Analysis of Extracellular Vesicles (EVs) Derived from CAR-T Cells

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Abstract:

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Analysis of extracellular vesicles (EVs) derived from CAR-T cells

For the degree of Master of Science

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CONTENT

ABSTRACT

Extracellular vesicles (EVs) conform a heterogeneous group of lipid bilayer membrane particles naturally released by cells. They vary in size, surface composition, mechanism of formation, biochemical content, and function within the human body. EVs are classified in 3 groups, depending on their size and biogenesis: exosomes (originated by the endolysosomal system, measuring 60-150nm in diameter), microvesicles (direct budding from the plasma membrane, measure 100 – 1000 nm size) and apoptotic bodies (released by dying cells, size >1μm). A very important feature of the EVs is their ability to deliver information by horizontal transfer between the origin cell and the recipient one, evidence suggests that the uptake of EVs by recipient cells can induce changes in their own characteristics and function.

CAR T-cells are genetically modified T-cells that express on their surface a Chimeric Antigen T-cell Receptor (CAR) specific for a unique antigen expressed in the surface of tumor cells. Upon activation with several molecules or cytokines, normal T-cells and CART-cells can express related co-receptors and derived active T-cell biomarkers that will help them to perform a successful immunological synapsis (IS) within the human body, and to induce a successful TCR activation for a subsequent intracellular signal and immune response.

For several years the study of EVs has brought to light one of their most important features, the acquisition of the characteristics originally native from the donor cell by means of the expression of specific proteins in their surface, and the subsequent phenotypical and genotypical modification on the recipient cell after the uptake of the EVs, using this property to our advantage, a successful EV protein surface analysis could lead to the identification and future use of different biomarkers expressed by them to be implemented as a diagnostic tool in the clinical field .

It is here where our work comes into play, the aim of this thesis is the analysis of extracellular vesicles (EVs) derived from CAR-T cells, by the establishment of a successful isolation method of EVs and by the assessment of their surface protein signature together with the expression of some molecular markers derived from T-cells.

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Finally, I would like to dedicate this work to my dad, there´s no day that passes by and I don't think about you. I know you were always with me; I hope you look down and smile to see me accomplishing this that was our dream. Que no se te olvide.

ABREVIATIONS AND SYMBOLS

EV / EVs	Extracellular vesicle / Extracellular vesicles
CAR	Chimeric Antigen Receptor
IS	Immunological synapsis
TCR	T-cell receptor
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
miRNA	Micro ribonucleic acid
ILV	Intraluminal vesicles
MVB	Multivesicular bodies
ESCRT	Endosomal sorting complex required for transport
CD	Cluster of differentiation
MVE	Multivesicular endosome
MHC	Main histocompatibility complex
TNF	Tumor necrosis factor
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
TEM	Tetraspanin enriched domains
GITR	Glucocorticoid-induced TNFR-related protein
NK	Natural killer
nm	Nanometer
APC	Antigen presenting cell
MMP	Matrix metallopeptidase
DC	Dendritic cell
scFv	Single chain variable fragment
CTL	Cytotoxic T lymphocyte

NTA	Nano tracking analysis
ELISA	Enzyme-linked immunosorbent assay
AFC	Automatic fraction collector
SEC	Size exclusion chromatography
TEM	Transmission electron microscopy
PBMC	Peripheral blood mononuclear cell
FCS	Fetal calf serum
LAG-3	Lymphocyte activating gene 3
PD-1	Programmed cell death protein 1
CCM	Cell culture media
pg/mL	Picogram per milliliter
SEM	Scanning electron microscopy
ddH2O	Double distilled water
CTLA	Cytotoxic T-lymphocyte-associated protein
TIGIT	T-cell immunoreceptor with Ig and ITIM domain
BTLA	B and T lymphocyte attenuator

INTRODUCTION

1. The emergence of the study of extracellular vesicles

In the late 1960s were published the first reports of what we today know as extracellular vesicles, or EVs. The very first publications introduce these small double membrane particles as "platelet-dust" or "matrix-vesicles", considering them as "waste released by the cells". It was not until 10 years later than the scientist started reporting them as "microparticles" and "microvesicles" with a possible biological impact within the human body. In the year of 1982, Harding *et al*. noticed the existence of small vesicles while studying the maturation of mammalian reticulocytes, and it was not until 1987 that Johnstone *et al*. came up with the term "exosome" to name them. This new nomenclature, together with the basic understanding of how and which are the intracellular biogenesis pathways responsible for their formation, were the foundations of the study of the EVs. [1]

From that day on, the interest on this vesicles has grown and it has expanded through different branches of science, addressing the most common questions asked on the field, such as: which are the pathways involved in the EV formation, how their biogenesis affects the molecular and proteomic signature of the EVs, which are the effects in the human body after EV exposure, and the possible novel applications of EVs (as therapeutic option in treatment of some diseases), due to this and other reasons the relevance in the study of extracellular vesicles keeps expanding each year.

2. Overview of Extracellular vesicles

2.1 EVs: What are they?

Extracellular vesicles or EVs, are lipid bound vesicles that are released by the cells into the extracellular space under normal and pathological conditions. These secreted EVs are heterogeneous in size, internal biochemical content and surface composition, the biogenesis pathways involved in their formation may also be different, leading to a further classification according to their subcellular origin and size. [1, 2, 3]

Normally, EVs are released by the donor cell and up taken by the recipient cell, this type of communication is known as "horizontal transfer", this delivery will translate in diverse biological effects on the recipient cell depending on the EVs cargo and the surface proteins expressed in their membrane (tetraspanins, integrins, receptors, etc.) [2,3] **(Fig 1)**. After their up-take by the recipient cells, the EVs will fusion with the plasma membrane releasing the inner encapsulated molecules inside the cell (such as transcription factors, DNA, RNA, proteins, lipids, oncogenes, miRNA, and long non-coding RNA) affecting in some level the state of the cell, either conferring them with new qualities or by

changing their migration and even growth. [4, 5, 6] The EVs can not only be up taken by recipient cells through direct membrane fusion but they can also use several mechanisms such as phagocytosis, endocytosis, pinocytosis, by using membrane nanotubes and tunnelling nanotubes, using the gap junctions of the cells, among other mechanisms.[6]

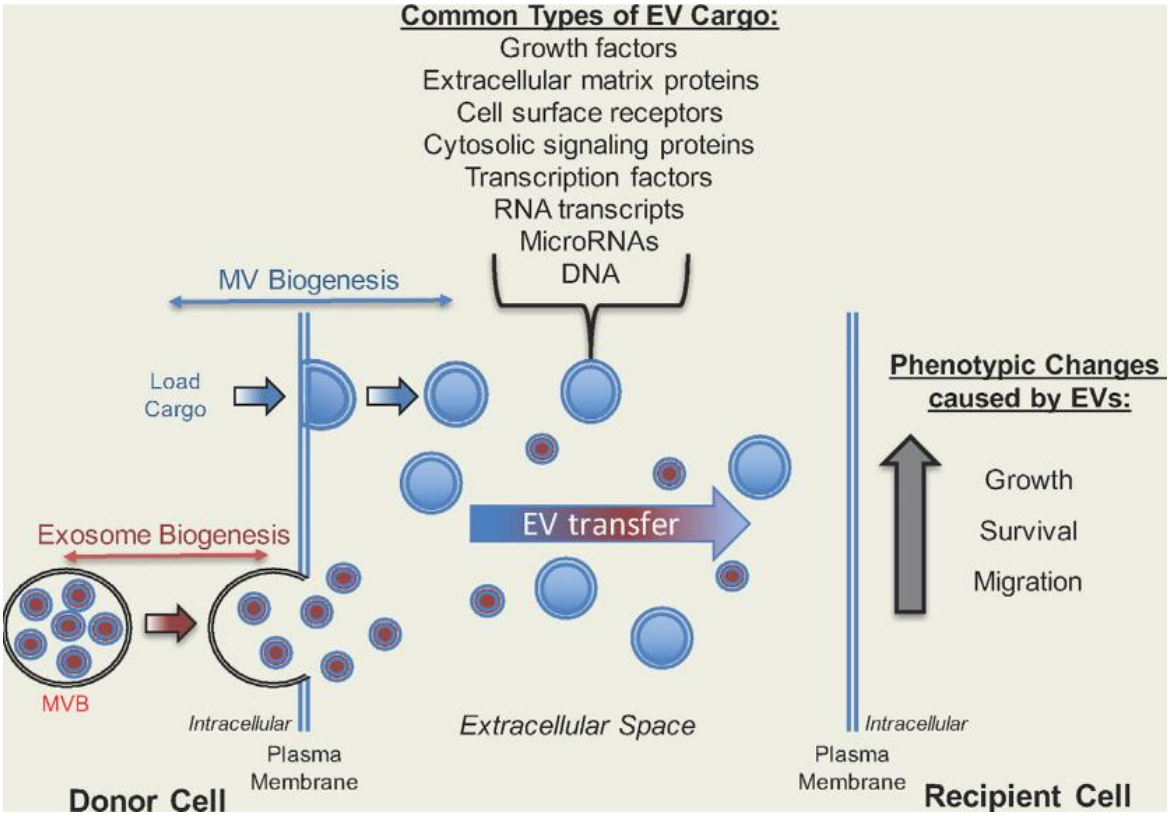

Fig 1. Representation of EVs role in intercellular communication.

EVs transfer information by horizontal communication. EVs are released by the donor cell into the extracellular space where they will be uptake by the recipient cells to finally induce a phenotypic change within the new cell.[2]

Another exciting property of the EVs is their broad reaching sites to perform a cell contact, EVs not only will reach the cells near their releasing site, due their nano-scale size EVs can travel through the entire human body reaching places where large molecules or cells can't (they can even cross the blood brain barrier), also, EVS can be found in the blood or in other bio-fluids such as saliva, breast milk, placenta, semen, just to name some examples. A very important feature of the EVs is their lipidic membrane, this will help them to protect their cargo from any degradation resulting of their trip allowing them to send the caring information to other parts of the human body.[7] Several studies have

shown that the transfer of vesicles from one cell to another not only is a novel mechanism of cell signal transduction, but it also plays an important role in intercellular communication. Commonly, the biological functions of these vesicles will be ruled by their cargo but it also depends on the state of the origin cell and the characteristics that they will confer them.[6, 7, 8]

2.2 EVs Biogenesis and size

The main difference between extracellular vesicles lies not only in the characteristics transferred by their cell of origin (donor cell), the heterogeneity of these vesicles also goes hand in hand with the mechanisms that help their formation and subsequent release from the donor cells.

To this day 3 different cellular routes by which vesicles can be released have been identified, each of these routes will confer the vesicles with unique characteristics and properties, allowing a more suitable nomenclature and classification in base of their size and mechanism of formation **(Fig 2)**.[7]

- Exosomes: Also known as intraluminal vesicles (ILVs), this are the smallest of all the membrane vesicles, they size range covers from 60 to 150nm of diameter, they originate by the endolysosomal system and they are released into the extracellular space with the help of the multivesicular bodies (MVB) after the fusion with the cell plasma membrane.[7]
- Microvesicles: The microvesicles or ectosomes are the medium size vesicles, their size comprises a range between 100 and 1000 nm, the microvesicles buds directly from the plasma membrane, leading to the direct shedding of this vesicles to the extracellular space.[7,9]
- Apoptotic bodies: These vesicles are released by the dying cells, they are formed when exist a rearrangement of the cytoskeleton during the cell membrane decomposition, they don't play a role in the intercellular communication process, and they are the biggest vesicles of all (800 to 5000 nm diameter). [7, 9]

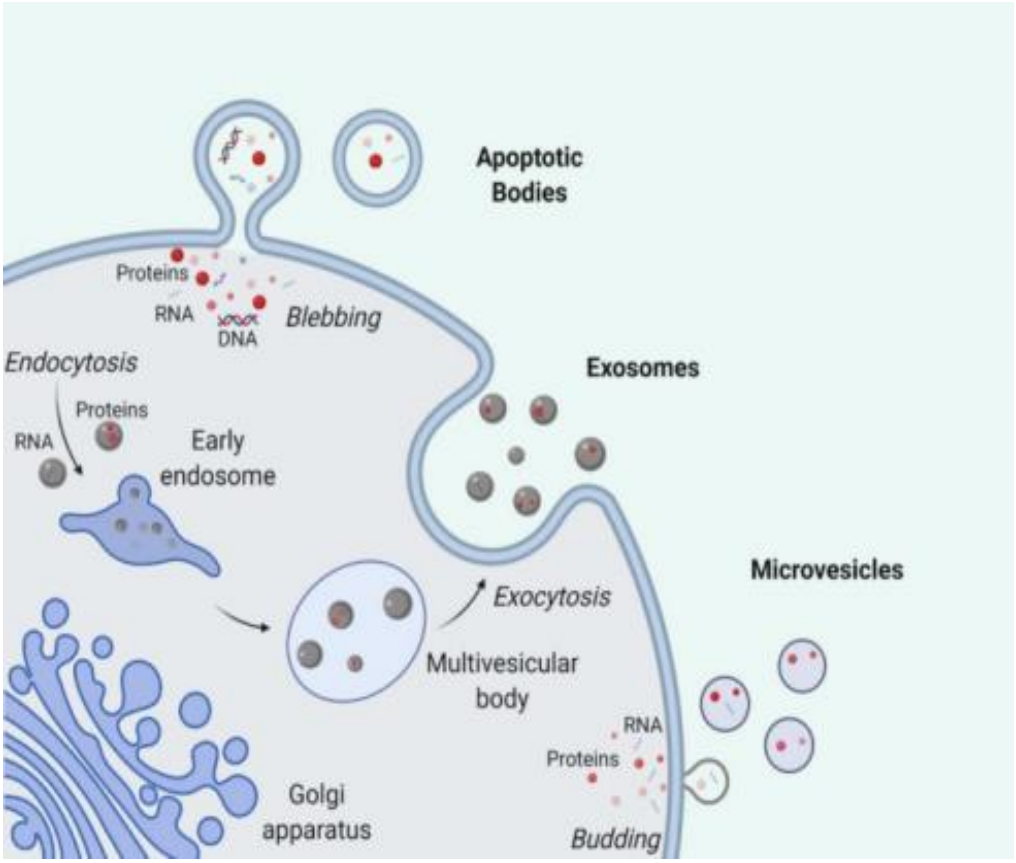

Fig 2. Biogenesis of extracellular vesicles (EVs) subtypes, exosomes, microvesicles and apoptotic bodies.

Exosomes are formed by early endolysosomal pathways and are released when a multivesicular body (MVB) fuses with the cell membrane through exocytosis, size of exosomes goes to 60 through 150 nm diameter. Microvesicles are formed by the outward shedding of the cell membrane to the extracellular space, size range 100 – 1000nm. Apoptotic bodies are generated by cells that undergo apoptosis, size range 800 – 5000nm.[9]

Several publications confirm that the mechanisms of biogenesis not only affect the size of the vesicles, but it can also aid in the understanding of their proteome composition. *Doyle* and *Wang*[8] confirm the tagging of exosomes by means of different molecules, for instance, during the exosomal formation and their transport by the multivesicular bodies (MVB) the process is regulated by the ESCRT proteins, the complex formed between these proteins and some other accessory proteins (tsg101 and Alix), are expected to be present on the exosomes despite of their cell of origin. This will help to differentiate the exosomes from the rest of the vesicles released by the cell. Despite this, there are some other mechanisms of formation independent to the ESCRT one that will induce a different tagging that will be showed in the surface of the exosomes.[10, 11] One of the molecules commonly expressed in the exosomes derived from T-cells is the cluster of differentiation 63 (CD63), which is reported to be present in the surface of exosomes but not for microvesicles, this is caused since this marker is found

in the limiting membrane of the T cell multivesicular endosome (MVE) helping to control their maturation and final release from the cell. The markers are linked to a network of proteins called tetraspanins, they will help vesicles not only to express receptors that allows the identification of their origin but will also confer them with activation or inhibition properties when get in contact with the receptor cells.[10]

In general, the molecular markers expressed on the surface of the vesicles will be dependent and vary accordingly to the type of the cell that release them, the activation state of the donor cells, and of the pathology of the cell of origin (normal or malignant).

2.3 EVs composition: Their cargo and surface molecules

As we previously mention, EVs carries inside them a variety of biological information referred as "cargo". The most common components found inside the EVs are DNA, RNA, proteins and lipids, and although exosomes and microvesicles cargo may vary, the nucleic acids, proteins, and lipids enclosed within their lipidic membrane will be involved in diverse biochemical and cellular processes specific for each vesicle. The cargo will vary depending on the cell of origin and the pathological state of the cell and will help to the transfer of new information among cells or to phenotypically modify the recipient cell after the EVs uptake. A well-known exosome database (exocarta) cites more than 9700 proteins that have been identified inside the vesicles, some of this includes membrane proteins, cytoplasmic proteins, and endoplasmic reticulum related proteins. There also have been reported some metabolism related enzymes, signal transduction proteins, some histocompatibility antigens, to name a few.[11]

As you already know by now, these proteins and cargo are not all the time the same for all the vesicles, in most of the cases the proteins will vary in accordance with the cellular source and the activation status of the cell during the EVs release. As an example, EVs may present specific receptors for antigen presentation (MHC class I and II), co-stimulatory molecules as CD83 and CD40, in the case of the B-cell derived exosomes the B-cell receptor (BCR) may also be expressed the same case for the T-cells (TCR). EVs also may contain cytokines together with their specific receptors (tumor necrosis factor-alpha (TNF-α) and tumor necrosis factor-alpha receptor (TNFαR)), and death receptors ligands this include FASL or TRAIL.[6, 11,12]

Despite all their variation, EVs can contain common proteins that will aid to their further identification; this are frequently referred as "markers of vesicles". Several proteomic and lipidomic analysis revealed that despite of the defined lipid and protein composition of EVs, they also carry highly rich domains of tetraspanins on their surface.[8, 9] In humans, the tetraspanin protein family consist of 33 members,

they interact with their own kind and with other cytosolic and membrane proteins to create a unique domain called tetraspanin-enriched microdomains (TEM). Tetraspanins oversee the mediation of a whole gamma of processes such as immune activation, cell adhesion, tumor and immune cell extravasation, intracellular trafficking regulation, even virus entry. Evidence suggests that the TEMs are an important key in the sorting of intraluminal cargo and the secretion of exosomes.[9, 12] This capacity of interact with the membrane molecules and to help with the organization of complexes in the cell membranes, confers them with the ability to modulate biological and pathological processes, in a few words, the tetraspanin web could be the key to decipher how genetic information and functional proteins are selected to cargo the EVs and to be transferred to the target cells.[12] Together with all their functions, tetraspanins had been proposed and used for some years now as a method to differentiate EVs from non-EV isolated particles. The most common markers used are the tetraspanins CD9, CD63 and CD81 and CD82. It must be taken into consideration that not all this tetraspanins will be expressed at once in all the types of EVs, and that they are mostly enriched on vesicles than cell lysates **(Fig 3)**.[8]

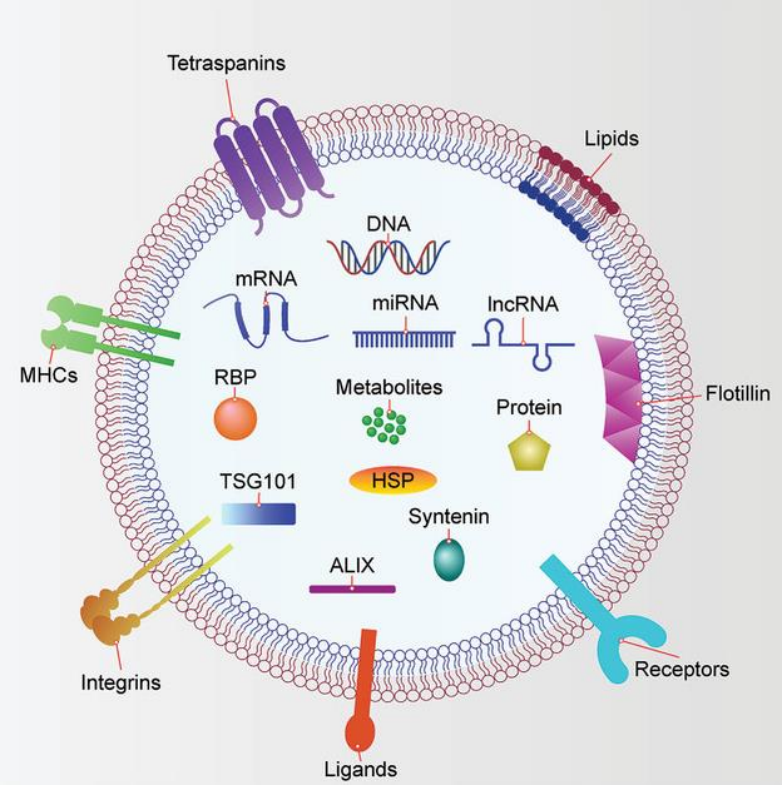

Fig 3. Representation of EV composition.

EVs contain several DNAs (dsDNA, ssDNA, mtDNA), RNAs (mRNA, miRNA, tRNA, snoRNA), proteins, lipids, and metabolites, at the same time may contain membrane proteins as tetraspanins (CD63, CD9, CD81), MHC molecules, integrins and ligands (Hh, PD-L1) among other components as EGFR or PDGFR receptors and flotillins derived from the donor cell.[5]

2.4 Characteristics and the immunological function of T-cell derived EVs

The surface proteins expressed on the vesicles derived from T-cell will play a role in basic cell processes such as cell activation, proliferation, antigen presentation and some other effector functions when they get in contact with another cell, this proteins can be transferred to the vesicle after the cell activation by cell-cell contact or by the stimulus of some interleukins such as IL-2, the exosomes derived from this step will bear the CD63, CD2 and CD3 molecule on their surface but will exclude the marker CD28 and CD45.[14] Other reports suggest that T-cell derived exosomes bear in their surface the cluster of differentiation CD2, CD4, CD8, CD11c, CD25, CD69, the glucocorticoid-induced tumor necrosis factor receptor (GITR), the MHC-I and MHC-II, among others.[11] But they may also present some common proteins, such as CD81 and CD63, some luminal proteins, annexins and proteins involved in several immunological processes such as HLA-I and TCR/CD3. Other reports cite that exosomes derived from activated T-cells can also express on their surface the Fas ligand which will induce cell death.[12,15, 16]

As is expected, once that the proteins expressed on the EV surface contact the recipient cell, will induce an immune response specific for the cell type and help to their regulation, for example Jurkat T-cell derived exosomes may express a ligand that will inhibit the cytotoxicity of natural killer cells (NK), but they can also regulate the proliferation and the physiological functions of the endothelial cells by the CD47 molecule. Also, T-cell can secrete CD63+ exosomes during the immune synapse formation (IS) to antigen-presenting cells (APCs) which will induce a unidirectional transfer of miRNAs. EVs of activated CD8+ T-cells will activate ERK and NF-kB in melanoma cells, leading to the increased expression of MMP9 and by this promoting a cancer cell invasion behavior on *in-vitro* studies. At the same time T-cell derived EVs can prime dendritic cells (DCs) for antiviral responses among other qualities.[8, 15, 16, 17]

3. Chimeric Antigen Receptors (CAR)-T cells

Chimeric antigen receptor (CAR)-T cell therapy is a personalized cancer treatment, were the T-cells from a patient are genetically modified to express an engineered T-cell receptor that will bind specifically to a surface tumor antigen. Although today exist a wide variety of chimeric antigen receptors (3rd generation so far), the extracellular domain of the first-generation CAR receptor is formed by the single-chain fragment variable (scFv) derived from variable monoclonal antibody and a spacer (**Fig 4**). The CAR´s recognize antigens that typically are expressed on the cell surface of the tumor cell, but with the advantage of not needing the antigen presentation by MHC as is the case of the common TCR´s.[18]

To produce the CAR-T, T-cells from the patient undergoing the treatment, are collected by leukapheresis followed by an isolation of the T-cells and a transfection with a non-viral or viral vector encoding for the CAR molecule. Finally, the transfected CAR T-cells are allowed to expand *in-vitro* and after some purification and quality control steps, the CAR T-cells are isolated to later be re-infused into the patient (**Fig 5**).[19, 20]

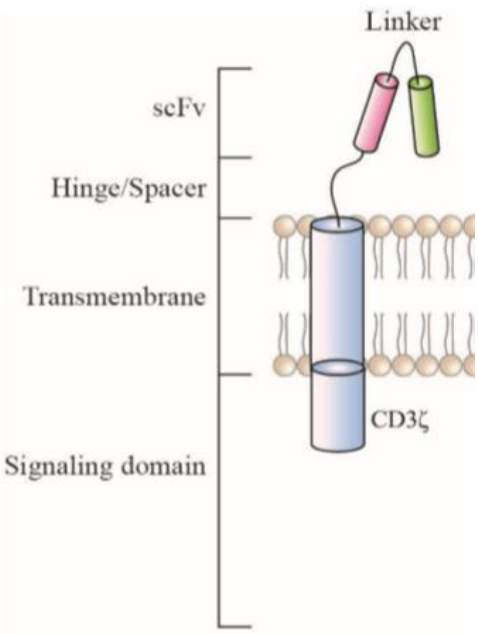

Fig 4. Schematic representation of chimeric antigen receptor (CAR) structure.

The extracellular domains of CARs consist of the single-chain fragment variable (scFv) from a monoclonal antibody, which will recognize the tumor-associated antigen (TAA). Although CARs molecules design has been evolving for several years, first generation CARs, are composed of the scFv extracellular domain, a hinge or spacer (to link the extracellular section to the transmembrane domain) and signal through the CD3ζ chain to confer T-cell activation.[18]

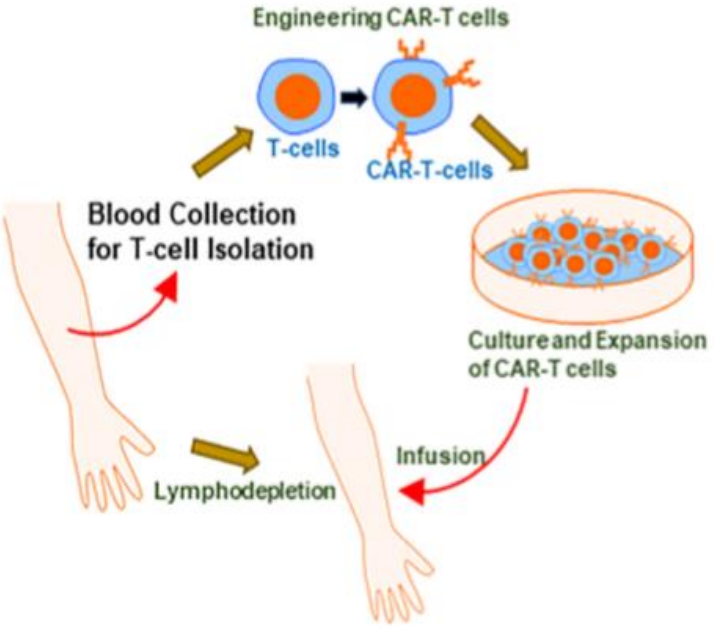

Fig 5. Schematic representation of the production of CAR-T cells for is use as a therapy.

The first step in CAR-T therapy is the collection of blood from the patient, the T-cells are harvested and transduced (by a viral or non-viral method) to create the "CAR-T cells". The engineered cells are then propagated and after accomplishing some quality controls, the cells are infused back into the patient, this step is mostly accompanied by lymphodepletion prior to the infusion. [19, 20]

Currently, the CAR-T cell therapy is mostly used as a treatment for several subsets of B-cell leukemia or lymphoma, and even with their higher rate of effectivity, this therapy can show some limitations as a treatment option and induce some negative effects on the patients undergoing it. Among the limitations of their use are the reduced anti-tumor activity, the antigen scape on the patient's body, the reduced CAR-T cell expansion during their production process, the restricted cell trafficking and limited tumor infiltration between the tissues, the host tumor microenvironment interactions with the CAR-T cells altering their normal function, among other aspects, to resume this, some of the most important considerations are explained in detail in ***Table 1***.[21]

Limitation of CAR-T cell therapy	Description	Possible solution
Antigen escape	Tumors become resistant to a single antigen targeting CAR construct	Optimize the selection of antigens to target, decreasing their escape mechanism and improve antitumor response
Systemic toxicity: On-target off- tumor effects	Solid tumors antigens are often expressed on healthy tissue at several levels	Target of tumor-restricted post-translational modifications. Select antigen capable of inducing sufficient antitumor efficacy with decreased presence in normal tissue
Systemic toxicity: Chemokine release syndrome (CRS)	Excessive cytokine production and massive in vivo T-cell expansion due extensively activated CAR-T cells, causing a severe hyper-inflammatory syndrome in the patient, nausea, headache, tachycardia, hypotension, among others.	
CAR-T cell trafficking and tumor infiltration	CAR-T cells limited ability to traffic and infiltrate in solid tumors (by immunosuppressive tumor microenvironment or natural barriers in solid tumors)	Improve delivery route (local administration instead of systemic)
Immunosuppressive microenvironment	Presence of tumor facilitating cytokines, chemokines, and growth factors; Immune checkpoints pathways (PD-1 or CTLA-4) decrease antitumor immunity	Immunotherapy with CAR-T cells and checkpoint blockades (PD-1/PD-L1) will ensure T-cell persistence and correct function. Combining immunotherapy strategies may be necessary

Table 1. Most common limitations of the CAR-T cell therapy.

Although CAR-T cells have a chance in the treatment of certain hematological malignances, some obstacles remain. Issues as antigen scape, systemic toxicity presented by the patients (on-target off-tumor and CRS), decreased CAR-T cell trafficking and tumor infiltration, and immunosuppressive microenvironment are the most common restrictions presented by this therapy.[21]

By all this reasons, the innovation of new treatments for several types of cancer, and the improvement of the existing therapeutic options that show a high anti-tumor activity with a decreased toxicity for the patients are more than necessary this days to overcome cancer.

3.1. The characteristics and function of CAR-T cell derived EVs

CAR-T cell derived exosomes not only may display on their surface the normal T-cell receptors and surface proteins than the regular EVs, but this vesicles can also show the same characteristics as their donor cell. Want *et al*. [22] reports that EVs derived from CAR-T cells can contain immunostimulatory RNA which could be up taken in by the cells of the immune system and enhance endogenous immunity against solid tumors. After the analysis of EVs derived from CAR-T cells Fu *et al*. [23] reported that some exosomes isolated directly from the medium of the CAR-T cells may lose their targeting specificity due they lack of the antibody-derived scFv in the CAR structure of the T-cells. The researchers also prove the presence of the CAR molecule expressed on the surface of the isolated EVs, as well as CD63, CD3 and CD57, they also report a high level of cytotoxic molecules, but the tetraspanins CD27 and CD28 were expressed in low level together with a total abolishment of the immunological check point PD-1 and CD45 RA.[23]

3.2. A look into the future: The therapeutic value of CAR-T derived EVs

As it was previously mentioned, CAR-T cell therapy is a promising method for cancer treatment, but it may present some major limitations. EVs are not only a unique way of intercellular communication, they also present some advantages, one of them (and maybe the most important one) is their nanoscale size, owing the fact that some released EVs can show the same anti-tumor properties as their donor cell, CAR-T cell derived EVs could also deliver anti-tumor agents to the malignant cells by reaching places whereas CAR-T cells can't, being a suitable option to treat solid tumors. In theory, the EVs derived from CAR-T cells may express the engineered CAR receptor in the surface of the vesicles, conferring them with the same targeting antigen-specificity as the CAR-T cells but without the systemic toxicities caused by their parental cells (Cytokine Release Syndrome) improving and establishing the usage of this treatment in the clinics.[24, 25] Although all this advantages of CAR-T cell derived EVs may look confident in their outcome, until today there is not enough information to support this hypothesis; Peters *et al.*[26] evaluate the role of human T-cell derived exosomes in cytotoxic T lymphocyte (CTL)-target cell interaction. In their study, CTL-derived exosomes ensure the unidirectional attack and further damage in the targeted tumor cells. Until this day, the exact effect of CAR-T cell derived EVs is anticipated, but their cytotoxic property in antigen-targeted tumors is highly expected to function thanks to the help of their membrane surface molecules and cytotoxic content guarded in their intraluminal space.[27] As an extra feature, in theory EVs can be designed at will and be loaded with additional molecules to induce a specific response to avoid tumor resistance and antigen escape.

OBJETIVES – AIM AND HYPOTHESIS

The main aim of this work is the analysis of extracellular vesicles (EVs) derived from the Chimeric Antigen Receptors (CAR)-T cells.

SPECIFIC OBJETIVES

a. Optimize a method for EV isolation from cell culture supernatants of un-transfected T-cells and CAR T-cells under activated and un-activated conditions, using Size Exclusion Chromatography (SEC).
b. Determine the SEC fractions containing higher particle yield using Nanoparticle Tracking Analysis (NTA) and asses the presence of non-EV contaminants.
c. Determine the EV containing fractions by analysis of the EV biomarkers using the Enzyme Linked Immunosorbent Assay (ELISA) and determine their morphology by Transmission Electron Microscopy (TEM).
d. Characterize the surface protein signature of EVs respecting the presence of functional biomarkers and immune check point receptors.

HYPOTESIS

- Activated T-cells and CAR-T cells produce an increased number of extracellular vesicles (EVs).

- EVs derived of activated T-cells and CAR-T cells present on their surface vesicle biomarkers, T-cell activation markers, immune check point receptors PD-1/LAG3, and T-cell derived vesicle markers.

MATERIALS AND METHODS

1. MATERIALS

1.1 Cell lines

Cell line	Description	Culture conditions
T-cells	T-cells derived from peripheral blood mononuclear cells (PBMCs) from two healthy donors, Centre of Chronic Immunodeficiency – CCI, Freiburg, Germany	Cultivation for 2 days prior to activation with anti-CD3/CD2/CD28 (stem cell technology).
CAR-T cells	Primary CAR-T cells were generated from peripheral blood mononuclear cells (PBMCs), Centre of Chronic Immunodeficiency – CCI, Freiburg, Germany	Culture media: 10% FCS EV-depleted, RPMI + 1% Penicillin and streptomycin + 1% HEPES buffer + 100µL IL-2, 50µL IL-15, 25µL IL-7), cultivation for 3 days before harvest of the supernatants.

1.2 Media and supplements

Product	Description	Source
Fetal Calf Serum (FCS)	FCS heart inactivated. USA, PDD	Institute for Transfusion Medicine and Gene Therapy, Uniklinik, University of Freiburg
Activation of T-cells	Beads α-CD3, CD2, CD28 Immunocult “stem cell technology”.	Institute for Transfusion Medicine and Gene Therapy, Uniklinik, University of Freiburg
Growing media	RPMI, 10% EV-depleted FCS, 1% Penicillin/streptomycin, 1% HEPES buffer, 100µL IL-2, 50µL IL-15, 50µL IL-7	Institute for Transfusion Medicine and Gene Therapy, Uniklinik, University of Freiburg

1.3 Chemicals and reagents

Product	Source
1x Phosphate Buffered Saline (PBS)	Molecular biology department, Uniklinik, University of Freiburg
NaOH 0.5M	Carl Roth, Germany
Sodium Azide 0,05%	Sigma Aldrich, Germany
0,1x Phosphate Buffered Saline (PBS)	Molecular biology department, Uniklinik, University of Freiburg
Glutaraldehyde 1%	Department Exosome and Tumor biology, Uniklinik, University of Freiburg
Uranyl acetate 1%	Department Exosome and Tumor biology, Uniklinik, University of Freiburg
1x HEPES buffer	Biochrom AG, Department Exosome and Tumor biology, Uniklinik, University of Freiburg
Ethanol 70%	Liquid Production, Germany
ddH2O Stakpure (0.2µm filter)	Uniklinik, University of Freiburg

1.4 Kits

Product	Source
qEV-original, SEC column (35nm)	Izon Science Ltd, Christchurch New Zealand
MicroBCA protein assay	Thermo Scientific, USA
Human CD9 antigen ELISA kit	MyBioSource, USA
Human CD63 antigen ELISA kit	MyBioSource, USA
Human Lymphocyte Activation Gene3 Protein (LAG-3) ELISA kit	MyBioSource, USA
Human Programmed cell death protein 1 (PD-1) ELISA kit	MyBioSource, USA
MACSPlex Exosome Kit, Human	Miltenyi Biotec, Germany

1.5 Instruments

Instrument	Manufacturer
Centrifuge Heraeus Megafuge 16R	Thermo Scientific, Germany
Ultracentrifuge CouITER Optima LE-80K	Beckman Coulter, USA
Hood SAFE2020	Thermo Scientific, USA
Automatic Fraction Collector (AFC)	IZON science, Christchurch New Zealand
Fume hood Secuflow	Waldner, Germany
PHmeter Inolab pH level 1	WTW Weilheim, Germany
Weighing scale	Sartorius, Germany
Eppendorf Pipettes Research Plus	Eppendorf, Germany
Multichannel pipette Research Plus	Eppendorf, Germany
Ice making machine	Ziegra, Germany
Infinite M Plex TECAN plate reader	Tecan, Switzerland
ZetaView NTA	Particle Metrix, Germany
Timer clock	Oregon scientific, USA
Vortex mixer	Heiddph Reax top, Germany
Cell culture incubator (37°C, CO2)	Thermo Scientific, USA
Fridge 4°C	Bosch, USA
-80°C freezer Eppendorf CryoCube	Thermo Scientific, USA
Electron microscopy	Anatomy department, University of Freiburg, Germany

1.6 Consumables

Product	Source
Falcon tubes (15 mL)	Corning Brand, Mexico
Polypropylene conical falcon tube (50 mL)	Corning Brand, Mexico
Syringe (20mL)	Braun, Germany
Pore filters millex (0.22µm)	Merck Millipore Ltd, Ireland
Disposable serological pipettes	Cellstar,USA
Serva proteus X-spinner 100kDa Ultra-filtration Tube	ProteinArk, Germany
Eppendorf tubes (0.5mL, 1.5mL, 2 mL)	Alpha Laboratory, UK
Micropipete plastic tips (200µL)	Brand, Germany
Micropipete plastic tips (1000µL)	Brand, Germany
Sterile insulin syringes (1mL), Omnifix 100 solo	BRAUN, Germany
Plastic microplates (96 wells)	Greiner bio-one, Germany
Parafilm Bermis	Sigma Aldrich, USA
Nitrite powder free examination gloves Micro-Touch	Nitra-Tex, China
Glass flasks	Schott, Germany
Grids TEM	Department Exosome and Tumor biology, Uniklinik, University of Freiburg

2 METHODS

2.1 Cell culture

2.1.1 Cell lines, activation, and culture conditions

Supernatants were produced and delivered by Dr. Jamal Al-ZuBi (Centre of Chronic Immunodeficiency – CCI, Freiburg, Germany). For their production, T-cells from peripheral blood mononuclear cells were isolated from 2 independent donors. T-cells were activated the 2nd day after collection with beads anti-CD3/CD2/CD28 (stem cell technology), $1x10^6$ cells were cultivated for 3 days before the transduction with the viral particles encoding the CAR construct (scFv, IgG hinge domain, CD28 transmembrane, CD3ʒ signaling domain and co-stimulatory domains).

CAR-T cells were cultured (10% FCS EV-depleted, RPMI + 1% Penicillin and streptomycin + 1% HEPES buffer + 100µL IL-2, 50µL IL-15, 25µL IL-7) for 3 days before 4mL media supernatant was collected. Before deliver, supernatants were centrifuge at 300xg for 5 minutes at 4°C to remove any cell presence. After supernatant collection, cells were stained with anti-CD25 antibody (Miltenyi) to assess T-cell activation (assessed by Dr.Jamal Al-ZuBi).

Medium activated and un-activated without cell presence was used as a control.

2.2 Isolation of fractions containing EVs using Size Exclusion Chromatography (SEC) method

As it was previously mentioned, all cells in the human body release extracellular vesicles; these EVs can vary in size and surface protein signature that provides them with unique properties. Another very important characteristic of EVs is their presence in a several biological fluids (such as blood, breast milk, semen, among others), at the same time each one of these fluids presents their own matrix characteristics as for example viscosity, density, etc.

Due to this reason, is crucial to select a suitable isolation method for culture cell supernatant that allow a high particle recovery, being this the first and most important step to lead a further EV analysis. To solve this inquiry the literature was reviewed, a summary of the currently ongoing EVs separation techniques is presented on ***Table 2***.[40]

After revision, discussion and evaluating the laboratory research capacities, it was decided to proceed with Size Exclusion Chromatography (SEC)-based method to isolate the particles present on CCM supernatant.

Size exclusion chromatography (SEC), also known as gel-filtration, is a separation method that allows the fractionalization of molecules or particles according to their molecular size. When the sample is charged to the top of the SEC column, the particles go through it in different times according to their sizes, by this, the larger molecules (larger than the pore) go through the interspaces of the gel and is eluted first in the void volume, the smaller molecules penetrate in the gel, presenting more retention time and presenting latter in the fractions. In other words, larger molecules elute first, and smaller ones will elute last.[67]

For preforming SEC, it was used the Automatic Fraction Collector (AFC) by IZON science. The AFC can collect the resulting fractions from the column by an automatic differentiation between the volume of void (no contain any EVs) and the volume of the fractions containing the EVs[68]. To perform the particle isolation, it was used the qEVoriginal 35nm column (IZON science) due the pore size range of the matrix is correspondent to the expected particle size of exosomes and microvesicles (capture range of the column: 35 to 350nm).

4mL of cell culture media (CCM) supernatant was collected after 4 days of incubation and centrifuged at 300g for 5 minutes at 4°C to remove any cell presence on the media; after the sample was delivered, a second centrifugation was performed at 2000xg for 10 min at 4°C to remove cell debris. The total volume was recuperated and concentrate up-to 1 mL using an Proteus X-spinner ultrafiltration tube 100kDa pore size, the centrifugation conditions were 1600xg for 10 min at 4°C. 500µL of the final concentrated volume was taken and charged into a SEC column (qEVoriginal 35nm, IZON science) to proceed with the SEC-based particle isolation, 3mL were directly eluted from the column and considered as void and the further 5 fractions (500µL total volume per fraction) were kept to assess.

The void volume, the 5 fractions, the un-used concentrate sample and X-spinner flow through waste were analyzed by Nano Tracking Analysis (NTA) to estimate the particle number present on each sample during every step of the isolation process, only the void and the 5 SEC fractions were analyzed by MicroBCA for total protein concentration quantification. The ***Figure 6*** shows the designed experimental workflow followed for each isolation.

Isolation technique	Principle	Advantages	Disadvantages
Sequential ultracentrifugation	-Particles present different density and size. -Particles show different sediment speed under centrifugal force	-Low cost -Low contamination risk -Suitable for large volume preparation	-High equipment requirement -Labor intensive -Protein aggregation -Not suitable for small volume diagnosis
Gradient ultracentrifugation	After centrifugation in a dense medium, objects in a tube could stay in the position of the medium with similar density	-High purity of products -Allows separation of sub-population of exosomes	-High equipment requirement -Time consuming -Labor intensive -Not suitable for small volume diagnosis
Ultrafiltration	Using filter membrane with defined size-exclusion limit or molecular weight cut-off	-Low equipment cost -Fast procedure -Good portability	-Moderate purity -Potential deterioration of particles induced by shear stress -Possible particle loss due to clogging of the membrane
Size-exclusion chromatography	After adding to porous materials, substances eluted out in accordance with their particle size, with big particles eluted earlier	-High purity -Fast preparation -Keep native state of EVs -Good reproducibility -Potential for both small and large sample capacity -Capable of processing all types of samples	-Relatively high device costs -Additional method for exosome enrichment is required.
Polymer precipitation	High hydrophilic water-excluding polymers can alternate the solubility of EVs	-Easy to use -Using ordinary equipment -Suitable for small and large sample volume -High efficiency	-Contaminants of proteins aggregates and polymeric contaminants -Extended processing time -Require complicated clean-up steps -Can be presence of downstream analysis and quantification
Immunoaffinity capture	Based on specific binding between exosome markers and immobilized antibodies (ligands)	-Suitable for separating various EVs -High purity EVs -Easy to use -No-chemical contamination	-High-cost antibodies -Exosome markers must be optimized -Low processing volume and yields -Extra step for EV elution may damage native EV structure

Table 2. Current methodologies for EV separation and isolation.[40]

Before isolation of particles, the literature was reviewed to assess and select the most suitable isolation method for the isolation of vesicles based on the sample characteristics and our laboratory capacities.

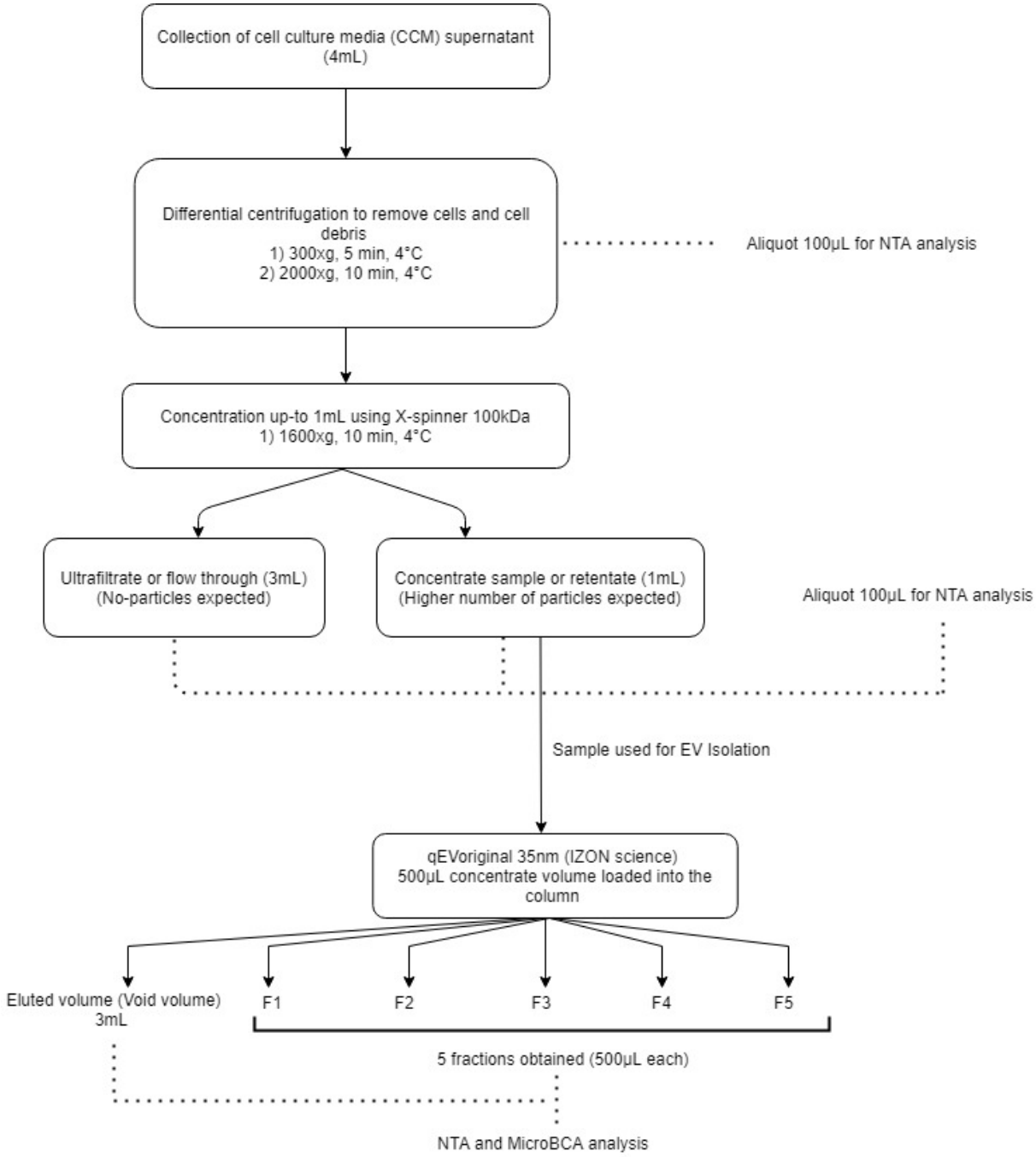

Fig 6. Schematic workflow followed of sample handling and isolation of fractions to evaluate.

4mL of CCM supernatant was obtained, two steps of differential centrifugation were performed to remove cells and cell debris present on supernatants. Proteus X-spinner 100kDa ultrafiltration tube was used for supernatants concentration up-to 1mL. Retentate was aliquoted on two Eppendorf's containing 500µL each; aliquots were used to perform size exclusion chromatography. SEC conditions were: 3mL eluted void volume (no-particles expected), 5 fractions of 500µL each (particles expected at different concentrations).

2.2.1 SEC – qEVoriginal 35nm column preparation, sample loading and SEC fractions acquirement

qEVoriginal 35nm columns were set in vertical position and equilibrated at room temperature for at least 40 minutes before use. Once it's ready, the columns are rinsed with 20 ml of sterile and filtered (0.22µm) 1x PBS, the flushing step must be repeated with at least 30mL of buffer.

500µL of concentrated EVs sample was charged into the column (minimal volume needed to preform SEC using qEVoriginal 35nm); 5 fractions of 500µL each were collected and 3mL of void was eluted and collected before the fractions isolation. After the fraction collection, the column was emptied and flushed with sterile and filtered (0.22µm) 1x PBS (45mL). Finally, 0.05% sodium azide was added into the column for further storage at 4°C until another use.

2.3 SEC fractions particle number quantification using Nanoparticle Tracking Analysis (NTA)

NTA is a method to visualize and measure nanoparticles in a suspension in a range from 10 – 1000 nm based on the analysis of Brownian motion. The particles in the sample are visualized by illumination with a laser beam. The size of each individually tracked particle is calculated, thus simultaneously allowing determination of their size distribution and concentration.[28]

A 100µL aliquot of the isolated SEC fractions and void volume were taken and diluted 1:100 in 0.1x PBS to a final volume of 1mL to be injected into the equipment. All particle number determinations were performed by triplicates under the same SOP (sensitivity: 90, shutter: 70, minimum size: 10, maximum size: 100 nm, brightness: 30, cycle: 1, positions: 11). The videos obtained by the machine were analyzed by the ZetaView Software to define the particle number and size distribution of the isolated particles contained on each fraction.

2.4 Total protein quantification of isolated SEC fractions using Micro BCA kit

Protein quantification was carried out in accordance with the manufacturer's instructions. MicroBCA kit uses bicinchoninic acid, which is a detergent-compatible formulation for the colorimetric detection (quantification) of total protein. In this reaction, the reduction of Cu^{+2} to Cu^{+1} of protein in an alkaline medium (biuret reaction) is measured. The cuprous cation (Cu^{+1}) is detected by a reactive containing bibinchoninic acid (giving a purple coloration) formed by the chelation of two molecules of BCA with one Cu^{+1} ion.[29] Preparation of the diluted albumin standard (BSA) was within the range of 200 – 0.5 µg/mL. The SEC fractions, void and standard curve were evaluated in duplicates. The plate was read at 562nm by Infinite M Plex (Tecan) plate reader. The results were obtained subtracting the average

562nm absorbance reading for the blank standard from the reading of all the other individual standards and samples.

2.5 Detection and quantification of surface vesicle biomarkers using the Enzyme Linked Immunosorbent Assay (ELISA)

2.5.1 Estimation of the presence of CD9 antigen in isolated particles using ELISA

One of the methods to differentiate EVs from non-EV particles is the detection of associated EVs biomarkers, the most common ones are CD9, CD63 and CD81. In this work is used the Human CD9 antigen ELISA kit (MyBioSource) to identify the presence of the CD9 in the isolated fractions.

The enzyme/linked immunosorbent assay (ELISA) is an immunological assay used to detect antigens, antibodies, and proteins in biological samples. The most common ELISA assay is the capture or "sandwich" ELISA, in were two antibodies (outer part of the sandwich) are used to detect the antigen or protein of interest (inner part of the sandwich). In this method, the ELISA plate is coated with a capture antibody specific to the antigen to interest present in the sample to analyze, any excess sample is washed from the plate to avoid misreading. After this reaction has occurred, the detection antibody is added to the plate, this antibody is labelled with an enzyme (Horse radish peroxidase or alkaline phosphatase). The function of the detection antibody is binding to any target antigen fixed to the plate. To make a final reaction, a substrate is added to the plate to produce a colored product which will be measured by a plate reader.[30] The determination of the protein concentration in the sample will be given by the standard curve that uses a known concentration of antigens that can be calculated using the optical density (OD) (**Fig 7**).

Each one of the SEC fractions was evaluated to quantify the presence of antigen of interest. The preparation of the reagents, samples and standard was carried out in accordance with manufacturer instructions, the Enzyme-linked immunosorbent assay (ELISA) plate has been pre-coated with the antigens of interest, the ELISA reagent was added to each well and incubated for 1 hour at 37°C. Washing step was provided 5 times, substrate solution A and B was added and incubate for 10 minutes at 37°C, finally acidic stop solution was added. The plate was read at 450nm by Infinite M Plex (Tecan) plate reader within 5 minutes after adding the stop solution to obtain the OD value. Standard curve and samples were evaluated in duplicates.

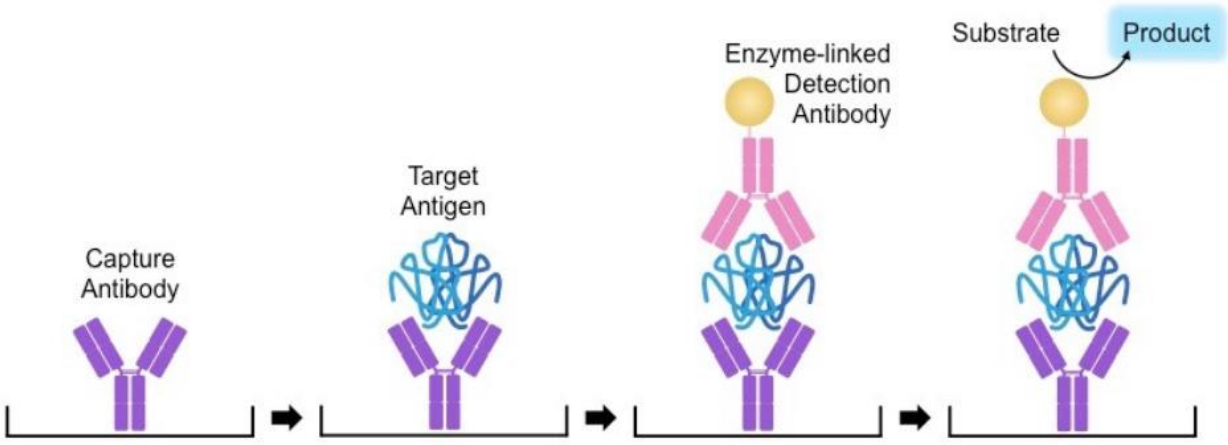

Fig 7. Summary of the principle of sandwich ELISA method.

This assay is based on four main steps. First, the target antigen located on the surface of the EV must be captured by the immunoplate pre-coated with the antibody of interest. The excessive and unbound particles are washed out to avoid misreading; the sandwich is formed by the biotinylated antibodies reactive to specific antigen present in the sample. This construct is bounded to streptavidin-HRP and after incubation the unbound streptavidin-HRP is washed away during the washing steps. Finally, the substrate solution is added, and a color will develop in accordance with the amount of antigen present in the sample, the reaction will be stopped by the addition of the acidic stop solution and the absorbance is measured at 450nm.

2.5.2 Estimation of the presence of CD63 antigen in isolated particles using ELISA.

To identify the presence of CD63 in the fractions it was used the Human CD63 antigen ELISA kit (MyBioSource). The ELISA plate has been pre-coated with CD63 Ab, the preparation of the reagents, samples and standard was carried out in accordance with manufacturer instructions. Each one of the SEC fractions and standard (range of 0 -2000 pg/mL) was evaluated by duplicate to quantify the presence of antigen of interest. 100µL of standards and samples were added to the plate, the plate was covered with a sealer and incubated for 2 h at 37°C. After removal of any unbound particles, 100µL of the biotin-conjugated antibody preparation specific for CD63 was added to all the wells followed by 1h incubation at 37°C. Washing step was provided 3 times by adding 300µL of the washing buffer and let it sit for 1:30 min, after that time any remaining buffer was removed by inversion of the plate. 100µL of Horseradish peroxidase (HRP) antibody was added and incubated for 1 h at 37°C, a second round of wash and decantation steps were repeated 5 times. 90µL of substrate solution was added to each well, the plate was sealed, protected from the light, and incubated for 10 min at 37°C, and finally 50µL of stop solution was added to each well. The plate was read at 450nm by Infinite M Plex (Tecan) plate reader within 5 minutes after adding the stop solution to obtain the OD value.

2.6 Assessment of the particles morphology using Transmission Electron Microscopy (TEM) technique.

Electron microscopy (EM) as emerged as one of the most used methods to determine characteristics on individual EVs as size and morphology. EM techniques can be divided in scanning electron microscopy (SEM) and Transmission electron microscopy (TEM). Both uses an electron beam to obtain high resolution images of nanoscale objects. SEM creates an image by detection of the reflected electrons, while TEM uses the electrons that pass through the sample to create an image.[31] Special specificity and features for both techniques are listed on ***Table 3.***

Feature	TEM	SEM
Electrons type	Transmitted electrons	Scattered, scanning electrons
Main provided information	Inner structure of the sample (morphology, crystal structure and stress state)	Sample´s surface analysis (roughness or possible contamination) and composition.
Max. magnification	More than 50 million times	Up to ~ 1-2 million times
Optimal spatial resolution	< 50pm have been reported	~ 0.5nm
Type of image	2D projections	3D image
Special requirements	Samples must be thin (<150 nm) if high-resolution imaging is required less than 30nm.	No specific requirement
Sample preparation	Complex preparation, trained/experienced users is required	Little or no effort for sample preparation, easy to use

Table 3. Summary of the TEM and SEM techniques.

Individual characteristics of TEM and SEM techniques, both methodologies are different in their requirements and resolution. Literature was reviewed to assess their implementation in this work.

The evaluation was performed only for the cell culture media supernatant correspondent for CAR-T cells under activated conditions, due the complexity and cost of this technique.

All the steps were performed at room temperature and under the extraction hood. Coper grids were held with forceps taking care of place them with the glossy side at the top. 10µL sample drop (10^9

particles/mL) was applied on top of the grid for 5 minutes to allow adsorption. Carefully the copper grid was taken and transferred from the sample drop to 20µL drop pf 1% Glutaraldehyde and left for 5 minutes. After this time the grid was washed with ddH2O by laying the grid on water drops for 30 seconds each. After the washing, the grid was incubated for 1 min with 20µL of 1% uranyl acetate; finally, the grid was dried with the help of nitrocellulose paper. All the procedure was repeated for each one of the samples to evaluate. Image acquisition was performed by Zeiss transmission electron microscope in collaboration with Mrs. Sigrun Nestel, of the Anatomy department, Freiburg, Germany.

2.7 Detection of surface functional biomarkers and immune check point receptors present on EV containing fractions

2.7.1 Detection of T-cell activation markers, T-cell exhaustion markers and the surface biomarkers of isolated EVs using beads-assisted Flow Cytometry by MACSPlex Human Exosomes kit

The EV analysis with any conventional flow cytometer can be a tricky technique to perform. The difficulty to studying EVs with flow cytometry lies in the small size of the EVs (**Fig 8**). Due EVs are smaller than the cells in size, flow cytometers can't detect the EVs and perform a former analysis, to solve this burden, most of the researchers find useful to bind EVs to beads that are large enough to be detected by the flow cytometer[32].

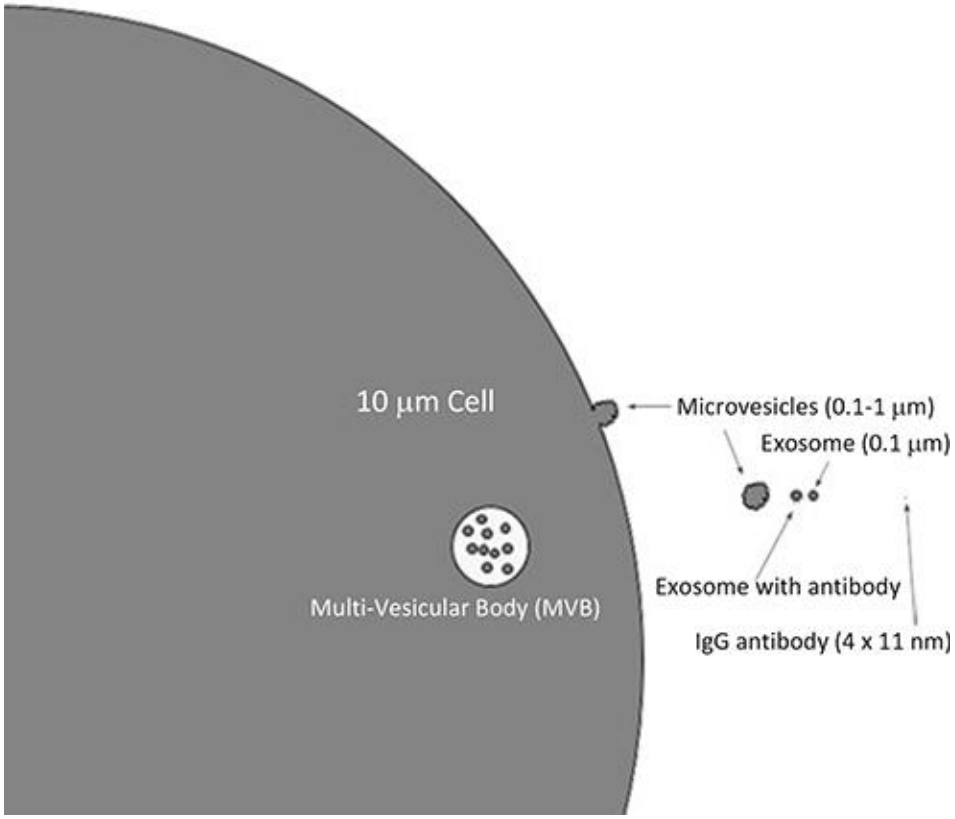

Fig 8. Graphical representation of the scale sizes of EVs, cells and antibodies.

Image describes the size difference of vesicles and antibodies. The typical size of a laser intercept at the point of flow cytometric analysis is 10 -20μm, while exosomes and similar EVS are ~0.1μm.[32]

A successful detection of EVs and EV-associated surface molecules requires the use of ligands that are specific to the EVs populations of interest; this will help to reduce the non-specific background caused by protein-binding beads or by other causes, ensuring a proper quantification of the ligands of interest present on each EV. *Morales-Kastresana* and *Jones*[32] describe a method for using streptavidin-coated beads to capture biotinylated antibodies, the beads are washed to remove any unbound antibodies, the EVs are captured and the bead-bound EVs is stained with directly conjugated antibodies this principle is represented in **Figure 9**.

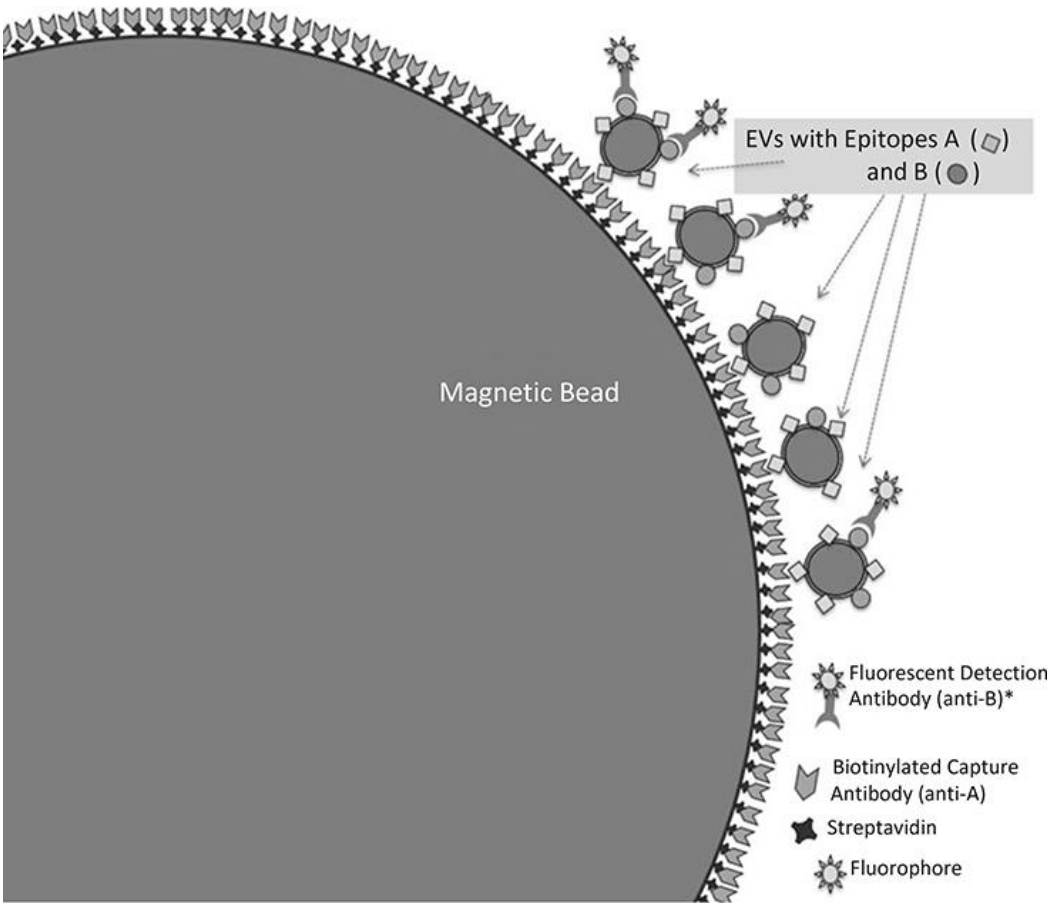

Fig 9. Representation of the EVs capture using a magnetic bead.

The magnetic bead is coated with streptavidin that will link to a biotinylated antibody specific for an antigen present on the EVs surface, helping to fixate the EV to the bead. A florescent detection antibody will be added; this will be specific for an antigen present on the EV surface and create the signal that will be read by the flow cytometer.[32]

The MACSPlex assay is formed by a cocktail of various fluorescently labeled bead populations that are defined as "capture beads" which will be identified by common flow cytometry technique. Each captured bead is coupled to a specific antibody that will bind to their corresponding exosomal surface epitope. The final formed complexes include the capture beads, the exosomes and the detection reagent included in the kit (**Fig 10**). The complexes are analyzed based on their fluorescence characteristics with the help of a flow cytometer (complexes and detection reagent). The obtained positive signals indicate the existence of specific surface epitopes in the exosomes contained on the sample.[33]

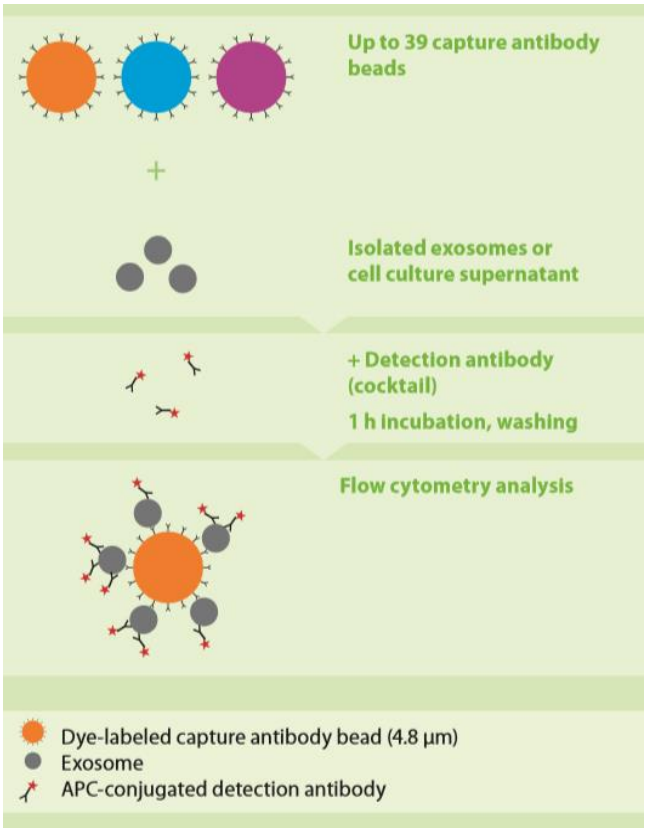

Fig 10. Schematic representation of the MACSPlex assay principle. [33]

Capture beads contained in the kit are coated with 39 different epitopes. Isolated vesicles are added to the beads together with the detection cocktail containing CD9, CD63 and CD81 conjugated antibodies. After an overnight incubation of the mixture, the complex bead-exosome-Ab is analyzed using flow cytometry. Data analysis was performed by subtracting the normalized signal of theCD9, CD63 and CD81 signal. Data obtained by duplicates.

The fractions that presented a higher particle yield (fractions 1 and 2) of all the supernatants were analyzed using MACSPlex. Due the particle number expected on those fractions wasn't high enough, we decided to pool together 4 different isolates derived from 3 different biological replicates, to ensure the presence of vesicles in our sample and to reach a detectable signal in our readings. The assay was normalized using 0.253 vesicles 10^9 per supernatant. The preparation of the reagents, samples and standards was carried out in accordance with manufacturer instructions. 120µL of the MACSPlex exosome capture beads and 15µL of the detection reagent (CD9, DC63 and CD81) was added. Tubes containing the mix were incubated overnight at room temperature in the dark and under constant agitation. After this time, two steps of incubation at room temperature for 15 min after adding 500µL of MACSPlex buffer were performed, samples were centrifuged at 300x g for 5 min to finally obtain a pellet of EVs + capture beads, the supernatant was removed; data acquisition was made following the normalization factor of the mean derived from the signals obtain by CD9, CD63

and CD81 for each sample. The determination of the relative exosome marker level was made by calculating the ratio of the signal intensities of each sample.

2.7.2 Detection of the immune check point receptor Programmed Death cell protein 1 (PD-1) present on EV containing fractions using the Enzyme Linked Immunosorbent Assay (ELISA)

Immune checkpoints are regulators of the immune system, and they play a crucial role in self-tolerance within the organism, which means that they prevent the self-attack of the immune system to the normal cells present in the body; however, some types of cancer have developed the quality to protect themselves from attack by stimulating immune checkpoint targets. The microenvironment of the tumor is normally infiltrated with many different types of immune cells whose immune surveillance functions are often suppressed. The suppression of the signals is translated as the ways as tumor cells downregulate the activity of stimulatory immunoreceptors and upregulate the inhibitory immunoreceptors.[34]

Until this day, several types of cancers are developing resistance to conventional treatment therapies as is chemotherapy. In most cases, the survival of these tumors is caused by checkpoint immunomodulation to maintain the imbalance between immune surveillance and the cancer proliferation.[35]

T-cell activation is regulated by different processes, upon activation; co-inhibitory checkpoints (as PD-1) are induced to regulate T-cells. PD-1 present an essential role in balancing the immunity, however during responses to tumors, PD1 expression can limit protective immunity.[37] Tumor cells can express in their surface the ligand of PD-1 (PD-1L) that will bind to PD-1 on T-cells avoiding the killing ability of the T-cell. When PD-1L or PD-1 is blocked, this will allow the normal T-cell targeting ability to kill the tumor cell (**Fig 11)**.

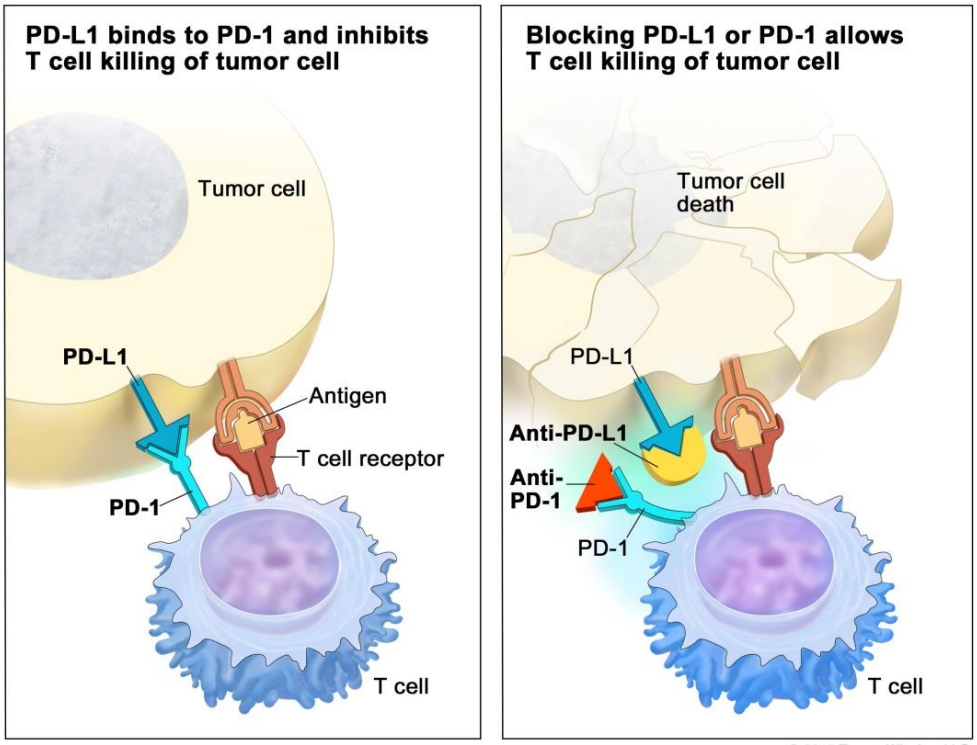

Fig 11. Representation of the function of Programmed Death cell protein 1 (PD-1) and its ligand PD-1L on final T-cell activity.[37]

T-cells might express in their surface the PD-1 receptor under normal conditions. After blockade of PD-1 receptor expressed on the T-cells by Anti-PD-1 protein, the attacking and possible destruction of the tumor by the T-cells is expected, due the complex PD-1 and PD-1L (expressed on the surface of some tumor cells) is not formed the signal cascade is not activated inside the tumor cell.

In 2011 the loss of immunologic control was confirmed as one of the emerging hallmarks of cancer, and in 1996 was proposed an immune checkpoint blockade as a strategy for treating cancer being anti-CTLA-4 antibody as the first one approved by the FDA to treat melanoma, the use of this blockers has been increasing till this days, extending the spectrum of inhibitory immunoreceptors used for therapy, some of this examples are PD-1, CTLA-4, LAG3, Tim3, TIGIT and BTLA to name a few.[35]

To quantify the presence of PD-1 on each of the fractions with higher particle yield, it was used the two-site sandwich enzyme-linked immune-sorbent assay (ELISA) Human PD-1 kit (MyBioSource). Selected samples and standard curve (range 250 – 3.9 pg/mL) were evaluated according to the manufacturer guidelines in duplicates. Aspirate and wash each well repeating the process twice for a total of three washes, for this step it was used the Wash Buffer (250µL), after this it was removed all liquid by decanting the plate and blot it against clean paper towels. 100µL of each diluted standard and sample was added to each well, plate was sealed and incubate for 2 hours at room temperature.

After incubation time, plate was washed 3 times as before. After this, 100µL of diluted HRP-conjugated Human PD-1 detect antibody was added to each well, plate was sealed an incubated for 1 hour at room temperature. Another washing step was performed; next 100µL of HRP substrate (TMB) was added to each well, plate was protected from de light and incubated 15 min at room temperature. Finally, 50µL of stop solution was added to each well and a change of coloration was present (shifting from blue to yellow). Determination of the optical density (OD value) was obtained 10 minutes after adding the stop solution. The plate was read with a correction of wavelength set a 540nm, subtracting the readings value from the reading at 450nm, measurements were performed by Infinite M Plex (Tecan) plate reader.

2.7.3 Detection of the immune check point receptor Lymphocyte Activating Gene 3 (LAG3) present on EV containing fractions using the Enzyme Linked Immunosorbent Assay (ELISA)

LAG-3 forms part of the immunoglobulin superfamily is an important immune checkpoint with higher relevance in cancer. LAG-3 inhibits the activation of its host cells and generally promotes a more suppressive immune response, as for example in T-cells, LAG-3 reduces cytokine production and proliferation, while encouraging T-cell differentiation.[38]

Immunotherapeutic ways to develop tumor-specific memory T-cells and overcome inhibitory mechanisms in the tumor microenvironment present the potential to increase anti-tumor immune and clinical responses. Until these days, one of the most effective therapeutic strategies is the modulation of the immune checkpoints, by regulating the balance between the immune response and the tolerance within the human body. The targeting the immune suppressive cells, blocking inhibitory molecules on suppressive/regulatory and tumor cells, and activating co-stimulatory molecules on effector cells also are a promising therapeutic approach to increase antitumor immunity and to improve therapeutic efficacy.[39] As this day, there are not enough information about this exciting molecule, nor the accurate function and response within the cancer patients, due to this reason it was chosen this surface protein to perform a further evaluation of its presence on the surface of the EVs.

To quantify the presence of LAG3 on each of the fractions with higher particle number, it was used the sandwich enzyme-linked immune-sorbent assay (ELISA) Human Lymphocyte activation gene 3 protein (LAG-3) kit (MyBioSource). Selected samples and standard curve (range 20 – 0.05 ng/mL) were evaluated according to the manufacturer guidelines in duplicates. 50µL of standard and 40µL sample were evaluated, only to the wells containing the sample were added 10µL of anti-LAG3 antibody, then 50µL of streptavidin-HRP was added to all wells (sample and standard), the plate was sealed and

incubate at 37°C for 1 hour. After incubation, the plate was washed 5 times with washing buffer (350µL) within a time of 30 seconds between each washing step. For the last washing step, the plate was decanted and blot into paper towels. 50µL of substrate solution A and B were added to each well, and plate was protected from the light and incubated for 10 min at 37°C. Finally, 50µL of stop solution was added until a change of coloration was present (from blue to yellow). Determination of the optical density (OD value) was obtained immediately after adding the stop solution. The plate was read at 450nm within 10 minutes after the final step using an Infinite M Plex (Tecan) plate reader.

2.8 Data analysis

Data analysis and statistics (unpaired T-test) reported in this work was performed using GraphPad Prism version 9 software, Department Exosome and Tumour biology, Unikilinik, University of Freiburg.

RESULTS AND DISCUSSION

1. Sample acquisition

The cell culture media (CCM) supernatant was obtained in collaboration with Dr. Jamal Al-Zu´bi and the Centre of Chronic Immunodeficiency (CCI) Freiburg, Germany.

2. Isolation method to purify particles present on cell culture media supernatant and particle number quantification

To have a better understanding of the state of the supernatants as a starting point and to confirm a proper concentration of X-spinner ultrafiltration tube, we decided to assess the particle number of each supernatant on each step of the isolation process, to achieve this we use the Nano Tracking Analysis (NTA).

The NTA analysis of the CCM supernatant samples after the centrifugation to remove cell debris, showed an enrichment of particles in comparison with the un-activated supernatants (***Fig 12. A***).

Proteus X-spinner 100kDa ultrafiltration tube helps to concentrate the supernatants and to decrease the loss of particles by the 100kDa pore size filter by reducing the final volume of the retentate (concentrated sample) up-to 1mL, the assessment of the particle number by NTA analysis confirmed the increment in the number of particles in comparison of the initial state of the sample (***Fig 12. B***), finally the ultrafiltrate waste (flow through) presented less than $1x10^8$ particles/mL, corroborating the proper concentration of the supernatants with minimal loss of particles on the waste (***Fig 12. C***).

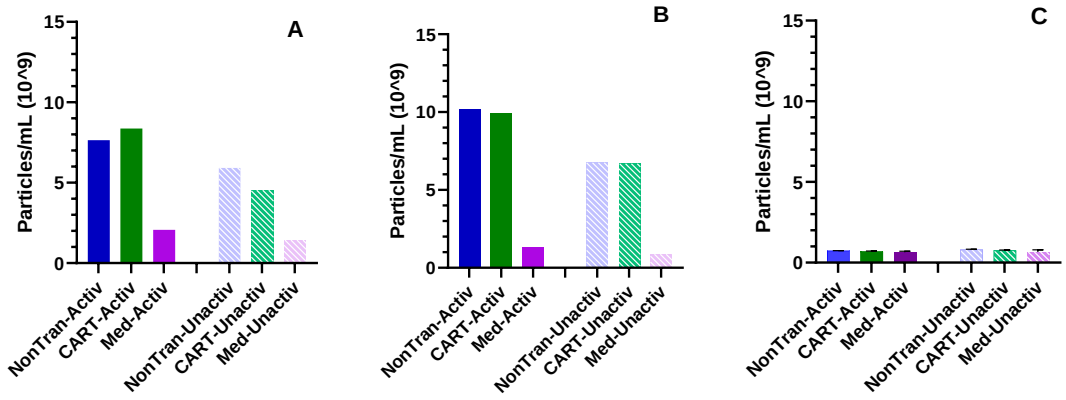

Fig 6. Concentration of particles (particles/mL 10^9) present on the sample after different steps of the isolation process.

A) Total particle number of the sample after cell debris removal (2000xg, 10 min, 4°C), B) Particle number of the concentrate supernatants after X-spinner 100kDa ultrafiltration tube, C) Particle number of the waste (flow

through) derived from X-spinner 100kDa ultrafiltration. The use of X-spinner 100kDa ultrafiltration tube helps to concentrate the particles present on supernatant samples with minimal particle loss in the waste of the tube.

Once the CCM supernatants were concentrated and the enrichment of particles was verified, SEC isolation was performed for each one of the samples (SEC settings were previously mentioned) using 500µL of retentate. The NTA analysis of the SEC fractions derived from the CCM supernatants revealed a successful isolation of particles confirming a proper performance of this method by showing a fractionalization of particles at different times. NTA revealed a different particle number per fraction and working together with SEC principle we can infer that each fraction contains particles with different sizes.

Moving forward in the assessment of the enrichment of particles contained on supernatants and fractions, NTA analysis confirmed that fractions 1 and 2 presented a higher particle yield, regardless of the activated or un-activated conditions. Even though those two fractions were the ones with more particle number, it draw to attention that the rest of the fractions are not similar between the activated and un-activated groups (**Fig 13A, 13B, 13C**), at naked eye the activated condition increase the particle concentration in the CCM supernatants, to associate if the activation was in fact the cause of this response a T-test was performed for both conditions (activated and un-activated) and for the two fractions (fractions 1 and 2) of all supernatants.

As is showed in the **Figure 14**, both fractions of the activated non-transfected T-cells and the CAR-T cells present a significant increase in the particle number when are compared with their control (activated media), this data support our hypothesis where the activation of CART and T-cells induces an increment in the particle number in both analyzed fractions. Unpaired T-test of the un-activated supernatants (NonTran and CART) reveals a significate increase in both analyzed fractions of the Non-transfected T-cells (**Fig 15 A1, A2**), effect only showed in fraction 1 of the CART-cells (**Fig 15 B1, B2**).

So far, our inferences of the activation step were supported by this data, and after a comparison between each non-activated cell group supernatant vs their activated one, the previous statement was once again confirmed. The fraction 1 from non-transfected T-cells (active vs non-active) showed a significant increase of particles in the activated condition (**Fig 16 A1, A2**), for the case of the CAR-T cells group (active vs non-active) this behavior was also observed in both analyzed fractions (**Fig 16 B1, B2**).

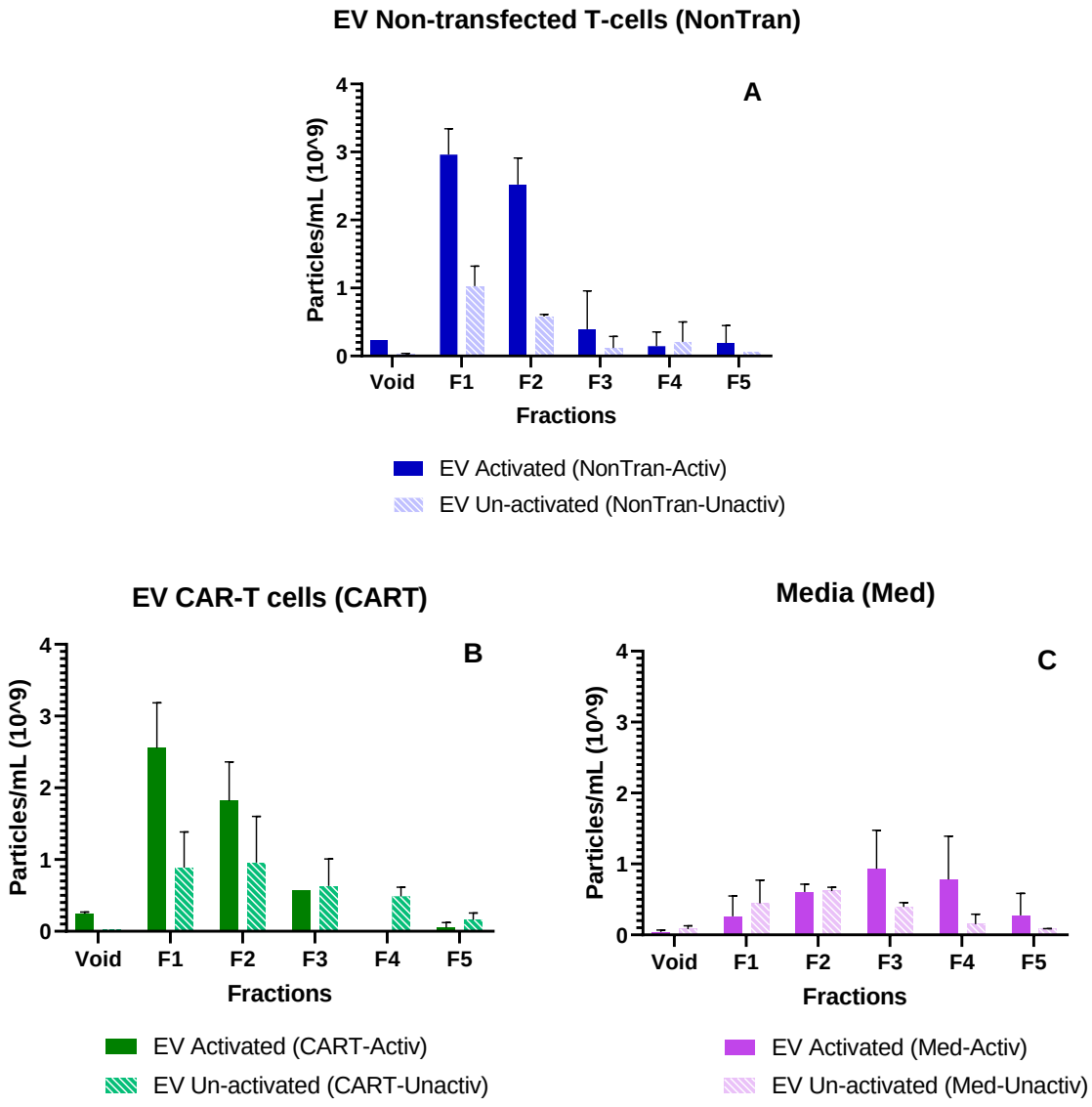

Fig 7. Particle distribution and total number of particles among the isolated SEC fractions derived from supernatants.

A) Total particle number derived from Non-transfected T-cells fractions under activated and un-activated conditions. B) Total particle number derived from CAR-T cells fractions under activated and un-activated conditions. C) Total particle number from fractions derived from the media under activated and un-activated conditions. Data was obtained by three biological and technical replicates; graphs represent the mean of the values.

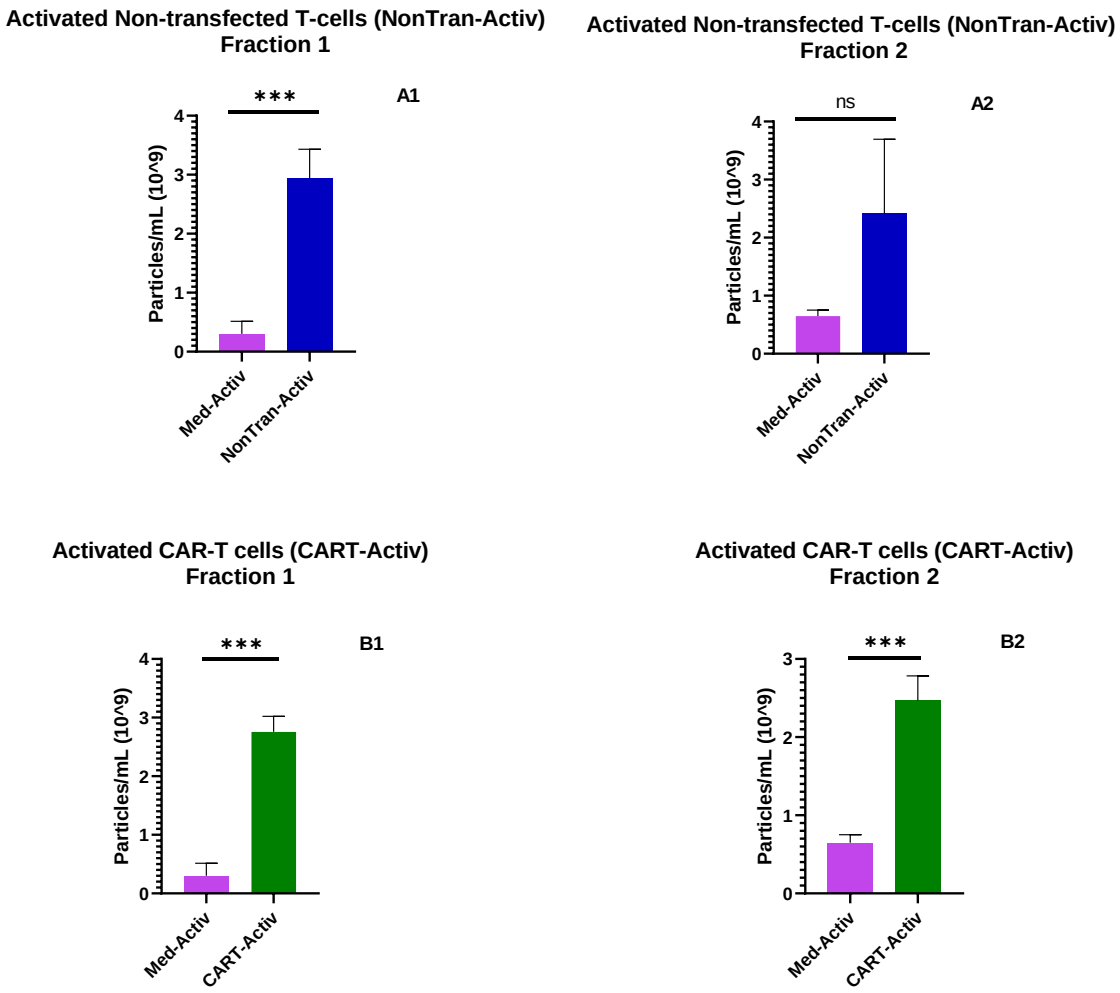

Fig 8. Fractions 1 and 2 particle number comparison between the activated non-transfected T-cells (NonTran-Activ) and CAR-T cells (CART-Activ).

T-test analysis of the fractions 1 and 2 of the activated non-transfected T-cells and CAR-T cells present a significantly increased difference between them when are compared with their activated control (activated media), A1) NonTran-Activ vs Med-Activ fraction 1, two-tailed p-value is 0.0010, 95% confidence interval. A2) NonTran-Activ vs Med-Activ fraction 2, two-tailed p-value is 0.0744, 95% confidence interval. B1) CART-Activ vs Med-Activ fraction 1, two-tailed p-value is 0.0002, 95% confidence interval. B2) CART-Activ vs Med-Activ fraction 2, two-tailed p-value is 0.0007, 95% confidence interval.

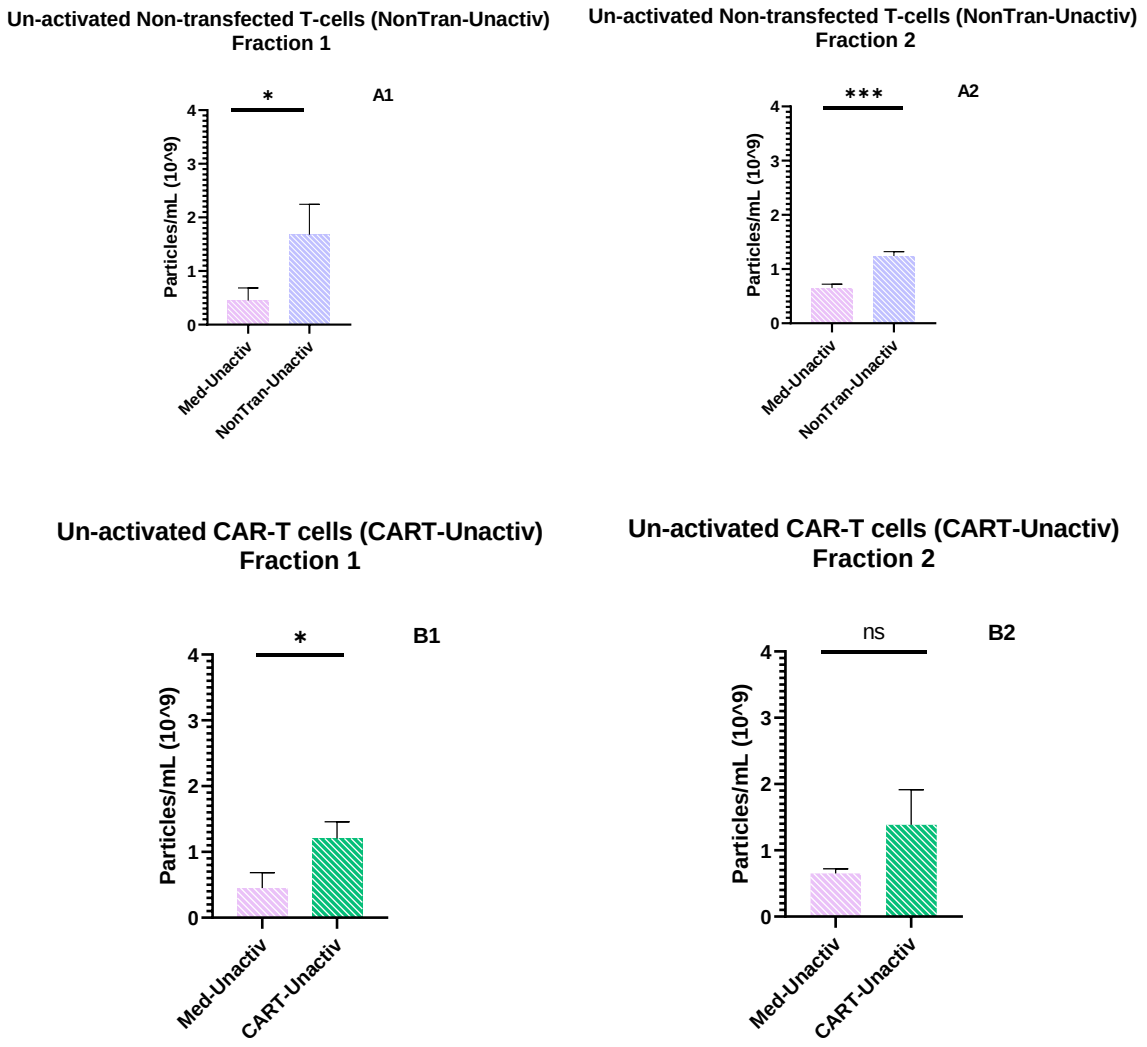

Fig 9. Particle number comparison between fractions 1 and 2 of the un-activated non-transfected T-cells (NonTran-Unactiv) and CAR-T cells (CART-Unactiv).

Unpaired T-test analysis of the fractions 1 and 2 of the un-activated non-transfected T-cells and CAR-T cells. Both fractions of the NonTran-Unactiv group present a significant increment of particles when are compared with their control (un-activated media). A1) NonTran-Unactiv vs Med-Unactiv fraction 1, two-tailed p-value is 0.0256, 95% confidence interval. A2) NonTran-Unactiv vs Med-Unactiv fraction 2, two-tailed p-value is 0.0006, 95% confidence interval. B1) CART-Unactiv vs Med-Unactiv fraction 1, two-tailed p-value is 0.0195, 95% confidence interval. B2) CART-Unactiv vs Med-Unactiv fraction 2, two-tailed p-value is 0.0767, 95% confidence interval.

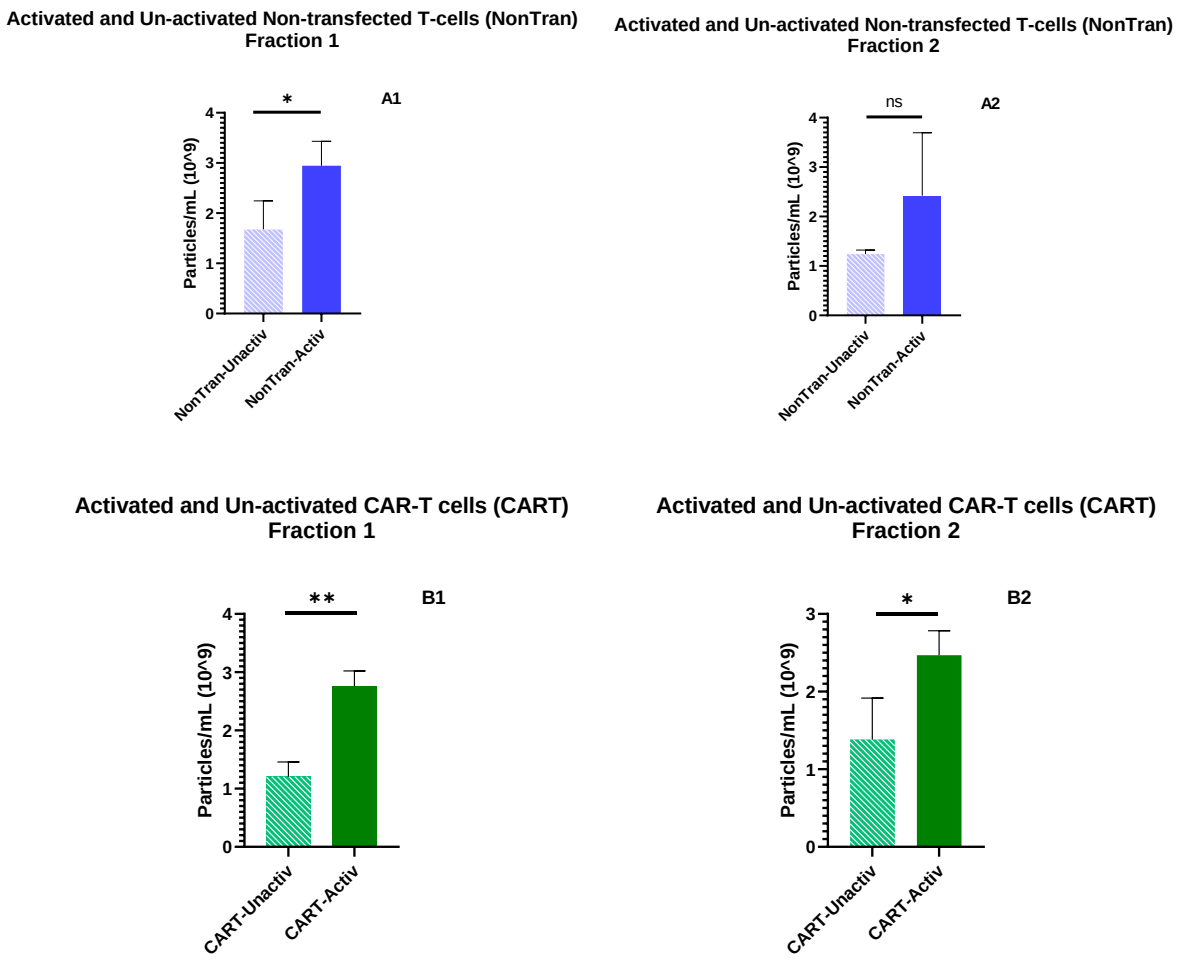

Fig 10. Particle number comparison between activated and un-activated Non-Tran and CART supernatants.

Activation by beads (anti-CD3, CD2, CD28) induces an increase in the production of particles in both non-transfected and CAR T-cells. A1) Fraction 1 of the Non-transfected T-cells showed a significant increase in particle number for the activated condition (two-tailed p-value:0.0423). B1) Fraction 1 of the CAR-T cells showed a significant increased particle number for the activated condition (two-tailed p-value: 0.0018).

This evidence confirms that after TCR activation by the beads anti-CD3, CD2 and CD28 used during the cells culture process leads to an increase in the production of particles in both cell lines. Fu W. *et al.*[23], Blanchard *et al*.[14], Tang XJ *et al*.[27], and Jasper G. *et al*.[41] reports the same behavior upon the activation of the cytotoxic T-cells under *in vitro* experimental conditions.

Related to the fact that during a normal T-cell activation by an antigen peptide presentation not only the T-cell receptor comes into play to create a proper T-cell response, the co-receptors (CD3 and CD28) located in the transmembrane part of the lipidic bilayer of the cell also plays a role in the formation of the immunologic synapsis transferring the signal to the inner part of the complex. It comes to a sense

that after the use of the activation beads the TCR complex might be fully active, inducing the production of this particles as a response to a proper intracellular signal[23], if we translate this action to an immunological reaction, we could expect the development of a cytotoxic response against strange peptides. Of course, this response will only be present if in fact this isolated particles are vesicles, although is too early to this speculation, the idea of a proper immunological function is on the table for further asses in the future.

Even though the presence of particles was distributed among all the 5 fractions, the increase of particles in fraction 1 and 2, together with the technical principle of SEC-methodology (fractionalization of particles in accordance with their size), support the idea that those isolated particles presented similar particle size distribution. To confirm this hypothesis, all the fractions were analyzed by the Dynamic Light Scattering (DLS) method.

Hartjes *et al*.[72] recommend the use of DLS as a method for physical analysis of vesicles together with NTA. The authors consider that DLS over other methods when performing quantitative analysis of samples, another positive point that the authors mention is the simplicity and the speed of measurements carried out by the machine.

Dynamic light scattering is a non-invasive technique for measuring the size of particles and molecules in a suspension measuring the Brownian motion principle. The DLS equipment measures particle size distributions of suspensions in a range of 0.8 – 10,000 nm and concentrations up to 40% by volume.[42]

For this analysis, an aliquot was taken from each one the SEC fractions and the void volume, 20 µL of the sample was loaded on the surface of the sensor to allow the laser beam to pass through the suspension volume. Samples were measured using the same settings program (standard, shape: spherical, run time: 60 seconds, transparent, runs: 5). The size distribution analysis of the isolated particles from the NonTran-Activated supernatant fraction 1 confirms that all the isolated particles correspond to a similar size, keeping in mind that microvesicles are vesicles in a range from 100 to 1000 nm in diameter[7,9], we can state that some of this particles present the same expected size of the microvesicles, the distribution of the particles was compared with their respective control (activated medium), the DLS analysis of the NonTran-Activ particles isolated from fraction 1 showed an bell shape distribution of the particles unlike the activated media particles (**Fig 17**). Of course, is still too early to draw any conclusions of any microvesicles presence so far, the size is compatible between them and our isolated particles, to confirm the existence of EVs in our fractions we must move forward in the characterization of these particles by other methodologies.

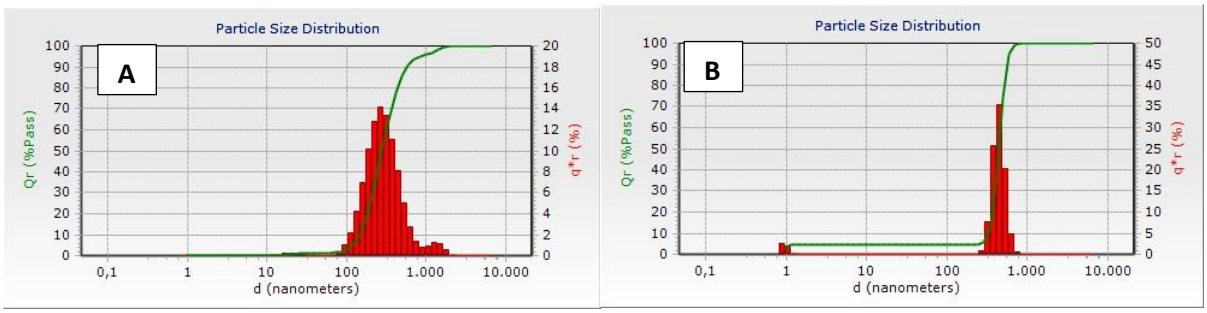

Fig 11. DLS particle size distribution of fraction 1 NonTran-Activ and Med-Activ isolated particles.

A) DLS analysis of isolated particles derived from the fraction 1 of the no-transfected T-cells under activated conditions (NonTran-Activ). B) DLS analysis of isolated particles derived from the fraction 1, medium under activated conditions (Med-Activ). Isolated particles obtained in NonTran-Activ are in a range from 100 to 1000nm diameter (vesicle expected size), in difference than the media only where the isolated particles don't distribute homogeneously through all the range of size. SOP: standard, shape: spherical, run time: 60 seconds, transparent, runs: 5.

3. Total protein quantification of isolated SEC fractions

After the quantification of the particles contained on each fraction, we continue the analysis by evaluating the total protein contained on them. Protein quantification among SEC fractions helps us to differentiate between a well performed particle isolation and an inadequate one, while NTA measurement only help us to quantify the total particle number present in a suspension, does not help us to differentiate the origin and composition of the isolated particles and do not distinguish between other co-isolated particles (protein aggregates and lipoproteins) and EVs.[69, 70]

To confirm that we are not isolating and measuring any other kind of particles such as protein contaminants, we performed a MicroBCA evaluation (Thermo Scientific, USA) to quantify the total free protein concentration. This assay detects the free protein and the protein of the vesicles itself, evidencing a successful and proper isolation of vesicles when decrease the protein levels contained per fraction.

Franquesa *et al*.[70], together with other authors state the importance to select a proper purification method to differentiate between non-EV material and pure isolated EVs. Although Brennan *et al.*[71], recommend the implementation of more than one purification method to remove any protein contaminants existing on the sample to analyze, they recognize the risk of this approach due the loss of particles caused by an extra purification step is highly expected. Based on the properties of our supernatants and the particle number contained on them, we decided to proceed with only one purification step implemented by the SEC column and the pore size of the column matrix (35 - 350nm).

As the ***Figure 18*** shows, the protein levels arise in fractions 4 and 5 in all supernatants despite the activation state. Our results suggest a proper purification of vesicles and separation of non-EV material contaminants on the assessed SEC fractions. This result was observed by a difference in the protein concentration on the fractions, when they contain an increased particle yield, they show a decreased protein concentration, and the other way around, when low particles are expressed, more protein is detected. In our isolates the two last fractions the protein concentration increases abruptly by two times fold.

It was also demonstrated to us that SEC method has an optimal performance during not only the isolation of particles but also to their purification. Total protein quantification not only help us to assess the supernatants content, but also lead us to a further analysis of the biochemical content of the fractions, both are huge advantages when analysis of EVs is carried on.[72]

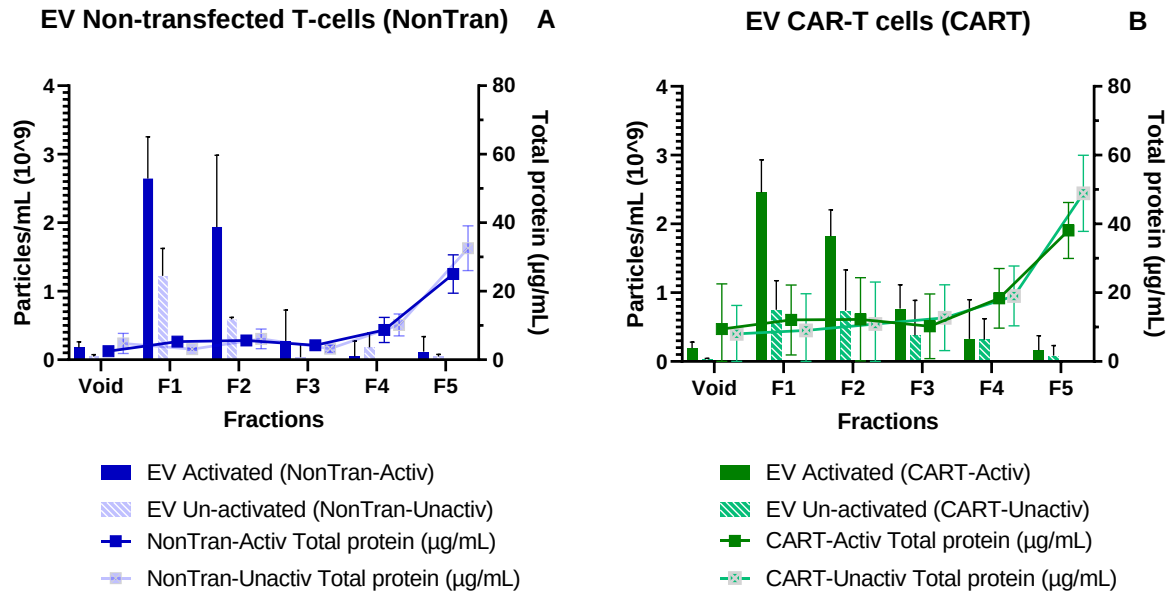

Fig 12. Total protein quantification (ng/mL) on isolated SEC fractions of activated and un-activated non-transfected T-cells and CART-cells.

Assay supports a proper separation of particles from protein contaminants contained on each fraction. The detected protein in fractions 1 to 3 correspond to the protein of the vesicles, while the protein detected in the last two fractions correspond to the free protein, both particle number and protein concentration are not increased at the same time, confirming a successful isolation and purification of particles. A) Total protein quantification of Non-Transfected T-cell fractions. B) Total protein quantification of CAR-T cells fractions.

4. Detection and quantification of surface vesicle biomarkers CD9 and CD63 of isolated SEC fractions

CD9 and CD63 are two of the most cited vesicle biomarkers; their presence on vesicles can help us to differentiate them from the rest of the co-isolated non-EV material. Due to this reason we wanted to detect and quantify the amount of both markers on each one of the isolated fractions to confirm the existence of vesicles and identify which fractions contains them.

In this quantification the analyzed fractions are derived from one single isolation (500µL retentate) of one biological replicate (***Fig 19***). The ELISA protocol for both biomarkers was followed step by step based on the manufacturer specifications.

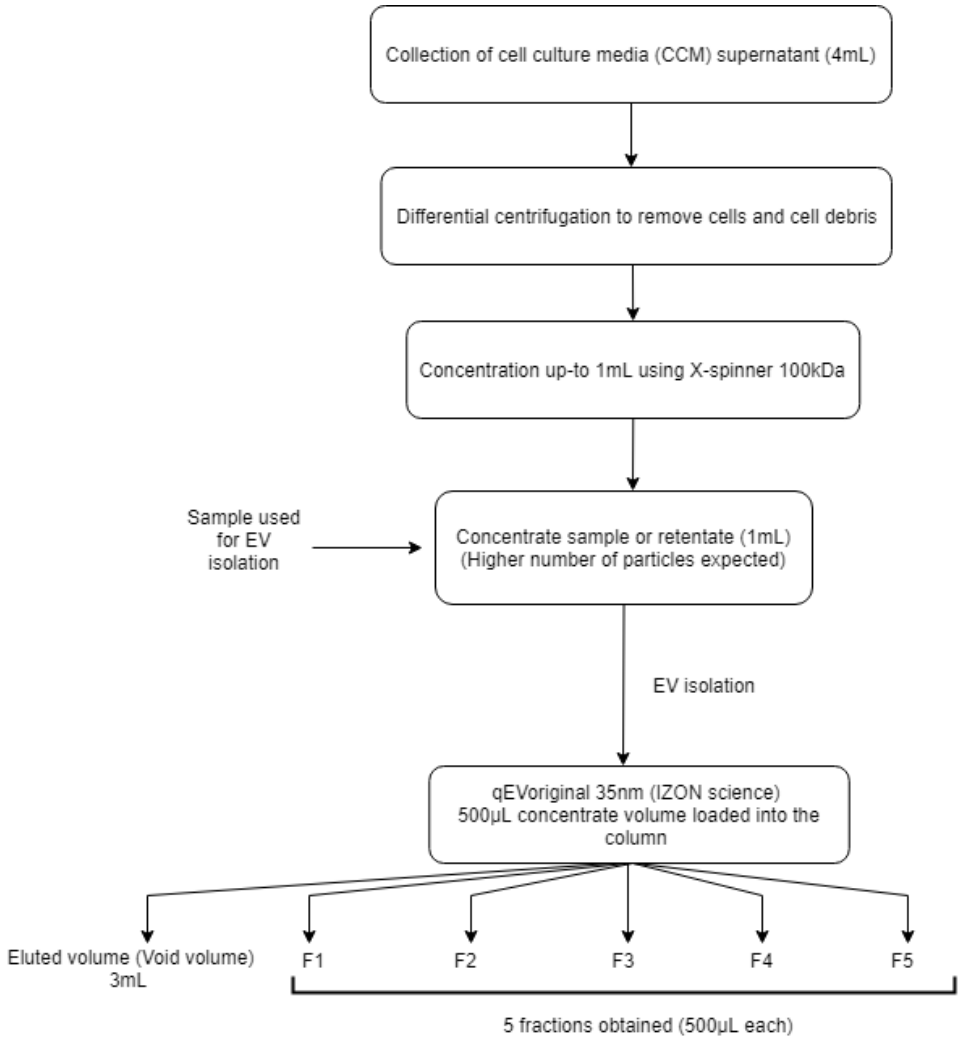

Fig 13. Isolation scheme of fractions used to evaluate CD9 and CD63.

To quantify CD9 and CD63 biomarkers the fractions to be analyzed were derived from one biological replicate of cell culture media supernatants for each one of the cells and activated and un-activated conditions. SEC conditions were: 3mL void volume, 5 fractions of 500µL each.

Figure 20 represents the amount of CD9 and CD63 contained on each fraction. The marker CD9 was observed on the fractions 2 and 3 in the NonTran-Activ group, and in the fractions 1 through 3 of the CART-Activ group, in both cases the signal was present in the fractions that showed an increased particle yield (***Fig 20 A***). In the case of the biomarker CD63, the signal was only detected in the fraction 1 for the non-transfected activated T-cells, in case of CART-cells fractions, the marker was observed on 1 and 2. As an only exception, the CART-unactive group showed a signal in fractions 1, 2 and 4 (***Figure 20B***). Both markers were undetectable for the rest of the un-activated fractions derived from supernatants.

Although the used ELISA method in this work had a detection sensitivity of 0.002 ng/mL for the quantification of both biomarkers, the low signal detected in CD63 ELISA together with the lack of signal of both markers in the non-activated supernatants made us think that the number of vesicles per fraction was not enough to ensure a proper detection by our method. Due to this reason, we decided to pool the fractions derived from 3 isolations and 3 different biological replicates, to achieve this we used once again the X-spinner 100kDa ultrafiltration tube to concentrate the entire volume (1.5mL total, 500µL per isolated fraction) to a final volume of 300µL, allowing us to ensure an enrichment of vesicles on each fraction, a further description of the isolation and pooling process is described by ***Fig 21***.

After the pooling of the fractions, CD9 was detected in all the un-activated supernatants. CD9 was present in fractions 1 through 4 of the NonTran-activated and un-activated in detectable limits (minimum detected 2 ng/mL), and it was detected in fractions 1 through 4 for the CART-Activ and in fractions 1 and 3 for the CART-Unactiv (***Fig 22A***). CD63 was detected also, for the no-transfected T-cells the signal was present on fraction 2 for both activation conditions, in the case of the CART- cells supernatants, the signal was only detectable for the active CART with a higher signal in the fraction 2 (***Fig 22B***).

So far, the signals of both markers correspond to the fractions that present a higher particle yield, supporting our hypothesis that vesicles are in fact isolated from the fractions with increased number of particles. Although the signal from the medium was subtracted on each data analysis, some of the vesicles derived from the FCS contained in the medium could emit the signal belonged to the fraction 4, where the higher number of vesicles derived from the media are isolated, explaining the detection of the marker in that fraction.

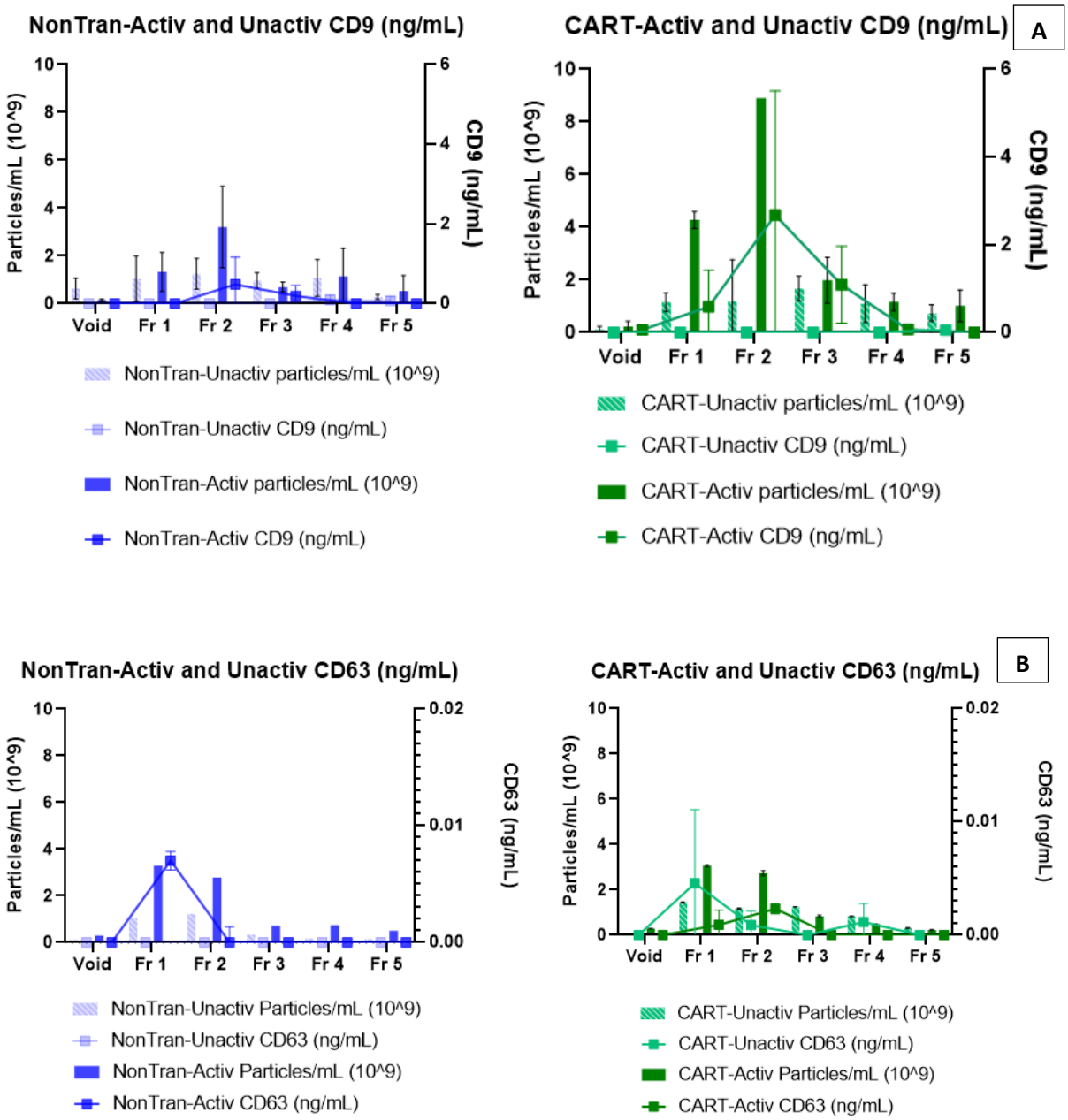

Fig 14. Quantification of CD9 and CD63 using sandwich ELISA.

CD9 and CD63 signals are obtained in the same fractions that contain a higher particle yield for the activated conditions in both cell lines. Isolated vesicles derived from non-activated conditions don't present any signal. Results obtained by one isolation of one biological replicate. Presented data correspond to the mean of the values, two technical replicates.

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Although the pooling and concentration of the fractions aids to the detection and to the improvement of the signal to in fact evidence the existence of both markers on each fraction, the amount of detected CD63 was not enough to strongly conclude the presence of this marker among the fractions in all the supernatants. Nevertheless, CD9 marker was present in detectable levels after the pooling and concentration of fractions. The low CD63 signal could not only be caused by lack of this marker on the vesicles but could also by the low specificity of the antibody used in this evaluation. It is recommended another assessment changing the antibodies and improving the detection limits, an extra isolation could be pooled to increase particle concentration as well.

Even though some reports identify the presence of CD63 for T-cell derived vesicles, Théry C. *et al*.[43] publications evidence that the expression of this marker on EVs is dependent of several factors, such as the cell state of the donor cell, even the cell type might modify their expression, not to mention that CD63 marker could be strongly related to the vesicle biogenesis pathway as is expressed by Cashikar *et al*.[73] We must remember that although CD9, CD63 and CD81 can help to discriminate EVs form non-EV material; this does not assure that all vesicles will contain these 3 proteins on their membrane, nor a defined concentration of any of these markers per individual vesicle.[43]

On the other hand, Reyes R. *et al*. [45] confirms the expression of CD9 in several subsets of leukocytes, due CD9 is a tetraspanin in charge of many cellular activities as signaling, proliferation, activation, migration, invasion, adhesion, among others. The authors report that CD9 expression in vesicles is not only defined by the characteristics of their cell of origin, this marker can be also attached to the EVs during their formation process by their biogenesis pathway when is released from the cell.

This idea is also confirmed by *Mathieu et al.* [44] they studied the nature of EVs derived from HeLa cells by the content of different tetraspanins expressed on their surface, in this publication the tetraspanins CD9 and CD81 were associated to the microvesicles, while CD63 was expressed on the vesicles derived from endosomes (or exosomes).

Other authors such as Jankovičová J. *et al*. [46] also emphasize in the large difference between the tetraspanin profile that vesicles can contain, their statement declare that EVs released by different cell types and/or the same cells can differ quantitively and qualitatively in their tetraspanin profile, this supports the fact that heterogeneity among individual EVs may contribute to the cellular responses caused after their uptake by the recipient cell.

As a final point, the gained experience of isolating vesicles from several biological replicates during the experimental work of this thesis demonstrate us more than once the fact that each biological replicate variates between them, not only in the particle concentration, but apparently it also varies in the number of vesicles contained per fraction and the characteristics that this vesicles might show. An alternative to overcome this situation in the future could be the enlargement of the produced supernatant by means of extension of cell cultures to obtain higher volumes of the same biological replicate supernatants, avoiding by this more variation in the vesicles content and surface protein signature.

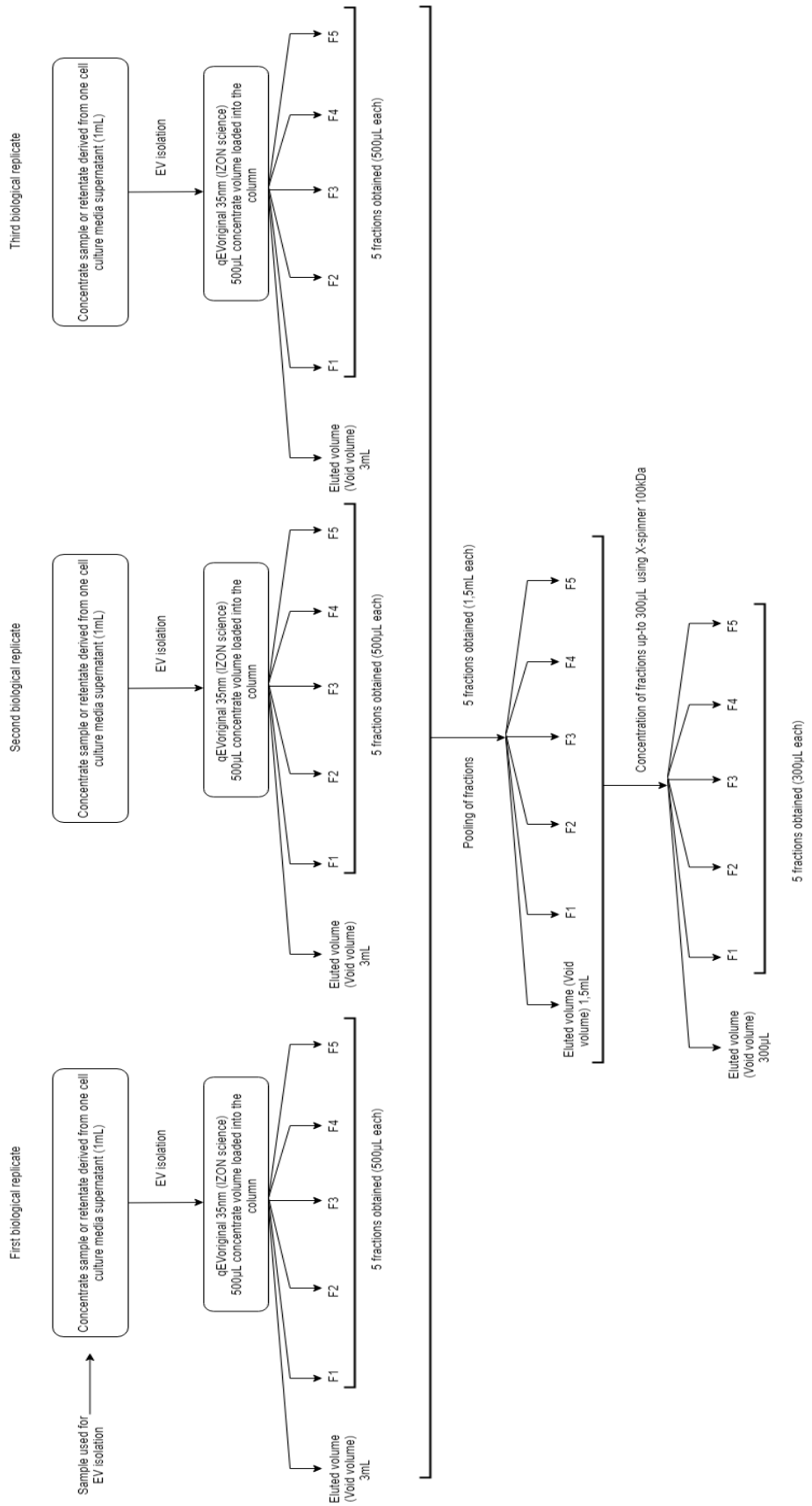

Fig 15. Pooling and concentration of fractions isolation scheme.

The analyzed fractions were derived from three different cell culture media supernatants, the fractions correspondent to the same cell line (Non-transfected T-cells, CART-cells) and activation condition were pooled together to achieve a total volume of 1.5mL. The pooled 1.5mL volume was concentrated up-to 300µL using an X-spinner 100kDa ultrafiltration tube, the concentrated retentate sample was used to perform CD9 and CD63quantification using the ELISA sandwich method.

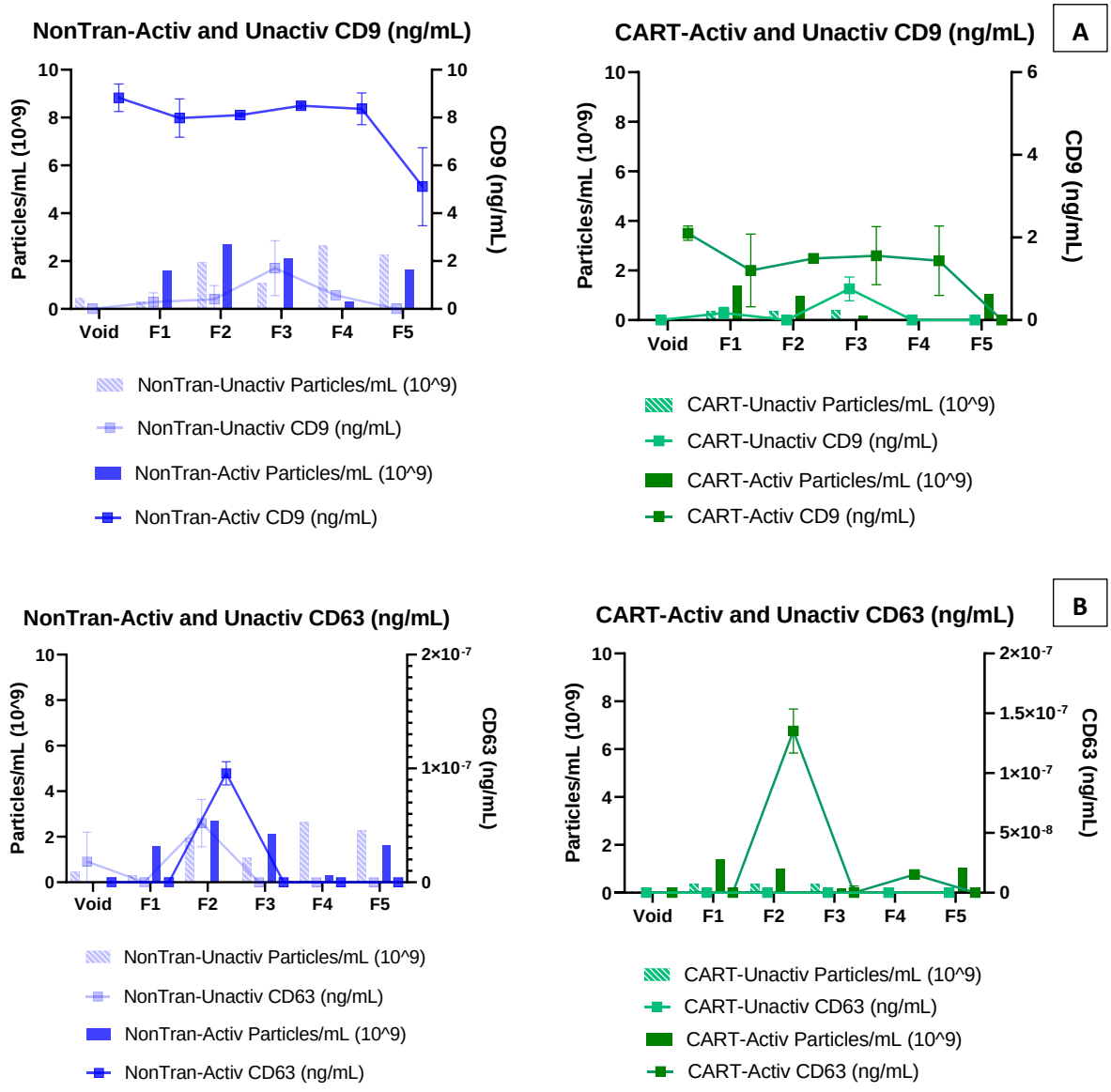

Fig 16. Quantification of CD9 and CD63 on pooled/concentrated fractions using sandwich ELISA.

Evaluated sample was obtained by pooling and concentration of three isolations obtained from three different biological replicates. Biomarker CD9 was detected in both cell lines and activation states. CD63 was detected under activated and un-activated conditions in Non-Transfected T-cells, while it's only detected in the CART-cells under activation state. Showed data correspond to two technical replicates; expressed data correspond to the mean of the values.

5. Assessment of the particles morphology using Transmission Electron Microscopy (TEM) technique

TEM is a technique that helps us to visualize and by this corroborate the vesicle presence in isolated samples. So far, we have only quantified the particle number and even though we already confirm with help of MicroBCA assay the successful isolation of particles with a diminishment of possible protein contaminants, we still need to confirm if these particles are in fact vesicles. We can achieve this aim by two different manners, we can analyze the biomarker presence on each fraction using different methodologies as we already proved by ELISA (other methods are: flow cytometry, western blot, ELISA, etc.) or we can confirm their existence by imaging of the vesicles. Both alternatives are approached in this thesis.

The use of TEM not only helps us to analyze the morphology of the vesicles, but it also helps us to define the size of the isolated particles.[31]

Chuo *et al*.[75] together with Rikkert *et al*.[76] confirms that TEM is the most common imaging methodology used for EVs visualization and further characterization. TEM is used to describe a detailed structural characteristics and further information of EV surface composition, not only size.

The reports of Noble *et al*.[77] and Cizmar-Yuana[78] describes the TEM imaging of the isolated EVs as double layer vesicles with heterogeneous sizes that will lead to a deeper classification after further assessment.

The assessment by Transmission Electron Microscopy of our supernatants, imaging revealed double membrane structures contained on fraction 1 of the CART-Activ supernatant, this fraction displayed an increased particle number and low protein concentration in previous assessments. Although no other double membrane structures were observed in fraction 2 or 3, the existence of vesicles is supported by this evidence.

As ***Figure 23*** reveals, the small double membrane particle is about 100nm diameter. If we focus only in the fact that the isolated vesicles are in a range of 100 – 200 nm[1,7,9], it will be easy to infer that our isolation method allows the recovery of microvesicles which are in the exact range of length, of course is still too early to conclude this statement based on the evidence we have so far, to do so we need more evidence and the use of other methodologies.

In our evaluation, the number of double membrane structures was not sufficient to strongly conclude the presence of the vesicles of interest; this might be caused by a lack of concentration and pooling of fractions during this evaluation. For improvement of further analysis in the future, it is recommended

the enrichment of particles as described before (CD9, CD63 quantification) to successfully observe more structures and strongly conclude the insolation and presence of vesicles in the selected fractions.

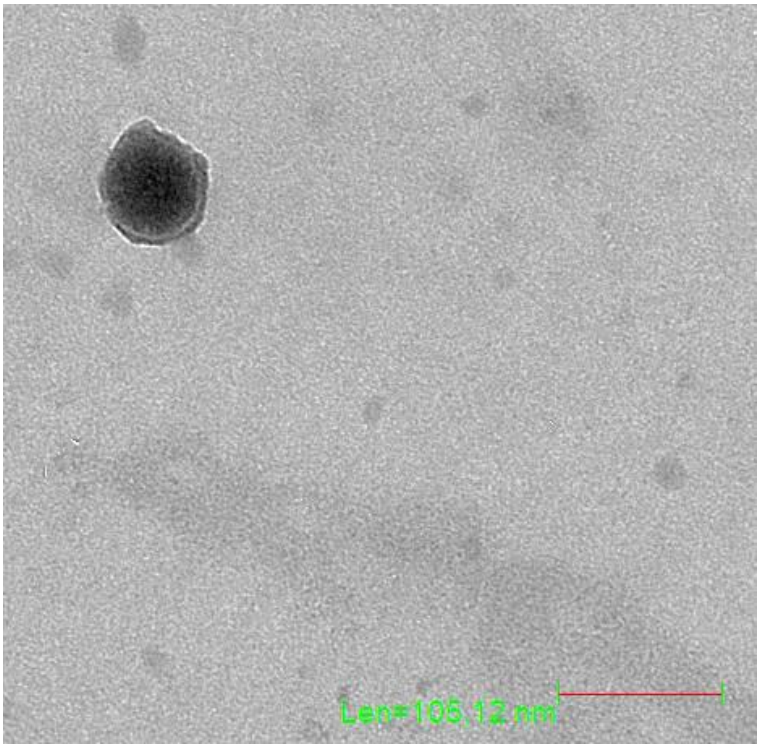

Fig 17. Isolated double membrane structure contained in fraction 1 of CART-Activ supernatant. Presence is confirmed by Transmission Electron Microscopy.

The isolated double membrane particle is in a range of 100 – 150nm, showing the same expected size than microvesicles. Particle was isolated from fraction 1 of the CART-Activated CCM supernatant. Particle isolated from one biological replicate, non-pooled nor concentrated sample.

6. Detection of surface functional biomarkers (T-cell activation markers, TCR co-receptors) and immune check point receptor presented on EV containing fractions

6.1.1 Detection of T-cell activation markers and TCR co-receptors.

To assess the presence of derived T-cell markers on the surface of the isolated EVs, we used a beads-assisted flow cytometry technique previously described. The results obtained demonstrate that the isolated vesicles from the 4 different CCM supernatants bear important T-cell biomarkers, at the same time vesicles derived from activated CART-cells and non-transfected T-cells possess specific T-cell biomarkers acquired upon activation by CD3 and CD25 beads, the same ones used in the culture of our cells. Negative controls were added to validate the obtained results; no signal was detected for the specific B-cells markers contained in the kit, confirming the stripe of our vesicles (***Fig 24***).

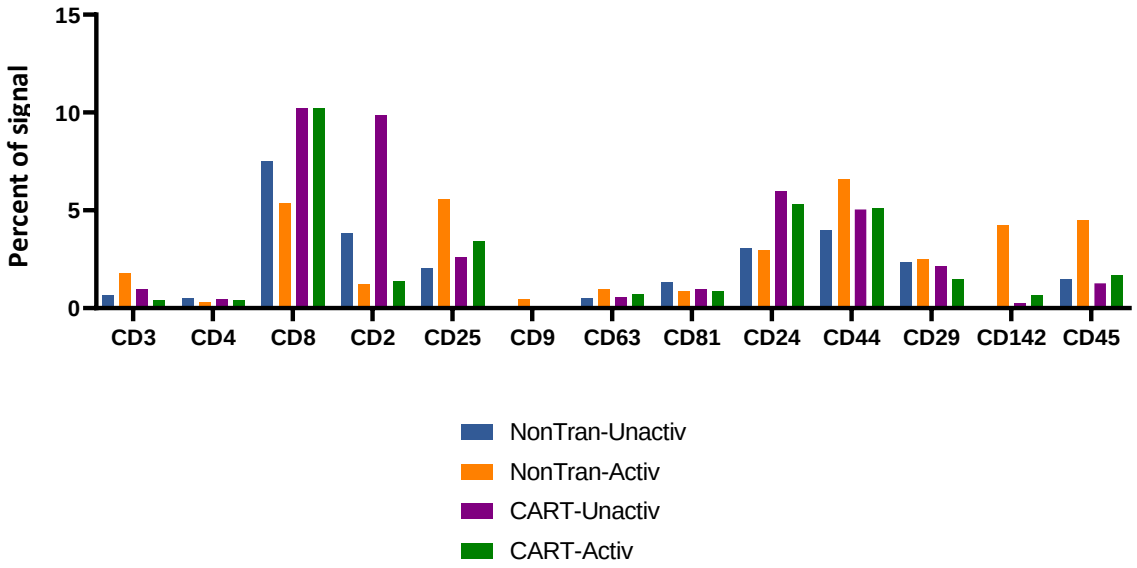

Fig 18. Detection of derived functional T-cell biomarkers on the surface of isolated vesicles.

Isolated particles express T-cell derived markers in charge of different T-cell cellular processes such as: activation (CD3, CD2, CD44, CD45 and CD25), cell adhesion (CD2), co-receptors of T-cells (CD4, CD24 and CD8) and inhibition of apoptosis (CD29), together with vesicle biomarkers such as CD9, CD63 and CD81. The determination of the relative exosome marker level was made by calculating the ratio of the signal intensities of each sample, the results are expressed by percent, and the assay was carried out using two technical replicates.

Reports of Oba, Ryutaro *et al*.[47] associate the cluster of differentiation CD3, CD4 and CD8 to function as co-receptors of T-cells and also to their couple with the T-cell receptors (TCR), in more detail, CD3 is in charge of inducing the activation of the cytotoxic T-cells (CD8+ naïve T-cells) and the T Helper T-cells (CD4+ naïve T cells) during TCR signal transduction, the co-receptor proteins CD4 and CD8 are in charge to bind to the MHC-II or MHC-I to facilitate the activation of T-cells after their adhesion with APCs as is described by Tak W. *et al*.[48] Our results demonstrate that CD8 (mostly co-receptor of T-cells) is highly expressed for the CART-cells regardless their activated condition, while CD3 is increased in the NonTran-Activ derived vesicles evidencing and confirming the activation effect caused by the beads during the cell culture.

As Hünig T. *et al*.[74] states in their publication both markers CD2 and CD25 are strongly related to the activation of the T-cells, in our case CD2 is more expressed in the vesicles derived from the un-activated cells, this could be related to the fact that CD2 is mostly located on the surface of the T-cells due their functionality as an adhesion molecule, this suggest that there is a slight possibility that those isolated vesicles could be in fact microvesicles, and due they bud directly from the plasma membrane

of the cells, the probability to bear CD2 is more likely than in the case of the exosomes freed by the activated cells.

Our isolated vesicles express an increased positive signal for CD25 in both active cell types; this corresponds with a previous activation assessment in cells performed by Dr. Jamal Al-ZuBi before the CCM supernatants were delivered to our department. The previous T-cell activation revealed that non-transfected T-cells (1.2 – 1.4x10^6 cells/mL) and CART-cells (1.5x10^6 cells/mL) before activation showed less than 10% of CD25+ cells, an effect that after activation with anti-CD3/CD2/CD28 beads, CD25+ cells increased up to an 80% in both cases, this assessment was performed using 3 independent experiments, the collection of transfected and non-transfected T-cells was made from 2 independent donors.

Our results suggests that CD25 marker can be transferred to the vesicles by the cells of origin, although we still don't know which type of vesicles are the ones bearing this tetraspanin, this opens a query for the study of intracellular trafficking of CD25 and their deliver to vesicle types. Reports by Akihiro T. *et al*. [49] confirm the presence of CD25 on vesicles and state the critical role that this molecule plays during activation and proliferation of T-cells[11].

Our results after the assessment of the vesicle biomarkers CD9, CD63 and CD81; indicates that CD63 and CD81 are expressed on the isolated vesicles; both markers were found increased on the non-transfected derived vesicles. Zoraida, *et al*. [13] and Blanchard, *et al*.[14] confirms that CD63 and CD81 are expressed on vesicles after activation with CD3. Even though CD9 was not entirely detected by this assay, there is a slight signal only in the NonTran-Activ vesicles. The obtained results using beads-assisted flow cytometry are more coherent and supported by the literature than the previously obtained by ELISA, due the CD9 biomarker is frequently reported to be expressed by T-cells, but not enough information is found regarding the transfer of the marker to the vesicles. The difference in our results could be caused by the unspecific binding of antibodies during our ELISA assays causing a false positive signal. This new information helps us to understand which one is the best approach for the assessment of EVs derived from CAR and normal T- cells.

A few information is founded regarding the presence of CD24 in vesicles derived from T-cells, normally CD24 is a marker for differentiation of cells and it can act as a co-stimulation for T-cells (non-lymphoid organs),other studies demonstrate that CD24 is an important genetic checkpoint for homeostasis and autoimmune diseases.[50] Keller *et al*. [51] studied the CD24 as a marker of exosomes secreted into urine and amniotic fluid, in this paper the authors report that CD24 is nor essential for the formation and consecutive release of exosomes, but they propose this marker as a convenient molecule to identify vesicles. In our case, this marker was expressed on all the isolated vesicles derived from all the

supernatants without any discrimination of their active state although vesicles derived from CART-cells express the higher levels of this marker, maybe this is related to the fact that CD24 is quickly induced after the activation of TCR-CD3 complex, despite the maturation state of our cells as is reported by Li O, *et al*.[52] In spite of all our speculations, the role of CD24 on T-cells is still largely unknown when literature is reviewed.

CD44 is also an important activation marker on T-cells, reports by Schumann J., *et al*.[53] and Baaten BJG, *et al*.[54] state that CD44 marker can help to differentiate memory and effector T-cells from the naïve ones, helps in the regulation of cell adhesion and migration, and it can amplify T-cell receptor signaling. Our results indicate that CD44 biomarker was considerably more expressed on the vesicles derived from both activated cell lines, although the difference is not pronounced enough in CART-cells. When we consider the fact that this molecule is also expressed by T-cells under normal conditions, their carriage on vesicles originated from cells without the activation is not a surprise.

CD29 is a marker related to activated T cells, the authors Zhu Y. *et al*. [55] and Monguió-Tortajada, *et al*.[56] consider this tetraspanin to be related in the mediation of proliferation and during inhibition of apoptosis in CD4+ T-cells, although not much information was found of the presence of this marker on vesicles, our results are linked to the fact that activated no- differentiated CD8+ T-cells could possess and at the same time shipping this marker to the vesicles. Our results demonstrate that CD29 is present after T-cell activation, and this feature is conserved over all cell lines despite their un-activated state.

CD142 is a marker most related to the endothelium, thrombosis and as one of the initiators of the coagulation during severe infections.[57,58,59] Although the literature doesn't deep down when referring CD142 and other vesicle related function outside the endothelium and infections, our results lead us to think that this marker could be endogenous of the activated T-cells, still many studies are need it to define more in dept this idea.

Finally, CD45 is a crucial tetraspanin for the activation of T cells via TCR, it can exist different isoforms with various levels of importance for the cells.[11,49,60] As we already mentioned, the presence of co-receptors is very important for a proper signal transfer through the cytosol of the cell which will finally lead to an activation response. Due CD25 was used for the activation of this T-cells and because CD45 can be found in vesicles after the activation of T-cells with CD25, our results confirm this statement when we observe how vesicles derived from both activated cell lines exhibit this marker.

6.2 Detection of immunological check point receptors.

For the assessment of a protective and proper functionality of the immune system, we evaluate the presence of the markers LAG-3 and PD-1 on the isolated SEC fractions that presented an increased particle yield (Fraction 1 and 2), for this evaluation 500µL of each fraction was concentrated using X-spinner 100kDa ultrafiltration column to reach a volume up-to 200µL (requires volume to assess technical duplicates). Our ELISA results of the quantification of LAG-3 were negative, in all tested fractions. Even though the second fraction of the NonTran-Unactiv isolated vesicles reported a signal of 0.0107ng/mL, it did not reach the delimited detection limit of our ELISA (sensitivity of 0.025ng/mL).

Although LAG-3 is reported to be borne by EVs derived from T-cells by the authors Ceeraz, *et al*. [61], Aiello, S., *et al*.[62] and Ali S., *et al*.[63] our findings could not confirm those statements. Our only hypothesis is the lack of vesicles on those fractions due this problem was recurrent on each evaluation, we suggest another assessment using an even more concentrated sample derived from more than one isolated, maybe 3 or 4 isolates pooled together to ensure enough vesicle presence.

As the reports of Riley[64], Freeman, G., et al. [65], and Kumagai, S., et al. [66] states that PD-1 is involved in the inhibition of T-cell proliferation after contact with their ligand PD-1L, being one of the most studied markers when we talked about T-cell exhaustion. During our experimentation, PD-1 was not expressed in our isolated vesicles although the sensibility of our assay was high (minimum detected dose was 3.9pg/mL). These results together with the literature make us infer that constant reactivation of the cells (by the beads or by normal re-culturing conditions) don't induce an enervation on the cells.

Riley[64] mentions the relationship between CD28 and PD-1 for T-cell signal transduction after TCR activation. Having this knowledge so far, we expected this increase in PD-1 after TCR activation by our beads anti-CD3/CD2/CD28, but a further analysis of this "relationship" carried out by *Riley[64]* brought to light the low activity of T-cell after the binding of PD-1, due both proteins (CD28 and PD-1) compete for PI3K a carrier for glucose on the cell. Our results suggest that cells not demonstrate to be exhausted after constant activation, reducing the expression of PD-1 and by this controlling the immune response in a conventional way.

Due our obtained results are slightly supported by this evidence, we still recommend another repetition of this assay with the same pooled and concentrated conditions, due LAG-3 might be present and by this, revealing the exhaustion activity of our cells by a different biochemical pathway.

CONCLUSIONS

In conclusion:

- We successfully developed and standardized a protocol for the isolation and enrichment of particles contained in cell culture supernatants of non-transfected T-cells and CAR T-cells under activated and non-activated conditions using an ultrafiltration tube and the size exclusion chromatography method.
- Cell activation induces a significant increased particle production in non-transfected T-cells and CAR T-cells.
- Isolated SEC fractions 1 and 2 of both non-transfected and CAR T-cell lines activated and un-activated showed an increased particle yield in comparison with the other isolated fractions.
- Total protein quantification of the isolated fractions demonstrated a successful abolishment of protein contaminants by SEC methodology.
- Analysis of the vesicle biomarkers CD9 and CD63 by ELISA assay confirmed the presence of vesicles contained in the same isolated SEC fractions who showed an increased particle yield.
- TEM imaging of CART-Activ fraction 1 revealed the presence of double membrane structures with similar morphology than the vesicles of our interest.
- Surface protein characterization of isolated particles demonstrate the expression of T-cell activation biomarkers (CD2, CD3, CD25, CD44 and CD45), co-receptors of T-cells (CD4, CD8, CD24) and vesicle biomarkers (CD9, CD63 and CD81), with no detectable presence of PD-1 and LAG-3.
- Further characterization and analysis of the vesicles derived from non-transfected and CAR T-cell is needed, is strongly recommended to pool, and concentrate the fractions to increase particle yield and aid the performance / proper detection of signals by the methodologies.

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BIBLIOGRAPHY

1. Willms E, Cabañas C, Mäger I, Wood MJA and Vader P (2018) Extracellular Vesicle Heterogeneity: Subpopulations, Isolation Techniques, and Diverse Functions in Cancer Progression. *Front. Immunol.* 9:738. doi: 10.3389/fimmu.2018.00738
2. Desrochers, Laura M *et al*. "Extracellular Vesicles: Satellites of Information Transfer in Cancer and Stem Cell Biology." *Developmental cell* vol. 37,4 (2016): 301-309. doi:10.1016/j.devcel.2016.04.019
3. Holdenrieder, S. Extracellular vesicles as gold mine for new diagnostics and therapeutic approaches in medicine. *Trillium Extracellular Vesicles* 2019; 1(1).
4. Marie, L. C.; Ricklefs, F. L. Tumor cell communication through EVs: new challenges and opportunities. *Trillium Extracellular vesicles* 2019; 1(1).
5. Teng, F. and Fussenegger M. Shedding light on extracellular vesicle biogenesis and bioengineering. *Advanced science 2020*; 8(1). https://doi.org/10.1002/advs.202003505
6. Gutiérrez-Vázquez, Cristina *et al*. "Transfer of extracellular vesicles during immune cell-cell interactions." *Immunological reviews* vol. 251,1 (2013): 125-42. doi:10.1111/imr.12013
7. Xavier, Cristina P.R., Hugo R. Caires, Mélanie A.G. Barbosa, Rui Bergantim, José E. Guimarães, and M. H. Vasconcelos 2020. "The Role of Extracellular Vesicles in the Hallmarks of Cancer and Drug Resistance" *Cells* 9, no. 5: 1141. https://doi.org/10.3390/cells9051141
8. Doyle, Laura M, and Michael Zhuo Wang. "Overview of Extracellular Vesicles, Their Origin, Composition, Purpose, and Methods for Exosome Isolation and Analysis." *Cells* vol. 8,7 727. 15 Jul. 2019, doi:10.3390/cells8070727
9. Fricke, F.; Buschmann D.; Pfafll M.W. Isolation and characterization of extracellular vesicles. *Trillium extracellular vesicles* 2019; 1(1).
10. Ventimiglia, Leandro N, and Miguel A Alonso. "Biogenesis and Function of T Cell-Derived Exosomes." *Frontiers in cell and developmental biology* vol. 4 84. 17 Aug. 2016, doi:10.3389/fcell.2016.00084

11. Lu J., *et al*., Role of T cell-derived exosomes in immunoregulation. Springer. Immunologic research (2018) 66:313-322. https://doi.org/10.1007/s12026-018-9000-0
12. Alonso, R., Mazzeo, C., Rodriguez, M. *et al.* Diacylglycerol kinase *α* regulates the formation and polarisation of mature multivesicular bodies involved in the secretion of Fas ligand-containing exosomes in T lymphocytes. *Cell Death Differ* 18, 1161–1173 (2011). https://doi.org/10.1038/cdd.2010.18
13. Andreu, Zoraida, and María Yáñez-Mó. "Tetraspanins in extracellular vesicle formation and function." *Frontiers in immunology* vol. 5 442. 16 Sep. 2014, doi:10.3389/fimmu.2014.00442
14. Blanchard, Nicolas *et al*. "TCR activation of human T cells induces the production of exosomes bearing the TCR/CD3/zeta complex." *Journal of immunology (Baltimore, Md. : 1950)* vol. 168,7 (2002): 3235-41. doi:10.4049/jimmunol.168.7.3235
15. Shao, Yuanyuan *et al.* "Role and Function of T Cell-Derived Exosomes and Their Therapeutic Value." *Mediators of inflammation* vol. 2021 8481013. 12 Nov. 2021, doi:10.1155/2021/8481013
16. Wang, Xiaochen *et al.* "Exosomes derived from exhausted CD8+ T cells impaired the anticancer function of normal CD8+ T cells." *Journal of medical genetics* vol. 56,1 (2019): 29-31. doi:10.1136/jmedgenet-2018-105439
17. van der Vlist, Els J *et al.* "CD4(+) T cell activation promotes the differential release of distinct populations of nanosized vesicles." *Journal of extracellular vesicles* vol. 1 10.3402/jev.v1i0.18364. 16 Apr. 2012, doi:10.3402/jev.v1i0.18364
18. Miliotou A. N.; Papadopoulou L.C. CAR T-cell therapy: a new era in cancer immunotherapy. Bentham science. *Current pharmaceutical biotechnology,* 2018; 19, 5-18. doi: 10.2174/1389201019666180418095526.
19. Ahmad, A.; Uddin S.; Steinhoff M. CAR-T cell therapies: An overview of clinical studies supporting their approved use against acute lymphoblastic leukemia and large B-cell

lymphomas. *International journal of molecular sciences*, 2020; 21, 3906; doi:10.3390/ijms21113906

20. Wang X., Riviere I., Clinical manufacturing of CAR T-cells: foundation of a promising therapy. Official journal of the American Society of Gene & Cell Therapy. Molecular Therapy — Oncolytics (2016) 3, 16015; doi:10.1038/mto.2016.15
21. Sterner, R.C., Sterner, R.M. CAR-T cell therapy: current limitations and potential strategies. *Blood Cancer J.* 11, 69 (2021). https://doi.org/10.1038/s41408-021-00459-7
22. Wang, M., Zhang, C. & Jiang, X. CAR-T: a potential gene carrier targeting solid tumor immune microenvironment. *Sig Transduct Target Ther* **6,** 393 (2021). https://doi.org/10.1038/s41392-021-00812-z
23. Fu, W., Lei, C., Liu, S. *et al.* CAR exosomes derived from effector CAR-T cells have potent antitumour effects and low toxicity. *Nat Commun* **10,** 4355 (2019). https://doi.org/10.1038/s41467-019-12321-3
24. Abhishek Dutta (2021) Exosomes-based cell-free cancer therapy: a novel strategy for targeted therapy, Immunological Medicine, 44:2, 116-123, DOI: 10.1080/25785826.2020.1818482
25. Gao D, Jiang L. Exosomes in cancer therapy: a novel experimental strategy. Am J Cancer Res. 2018 Nov 1;8(11):2165-2175. PMID: 30555736; PMCID: PMC6291654.
26. Peters PJ, Geuze HJ, Van der Donk HA, Slot JW, Griffith JM, Stam NJ, Clevers HC, Borst J. Molecules relevant for T cell-target cell interaction are present in cytolytic granules of human T lymphocytes. Eur J Immunol. 1989 Aug;19(8):1469-75. doi: 10.1002/eji.1830190819. PMID: 2789142.
27. Tang XJ, Sun XY, Huang KM, Zhang L, Yang ZS, Zou DD, Wang B, Warnock GL, Dai LJ, Luo J. Therapeutic potential of CAR-T cell-derived exosomes: a cell-free modality for targeted cancer therapy. Oncotarget. 2015 Dec 29;6(42):44179-90. doi: 10.18632/oncotarget.6175. PMID: 26496034; PMCID: PMC4792550.

28. Introduction to Nanoparticle tracking analysis (NTA), Particle Metrix web page, searched in the web: 07.052021. https://www.particle-metrix.de/en/technologies/nanoparticle-tracking/articles-nanoparticle-tracking/introduction-to-nanoparticle-tracking-analysis-nta

29. John M. Walker. The protein protocols Handbook. Second edition. Chapter 3, 11 -14 University of Hertfordshire, Hatfield, UK. *Humana Press*, 2002. https://d1wqtxts1xzle7.cloudfront.net/46585760/Springer_Protocols_Handbooks20160617-20673-m8axov.pdf?1466229911=&response-content-disposition=inline%3B+filename%3DMethods_and_Techniques_for_Research_on_t.pdf&Expires=1625653802&Signature=eHsO~7pCBykvbLHFL5G5~1NZSPqQyr5ZbXlWy4PbRHN-wzB4tpHq~lm6NcrW90telNi0NuC5~GGsoiqf8PdXfrTju0~jE7Vw975L20xvTYKeHDE5LC0QOGEbE3OP8OkzIJAI6pCgVXKmnraqsoCEXS25BXf2ZrJZTLQBtlnoIbAUPViV3VMr8PvEyKJzsjH8u31RHgWQTLOn4T1P1cyZMm3C6LyBkyvRyutyIxH4NWOGRncEU~MG2qjPRD7DuZ7JeGG6mjrjA7t8WM2odhD-GY8xnh2kW8KqmTsk0~TzAhVCrZevDNCC47GaSP-hWEU2KbTRHCPGNsxxu06JRW1U8w__&Key-Pair-Id=APKAJLOHF5GGSLRBV4ZA#page=36

30. Alhajj M, Farhana A. Enzyme Linked Immunosorbent Assay. [Updated 2021 Feb 6]. In: StatPearls [Internet]. Treasure Island (FL): *StatPearls Publishing*; 2021 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK555922/

31. Hartjes, T.A.; Mytnyk, S.; Jenster, G.W.; van Steijn, V.; van Royen, M.E. Extracellular Vesicle Quantification and Characterization: Common Methods and Emerging Approaches. *Bioengineering* 2019, *6*, 7. https://doi.org/10.3390/bioengineering6010007:

32. Morales-Kastresana A, Jones JC. Flow Cytometric Analysis of Extracellular Vesicles. *Methods Mol Biol*. 2017; 1545:215-225. doi:10.1007/978-1-4939-6728-5_16

33. Data sheet MACSPlex exosome kit human. Miltenyi Biotec. www.miltenyibiotec.com

34. He, X., Xu, C. Immune checkpoint signaling and cancer immunotherapy. *Cell Res* 30, 660–669 (2020). https://doi.org/10.1038/s41422-020-0343-4

35. Alsaab HO, Sau S, Alzhrani R, et al. PD-1 and PD-L1 Checkpoint Signaling Inhibition for Cancer Immunotherapy: Mechanism, Combinations, and Clinical Outcome. *Front Pharmacol*. 2017;8:561. Published 2017 Aug 23. doi:10.3389/fphar.2017.00561

36. *Web page: NIH – National Cancer Institute. https://www.cancer.gov/publications/dictionaries/cancer-terms/def/immune-checkpoint-inhibitor*

37. Sharpe, A., Pauken, K. The diverse functions of the PD1 inhibitory pathway. Nat Rev Immunol 18, 153–167 (2018). https://doi.org/10.1038/nri.2017.108

38. Graydon CG, Mohideen S, Fowke KR. LAG-3's Enigmatic Mechanism of action. Front Immunol. 2021. 11:615317. doi: 10.3389/fimmu.2020.615317

39. Bae, J., Accardi, F., Hideshima, T. *et al.* Targeting LAG3/GAL-3 to overcome immunosuppression and enhance anti-tumor immune responses in multiple myeloma. *Leukemia* (2021). https://doi.org/10.1038/s41375-021-01301-6

40. Yang D, Zhang W, Zhang H, et al. Progress, opportunity, and perspective on exosome isolation - efforts for efficient exosome-based theranostics. *Theranostics*. 2020;10(8):3684-3707. Published 2020 Feb 19. doi:10.7150/thno.41580

41. Jasper G. van den Boorn, Juliane Daßler, Christoph Coch, Martin Schlee, Gunther Hartmann. Exosomes as nucleic acid nanocarriers. Advanced Drug Delivery Review: Vol. 65, Issue 3, 2013. Pages 331-335, ISSN 0169-409X, https://doi.org/10.1016/j.addr.2012.06.011

42. Technical Note. Particle size distribution laboratory & inline. Zeta potential and charge titration. Colloid Mextrix, 2021. www.colloid-metrix.de

43. Théry C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, Antoniou A, Arab T, Archer F, Atkin-Smith GK, Ayre DC, Bach JM, Bachurski D, Baharvand H, Balaj L, Baldacchino S, Bauer NN, Baxter AA, Bebawy M, Beckham C, Bedina Zavec A, Benmoussa A, Berardi AC, Bergese P, Bielska E, Blenkiron C, Bobis-Wozowicz S, Boilard E, Boireau W, Bongiovanni A, Borràs FE, Bosch S, Boulanger CM, Breakefield X, Breglio AM, Brennan MÁ,

Brigstock DR, Brisson A, Broekman ML, Bromberg JF, Bryl-Górecka P, Buch S, Buck AH, Burger D, Busatto S, Buschmann D, Bussolati B, Buzás EI, Byrd JB, Camussi G, Carter DR, Caruso S, Chamley LW, Chang YT, Chen C, Chen S, Cheng L, Chin AR, Clayton A, Clerici SP, Cocks A, Cocucci E, Coffey RJ, Cordeiro-da-Silva A, Couch Y, Coumans FA, Coyle B, Crescitelli R, Criado MF, D'Souza-Schorey C, Das S, Datta Chaudhuri A, de Candia P, De Santana EF, De Wever O, Del Portillo HA, Demaret T, Deville S, Devitt A, Dhondt B, Di Vizio D, Dieterich LC, Dolo V, Dominguez Rubio AP, Dominici M, Dourado MR, Driedonks TA, Duarte FV, Duncan HM, Eichenberger RM, Ekström K, El Andaloussi S, Elie-Caille C, Erdbrügger U, Falcón-Pérez JM, Fatima F, Fish JE, Flores-Bellver M, Försönits A, Frelet-Barrand A, Fricke F, Fuhrmann G, Gabrielsson S, Gámez-Valero A, Gardiner C, Gärtner K, Gaudin R, Gho YS, Giebel B, Gilbert C, Gimona M, Giusti I, Goberdhan DC, Görgens A, Gorski SM, Greening DW, Gross JC, Gualerzi A, Gupta GN, Gustafson D, Handberg A, Haraszti RA, Harrison P, Hegyesi H, Hendrix A, Hill AF, Hochberg FH, Hoffmann KF, Holder B, Holthofer H, Hosseinkhani B, Hu G, Huang Y, Huber V, Hunt S, Ibrahim AG, Ikezu T, Inal JM, Isin M, Ivanova A, Jackson HK, Jacobsen S, Jay SM, Jayachandran M, Jenster G, Jiang L, Johnson SM, Jones JC, Jong A, Jovanovic-Talisman T, Jung S, Kalluri R, Kano SI, Kaur S, Kawamura Y, Keller ET, Khamari D, Khomyakova E, Khvorova A, Kierulf P, Kim KP, Kislinger T, Klingeborn M, Klinke DJ 2nd, Kornek M, Kosanović MM, Kovács ÁF, Krämer-Albers EM, Krasemann S, Krause M, Kurochkin IV, Kusuma GD, Kuypers S, Laitinen S, Langevin SM, Languino LR, Lannigan J, Lässer C, Laurent LC, Lavieu G, Lázaro-Ibáñez E, Le Lay S, Lee MS, Lee YXF, Lemos DS, Lenassi M, Leszczynska A, Li IT, Liao K, Libregts SF, Ligeti E, Lim R, Lim SK, Linē A, Linnemannstöns K, Llorente A, Lombard CA, Lorenowicz MJ, Lörincz ÁM, Lötvall J, Lovett J, Lowry MC, Loyer X, Lu Q, Lukomska B, Lunavat TR, Maas SL, Malhi H, Marcilla A, Mariani J, Mariscal J, Martens-Uzunova ES, Martin-Jaular L, Martinez MC, Martins VR, Mathieu M, Mathivanan S, Maugeri M, McGinnis LK, McVey MJ, Meckes DG Jr, Meehan KL, Mertens I, Minciacchi VR, Möller A, Møller Jørgensen M, Morales-Kastresana A, Morhayim J, Mullier F, Muraca M, Musante L, Mussack V, Muth DC, Myburgh KH, Najrana T, Nawaz M,

Nazarenko I, Nejsum P, Neri C, Neri T, Nieuwland R, Nimrichter L, Nolan JP, Nolte-'t Hoen EN, Noren Hooten N, O'Driscoll L, O'Grady T, O'Loghlen A, Ochiya T, Olivier M, Ortiz A, Ortiz LA, Osteikoetxea X, Østergaard O, Ostrowski M, Park J, Pegtel DM, Peinado H, Perut F, Pfaffl MW, Phinney DG, Pieters BC, Pink RC, Pisetsky DS, Pogge von Strandmann E, Polakovicova I, Poon IK, Powell BH, Prada I, Pulliam L, Quesenberry P, Radeghieri A, Raffai RL, Raimondo S, Rak J, Ramirez MI, Raposo G, Rayyan MS, Regev-Rudzki N, Ricklefs FL, Robbins PD, Roberts DD, Rodrigues SC, Rohde E, Rome S, Rouschop KM, Rughetti A, Russell AE, Saá P, Sahoo S, Salas-Huenuleo E, Sánchez C, Saugstad JA, Saul MJ, Schiffelers RM, Schneider R, Schøyen TH, Scott A, Shahaj E, Sharma S, Shatnyeva O, Shekari F, Shelke GV, Shetty AK, Shiba K, Siljander PR, Silva AM, Skowronek A, Snyder OL 2nd, Soares RP, Sódar BW, Soekmadji C, Sotillo J, Stahl PD, Stoorvogel W, Stott SL, Strasser EF, Swift S, Tahara H, Tewari M, Timms K, Tiwari S, Tixeira R, Tkach M, Toh WS, Tomasini R, Torrecilhas AC, Tosar JP, Toxavidis V, Urbanelli L, Vader P, van Balkom BW, van der Grein SG, Van Deun J, van Herwijnen MJ, Van Keuren-Jensen K, van Niel G, van Royen ME, van Wijnen AJ, Vasconcelos MH, Vechetti IJ Jr, Veit TD, Vella LJ, Velot É, Verweij FJ, Vestad B, Viñas JL, Visnovitz T, Vukman KV, Wahlgren J, Watson DC, Wauben MH, Weaver A, Webber JP, Weber V, Wehman AM, Weiss DJ, Welsh JA, Wendt S, Wheelock AM, Wiener Z, Witte L, Wolfram J, Xagorari A, Xander P, Xu J, Yan X, Yáñez-Mó M, Yin H, Yuana Y, Zappulli V, Zarubova J, Žėkas V, Zhang JY, Zhao Z, Zheng L, Zheutlin AR, Zickler AM, Zimmermann P, Zivkovic AM, Zocco D, Zuba-Surma EK. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J Extracell Vesicles*. 2018 Nov 23;7(1):1535750. doi: 10.1080/20013078.2018.1535750. PMID: 30637094; PMCID: PMC6322352.

44. Mathieu, M., Névo, N., Jouve, M. *et al.* Specificities of exosome versus small ectosome secretion revealed by live intracellular tracking of CD63 and CD9. *Nat Commun* **12,** 4389 (2021). https://doi.org/10.1038/s41467-021-24384-2

45. Reyes R, Cardeñes B, Machado-Pineda Y and Cabañas C (2018) Tetraspanin CD9: A Key Regulator of Cell Adhesion in the Immune System. *Front. Immunol.* 9:863. doi: 10.3389/fimmu.2018.00863

46. Jankovičová J, Sečová P, Michalková K, Antalíková J. Tetraspanins, More than Markers of Extracellular Vesicles in Reproduction. *International Journal of Molecular Sciences*. 2020; 21(20):7568. https://doi.org/10.3390/ijms21207568

47. Oba, Ryutaro et al. "Circulating $CD3^{+}HLA\text{-}DR^{+}$ Extracellular Vesicles as a Marker for Th1/Tc1-Type Immune Responses." *Journal of immunology research* vol. 2019 6720819. 8 May. 2019, doi:10.1155/2019/6720819

48. Chapter 8 - The T Cell Receptor: Proteins and Genes, Editor(s): Tak W. Mak, Mary E. Saunders, Bradley D. Jett, Primer to the Immune Response (Second Edition). Academic Cell, 2014. Pages 181-196, ISBN 9780123852458. https://doi.org/10.1016/B978-0-12-385245-8.00008-X.

49. Akihiro T, Mitsutoshi T, Hitoshi H, Yoshinori M, Shuji K., Involvement of SLC17A9-dependent Vesicular Exocytosis in the Mechanism of ATP Release during T Cell Activation. Journal of Biological Chemistry, Volume 285, Issue 23, 2010. (17406-17416), ISSN 0021-9258. https://doi.org/10.1074/jbc.M110.112417

50. Yang Liu, Pan Zheng. CD24: a genetic checkpoint in T cell homeostasis and autoimmune diseases. Trends in Immunology, Volume 28, Issue 7, 2007. (315-320), ISSN 1471-4906. https://doi.org/10.1016/j.it.2007.05.001.

51. S. Keller, C. Rupp, A. Stoeck, S. Runz, M. Fogel, S. Lugert, H.-D. Hager, M.S. Abdel-Bakky, P. Gutwein, P. Altevogt. CD24 is a marker of exosomes secreted into urine and amniotic fluid. Kidney International. Volume 72, Issue 9, 2007. (1095-1102), ISSN 0085-2538. https://doi.org/10.1038/sj.ki.5002486.

52. Li O, Zheng P, Liu Y. CD24 expression on T cells is required for optimal T cell proliferation in lymphopenic host. *J Exp Med*. 2004;200(8):1083-1089. doi:10.1084/jem.20040779

53. Schumann J, Stanko K, Schliesser U, Appelt C, Sawitzki B. Differences in CD44 Surface Expression Levels and Function Discriminates IL-17 and IFN-γ Producing Helper T Cells [published correction appears in PLoS One. 2015;10(11):e0143986]. *PLoS One*. 2015;10(7):e0132479. Published 2015 Jul 14. doi:10.1371/journal.pone.0132479

54. Baaten BJG, Tinoco R, Chen AT and Bradley LM (2012) Regulation of antigen-experienced T cells: lessons from the quintessential memory marker CD44. *Front. Immun.* 3:23. doi: 10.3389/fimmu.2012.00023

55. Zhu Y, Feng Y, Liu H, et al. CD4+CD29+T cells are blamed for the persistent inflammatory response in ulcerative colitis. *Int J Clin Exp Pathol*. 2015;8(3):2627-2637. Published 2015 Mar 1.

56. Monguió-Tortajada M, Roura S, Gálvez-Montón C, et al. Nanosized UCMSC-derived extracellular vesicles but not conditioned medium exclusively inhibit the inflammatory response of stimulated T cells: implications for nanomedicine. *Theranostics*. 2017;7(2):270-284. Published 2017 Jan 1. doi:10.7150/thno.16154

57. Carolina Balbi, Jacopo Burrello, Sara Bolis, Edoardo Lazzarini, Vanessa Biemmi, Enea Pianezzi, Alessio Burrello, Elena Caporali, Lorenzo Gauthier Grazioli, Gladys Martinetti, Tanja Fusi-Schmidhauser, Giuseppe Vassalli, Giorgia Melli, Lucio Barile. Circulating extracellular vesicles are endowed with enhanced procoagulant activity in SARS-CoV-2 infection. EBioMedicine, Volume 67, 2021. 103369, ISSN 2352-3964. https://doi.org/10.1016/j.ebiom.2021.103369.

58. J.I. Zwicker, C.C. Trenor 3rd, B.C. Furie, B. Furie. Tissue factor-bearing microparticles and thrombus formation. Arterioscler Thromb Vasc Biol, 31 (4) (2011), pp. 728-733.

59. F. Dignat-George, C.M. Boulanger. The many faces of endothelial microparticles. Arterioscler Thromb Vasc Biol, 31 (1) (2011), pp. 27-33.

60. Altin, J G, and E K Sloan. “The role of CD45 and CD45-associated molecules in T cell activation.” *Immunology and cell biology* vol. 75,5 (1997): 430-45. doi:10.1038/icb.1997.68

61. Ceeraz, Sabrina et al. “Immune checkpoint receptors in regulating immune reactivity in rheumatic disease.” *Arthritis research & therapy* vol. 16,5 (2014): 469. doi:10.1186/s13075-014-0469-1

62. Aiello, S., Rocchetta, F., Longaretti, L. *et al.* Extracellular vesicles derived from T regulatory cells suppress T cell proliferation and prolong allograft survival. *Sci Rep* **7,** 11518 (2017). https://doi.org/10.1038/s41598-017-08617-3

63. Ali S, Toews K, Schwiebert S, Klaus A, Winkler A, Grunewald L, Oevermann L, Deubzer HE, Tüns A, Jensen MC, Henssen AG, Eggert A, Schulte JH, Schwich E, Rebmann V, Schramm A and Künkele A (2020) Tumor-Derived Extracellular Vesicles Impair CD171-Specific $CD4^+$ CAR T Cell Efficacy. *Front. Immunol.* 11:531. doi: 10.3389/fimmu.2020.00531

64. Riley, James L. “PD-1 signaling in primary T cells.” *Immunological reviews* vol. 229,1 (2009): 114-25. doi:10.1111/j.1600-065X.2009.00767.x

65. Freeman, Gordon J et al. “Reinvigorating exhausted HIV-specific T cells via PD-1-PD-1 ligand blockade.” *The Journal of experimental medicine* vol. 203,10 (2006): 2223-7. doi:10.1084/jem.20061800

66. Kumagai, S., Togashi, Y., Kamada, T. *et al.* The PD-1 expression balance between effector and regulatory T cells predicts the clinical efficacy of PD-1 blockade therapies. *Nat Immunol* 21, 1346–1358 (2020). https://doi.org/10.1038/s41590-020-0769-3

67. Y. Sun, Q.-H. Shi, L. Zhang, G.-F. Zhao, F.-F. Liu, 2.47 - Adsorption and Chromatography, Editor(s): Murray Moo-Young, Comprehensive Biotechnology (Second Edition), *Academic Press*, 2011, 665-679, ISBN 9780080885049, https://doi.org/10.1016/B978-0-08-088504-9.00123-9.

68. Automatic Fraction Collector, User manual V1 Revision G. *IZON science*, 2018. www.izon.com

69. Jana Klimentová, Jiří Stulík. Methods of isolation and purification of outer membrane vesicles from gram-negative bacteria, Microbiological Research, Volume 170, 2015, Pages 1-9, ISSN 0944-5013, https://doi.org/10.1016/j.micres.2014.09.006.

70. Franquesa M, Hoogduijn MJ, Ripoll E, Luk F, Salih M, Betjes MGH, Torras J, Baan CC, Grinyó JM and Merino AM (2014) Update on controls for isolation and quantification methodology of extracellular vesicles derived from adipose tissue mesenchymal stem cells. *Front. Immunol.* **5**:525. doi: 10.3389/fimmu.2014.00525

71. Brennan, K., Martin, K., FitzGerald, S.P. *et al.* A comparison of methods for the isolation and separation of extracellular vesicles from protein and lipid particles in human serum. *Sci Rep* **10,** 1039 (2020). https://doi.org/10.1038/s41598-020-57497-7

72. Hartjes, Thomas A et al. "Extracellular Vesicle Quantification and Characterization: Common Methods and Emerging Approaches." *Bioengineering (Basel, Switzerland)* vol. 6,1 7. 16 Jan. 2019, doi:10.3390/bioengineering6010007

73. Cashikar, Anil G, and Phyllis I Hanson. "A cell-based assay for CD63-containing extracellular vesicles." *PloS one* vol. 14,7 e0220007. 24 Jul. 2019, doi:10.1371/journal.pone.0220007

74. Hünig, T., Tiefenthaler, G., zum Büschenfelde, KH. *et al.* Alternative pathway activation of T cells by binding of CD2 to its cell-surface ligand. *Nature* **326,** 298–301 (1987). https://doi.org/10.1038/326298a0

75. Chuo, S.TY., Chien, J.CY. & Lai, C.PK. Imaging extracellular vesicles: current and emerging methods. *J Biomed Sci* **25,** 91 (2018). https://doi.org/10.1186/s12929-018-0494-5

76. Rikkert, L G et al. "Quality of extracellular vesicle images by transmission electron microscopy is operator and protocol dependent." *Journal of extracellular vesicles* vol. 8,1 1555419. 9 Jan. 2019, doi:10.1080/20013078.2018.1555419

77. Jade M. Noble, LaDeidra Monét Roberts, Netta Vidavsky, Aaron E. Chiou, Claudia Fischbach, Matthew J. Paszek, Lara A. Estroff, Lena F. Kourkoutis, Direct comparison of optical and electron microscopy methods for structural characterization of extracellular vesicles, Journal of Structural Biology, Volume 210, Issue 1, 2020, 107474, ISSN 1047-8477, https://doi.org/10.1016/j.jsb.2020.107474.

78. Cizmar, Petr, and Yuana Yuana. “Detection and Characterization of Extracellular Vesicles by Transmission and Cryo-Transmission Electron Microscopy.” *Methods in molecular biology (Clifton, N.J.)* vol. 1660 (2017): 221-232. doi:10.1007/978-1-4939-7253-1_18