasma membrane. Larger microvesicles with diameter of 100 nm–1 mm result from the direct outward budding and fission of the plasma membrane (2).

In the 1980s, exosomes were thought to act as 'garbage bags' with the main function to discard the cellular waste, now they are considered as important intercellular communication devices (3).

Evs have been detected in several biological fluids such as blood, saliva, urine, bronchoalveolar lavage fluid, synovial fluid, breast milk, amniotic fluid and malignant ascites (4).

EVs carry a plethora of molecules including proteins, lipids, small and long, coding and non-coding RNAs that interact with local and distant cellular targets, thus modifying cell phenotypes. EVs add an alternative to the paracrine and endocrine cellular communication (5). EVs can interact with recipient cells with different endocytic mechanisms including clathrin-mediated endocytosis, clathrin-independent endocytosis, phagocytosis and macropinocytosis. The selected pathway depends on the expression of surface proteins on the recipient cells and on proteins present in EVs (6). After internalization by target cells, the mechanism by which EV cargos escape the degradative pathway of the recipient cells is still unclear (7).

EVs have a key role in several biological processes, they are considered as a new paradigm in cell signaling. EVs

carry molecules that can activate downstream canonical pathways in the target cells (8).

The widespread research in the field of the vesicle role in cancer highlights the effects of EVs in modulation of the microenvironment. Increasing evidence shows that tumor-derived exosomes have multiple roles in tumorigenesis; they can contribute to cancer growth inducing anti-apoptotic and oncogenic pathways such as invasion, metastasis and angiogenesis. Exosomes can promote tumor immune evasion with T-cell apoptosis induction (9-11).

Recently, exosomes attracted the interest of the scientific community for their potential use in therapy as a targeted and non-immunogenic delivery system for drugs or biological molecules (12).

EVs are involved in the maintenance of tissue homeostasis and they contribute to tissue repair and regeneration (13) as extensively showed in the review of Silva and colleagues (14).

Because Evs are involved in pathological processes and their presence in biological fluids allows their use for easy liquid biopsies (10), EVs have also attracted great interest for the potential use as disease biomarkers (15).

The authors of the review "Extracellular vesicles: Immunomodulatory messengers in the context of tissue repair/regeneration" preferred to indicate the nanovesicles discussed in the paper as EVs, in order to review studies that investigate a broad range of different population of vesicles (exosomes, microvesicles, ectosomes) according to the nomenclature guidelines proposed by ISEV.

In this review, the authors discussed the role of EVs in tissue repair and regeneration and the potential therapeutic effects of natural, modified and artificial EVs in control of inflammation and regenerative medicine. The authors described effects of EVs in tissue repair as mediators of cell proliferation and differentiation and they reported the effects of EVs in the extracellular matrix remodeling. Moreover, they focused the attention on the role of EVs in therapy as vehicles of new drugs and as a novel drug-delivery system.

After isolation, EVs could be utilized in regenerative medicine through several methods, in combination with cells and/or other therapeutics or separately. It was described that EVs can be directly injected into tissue or circulation. EVs can be introduced in regenerative therapies, mixed with hydrogels, or it is possible to coat scaffolds using fibrin gels.

Since EVs are complex devices with a pleiotropic role, it is necessary to understand their advantages and disadvantages before to candidate them as therapeutic agents, considering the limitations that have emerged in the clinical trials for their use.

In this perspective, we analyzed the dual role of EVs in immune system, the technical limitations of a non-standardized procedure for the purification and quantification, necessary to utilize the vesicles in therapy.

The authors in this review focus on the use of EVs, as a promising candidate for a "novel cell-free" therapy, in the resolution of inflammation during the repair of tissue damage. Resolution of inflammation is one-step of tissue repair and regeneration.

The role of EVs in regenerative medicine is supported by several experimental data. It was demonstrated that exosomes released by mesenchymal stem cells (MSCs) have been tested in preclinical settings for the treatment of cardiovascular diseases (15), kidney injury (16), osteochondral regeneration (17), skeletal muscle regeneration (18) and neurological disease (19). EVs can modulate the immune-activity (20), through the regulation of cytokine expression. EVs can also affect the infiltration of immune cells into the damaged tissue, inhibiting the pro-inflammatory processes and immune cells activity.

It has been also demonstrated the role of EVs in immune suppression and/or immune stimulation. Several papers reported preliminary application of EVs as immunotherapeutic agents for the treatment of inflammation triggered by the injury. Blazquez *et al*. demonstrated the immunomodulatory and anti-inflammatory effects of human adipose MSCs derived exosomes (exo-hASCs) in *in vitro* stimulated T cells. hASCs exosomes inhibit the activation and differentiation of cytotoxic and helper T cells, as well as reduced T cell proliferation and IFN-γ release on *in vitro* stimulated cells (21).

Zhang *et al*. also demonstrated an immunosuppressive role for exosomes isolated from embryonic stem cells (ESC). Exosomes were shown to induce high levels of anti-inflammatory IL-10 and tumor growth factor-β1 (TGF-β1) in human monocytes, and they reduced the levels of pro-inflammatory IL-1B, IL-6, tumor necrotic factor α (TNFα), and IL-12P40 (22).

Wen *et al*. demonstrated that exosomes released by human bone marrow mesenchymal stem cells (hBMSCs) suppress immune reaction during Langerhans islet transplantation, promoting islet function and inhibiting immune rejection (23).

On the other side, data in the literature report the role of exosomes in immune system stimulation. Rahman *et al*.

demonstrated the role of Islet-derived MSCs exosomes as autoantigen carrier in the promotion of local autoimmune response. The cells of Langerhans islet of NOD mice (MSC-like cells) release immunostimulatory exosomes that could activate autoreactive B and T cells endogenously. The immunization with exosomes accelerated the effector T cell-mediated destruction of islets (24).

The exosomal features and their potential role in immune regulation suggest a possible clinical use of EVs as adjuvant or therapeutic agents, but several problems limit the clinical use of EVs.

Clinical Good Manufacturing Practice (cGMP)-grade standards for EV clinical use require standardized criteria for EV isolation and storage methods. It is necessary to standardize a long-term storage method, in order to preserve the EV functions and minimize the variability of EVs stability and composition.

Nowadays, a reliable quality control assay to test EV membrane integrity after their preservation is lacking (25). This limit does not ensure the reproducibility of the effects obtained after the EV administration.

Another problem to use the EVs as therapeutic agents or drug delivery vehicle is the lack of an optimal method for their isolation. The techniques most commonly applied for EV isolation, ultracentrifugation and polymeric precipitation, are not ideal due to several reasons: (I) the technique is cumbersome; (II) it does not avoid the co-precipitation of culture medium components (serum or bovine albumin) with EV pellets; this could affect the EV biological effects; (III) recovery is low and the scale up process not easy to standardize. The culture medium components could induce modulation in target cells that can be confused with the effects assigned to EVs. Therefore, it is necessary the standardization of a unique EV isolation technique that could prevent the presence of other components in EV pellets.

The clinical application of EVs also requires the standardization of the administered dose, but actually does not exist a unique technique of quantification, the achievement of comparable results is therefore difficult to obtain. To date, the amount of EVs is calculated in two different ways: in several studies, EVs released by a defined cell number are used, others scientists utilize the count of the EV number (11).

Furthermore, the characterization of the active molecules contained in EVs is a fundamental step for EV approval in clinical practice by the National Agencies for Drug Regulation. It is also important to consider the oncogenic potential of EVs that could limit the clinical use of vesicles as therapeutic agent. EVs contain oncogenic proteins, mRNAs, miRNAs and transcription factor that can potentially alter the genome and the proteome of recipient cells, thus favoring angiogenesis, proliferation and metastasis (16-20).

In the review, the authors discussed about the effects of EVs in regenerative medicine, but it is important to consider the dual effect of EVs on the immune system. There are benefits and disadvantages about the use of EVs as therapeutic immune-modulators in the context of tissue repair/regeneration.

Although, MSC derived EVs have immune-modulating activity (20), the effects of long-term EV administration are unknown. Since EVs display MHC (26) and carry several allogeneic proteins, they could stimulate an auto immune response in the patient in which they are injected.

Few data support the use of allogenic and xenogeneic EVs as therapeutic application to enhance the amount of EVs injectable in therapy. It was demonstrated that allogenic EVs are tolerated in immune–competent animals (5,7).

Nowadays, one clinical trial reported the use of allogenic EVs in human therapy. EVs released by MSCs have been first administered for compassionate use in a patient with steroid refractory graft versus host disease (27). Multiple injections (every 2–3 days) of EVs released by MSCs of unrelated bone marrow donors showed anti-inflammatory effects and ameliorated the clinical symptoms of the patient without adverse effects, indicating the safeness of EV administration. The first trial on MSC-derived microvesicles and exosomes on β-cell mass in type I diabetes mellitus is now enrolling (NCT02128331).

Arslan *et al*. also demonstrated that xenogeneic EVs might be tolerated in mice, *in vivo* (28), but nowadays there are not data about the potential time-dependent effect on the immune system modulation, following repeated injection of EVs in human.

In order to use EVs for therapeutic applications might be optimal to derive therapeutic EVs from an autologous source, to reduce the side effects and cost of EVs therapies.

Escudier *et al*., demonstrated the role of exosomes isolated from conditioned medium of autologous dendritic cell (DC) of patients with advanced metastatic melanoma, incubated with melanoma peptide antigens to induce presentation of the antigen on the cell surface, in association with MHC. EVs reintroduced into patients promoted an immune response against melanoma. Some patients reported minor inflammatory responses at the

site of exosome injection (mild swelling, redness, and DTH responses) and low-grade fever were observed after exosome administration. The patients tolerated administration of exosomes for up to 21 months (29), but it is necessary to evaluate the exosome effects in a long-term treatment.

Jefferson *et al*., in the pilot immunotherapy trial for recurrent malignant gliomas, suggested the use of autolog exosomes isolated from tumor cells removed during surgical craniotomy. The isolated cells, treated with an investigational new drug (an antisense molecule), that target surface receptor protein, are re-implanted (encapsulated in a small diffusion chamber) in the abdomen of patient, within 24 hours after the surgery. The lack of the surface receptor led tumor cells, treated with the antisense molecules, to apoptosis. The authors supposed that released exosomes are full of tumor antigens that together with the antisense molecules could stimulate the immune system against tumor recurrence. The patient recruitment of this trial is currently ongoing. (NCT02507583) (www.clinicaltrial.gov).

One of the most important advantage in the use of a cell-free therapy is the possibility to inject EVs locally thus minimizing the side effects of cells administration. Moreover, EVs can be engineered and addressed to specific organs. The use of EVs in therapy is more attractive than the injection of the cells, in fact, injected cells may die or not fully home into the site of damaged tissue whereas EVs can be locally administered with a controlled dosage (30).

The authors in the review also discuss about the use of EVs as drug delivery vehicles in tissue repair and regeneration, taking advantage of their natural biocompatibility and cell targeting.

Recent studies suggest the production of engineering artificial lipid vesicles that incorporate EV components such as specific lipids or proteins in order to increase stability, targeting, and uptake. Engineered EVs with high levels of phosphatidylserine result in vesicles with a rigid membrane, resistant to lipolytic and proteolytic degradation in the circulation (31). The engineered structure of the EV membrane could enhance the stability of the vesicles *in vivo* and increase the probability to target the specific organ before to be cleared or degraded. The authors indicate that EVs may be regarded as a promising candidate for a novel cell-free therapy for tissue repair damage. Unfortunately, the data present in the literature does not suggest clear advantages of the EVs for the therapeutic applications. Several problems could be related to the use of EVs as immunomodulatory messengers in the context of tissue repair/regeneration. In the next future, the goal of the researchers should be to standardize the technique to manipulate the EVs for their use in therapy and demonstrate the long-term EVs effectiveness and safety for the treatment of human tissue damage.

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Footnote

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Nexus between extracellular vesicles, immunomodulation and tissue remodeling: for good or for bad?

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Introduction

Over the past three decades or so, the field of immunology has advanced hugely with profound understandings on molecular regulation of immune cells and their contribution to various biological processes. The elaborative tools, *in vitro* assays, and refined animal models have favorably anticipated the immunomodulatory (immune suppression or activation) mechanisms elicited during the course of disease progression. The projected roles of immune cells are widely attributed to inflammatory diseases, autoimmune diseases, defense against infections, repairing injuries and progression to cancer among others.

Perhaps, the most widespread explanation to activated immune responses is the pattern recognition (1), generally through surface presentation of receptors and antigen presentation prerequisite to communicate direct messages. Antigen presenting cells (APCs), such as dendritic cells (DCs), B cells, macrophages, and mast cells contribute greatly to antigen presentation through major histocompatibility complexes (MHCs) on their surface which is recognized by T cells and favor a cellular cross-talk conferring immune responses (2,3). The immune responses are not exclusively relied on direct cross-talk, however, cells could also extend their messages through secreted trophic factors, such as cytokines, growth factors, transcriptional factors and non-coding RNAs (4), through extracellular vesicles (EVs)—that all may collectively serve as paracrine messengers of cellular cross-talk (5).

EVs are nanosized membrane vesicles (including exosomes and microvesicles) secreted by virtually all cell types including APCs such as B and T lymphocytes, DCs, and mast cells. Interestingly, EVs from APCs contain MHC class I and II, as well as T-cell costimulatory molecules (6-8). EVs have been thought to play unprecedented role in functional transfer of bioactive molecules such as nucleic acids and proteins between cells (9) and enable cell-to-cell communication (10,11). Largely due to their role in intracellular communication they enable, and due to the exchange of bioactive content between cells, EVs have been implicated in the pathogenesis of variety of diseases such as neurodegenerative and cardiovascular disease, immune diseases, and cancer development.

The immunomodulatory and inflammatory roles of EVs have recently been suggested in huge body of evidence (12-15). The most profound aspect of EVs elicited in triggering immune responses or provoking pro-inflammatory responses owe greatly to the presence of MHC-I and -II complexes. This renowned evidence for the first time came from the description of EVs secreted from APCs, for their extended roles to immune responses (6,7). Interestingly, the MHC-complexes carried by DC-derived EVs were capable for the induction of antitumor immune responses in order to facilitate the eradication of tumor cells in *in vivo* mice models. Such EV-mediated extended functions of APCs, as well as role of EVs in central tolerance, and their

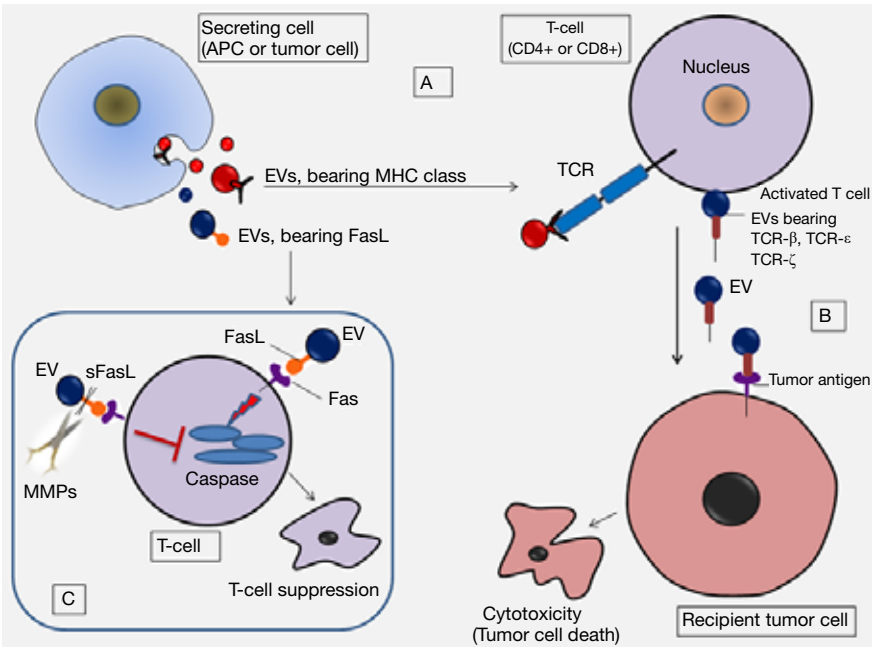

Figure 1 Extracellular vesicle mediated immune activation and immune suppression. (A) EVs containing MHC class from donor cells (either from tumor cells or from APCs) could bind with T cell receptor thereby inducing downstream signals to the T cell; (B) the activated T cells are further capable to release EVs loaded with TCR-β, TCR-ε, TCR-ζ that promote enhanced immune action and cytotoxicity in recipient cells; (C) EVs harboring FasL may have inherent ability to interact with corresponding Fas receptor present on T cells and induce signals down to T cell allowing the activation of downstream caspases which ultimately result into caspase induced T cell apoptosis. (C) Conversely, the caspases activity could be minimizing by EV-associated MMPs which convert membranous FasL into soluble FasL. This inhibits the interaction of FasL to Fas receptor, thus blocking the signals to caspases (17). EVs, extracellular vesicles; MHC, major histocompatibility complex; APC, antigen presenting cell; TCR, T cell receptor; FasL, Fas ligand; sFasL, soluble Fas ligand; MMPs, matrix metalloproteinases.

contribution to activation or suppression of the immune responses could be exploited for developing prospective immunotherapies (16,17).

The immunomodulatory features of EVs elicited in the context of regenerative processes are scarce in the literature, and are only more recently started being explored (18). Silva *et al.*, in a recent issue published in *Eur J Pharm Sci* (19), demonstrated the immunomodulatory features of EVs in the context of tissue reparative programs through their ability to participate in immune regulation and inflammation resolution. These features of EVs allow injured tissue to undergo tissue remodeling phase that is perquisite for reparative process. The current study serves as source of valuable knowledge for tissue regenerative biology; however translating this knowledge into therapeutic applications will require deeper understandings on such mechanisms. Moreover, in a certain resident tissue the detrimental effects of EVs conferred through their immunomodulatory properties making EVs good or bad, must also be determined.

This is important to consider that EVs by themselves do not show a uniform molecular pattern; instead they act as conveners and mediators of cellular responses through their cargo shipping ability. This implies that the molecular patterns contained by EVs and the cargo strictly depend on the external conditions, cell state as well as nature and type of the secreting cells which allow immunoreactive or immuno-suppressive consequences in several different ways (*Figure 1*) (17). Therefore, the immune regulatory features of EVs could be considered in both good and bad. Silva and colleagues propose that knowing the conditions linked to the production of EVs which foster inflammation resolution, could allow manipulation of the inflammatory processes to benefit tissue repair programs (19).

The authors of this study anticipate that EV-mediated transmission of damage-associated molecular patterns to the injury zone could activate certain immune cell populations thereby allowing the onset of the inflammatory

response against the injury (19). This proposition supports the idea that the activation of resident immune cells or the homing of additional immune cells at the site of injury/infection could provoke an inflammatory response, which is considered the seeding phase of tissue repair. In fact, tissue repair and regeneration develop along three major phases which are dependent on each other and include stepwise: inflammation resolve → repair → remodeling. During the course of infection or injury the exposed cells of the local tissue get activated that may induce local inflammation. These may include exposed resident epithelial cells, activated immune cells and the fibroblasts that further may promote the recruitment of circulating immune cells as well as growth factors to the site of injury. This reaction is thought to be the first phase in removing pathogens and washing out damaged cells from the injury zone.

The inflammation resolve allows reparative process to progress into the remodeling phase, which is characterized by enhanced angiogenesis facilitated by growth factors, and fibroblast activation. These processes are functionally linked with extracellular matrix (ECM) turnover and the deposition of new ECM, as well as damaged tissue cell replacement that is facilitated by cell proliferation and differentiation. Keeping in view the aforementioned three phases; authors arguably count on the fact that the inflammation resolve is a key phase in the context of tissue repair and regeneration whereby EVs are playing key role. Interestingly, the evolving roles of EVs in tissue repair and regeneration are mainly reliant on their features mimicking stem cell properties and promoting tissue's intrinsic regenerative programs within recipient cells in a paracrine manner (5). The most profound and relevant therapeutic implications in regenerative medicine that the past two decades have witnessed are those achieved through stem cell assisted tissue regeneration. In this context, stem cell-derived secreted trophic factors such as growth factors, cytokines and EVs could contribute greatly to inducing tissues intrinsic regenerative programs (5). Moreover, the tissue undergoing reparative program requires population equilibrium between cells, which could be accomplished by EV-assisted stem cell proliferation, differentiation and bi-directional communication established between injured tissues and stem cells i.e., injured cells send signals back to stem cells for producing more progenies (5). In this context, authors elaborate that EVs may influence the repopulation of regenerated tissue and functional differentiation of cells. What more can be expected on the beneficial effect of EVs, is their ability to promote angiogenesis—an integral element in healing process, which can be promoted by EV-mediated transportation of pro-angiogenic growth factors to the injury site. Of particular note, the stem cell-derived EVs such as those secreted from mesenchymal stem cells (MSCs) might have immunosuppressive role (16). In this regard Silva *et al*. envisaged such role of EVs as a major cause of inflammation resolution (19).

The third phase, in the context of repair and regeneration is the ECM turn over and tissue remodeling—that overlaps with above mentioned tissue repair phase. Authors continue to assess the tendency of EVs harboring matrix remodeling molecules which modulate the extracellular environment, as well as matrix deposition at the site of injury (19). One of the class of vesicles known as matrix vesicles have been reported previously for their selective distribution at the sites of initial calcification in cartilage, bone, and predentin and are thought to have role in mineralizing of vertebrate tissues during bone development (20). This indicates the importance of calcification and mineralization in developing matrix. Considering the fibroblasts activity in ECM environment, it is also notable that fibroblasts could be differentiated into cancer associated fibroblasts (CAFs) [reviewed elsewhere (16)]. However, despite the evidences for the involvement of EVs in ECM degradation, the relevance of their enzymatic activity in ECM turnover during tissue repair has not been fully explored.

Discrepancy of EVs in eliciting immune responses and immunotherapy

Hitherto, the concept of EV-mediated immune exploitation of target cells is extremely attractive, nevertheless several questions of such process requires critical considerations. The most important consideration would be to determine the relationship between two different aspects of immune responses such as immunosuppressive features versus immune provoking potentials of EVs which depend on several factors (*Box 1*). These discrepancies may represent EVs with variable outcomes in therapeutic perspectives. Presumably, EV-mediated overwhelming immune activation and pro-inflammatory cascades occurring at the site of injury may have undesirable and devastated effects. An example could be seen in the down-regulation of NK and B-cell proliferation by inflammatory cytokines (21). It is anticipated that a pro-inflammatory environment could not only modify the composition of EVs but also the consequent biological activities of immune effector cells, with possibility of increased risks of unpredictable effects (14).

Box 1 EV-mediated immune modulation: for good or for bad?

The nexus of EVs in stimulating immune responses largely depends on type and state of secreting cells e.g., cancer cells or immune cells, maturation state of APCs, as well as the content of EVs
Presumably, EVs may educate immune cells to be recruited at injured niche and further stimulation of local immune cells. This increased number of immune cells will foster local inflammation
EVs secreted from APCs or immune cells could stimulate the secretion of inflammatory or anti-inflammatory cytokines that may have opposite roles to inflammation resolve
EVs bearing (matrix metalloproteinases) MMPs may have a role in matrix remodeling that may either favor the tissue repair or cancer metastasis. Similarly, EV-mediated activation of fibroblasts and epithelial or endothelial cells may either have role in repairing tissue or may differentiate into cancer associated fibroblasts that may initial cancer instead of repair
EV-assisted recruitment of pro-angiogenic growth factors may have role for angiogenesis required for healing process, may also favor tumor angiogenesis
Activated T-cells may secrete EVs, bearing TCR-β, TCR-ε, and TCR-ζ that may enhance activities of NK cells or T- cell effectors; can foster cytotoxicity to kill cancer cells (*Figure 1*). If similar mechanism may apply to cytotoxicity at injured cites it may reflect adverse effects of EVs
EVs could inhibit and impair the maturation of B and T lymphocytes or natural killer (NK) cells: for this cancer cells use EVs to inhibit or suppress immune cells and evade immune surveillance
Apoptosis of T-cells: for their survival, the cancer cells secrete EVs with apoptotic molecules (such as those bearing FasL) which stimulate intrinsic or extrinsic apoptosis cascades in T-cells (*Figure 1*)
Cancer cell-derive EVs may have role in suppression or down regulation of T cell receptors (TCRs): this inhibits the recognition of MHCs by T cells
EVs stimulate the secretion of cytokines that may suppress or activate immune response presumably conferring different roles in tumor environment as compared to injured tissue environment
Collectively, these observations indicative that EV-mediated modulation of cellular responses could be considered both good and bad

APC, antigen presenting cell; EV, extracellular vesicle; MMP, matrix metalloproteinase; MHC, major histocompatibility complex.

Tissue regeneration therapy generally requires an immunosuppressive environment, in particular during organ implants; whereas the cancer immunotherapy largely relies on evoking the host immune system to fight against cancer cells (16). This reflects that for the purpose of repair programs the EV-mediated immune responses will need to be manipulated differently from those manipulated for the purpose of tumor eradication. The immunosuppressive tumor microenvironment is considered a major barrier to the effectiveness of anti-tumor immune activities, since it offers lower immunogenicity of immune cells against the cancer cells. However, growing literature on EVs functional roles continue to provide us with new insights in understanding such discrepancies.

Other therapeutic applications of EVs

In parallel to other beneficial effects resulted from transport of bioactive molecules and intercellular communication—EVs could also be applied as drug delivery vehicles. This is largely due to their natural tendency to transport biological molecules as well as their biocompatibility with the target cells. In the context of drug delivery vehicles, a relatively different but potentiating proposition of EVs—is their pharmacokinetics and pharmacodynamics (19), which could be tailored for pharmaceutical purposes in *in vivo* animal studies.

However, there remain several potent issues to be solved. For instance, loading efficacy and stability of a certain drug is a major concern, as has been observed with other delivery vectors. The additional consideration is the specific targeting issues, since EVs having surface chemistries compatible with cell receptors, could interact with unpredicted cells/tissues that may give undesired results/effects. Moreover, donor cell derived EV-cargo could provoke immune responses in recipient cells with a possibility to confer cross-reactivity.

Considering these facts, one of the important aspect

of the description by Silva and colleagues could be *in vivo* administration of EVs, biodistribution and the delivery of EV-cargo to targeted destinations (19). However, the targeted uptake and internalization of EVs by proposed target recipient cells remains an impeding question. Some of the strong clues provided by Hoshino *et al.*, offer interesting information with the arguments that EVs could seek target organs through different forms of surface integrin's presented on their surface (22). This knowledge could guide researchers for *in vivo* delivery of EV-loaded drugs, however, further studies will warrant translating this knowledge into targeted and organ guided drug delivery.

From bench to bedside

Pertaining to therapeutic applications in the context of tissue regeneration—the feasibility of EV-based therapies have not been eventuated in clinical trials (19). However, there is initial evidence for applying EVs to tissue healing process in an individual patient case. Kordelas *et al.* showed that MSC-derived EVs are well tolerated in patients during the treatment of graft-versus-host disease (GVHD) (23). Moreover, MSC-derived EVs treatment significantly reduced the pro-inflammatory cytokine response in patients' peripheral blood mononuclear cells (PBMCs) *in vitro*, as well as the clinical symptoms of GVHD were improved significantly shortly after the start of MSC-derived EV therapy to the patient (23). It was proposed that the donor derived EVs could recapitulate the immunomodulatory properties of MSCs. Therefore, the applications of immunosuppressive EVs could be of great therapeutic value for future clinical consideration due to the fact that such EVs are well tolerated in patients.

Despite improvements, both in the clinical procedures intended for tissue repairs, organ transplantation and cell-based therapies over the last decade, current methods present potential complications (for example, an increased risk of infection, toxicity, and graft rejection). In this context, compared with traditional stem cell therapies, EV-based cell-free therapies may improve patients' outcomes considerably with reduced complications as compared to cell-based therapy (16). However, stem cell-based therapies also need to consider potent risk factors and technical complications such as: culture-induced senescence, genetic instability, loss of functional properties, immune-mediated rejection, and the risk of transformation of resident cells into malignant phenotypes which presumably limit the applications of stem cells in tissue regeneration (5). Therefore, steering traditional stem cell-based therapy toward EV-based therapy is still a debated issue. In this regard, this could be of interest to applying combination of EV-based therapies with existing approaches in order to improve the therapeutic benefits.

Parallel to clinical trials on tissue regeneration, the evaluation of EVs for clinical trials in other human diseases is also very limited (24,25). However, this interesting to consider that in spite of very small number of patients included in these clinical trials, yet the potential of EVs for their prospective translation from bench to bedside is thought be promising. However, several technical hurdles still require an explicit attention. A potential challenge in the field exists largely due to the limitations of standardizing the existing technologies. In particular, standard protocols for EV isolation, purification and characterization are still a debated issue (9,26). It has been argued that the development of high-throughput approaches and robust capture platforms will warrant the implications of EVs in routine biomarker development, and therapeutic implications with a proposed workflow sheet to applying for USA food and drug administration (FDA) approval (27). Since there is intensive interest in the field both in basic research as well as therapeutic point of view—it is anticipated that in the next decade, EVs arena will see significant advances in clinical pipelines.

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Footnote

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Cell secretome based drug substances in regenerative medicine: when regulatory affairs meet basic science

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"Regenerative medicine" or "tissue regeneration" had become to an inspired term recently since it has been realized in the last decade that many organs such as the skin display morphological plasticity and possess healing capacity instead of being a post-mitotic terminally developed organ (1,2).

In the light of these findings researchers as well as clinicians have turned their therapy aims from a passive reduction of tissue damage towards an active improvement of tissue regeneration and function. Whereas for almost two decades cell based therapies were thought to be the philosophers' stone to enhance tissue regeneration this concept began to totter after the work of Gnecchi *et al.* in 2005 (3). His group was one of the first showing that paracrine factors released from mesenchymal stem cells are able to promote tissue regeneration. In the following years subsequent work from all over the world supported the hypothesis that rather the paracrine factors than the cells were indeed responsible for the beneficial effects (4-8).

The cell-secretome consists of all factors actively or passively released from cells and contains among others soluble proteins (e.g., cytokines, chemokines and growth factors), lipids, free nucleic acids and extracellular vesicles (EV). The latter can be further subdivided mainly based on their size, density, surface markers and origin into apoptotic bodies, microparticles and exosomes. Especially exosomes have recently come into focus of research based on their high capacity to interact with target cells and their ability to selectively modify cell signalling (9).

One of the most comprehensive article summarizing the role of EV in the field of tissue regeneration and regenerative medicine was recently published by Silva and co-workers (10). The authors explained the principles of EV formation and built a bridge between preclinical work and putative application of EV in the clinics. The authors summarized the major effect of EV on immune cell activity modulation as well as their putative role in extracellular matrix remodelling, both of which linked to tissue repair. Finally, the authors provide an excellent overview of the current literature of EV as therapeutic drug-delivery vehicles. This is of special interest for both clinically orientated scientists as well as scientifically interested clinicians for the following reasons: (I) EV can be seen as a natural "off-the-cell" products released from almost all cell types *in vitro* and *in vivo* (11); (II) modified EV loaded with drugs such as chemotherapeutics, growth factors or tumour antigens might serve as targeted therapies or immune modulatory therapies in several clinical indication. When applying autologous EV the risk of pathogen transmission and immunological intolerances can be strongly reduced. In our opinion this is of special significance in case of any clinical testing of exosomes in phase I or II studies. Especially if allogeneic products are used the authorities require two different virus clearance steps, such as high dose ionizing radiation or methylene blue inactivation,

before approving any clinical testing (7). Currently EV-based therapeutics are classified as biologicals and their active substance determined their pharmaceutical classification (12). It remains to be elucidated whether this mandatory pathogen reduction steps during purification and storage or EV might affect morphology, vesicular integrity, interaction with target cells and finally the biological activity.

A further issue that has to be considered before planning any clinical application is that the production of significant amounts of EV is technically extremely challenging, time consuming and therefore expensive. Commercially available EV purification kits are not suitable to produce enough EV for a routine clinical application. On the other hand ultracentrifugation methods have the drawback that a production according to good manufacturing practice (GMP) guidelines (e.g., viral free environment) would be difficult to establish and costly. Centrifugation steps require several manual processing steps (e.g., removing the supernatant during repetitive centrifugation steps), which have to be conducted in a GMP facility.

Beside the technical production hurdles, probably one of the most important aspects one has to consider is the mode of action (MoA) or mechanism of action (MoA) of EV in the field of regenerative medicine. In principle the MoA describes the interaction of a drug with the organism/cell and its biological or molecular effects. As summarized by Silva *et al.* and others EV display multiple biological effects depending on the one hand on their donor cells and how these cells are stressed an on the other hand on the target cells, their mode of application (i.v., i.p., s.c., topical) and duration of application (13). Although individual groups have highlighted the role of selected EV molecules for their regenerative potential (14,15) it still remains a miracle whether a single component alone or the interaction of different factors present in EV are responsible for their biological effectiveness (12). EV contain distinct classes of molecules such as functional mRNAs, small RNAs, such as microRNAs (miRNAs), lipids and proteins, which alone or in combination are able to promote tissue regeneration. miRNAs and mRNAs can be transferred from donor to recipients cells, thus regulating signalling pathways (16). In addition, selected lipid classes, such as ceramides, have been shown to modulate cell death pathways and induce cell death in an oligodendroglioma cell line (17). The regenerative capacity of EV is furthermore attributed to the cell type from which they are released. It remains unclear which donor cell source provides the most potent EV subtypes in the field of regenerative medicine. Beside stem cell derived EV, peripheral blood mononuclear cell (PBMC) derived or endothelial derived cell types can promote tissue regeneration and cytoprotection (7,18). The combination of pleiotropic EV components that are different between cell and cell type exacerbate a clear description of the EV's role in the field of regenerative medicine. As compared to the whole cell secretome, also EV contain a mixture of proteins, lipids, an regulatory RNAs which probably orchestrate their biological activity by the interaction with each other and with target cell molecules. In our opinion the identification of a single molecule, which is responsible for the distinct regenerative aspects is rather unlikely.

The same may be the case for secretome based therapies. While the beneficial effects of these therapies are increasingly investigated the exact bioactive components still remain unclear in large areas. It seems more reasonable for us that pleiotropic factors of EV or of secretome based therapies should be seen as a whole bioactive drug ready to use instead of trying to find one singe responsible molecule.

Therefore, further research should increasingly address questions concerning the development of a production workflow according to the GMP guidelines and stability and potency aspects of proven biological active EV to move forward to a clinical application, as the authorities will demand these data before approving any clinical testing.

Outlook

Taken together, the number of peer-reviewed articles focusing on the use of EV in regenerative medicine has increased exponentially in the last years. EV contain diverse factors with pleiotropic different biological activities, in part supporting tissue regeneration. Depending on the cell type, experimental setup, and purification method EV consist of different components with different biological effects. However, instead of focusing on the identification of "the" single active factor we—as clinically oriented researchers—should increasingly face regulatory demands and consider or incorporate them into our experimental design. The establishment of production protocols according to the GMP guidelines including product characterization, preclinical proof of efficacy, production characterization, stability assays and the identification of the MoA will facilitate the entry of EV into the clinics. In our opinion a close cooperation between the authorities and researchers might be the key to success.

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Extracellular vesicle-mediated communication in host-parasite interactions: insight from *Fasciola hepatica*

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Extracellular vesicles in the host-parasite interaction

In recent years, extracellular vesicles (EVs) have been accepted as a new intercellular communication system that mediate the transfer of proteins, lipids, mRNA, microRNA and other non-coding RNA species. Special attention has been paid to the role of EVs in the establishment and progression of human diseases. Indeed, perturbing EV production to modulate their pathological effects is an attractive therapeutic option that has been successful in a number of diseases, including cancer (1). To the same extent, several studies have described the contribution of parasite-derived EVs to the modulation of the host immune system (2-4) or the pathological effects on host cells (5). Tools such as transcriptomics and proteomics, have been particularly useful for identification of the immunomodulatory molecules that parasites package into EVs (6). A better understanding of how parasite EVs are produced and interact with host cells may open new avenues for parasite control, since the selective inhibition of these would prevent the delivery of potent immunomodulators that induce a host immune phenotype that favors parasite survival.

It is in this context that we established a definitive characterization of the total secretome of the zoonotic parasite *Fasciola hepatica* (6), one of the causative agents of the trematode foodborne disease known as fascioliasis. Whilst primarily regarded as a disease of livestock, it is estimated that *F. hepatica* infects between 2 and 17 million people worldwide, with a further 180 million living at risk of infection (7). Resistance to triclabendazole, the frontline chemical treatment against *Fasciola*, is rapidly spreading and highlights the need for novel control strategies against the parasite (8).

Characterization of the EVs released by *Fasciola hepatica*

EVs released by the parasite during *in vitro* culture were isolated using ultracentrifugation and ultrafiltration and subsequently analyzed by transmission electron microscopy (TEM) and mass spectrometry. One of the pivotal findings was that *Fasciola* secretes at least two sub-populations of EVs of varying size that bear different cargo molecules and may be released from distinct sites within the parasite. TEM revealed that the larger EVs are released from the specialized cells that line the parasite gastrodermis and are specifically enriched in the zymogen of the 37 kDa cathepsin L peptidase, which mainly performs a digestive function (9). Proteomics and transcriptomics data provided insight into molecular origin of the smaller exosome-like EV population. Whilst a previous exosome characterization study described only the total vesicular content (10), we wanted to obtain a more detailed picture of the vesicle architecture. Thus, we performed a differential extraction of membrane associated proteins—more likely to participate in interactions with

host cells—and those packaged as cargo—envisaged to be delivered into host cells. Mass spectrometry analysis revealed a significant number of proteins belonging to the ESCRT pathway of EV biogenesis and vesicular transport. Together with the abundance of shared tegumental proteins (11), these results suggested that at least some EVs from *Fasciola* originate from multivesicular bodies within the tegumental syncytium before being shed from the apical plasma membrane. Furthermore, transcriptomics analysis indicated that whilst the molecular "machinery" required for EV biogenesis is constitutively expressed (albeit at low levels) across the intra-mammalian developmental stages of the parasite, the cargo molecules packaged within the EVs are developmentally regulated. This suggests that there is a constant release of EVs containing effector molecules finely tuned to the defensive needs of the developing parasite as it migrates through various host tissues.

Future research directions

Although this study provided insight into the mechanisms that helminth parasites use to produce EVs, it raised a considerable number of questions that need to be addressed before designing a rational therapeutic approach for this or other helminth parasites (12). Our proteomics data largely supports an ESCRT-dependent origin for the exosome-like EVs released by *F. hepatica* (*Table 1*). However, further research is needed to determine the specific roles of individual pathway members during exosome biogenesis in liver fluke—e.g., by RNAi mediated gene silencing, which is functional in this parasite (13). Additionally, before members from these pathways can be selected as possible targets for anti-parasite drugs, it remains to be elucidated whether mammalian exosome biogenesis pathways are conserved in *F. hepatica* or if novel routes are used by the parasite. The presence of orthologues from ESCRT-independent pathways, such as sphingomyelinase and various members of the tetraspanin family in its secretome (6) (*Table 1*), could indicate that *F. hepatica* uses hybrid routes for EV release as have been described in some mammalian cell types (14). However, this may also be due to the heterogeneity of vesicle populations in the isolated EVs. The lack of specific markers to distinguish EV sup-populations is a common issue in the field (15) and therefore, to establish a broader set of markers would help to discriminate EV populations and track down their site(s) of production and release from the parasite. We found that the zymogen of cathepsin L, specifically enriched in the

Table 1 Summary of proteins identified in adult *Fasciola hepatica* extracellular vesicles that are involved in EV biogenesis in mammalian cells

Protein	Function	EV subtype
ESCRT-dependent pathway		
TSG101	ESCRT-I component	Exo & MV
CHMP2A	ESCRT-III component	Exo
CHMP5	ESCRT-III component	Exo
CHMP1A,B	ESCRT-III component	Exo & MV
IST1	ESCRT-III component	Exo
VPS4	EV abscission	Exo & MV
VTA1	VPS4 cofactor	Exo
ALIX/PDCD6IP	ILV formation/cargo sorting	Exo
Syntenin	ILV formation/cargo sorting	Exo
ESCRT-independent pathway		
Sphingomyelinase	Ceramide-dependent ILV formation	Exo & MV
CD63 antigen	ILV formation/cargo sorting	Exo
Vesicle trafficking and membrane fusion/remodelling		
Rab8a	Fusion of MVB with the PM	Exo
Rab11	Fusion of MVB with the PM	Exo
Rab27	Fusion of MVB with the PM	Exo
Ral-1/Ral-A	Fusion of MVB with the PM	Exo
Rho1 GTPase	Signal-induced cytoskeletal regulation	MV & Bleb
Synaptotagmin	t-SNARE	Exo
VAMP7	v-SNARE	Exo
Phospholipid translocases	PM curvature	MV
Phospholipases	Signal-induced cytoskeletal regulation	Exo & MV
vATPase (V0)	Fusion of MVB with the PM	Exo

Exo, exosomes; MV, microvesicles; ILV, intraluminal vesicle; MVB, multivesicular body; PM, plasma membrane.

larger EVs released by the parasite, constitutes a potential marker for this type of vesicle. Our differential extraction approach, which separated membrane-associated proteins from those packaged into the lumen of exosome-like vesicles, helped to identify exosomal markers common to many species as well as potential parasite-specific molecules, such as tetraspanins.

Transcriptome analysis indicated that members of EV biogenesis pathways are constitutively expressed during the intra-mammalian developmental stages of the parasite. This is in agreement with reports of constitutive release of exosomes via the endosomal pathway in mammalian cells (16). On the other hand, shedding of microvesicles from the plasma membrane usually occurs in response to a stimulus. It is well documented that *F. hepatica*, as well as other platyhelminth and nematode parasites, shed vesicles (usually referred to as blebs) from their cuticle/tegument in response to drug treatment or humoral immune challenge (17,18). Although it has been suggested that blebbing is an attempt by helminths to replenish tegument that has been lost/damaged due to drug action (19), this mechanism might constitute a defensive response of the parasite to reduce drug effective concentrations by packing them into vesicles that are immediately disposed of. To determine whether the molecular pathways involved in bleb production are the same as microvesicle production could provide a better understanding of drug resistance in helminth parasites, and a means to counter it.

Whilst EVs secreted by helminths can be internalized by host cells and regulate host immune and inflammatory responses (2-5,10), it is unclear to what extent *Fasciola* EVs contribute to maintaining a Th2/regulatory environment that is permissive to fluke survival and reproduction. Once we gain a better understanding of these issues, the selective disruption of key pathways involved in EV biogenesis, or blocking the EV-driven delivery of parasite immunomodulators to host cells, might prove to be an efficient way to achieve parasite control in the future.

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Footnote

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Exosome-like vesicles of helminths: implication of pathogenesis and vaccine development

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Helminthes are multicellular parasitic pathogens including nematodes (roundworms), cestoda (tapeworms) and trematoda (flukes) (1). These parasites cause hosts' weakness and diseases by living in and feeding on living hosts, by receiving nourishment (1). Deep understanding of mechanism between parasites and hosts may help to develop a novel and effective intervention against parasitic diseases.

Extracellular vesicles (EVs) are small membrane bounded vesicles that formed via the invagination of endocytic compartments generating multivesicular bodies (MVBs) and then released to the extracellular space after fusion of the MVBs with the plasma membrane. Consequently, various proteins, lipids, and nucleic acids are capsulated into EVs. It has been demonstrated that exosomes not only contribute to importantly biological functions such as tissue repair, neural communication, immunological response and the transfer of pathogenic proteins (2) but also regulate cellular functions, including motility and polarization, immune responses and development, and contribute to diseases such as cancer and neurodegeneration (3). In parasites, the pioneering study of parasitic exosomes was determined in *Trypanosoma brucei* in 2001 (4). Subsequently, several protozoa such as *Trichomonas vaginalis*, *T. cruzi*, *Leishmania spp.*, and helminths can also found to secrete EVs into living hosts (5). In helminths, secreted EVs have important roles in establishing and maintaining infection. There are two groups of EVs associated with parasitic infection: EVs secreted from extracellular pathogens and EVs produced by host cells infected by parasitic pathogens. Accumulated evidences indicated that pathogenic EVs can act as signal molecules both in parasite–parasite inter-communication as well as in parasite-host interactions for maintaining normal parasitic physiology and leading to the pathogenesis of host (6,7).

Firstly, Marcilla and co-worker demonstrated that two trematodes, *Echinostoma caproni* (*E. caproni*) and *Fasciola hepatica* (*F. hepatica*) can secrete exosome-like vesicles and these EVs can be taken up by host cells, suggesting their potential role in the communication between the parasites and the host (6). In addition, Cwiklinski *et al.* characterize the EVs that involved in pathogenesis and migration of parasite through host tissue, from *F. hepatica* (8). This was one of the first studies to compare soluble and vesicular secretome of *F. hepatica* and describe the EV biogenesis, cargo sorting, and membrane trafficking and cytoskeleton regulation in *F. hepatica*. EVs secreted from *F. hepatica* are enriched in miRNAs and are associated with immune regulatory function (9). In flatworm, earlier study examining the glycocalyx of *Schistosoma mansoni* (*S. mansoni*) cercariae indicated the potential presence of structures similar to MVBs around schistosomula tegument (10). Recent studies in *S. japonicum* demonstrated that adult schistosomes secrete exosome-like vesicles that are able to uptake by mammalian cells and their miRNA cargos could potentially regulate

the expression of host's genes (11,12). Similarly, studies in *S. mansoni* also demonstrated that schistosomes can secrete EVs enriching small non-coding RNAs and proteins that some of them are similar to vaccine candidate (13,14). In nematodes, Buck and coworker demonstrated that *Heligmosomoides polygyrus* (*H. polygyrus*) secretes exosomes that are internalized by host cells. These are enriched in specific proteins, including those associated with exosome biogenesis (e.g., alix, enolase, HSP70), as well as many proteins of unknown function and contain miRNAs and other classes of non-coding RNA. In addition, they further demonstrated that the *H. polygyrus* exosomes could suppress immunological response *in vivo* (15). Overall, EVs from helminths could play important roles in the regulation of host immunological responses to tolerate the parasitic living in a final host.

As EVs carry proteins, lipids and nucleic acids from originating cells, these parasitic exosomes or exosome-like vesicles may express numerous proteins of their originating pathogen, and also release several molecules that involved in origination of these vesicles (16). These proteins and molecules could be potential biomarkers for diagnosis of helminths. Meanwhile, the concept for the use of exosomes as vaccine was first begun in the field of cancer where exosomes released from dendritic cells (DC) were used to mobilize immune system (17). Recent advances in disease control making scientists more confident for the use of antigen origin exosomes in vaccine progress. For instance, *Toxoplasma gondii* (*T. gondii*) infected DC-derived exosomes were used to protect against *T. gondii* infection in mice (18). DC derived exosomes from cells infected with the parasite *Eimeria* were found to convey protection in a poultry model (19). Furthermore, DC derived exosomes were also found to confer protective immunity against *Leishmania* in mice (20). In addition, exosome associated protein, CD63-like tetraspanins, from cestode *Echinococcus granulosus* has been implicated for the targeting of recipient cells and considering the use of exosomal protein as a promising tool for vaccination against alveolar echinococcosis (21). Moreover, mice immunized with purified exosomes from *E. caproni* reduced the symptom severity and mortality and increase the level of IFN-γ, IL-4 and TGF-β (22). Cumulative evidence suggests that exosomes based vaccine may be an important strategy for developing novel vaccine against parasitic diseases.

Although current studies of helminths EVs significantly expand our knowledge of host-pathogen interaction, and our general understanding for roles of EVs involved in biogenesis and pathogenesis of helminths is steadily increasing. By deeply characterizing the functions of EVs may result in the identification of novel biomarkers and therapeutic strategies against neglected tropical diseases.

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Footnote

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Vesicles & Clinical Application

A novel role of exosomes in the vaccination approach

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Exosomes are the type of extracellular vesicles (EVs) derived from inward budding of the inner endosomal membrane to form multivesicular bodies, followed by the fusion of multivesicular bodies with the plasma membrane (1). These vesicles range in size from 30 to 150 nm. Composed of a lipid bilayer, they contain both transmembrane and cytosolic proteins and enclose miRNAs, mRNAs, long non-coding RNAs and DNA (2). Cells use these vesicles to communicate with both adjacent and distant cells. Exosomes have biological activities *in vivo* and exert significant roles in various pathological conditions such as cancer, autoimmune diseases, infectious and neurodegenerative diseases (3). The presence of exosomes in body fluids, along with the plasticity of exosomal content in response to various physiological stimuli and pathological states render exosomes an ideal biomarker for disease state. Exosomes secrete from immune cells and their immunomodulatory functions are very well characterized (4,5). The finding that EVs released by B-cell lines carry MHC class-II, co-stimulatory and adhesion molecules suggested that such vesicles could directly stimulate CD4 T-cell clones (6). This idea received further support with the demonstration that vaccination of mice with tumor peptide-pulsed dendritic cells (DCs) exosomes primes tumor-specific CTLs and suppresses tumor growth in a T-cell dependent manner (7). Similarly, EVs pulsed with peptides from Epstein-Barr virus, cytomegalovirus and influenza virus have been shown to directly trigger *in vitro* IFNγ secretion by a small percentage of human peripheral CD8 T cells, and probably memory T cells (8). Exosome-based cancer immunotherapy is an attractive approach against cancer as tumor derived exosomes carrying tumor associated antigens are reported to recruit the immune responses (9).

In the context of immune responses against pathogens, exosomes also carries pathogen antigens and known to evoke immune responses. *Mycobacterium bovis* BCG-infected macrophages release EVs containing mycobacterial antigen that, in the presence of DCs, promote T-cell immunity in mice (10). Certain viral antigens are also targeted into the exosome pathway. T cells segregate the HIV Gag protein into plasma membrane-derived EVs (11), and CMV-infected endothelial cells release EVs containing CMV gB protein that stimulate memory CD4 T cells in the presence of APCs (12). In a recent issue of *Veterinary Research*, Montaner-Tarbes *et al.* described the presence of porcine respiratory and reproductive syndrome virus (PRRSV) antigens in serum derived exosomes isolated from both viremic (V) and non-viremic (NV) pigs (13). Moreover, immune sera from pigs previously exposed to PRRSV specifically reacted against exosomes purified from NV pig sera. The presence of antigenic viral protein in serum-derived exosomes free of virus, suggested the possibility of these exosome derived viral antigens as a novel vaccine approach against PRRSV. They first isolated the exosomes by size exclusion chromatography and characterized the exosomes from serum of naive control (CN) pigs (PRRSV negative), V animals (PRRSV RNA positive and seropositive) and NV animals (PRRSV RNA negative and seropositive). The phenotypical characterization of exosomes was based on the classical exosome markers, CD63 and CD81. Nanoparticle tracking analysis (NTA) and cryo-electron microscopy revealed

the size and concentration of exosomes. Exosomal protein characterization by liquid chromatography and mass spectrometry identified the unique pattern of PRRSV proteins associated with the exosomes. Both the NV and V group of animal exosomes contained the peptides from major envelope glycoproteins GP5-Tm:pFc (a fusion protein of GP5 with no transmembrane domain and pig fragment crystallizable portion), from envelope glycoprotein GP3, NSP2 and partial ORF2b. Apart from these common peptide sequences, more interestingly, the exosomes from NV animals showed peptides from RNA-dependent RNA polymerase and nucleocapsid protein N. The proteomic analysis also identified for the first time the porcine proteins contained within the exosomes, which were related to exosome composition and function. Importantly, this will facilitate the future studies between host and pathogens in PRRSV and other animal diseases.

Furthermore, the authors also tested the antigenic properties of exosome derived viral proteins and demonstrated that immune sera from pigs previously exposed to PRRSV, specifically reacted to the exosomes from NV animals. This exosomal protein mediated antigenic activity was very similar to the antigenic activity contained in the commercially available vaccine (Porcilis PRRSV vaccine, Intervet, Boxmeer, The Netherlands). Though the immunogenic properties of pathogen derived exosomal antigens have been tested in several preclinical models and diseases of parasitic and viral origin (14,15), the circulation of viral antigens through the exosomes in the serum of the host with no pathogen load detected in peripheral circulation (NV) suggested the importance of this study for a novel vaccine approach.

Since the physical and chemical characteristics of many EVs, including exosomes as well as their biogenesis pathways, resemble those of retroviruses (16), the authors used the polyethylene-glycol (PEG) precipitation and size exclusion chromatography process to scale up the exosome isolation based on retrovirus isolation method (17). The scaling up process did not affect the immunological property of exosomes. Thus, collectively the authors demonstrated the isolation, characterization, antigenicity and scaling-up process of serum-derived exosomes from pigs previously infected with PRRSV and warranted the further exploration of this study as a novel vaccination approach to eradicate PRRSV. This approach will overcome the current limitations of current conventional vaccines against PRRSV.

However, it should be noted that there are certain areas remain to be explored. It is tempting to speculate that why the viral RNA dependent RNA polymerase and nucleocapsid protein are circulating in the serum derived exosomes from NV animals without the ongoing viral replication. Whether these viral proteins in exosomes will act as immune modulators to establish the viral infection by immune evasion mechanisms (18) or the proteins like RNA dependent RNA polymerase and nucleocapsid will aid in enhancement of viral RNA replication in immunized animals already infected with PRRSV still remains elusive. Recently, Li *et al.* found that the viral RNA dependent RNA polymerase and helicase increase the virulence of the atypical highly pathogenic HP-PRRSV emerging in China (19), but information regarding mechanisms by which they could contribute to pathogenicity remains unknown. Similarly, the nucleocapsid protein interacts with different cellular factors of the host to facilitate virus infection. The N protein and three non-structural (Nsps) PRRSV proteins have been identified as playing an important role in type I IFN suppression and modulation of the NF-κB pathway as it is translocated to the nucleus during early stages of infection (20). Continuous studies to evaluate these mechanisms will provide more specific insights to understand the encapsidation of viral proteins in exosomes and their role as a novel vaccine approach.

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Not all extracellular vesicles were created equal: clinical implications

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Provenance: This is a Guest Editorial commissioned by section Editor Mingzhu Gao, MD (Department of Laboratory Medicine, Wuxi Second Hospital, Nanjing Medical University, Wuxi, China).

Comment on: Muhsin-Sharafaldine MR, Saunderson SC, Dunn AC, *et al.* Procoagulant and immunogenic properties of melanoma exosomes, microvesicles and apoptotic vesicles. Oncotarget 2016;7:56279-94.

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Extracellular vesicles are lipid membrane-bound particles containing cytoplasmic and transmembrane proteins, DNA and RNA that are released from cells in a variety of circumstances and contribute to cell-to-cell communication and other processes. Recent research has emphasized the potential role of microvesicles in cancer cell signaling and tumor biology, from metastasis to cancer-associated thrombosis (1,2). In this regard, Muhsin-Sharafaldine *et al.* report in *Oncogene* on the contribution of diverse types of microvesicles (exosomes, microvesicles and apoptotic vesicles) to the procoagulant and immunogenic properties of melanoma (3).

Different types of extracellular vesicles

Mammalian and non-mammalian cells release different types of extracellular vesicles (4). Three main pathways for the generation of microvesicles are recognized (*Figure 1A*).

Exosomes are small particles (<150 nm) generated intracellularly inside multivesicular endosomes or multivesicular bodies (*Figure 1B*). Extracellular vesicles may be produced by budding from the extracellular membrane yielding particles from 100 to 1,000 nm known as microvesicles, ectosomes or microparticles. There are some specialized "microparticles". Thus, platelets may be considered a very specialized form of extracellular vesicles released from megakaryocytes as the end products of membrane protrusions that extend into the sinusoidal vessels, where they are sheered off by blood flow in the process known as thrombopoiesis or platelet biogenesis (5). Finally, apoptotic vesicles are released during apoptotic cell death and are generally larger (100–5,000 nm in diameter). During apoptosis, proteases such as caspases are activated and breakdown intracellular proteins, resulting in nuclear condensation and fragmentation and cell fragmentation, with preservation of cell membrane integrity until very late stages of the process (6). Indeed, apoptotic cells are usually engulfed by adjacent cells before lysis, and apoptosis is usually considered a non-inflammatory form of cell death. Erythropoiesis shares some features with apoptosis, such as reduction in cell size, chromatin and nuclear condensation, and enucleation that require caspase-3 activity (7).

Extracellular vesicles in inflammation

Extracellular vesicles contribute to several pathophysiological responses. Thus, extracellular vesicles derived from epithelium activate wound repair circuits (8). However, they may also contribute to the pathogenesis of infection and sterile inflammatory conditions (9). Danger-associated molecular patterns (DAMPs), such as extracellular ATP, are released during tissue damage and activate the inflammasome in macrophages, amplifying inflammation. Inflammasome-induced activation of an intracellular caspase-1/calpain cysteine protease cascade facilitates the formation and release of phosphatidylserine-positive, highly procoagulant microparticles (10). Thus, galectin-3, a molecule contributing to atherothrombosis,

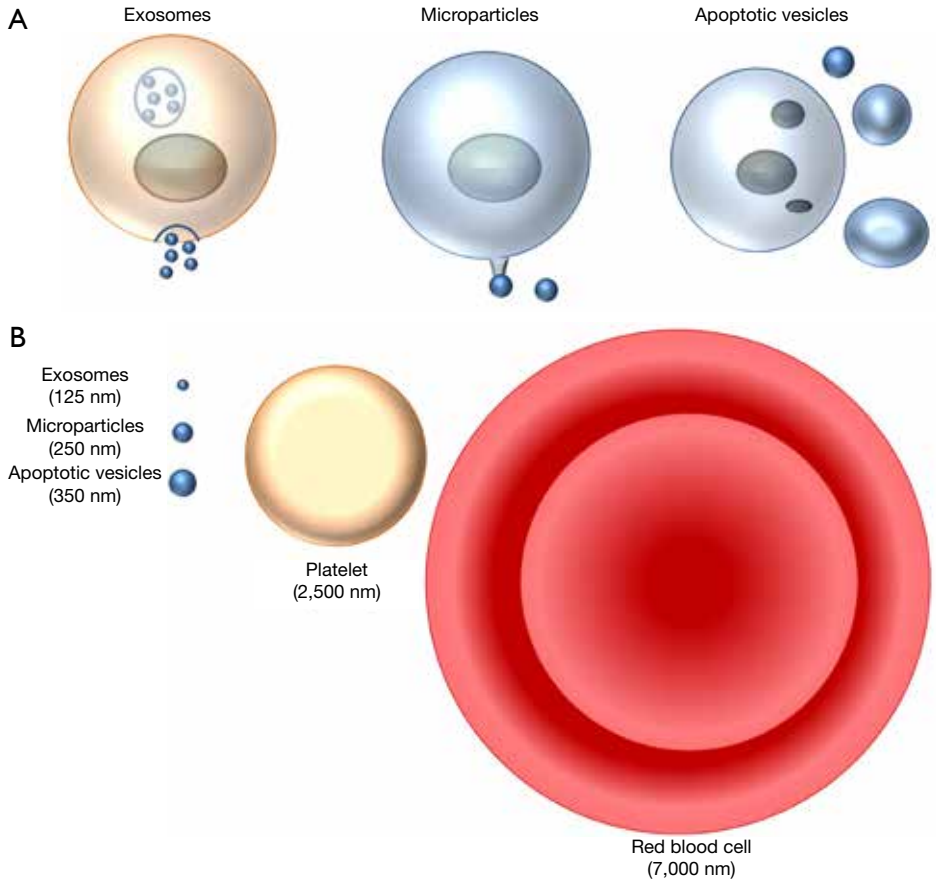

Figure 1 Different types of extracellular vesicles. (A) According to the mechanism of generation. Exosomes are generated intracellularly inside multivesicular bodies. Microvesicles are produced by budding from the extracellular membrane. Apoptotic vesicles are released upon cell fragmentation during apoptotic cell death; (B) comparative size of diverse extracellular vesicles as compared to platelets and red blood cells. The size range is <150 nm for exosomes, 100 to 1,000 nm for microvesicles and 100–5,000 nm in diameter for apoptotic vesicles. Representative sizes are shown.

is released from monocyte macrophages in exosomes (11). Parenchymal cells, such as kidney tubular cells, also release exosomes constitutively (12). Exosomes may be used as biomarkers (9). Thus, urinary exosomes from diabetic nephropathy patients differentially expressed a panel of 3 proteins (AMBP, MLL3, and VDAC1) when compared to non-diabetic controls (13), while osteoprotegerin was increased in urinary exosome-like vesicles from patients with autosomal dominant polycystic kidney disease (12). Furthermore, release of extracellular vesicles from non-mammalian cells may be involved in the pathogenesis of infectious disease. Thus, extracellular vesicles from Trypanosoma brucei mediate virulence factor transfer and erythrocyte remodeling, causing anemia (14).

Extracellular vesicles and cancer

Cancer cells also release several types of extracellular vesicles that are uptaken by other cells, potentially leading to transfer of functional mRNA and to altered cellular behavior (15). Indeed, extracellular vesicles released by malignant tumor cells are taken up by less malignant tumor cells located within the same and within distant tumors *in vivo* (15). Extracellular vesicles carry mRNAs involved in migration and metastasis and may indeed increase the migratory behavior and metastatic capacity of less malignant cells *in vivo* (15). However, the exact microvesicles that display this activity remain to be clearly identified (4).

Exosomes from lung-, liver- and brain-tropic tumor cells

express specific integrins that allow preferential uptake by resident cells at their predicted destination, promoting the preparation of the pre-metastatic niche (16). Conversely, normal cell-derived exosomes mediate an intercellular transfer of miRNAs that specifically suppresses expression of tumor-related genes, as it is the case for astrocyte-derived exosomes and PTEN expression by brain metastasis (17). Melanoma-derived exosomes reprogrammed bone marrow progenitors toward a pro-vasculogenic phenotype (18). Reducing Met expression in exosomes diminished the pro-metastatic behavior of bone marrow cells. RNA interference for RAB27A, a regulator of membrane trafficking and exosome formation, decreased exosome production by melanoma cells, preventing bone marrow education and reducing tumor growth and metastasis. This information may be useful in designing novel therapeutic strategies. In addition, specific expression of certain proteins in tumor-derived exosomes may allow non-invasive, early diagnosis of tumors such as pancreatic cancer (19).

Extracellular vesicles and the immune response

Extracellular vesicles play a key role in immune regulation (20). During transplant rejection, exosome-like extracellular vesicles from donor dendritic cells interact with recipient dendritic cells, leading to their activation and triggering of alloreactive T cell activation (21). Dendritic cells are also thought to be important for immune cell-dependent tumor rejection and early clinical trials have demonstrated the feasibility and safety of the approach (22). Extracellular vesicles from cytotoxic T cells are loaded with cytokines promoting cell death. By contrast, extracellular vesicles also contribute to tumor-mediated immune suppression. Tumor-derived exosomes carry immunosuppressive molecules and factors that interfere with immune cell functions (23). Melanoma-derived extracellular vesicles disseminate via lymphatics and preferentially bind subcapsular sinus $CD169^+$ macrophages in tumor-draining lymph nodes (24). $CD169^+$ macrophage may act as tumor suppressors by containing the spread of vesicles. In this regard, they prevent the interaction of melanoma-derived vesicles with lymph node cortex B cells, an interaction that may foster tumor-promoting humoral immunity (24).

Properties of diverse melanoma-derived extracellular vesicles

As discussed above, extracellular vesicles derived from tumor cell may contribute to multiple tumor-related complications, from metastasis to suppression of the anti-tumor immune response to promotion of this immune response to the thrombotic complications of cancer. A correct understanding of the specific molecule and the specific vesicles involved in each of these complications may be used to design therapeutic approaches that differentially modulate the amount and properties of the different vesicles to optimize the therapeutic benefit, potentiating some functions of extracellular vesicles while dampening others. Muhsin-Sharafaldine *et al*. have now explored the specific molecular composition and properties of exosomes, microvesicles and apoptotic vesicles derived from melanoma cells (3). The pattern of surface and cytoplasmic molecule expression differed between the vesicle types and this was associated with different procoagulant and immunogenic functions.

Melanoma cells were either killed by adding doxorubicin to generate apoptotic vesicles or cultured for 48 hours to generate exosomes or microvesicles. As a result, there is no information of the relative number of the different vesicles released under the same culture conditions. An approach based on amount of protein suggested that the bigger the vesicles, the higher the amount of protein, but it did not provide information on number of vesicles.

The composition of the vesicles was assessed using proteomic approaches and the main findings are summarized in *Table 1*. Exosomes were enriched in histones and heat shock proteins, and the ten most abundant ion scores in exosomes included histones (H2A, H2B, H3.1 and H4), heat shock proteins (GRP78 and HSC71) and the tetraspanin CD81.

From a functional point of view, apoptotic vesicles had the highest functional impact, assessed either as immunogenicity or as a prothrombotic potential (*Table 1*). Apoptotic vesicles were more immunogenic, and mice immunized with antigen-pulsed apoptotic vesicles and challenged with melanoma cells were protected up to 60 days, while lower protection rates were afforded by microvesicles and exosomes. In this regard, only apoptotic vesicles showed enrichment for the melanoma associated antigen PMEL, thus potentially contributing to generate tumor-specific immunity. However, the immunogenicity of apoptotic vesicles is a surprising finding, since apoptosis is, in general, a non-inflammatory, non-immunogenic form of cell death. The observed immunogenicity of apoptotic vesicles may be due to the fact that under the culture conditions, these vesicles were not rapidly engulfed and

Table 1 Key differential characteristics of eh three extracellular vesicle types studied in reference (3)

Characteristics	Exosomes	Microvesicles	Apoptotic vesicles
Histone H4	++++*	+	+/–
HSPA5	+++*	+	+/–
α4	+++*	++	+
CD9	++*	+	–
CD81	+	++*	+
β1	++	+++*	+
CD44	+	++	+++*
PMEL	–	–	+++*
Immunogenicity#	+	++	++++*
Phosphatidylserine	+	++	+++*
Coagulation#			
Fibrin generation#	+/++	++	+++*
Thrombin generation#	+	++*	++*

*, mark the extracellular vesicle type with the highest expression or function; #, functions. HSPA5, heat shock protein A5; PMEL, premelanosome protein; CD9, CD81, tetraspanins; α4, β1, integrins.

cleared by adjacent cells, as would occur *in vivo*, in which different cell types, including macrophages are present. Rather, the cell culture conditions of a homogenous cell type exposed to a toxic that kills or injures all cells in the culture may impair engulfment of apoptotic vesicles. If this were the case, the immunogenicity observed under the experimental conditions may not occur *in vivo* due to rapid clearance of apoptotic vesicles. Another possibility is that doxorubicin also caused immunogenic forms of cell death, such as necroptosis. In this regard, lethal stimuli may cause both apoptosis and necroptosis within the same cell culture (25,26). While the generation of vesicles by necroptotic cells has not been described, it is conceivable the simultaneous occurrence of apoptosis and necroptosis may result in the generation immunogenic apoptosis vesicles. This may occur through adsorption of immunogenic components on the surface of apoptotic bodies. This hypothesis merits further exploration. The observed immunogenicity of apoptotic vesicles may be used to develop anti-tumor vaccines. In addition, it may identify a further mechanism by which chemotherapeutic agents may promote anti-tumor immunity *in vivo*, especially if these agents also induce necroptosis.

Microvesicles and apoptotic vesicles, especially the latter in some assays, were more procoagulant than exosomes and functional studies disclosed that tissue factor and phosphatidylserine were critical for procoagulant activity. In this regard, this may be an untoward effect of chemotherapeutic agents. A better understanding of the mechanisms involved may result in the development of novel therapeutic approaches that target specifically this well-known complication of cancer and its therapy.

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Footnote

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