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IMMUNOREGULATORY PROPERTIES OF BONE MARROW MESENCHYMAL STROMAL CELL-DERIVED EXTRACELLULAR VESICLES

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ABSTRACT

Mesenchymal stromal cells (MSCs) are adult stem cells of mesodermal origin that can be isolated from various tissues, including bone marrow (BM), adipose tissue and amniotic fluid. MSCs express mesenchymal markers, i.e. CD73, CD90, and CD105, and lack expression of hematopoietic markers, such as CD45, CD34, CD11b and CD14. In addition to their tri-lineage differentiation towards adipocytes, chondrocytes and osteoblasts, MSCs modulate the immune response. In fact, MSCs can regulate the proliferation and activation of different immune effector cells (IECs), including T, B and NK cells. The biological effects of MSCs are not exclusively related to their close interaction with target cells by cell-to-cell contact, but can be mediated by molecule release. For instance, MSC immunomodulation may occur through paracrine mechanisms, including indolamine 2,3 dioxygenase, prostaglandin E2, heme-oxygenese-1, and TGF-β. In the last decade, a key mechanism of cell-to-cell communication of MSCs through extracellular vesicles (EVs) has been clarified. The potential therapeutic role of MSC-derived EVs has been described in different diseases, including cardiovascular disease, acute kidney injury, and lung injury. EVs are molecular shuttles consisting of a phospholipid bilayer containing different molecules, including proteins and different types of RNAs (mRNA and miRNA). EVs are a family of different shedding vesicles, including exosomes (EXs, 50-100 nm), microvesicles (MVs, 100-1000 nm), and apoptotic bodies (ABs, 50-500 nm). EXs originate by multivesicular body and express specific markers, such as CD63, CD9 and Alix. MVs result from the plasmatic membrane and express specific proteins of the cells of origin. To understand whether the MSC immunomodulatory effects are mediated by EV release, we characterized the protein content and immunomodulatory functions towards different immune effector cells of EVs derived from BM-MSCs. In addition, we evaluated the capability of unfractionated PBMCs to internalize MSC-derived EVs. We observed that the rate of EV internalization was higher in B cells and correlated with their capability to reduce B cell proliferation. By using a reproducible and standardized method we showed a new mechanism

of MSC-mediated immunosuppression, thus characterizing better the biological function of MSC-derived EVs and paving the way to a possible clinical application of EVs as alternative cell-free therapy.

PREFACE

Experiments presented in this thesis are a part of the published work below.

Mariano Di Trapani, Giulio Bassi, Martina Midolo, Alessandro Gatti, Paul Takam Kamga, Adriana Cassaro, Roberta Carusone, Annalisa Adamo & Mauro Krampera. **Differential and transferable modulatory effects of mesenchymal stromal cell-derived extracellular vesicles on T, B and NK cell functions.** Scientific Reports | 6:24120 | DOI: 10.1038/srep24120.

The data presented in the article above have been here reproduced/adapted with the permission of all the article's authors.

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I. INTRODUCTION

I.1. Mesenchymal stromal cells (MSCs)

Mesenchymal stromal cells (MSCs) belong to adult stem cells. They are non-hematopoietic precursors isolated as adherent cells, with self-renewal and great proliferative capacity and attitude to differentiate into mesenchymal tissues, such as stroma, adipose and bone tissue, and cartilage. MSCs were firstly described more than a century ago in studies with bone marrow cells (Cohnheim J. Arch Path Anat Physiol Klin Med 1867), but the presence of adherent cells differentiating into fibroblasts was shown around 1975 by A.J. Friedenstein. Bone marrow samples were seeded on culture plates and non-adherent cells were removed after 4 hours; the remaining adherent cells displayed elongated shape and formed aggregates of a few cells called fibroblast colony-forming units (CFUs-F) [1]. After several passages in culture, cells became increasingly homogeneous, showing the ability to differentiate into bone and cartilage tissues.

Several soluble factors are added in the culture medium to demonstrate MSC multipotency; in fact, they can differentiate into adipocytes (with vacuoles containing lipids), osteoblasts (with hydroxyapatite deposits) and chondrocytes (with synthesis of cartilage matrix proteins)[1-4]. Furthermore, MSCs can synthesize and secrete cytokines (IL-7, IL-8, IL-11), stem cell factor (SCF) and stromal-derived-factor-1 (SDF-1). They are also able to drive the migration of bone marrow hematopoietic stem cells [1, 3-5], playing a key role in the homeostasis of bone marrow stromal niches. MSCs represent a rare cell population of bone marrow with great proliferative capacity once seeded in culture *in vitro* [6, 7]. Although some *in vitro* studies showed evidence of *in vitro* and *in vivo* pluripotency leading to the definition of Multipotent Adult Progenitor Cells (MAPCs) [4], MSCs are currently considered multipotent stem cells [8], which may differentiate into lineages of strictly mesodermal origin.

In 2006 the International Society for Cellular Therapy (ISCT) defined the main criteria to characterize MSCs [9]:

1. Expression of a combination of membrane markers, such as CD90, CD105, CD73 and lack of leukocyte or endothelial markers, such as CD31, CD45, CD34 and CD14;
2. Adhesion to culture plates once seeded in culture;
3. Ability to differentiate into adipocyte, osteoblast and chondrocyte tissues (Fig. 1).

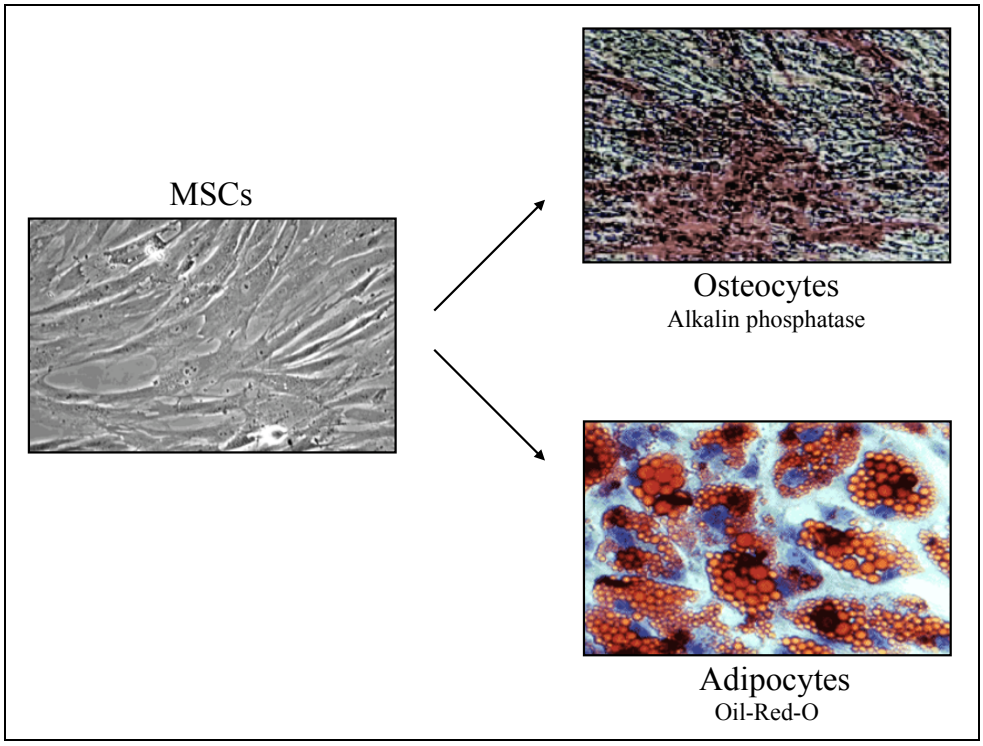

Fig. 1: Mesenchymal stromal cell differentiation

MSCs can make fat and bone cells *in vitro*, with specific stimuli. In the osteocytes mineral deposits are revealed through alkalin phosphatase staining; while in adipocytes lipid vacuoles are revealed through Oil-Red-O stainig.

MSCs can be isolated from non-hematopoietic tissues, such as adipose tissue, placenta, dermis, dental pulp and lung; by contrast, peripheral blood is a very poor source of MSCs (Fig. 2) [10-14].

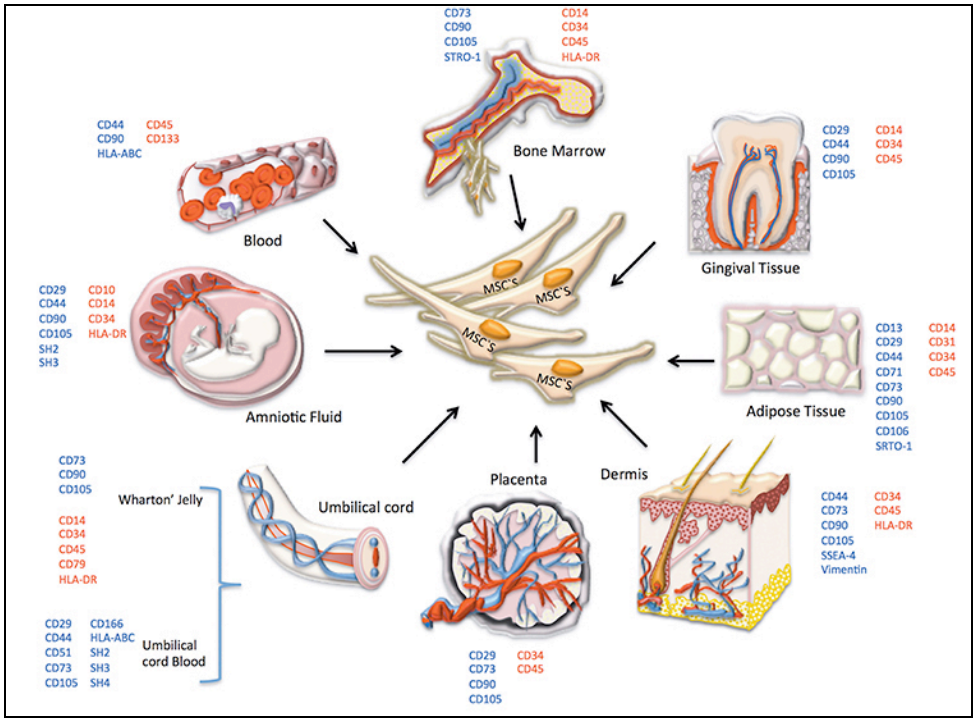

Fig. 2: Phenotype, tissue origin, and immune system regulation of MSC

In embryonic tissues, MSCs can be identified in the amniotic fluid, the wharton's jelly from the umbilical cord, the umbilical cord blood, and in the placenta. In adults MSCs are present in the bone marrow and can migrate to peripheral blood, propagating to several tissues including gingival tissue, adipose tissue, and dermis. Surface markers used to identify MSCs in different locations are indicated; positively expressed markers are shown in blue, negative markers are shown in red. (Image modified from Front Physiol. 2016 Feb 9;7:24. doi: 10.3389/fphys.2016.00024).

I.1.1. Tissues of origin for MSCs

Human MSCs derive from the mesoderm, the germ layer from which all connective tissues originate [15], however, their development in fetal and postnatal age is not widely known. MSCs have been described as a homogeneous population of rounded cells in the mesenchyme expressing extracellular matrix proteins, devoid of hemopoietic and endothelial markers and capable of supporting embryonic and adult hematopoiesis. The stromal component surrounds the primitive hematopoietic layer of the dorsal aorta in

the aorto-gonado-mesonephrical region of the human fetus [6, 15]. Fibroblastic cells were isolated, with conflicting results, from human peripheral blood of healthy donors in presence of FBS (Fetal Bovine Serum) without additional growth factors [10]. These cells express Vimentin, Collagen I and an Endoglin epitope (CD105), which is a mesenchymal marker; they also express Osteocalcin and Alkaline Phosphatase [16-18]. A substantial proportion of cells with similar phenotypic characteristics of bone marrow MSCs, but with greater differentiation potentials, can be detected in the peripheral blood of human fetus at the 7th week of pregnancy [19]. From embryonic development until adult age several body tissues maintain a MSC reservoir, thus suggesting that a broad mesenchymal system aimed at tissue repair and regeneration is persistently operational [7].

I.1.2. Isolation and functional characteristics of MSCs

MSCs are obtained from *ex-vivo* cell suspensions derived from bone marrow samples or from disaggregated tissues resuspended in culture medium. Cells can be seeded in culture flasks a concentration of 2.000 cells/cm^2, in modified Eagle medium (α-MEM) or Dulbecco's modified Eagle medium (D-MEM), 18% of fetal bovine serum (FBS) and 1% of antibiotics. After about 72 hours non-adherent cells are removed and the medium is changed twice a week. A few days later the aggregates of proliferating and adherent cells form spindle shaped fibroblast-like colonies, named colony forming units-fibroblasts (CFU-F); this assay is used to enumerate the MSCs in the starting material, by counting the colonies containing at least 50 cells. When proliferating cells form an almost confluent monolayer (70-80% of confluence), they are detached and reseeded into larger flasks, thus keeping on proliferating spontaneously for up to 40 generations.

Different mesodermal (i.e. chondrogenic, adipogenic and osteogenic) differentiation can be obtained with specific stimuli [3, 4, 15, 20]. Osteogenic differentiation is induced for three weeks with a medium containing dexamethasone, β-glycerophosphate and ascorbic acid; cells show a

geometric-like shape with mineral deposits highlighted with specific stainings, such as Von Kossa and Alizarin Red dyes.

Adipocyte differentiation is induced after 14 day-culture with a medium containing higher dexamethasone concentrations. Cells accumulate lipid droplets, revealed at the microscope following Oil-Red-O specific staining.

For chondrocyte differentiation, dexamethasone, proline, ascorbic acid, TGF-β1 (transforming growth factor-β1) are added to pelleted cells growing in the culture medium. Cells display rounded shape and are specifically stained with Toluidine Blue dye [14].

Most studies are focused on MSCs obtained from bone marrow, but many data are available in literature for other MSC sources, such as adipose tissue and umbilical cord blood [21].

I.1.3. Immunophenotypic characteristics

Human MSCs are recognized by the lack of hematopoietic stem cell markers (CD45 and CD34) and endothelial markers (CD31/PECAM-1), and the expression of surface molecules such as CD90 (Thy1), CD54 (ICAM-1), CD106 (VCAM-1), CD73, CD105 (endoglin), CD44 (hyaluronic acid receptor) and CD29 [14, 19, 22]. Other markers expressed by MSCs include molecules involved in immune responses (MHC I and II, CD119), receptors for cytokines, chemokines, adhesion molecules, and molecules of epithelial origin such as EGFR or HER-1 [20] (Tab. 1).

CD 105 (SH2/TGF-beta-R)	++	CD 11c	-
CD 73 (SH3 ed SH4)	++	CD 18	-
CD 29	++	CD 54 (ICAM-1)	+
CD 44	+++	CD 49°	+
CD 90	+++	CD 49b	+
CD 106	±	CD 49c	+
CD 31	-	CD 49d	-
CD 45	-	CD 49e	+
CD 34	-	CD 62L	+
CD 14	-	CD 166	+
CD 10	-	CD 120°	+
CD 56	-	CD 120b	+
HLA class I (A,B,C)	++*	CD 30	-
HLA-DR	-*	CD 30L (CD 153)	±
CD 80	-	CD 40	-
CD 86	-	CD 40L (CD 154)	-
CD 117	-	FasL	-/±
CD 119	+	Fas (CD 95)	++
CD 25	-	TRAIL	-
CD 122	-	TRAIL-R	-
CD 124	-	CXCR 4	-/±
EGFR-1 (HER-1)	+	CXCL 12	-
EGFR-4 (HER-4)	-	HB – EGF	-

Tab. 1: Immunophenotypical MSCs layout

+ And - mean the intensity of expression compared to negative controls and *expression induced by IFN-γ (modified from "Mesenchymal stem cells", Oncology Hematology Seminars, 3(2):131-153, 2006, Krampera M and Pizzolo G).

I.1.4. Immunoregulatory capabilities of MSCs

MSCs are capable of modulating the activity of different effectors involved in both innate and acquired immune responses, such as T, B and NK cells, dendritic cells, macrophages, and are also involved in the activation of the complement system [22-28]. As the immune system plays a key role in the onset and progression of several degenerative diseases, the immunoregulatory activity of MSCs coupled to their differentiation capability makes these cells potential candidates for regeneration of injured tissues in inflammatory and autoimmune diseases. Immunoregulatory properties are shared by MSCs of different tissue of origin [29, 30] and is a consequence of a priming by inflammatory cytokines, such as IFN-γ, TNF-α and IL-1 α/β, released in the microenvironment during the early phases of inflammation [22, 28]. The immunosuppressive capability is mediated by molecules expressed and

released by MSCs after inflammatory priming, such as indoleamine 2,3-dioxygenase (IDO), nitric oxide (NO), prostaglandin E2 (PGE2), TSG6, heme-oxygenase-1 (HO-1) and IL-10 [27, 28]. Although immunosuppression is a conserved mechanism, there are some differences in the mechanism of inhibition between the various species. Under the action of inflammatory cytokines (IFN-γ, TNF-α and IL-1), murine MSCs express NO-synthase, responsible for the production and subsequent release of NO, thus leading to the inhibition of T cell proliferation both *in vitro* and *in vivo* [31]. As NO has a very short-term effect, its activity depends on cell-to-cell contact and can be implemented by some adhesion molecules over-expressed following MSCs inflammatory priming, i.e. ICAM-1 and VCAM-1 [32]. Conversely, human MSCs show both contact-dependent and paracrine mechanisms. However, the main mechanism is mediated by IDO, at least in T and NK cells: this enzyme catalyzes and degrades the tryptophan amino-acid (essential for cell viability) present in the microenvironment, leading to production of metabolites with immunosuppressive activity, such as kynurenin. MSCs do not express IDO at basal conditions, but its expression is induced by IFN-γ [22]. IDO inhibition through specific inhibitors (L-1MT) determines the complete recovery of proliferation in T cell-MSC co-cultures [22, 33] (Fig. 3).

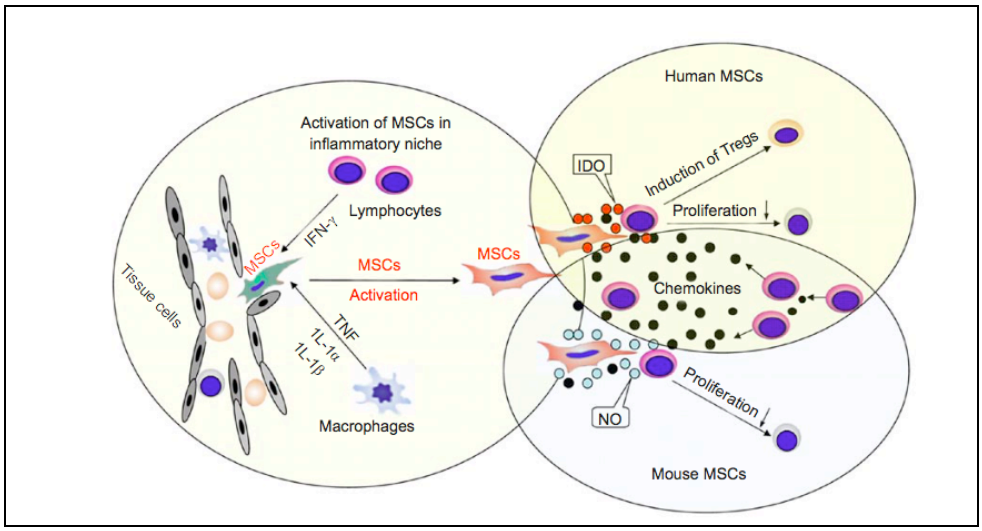

Fig. 3: Activation of MSCs in inflammatory niche and immunosuppression on lymphocytes

The recruited MSCs are readily activated by inflammatory cytokines produced by activated lymphocytes and monocytes, and release chemokines, NO or IDO. Lymphocytes are attracted in close contact with MSCs by

chemokines; mouse MSCs inhibit activation/ proliferation of lymphocytes by releasing NO, while human MSCs inhibit proliferation of lymphocytes by producing IDO and/or inducing T regulatory cells (Tregs). (Cell Res. 2010 May;20(5):510-8. doi: 10.1038/cr.2010.44).

MSC immunoregulatory capability has also been shown in animal models for the study of autoimmune diseases, such as graft versus host disease (GvHD), multiple sclerosis, collagen-induced arthritis, systemic lupus erythematosus and type 1 diabetes, with sometimes contradictory results related to a number of factors, such as the administration schedule and MSC tissue of origin. Unprimed MSCs administered at the initial phase of inflammation are ineffective, probably due to the lack of inflammatory microenvironment and the poor MSC survival in the tissue [34]. Immunodeficient NOD/SCID mice receiving multiple doses of MSCs derived from cord blood show improvement of the symptoms of GvHD [35]. Although the immunosuppressive activity of MSCs has been demonstrated in *in vivo* skin graft studies [26] and clinical setting [36], whether MSCs are effective for treating GvHD is still debated. Tisato et al. showed that the systemic infusion of cord blood MSCs before but not after the onset of GvHD markedly reduced human T-cell proliferation and significantly improved the survival of xeno-GvHD-model mice [37]. A study based on an allogeneic model of GvHD with mouse bone marrow MSCs suggested that MSCs could effectively increase survival rates after GvHD onset [38]. Zhou DH et al. assessed the optimal time intervals for administering MSCs derived from human umbilical cord blood, showing that they were effective both in preventive and in treatment phase. In fact, after systemic intravenous infusion, MSCs are trapped, initially, in the lungs and are redistributed to other organs, although they can influence the regeneration of injured sites or the engraftment of stem cells [39, 40]. However, when the distribution of MSCs was observed by means of bioluminescence imaging and qPCR between 1 and 7 days after injection, the fluorescence signals and messenger RNA transcripts rapidly decreased during the first 24 hours and gradually disappeared by day 7 [41]. Jang YK et al infused hUCB-MSCs at 3- or 7-day intervals (before and after in vivo clearance) and found that the infusion of

hUCB-MSCs at 3-day intervals was more effective in preventing GvHD. This result suggests that before hUCB-MSCs encounter inflammatory cytokines, cells should be repeatedly infused within a short time and at 3-day intervals because of rapid clearance. This protocol resulted in the prevention of the onset of GvHD. In contrast, infusion of hUCB-MSCs after the onset of GvHD significantly increased the survival rate for single and repeated injections because the MSCs were exposed to inflammatory cytokines on infusion [42]. As described so far it is clear that the MSCs communicate to other cells through paracrine signals; in fact, the paracrine effect of MSCs was the focus of many recent studies. MSCs can secrete many growth factors and proteases [43], including vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), insulin-like growth factors (IGF)-1, stromal cell-derived factor (SDF), basic fibroblast growth factor (bFGF), matrix metalloproteinases (MMP), transforming growth factor (TGF)-β, and platelet derived growth factor (PDGF). Some groups evaluated the beneficial effect of MSC supernatant, showing, for example, a reduction of cardiomyocyte apoptosis triggered by hypoxia/reoxygenation *in vitro* [44]. *In vivo* studies have shown the kidney-protective effects of MSC, mediated by complex paracrine actions that are able to significantly protect and regenerate the damaged vasculature in AKI [45]. Many studies suggested that the paracrine effect of MSCs can be mediated by EVs, like “signal carriers” in tissue regeneration and immunomodulation.

I.2. Extracellular vesicles (EVs)

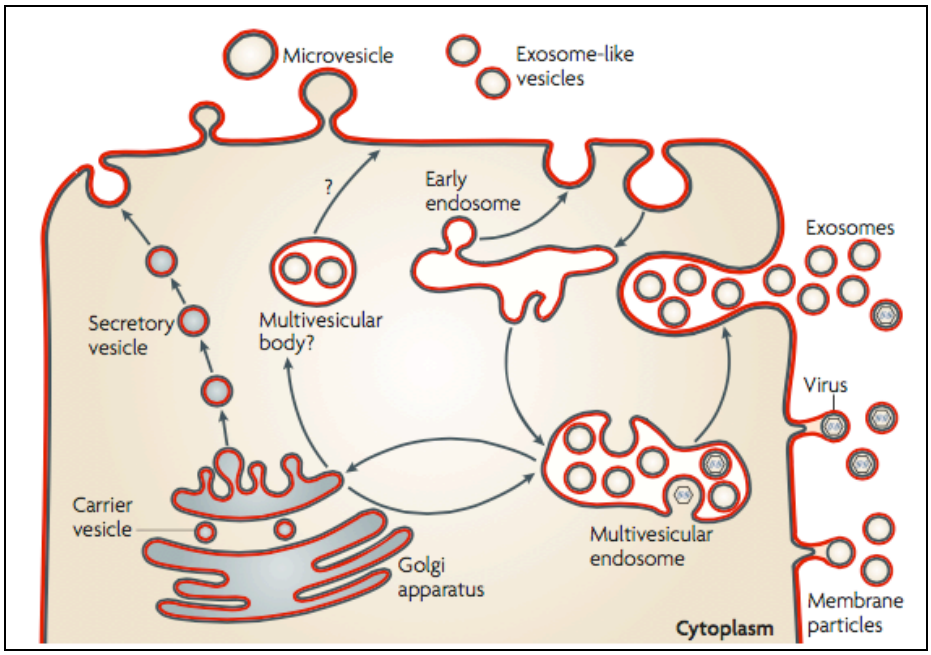

Fig. 4: Different types of secreted membrane vesicles

Intracellular trafficking either between subcellular compartments or towards the plasma membrane for secretion of soluble proteins occurs through carrier and secretory vesicles that contain intraluminal components. Secreted vesicles can form inside internal compartments from where they are subsequently secreted by fusion of these compartments with the plasma membrane. Vesicles generated in multivesicular endosomes are called exosomes once secreted. (Nat Rev Immunol. 2009 Aug;9(8):581-93. doi: 10.1038/nri2567).

Cellular communication is the main way of communication between neighbouring cells and usually occurs by exchange of soluble factors; literature focus has recently shifted to another intercellular communication method, both for short and long-range, i.e. the exchange of EVs [46, 47]. EVs measure from 100 nm to 2 μm, have a spherical shape, are composed of a membrane with a bilayer of phospholipids that contains transmembrane proteins derived from the cytosol of the donor cell, with a rich content of cellular DNA, RNA, proteins, lipids and active metabolites. EV release occurs through both physiological and pathological processes [48-50]. Numerous molecules that have shed through EVs are involved in the control of cell migration [51], proliferation, differentiation [52, 53], and apoptosis, as well as in carcinogenesis [54-56]. EVs shedding is a physiological phenomenon that includes cell activation and growth; furthermore, the presence of many stimuli, such as hypoxia, oxidative stress, and exposure to shear stress, can increase vesicle shedding [57, 58].

The term "exosome" was used for the first time in 1981 by *Trams* and colleagues [59] to indicate EVs ranging from 40 to 1000 nm, derived from various cellular sources in culture, but of dubious origin. The term "exosome complex", instead, was used for a totally different entity: namely, the intracellular particle involved in RNA editing [60]. Now the EVs have been carefully studied, described and divided by size, biogenesis and complexity into three large families: exosomes, microvesicles and apoptotic bodies [47].

I.2.1. Biogenesis and composition of exosomes, microvesicles and apoptotic bodies

Exosomes (EXs) measure from 50 to 100 nm, have know density (1.13-1.19 g/ml), are derived from Multivesicular Endosome (MVE) fusion with plasmatic membrane, thus enabling the release of molecules into the extracellular space. It is thought that the latter process of secretion of EXs is determined by the presence of Endosomal Sorting Complex Required for Transport (ESCRT) and includes four protein complexes: ESCRT- 0, I, II and III, which is involved in binding, sorting, and clustering of ubiquitinylated proteins and receptors [61]. There is also an exosome shedding independent of ESCRT machinery. Production of EXs occurs in endocytic pathway in ESCRT-depleted cells, although the mechanism is not yet clear, while a dramatic alteration in the EXs morphology is observed [62]. EXs carry various molecules of the cell lumen, such as RNAs, proteins and lipids from bilayer membrane. The scientific community has developed the online database, accessible free of charge, that has permitted researchers to archive the molecules identified in EXs to better understand their molecular composition. *ExoCarta* (available online: http://www.exocarta.org), a manually compiled and maintained database that lists proteins, RNAs (miRNA, mRNA, etc) and lipids identified in EXs, and *Vesiclepedia* (available online: http://microvesicles.org) a list of annotations useful for research groups working on secretoma [63-66]. The International Society for

Extracellular Vesicles (ISEV) and its official journal JEV (Journal of Extracellular Vesicles) were established to clarify, standardize and understand the methods of isolation and characterization of the EVs; EXs contain common protein families such as chaperones (Hsp70 and Hsp90), cytoskeletal proteins (actin, myosin and tubulin), ESCRT proteins (TSG-101 and Alix), proteins involved in transport and fusion (Rab11, Rab7, Rab2 and Annexines) as well as tetraspanin proteins (CD9, CD63, CD81 and CD82) and have also been shown to contain cell-specific proteins which are positive for specific markers such as alix, tetraspanin (CD9 and CD63) and TSG-101 [46, 50].

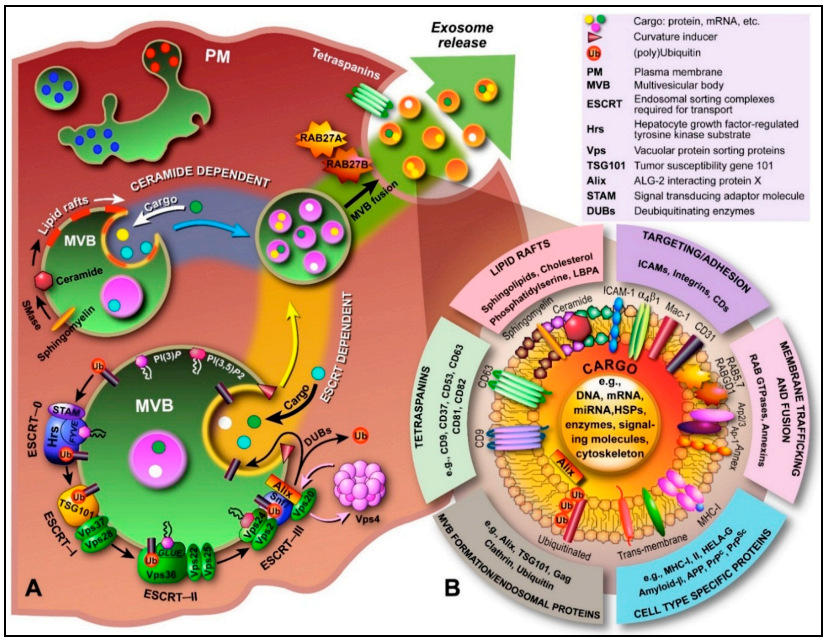

Fig. 5: Biogenesis, secretion and composition of exosomes

(A) The biogenesis and secretion of exosomes is believed to be mediated via a ceramide and/or ESCRT-dependent pathway. (B) Exosomal luminal cargo predominantly consists of mRNA, miRNA and gDNA fragments, and different proteins depending on the cell of origin. (Int J Mol Sci. 2016 Feb 6;17(2):170. doi: 10.3390/ijms17020170).

Microvesicles (MVs) or ectosomes, measure from 100 nm to 1 µm, derive directly from the plasma membrane, through a process called "membrane blebbing" and expose phophatidylserine. Membrane blebbing is a processing step that enables the outsourcing of phosphatidylserine in MV membranes. At first the transmembrane proteins and lipids are grouped in various domains,

and additionally, Ca2+ release/accumulation of enzymes induces degradation of cytoskeletal components. Finally it has been observed the release of MVs with externalization of phosphatidylserine in the MV membranes through the activation of floppase and scramblase proteins. The MVs release into extracellular environment is given by proteins such as GTP-binding protein, ADP-ribosylation factor 6 (ARF6), which promote the contraction of the cytoskeleton for the transport and the final abscission of MVs [67]. MVs contain a diverse population of proteins, including matrix metalloproteinases (MMPs), glycoproteins (as GPIb, GPIIb-IIIa), integrins, receptors (e.g., EGFRvIII) and cytoskeletal components such as β-actin and α-actinin-4 and RNAs [46, 62, 67].

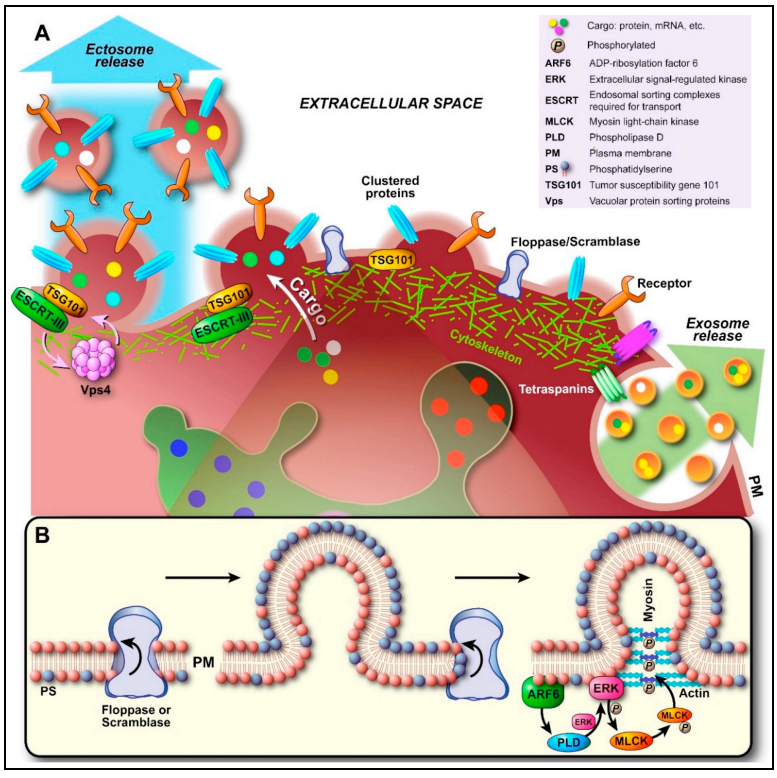

Fig. 6: Biogenesis and secretion of ectosomes

(A) Initial nucleation at the plasma membrane (PM) starts with clustering of transmembrane proteins and lipids in distinct domains. Outward budding is promoted by externalization of phosphatidylserine (PS) by specific translocases such as floppase, scramblase, see also (B). (Int J Mol Sci. 2016 Feb 6;17(2):170. doi: 10.3390/ijms17020170).

Finally, *Apoptotic Bodies* (ABs) have heterogeneous sizes, from 50 to 5000 nm, with a density of between 1.16-1.28 g/ml, are very frequent as they derive from the programmed cell death system [47, 54, 68], and carry DNA molecules. Cell apoptosis can be induced by chromatin condensation, internucleosomal DNA fragmentation, nuclear rupture, mitochondrial swelling and Cytochrome C release, proteolytic cleavage of the cytoskeleton and focal adhesion complexes, phosphatidylserine externalization, plasma membrane blebbing, etc.; eventually all the apoptotic cells culminate with packaging in ABs, their release and digestion by phagocytes [69, 70]. ABs are characterized by the presence of histones. Consequently, cell dismantlement and AB formation are controlled mechanisms to prevent leakage of potentially toxic, enzymatically active or immunogenic components of dying cells into tissues, thereby preventing tissue destruction, inflammation, and autoimmune reaction thrugh cytoskeleton weakening and activation of caspase enzymes. Initiator caspases (caspases-8, -9 and -10) activate effector caspases (caspases-3, -6 and -7) through proteolytic cleavage and starts cell dismantlement. Bleb formation follows a number of distinct steps involving other enzymes and protein families, such as ROCK1, scramblase and floppase adenosine triphosphate type 11C (ATP11C) [71, 72]. However, more studies are needed to understand this highly regulated process, as the formation of ABs is cell type dependent.

Literature on *Vesiclepedia* shows no information about the structure and composition of ABs, because they are involved in programmed cell death, already widely studied and discussed in various articles, while the characteristics and functionality of EXs triggers more interest in research.

I.2.2. EVs isolation methods

EVs, particularly EXs, have been isolated from different biologic fluids, such as blood [73], urine [74], amniotic fluid [75], cerebrospinal fluid [76], and bile [77]. EV isolation methods are many and depend on different factors,

such as the amount of starting sample, EV type and their physical properties (density, size and composition). However, with the available techniques, it is not currently feasible to separate any single EV subtype to obtain a homogeneous sample [78].

I.2.3. Exosome isolation methods

Ultracentrifugation at 100000-120000x g is the most commonly used method for exosome isolation [79, 80]. Despite the two steps of differential centrifugation, this method is inefficient in separating EV subpopulations. To fix this problem most groups of research add one step of filtration (0.1 or 0.2 μm pore size) and/or another differential centrifugation (medium speed 10000-20000x g), for ABs and larger ectosome. Some groups use ultrafiltration and microfiltration to isolate EXs rapidly from urine [81, 82]. The best technique to isolate EXs is density gradient centrifugation (sucrose, sucrose-deuterium oxide (D2O), or OptiPrep™ (Sigma Aldrich), because EXs have a known density and a homogeneous sample can be obtained [79]. The density gradient separation is the best enrichment technique that is currently in use for exosome isolation, even if it is not applicable to large volumes of initial samples. In addition to density gradient centrifugation, immune affinity-based methods (immune-beads and FACS), have also been employed to isolate EXs. Multiple exosomal membrane molecules have been used for this purpose, including CD63, CD9, and HER2. Special kits for rapid exosome isolation are now available, which then could serve as the ideal choice for the identification of exosomal biomarkers linked to specific diseases [83].

I.2.4. Ectosomes isolation methods

Ectosome isolation methods are similar to that for EX collection. However, a homogeneous final sample of ectosomes cannot be obtained because of the wide measuring range overlap with EXs. In addition, ectosomes cannot be

separated with density gradient separation, because they do not possess a specific density. Ectosomal fraction could be obtained through affinity-based methods, as they have distinctly different surface compositions, but at present no specific membrane markers for ectosomes are known.

I.2.5. Apoptotic bodies isolation methods

ABs are preferentially studied in well-defined cellular models of apoptosis. When other EV subpopulations are isolated, the fraction of ABs is discarded through a centrifuge at low g values. A general approach would start with a low speed spin at ~300-500x g to remove cells and followed by a short centrifugation at ~1000-2000x g to remove cellular debris and ABs. Further AB purification steps, such as immune-affinity purification or filtration, might be necessary. Moreover, ABs are not stable for long time periods and hence long isolation methods cannot be employed [84, 85].

I.2.6. General isolation method problems

Isolation methods may significantly vary among different groups; therefore the main problem is definitely the standardization of methods. *Thery* and colleagues have developed a protocol for isolation that provides a centrifuge step at 300x g for 10 minutes, then 200x g for 20 minutes and at the end two steps of ultracentrifugation at 100000x g for 40 and 90 minutes [83]; *Nguyen* and coll. isolated EVs through a centrifugation step at 1500x g for 10 minutes followed by centrifugation at 3000x g for 15 minutes twice to remove intact cells, cell debris, and apoptotic blebs. The supernatants were collected and further centrifuged at 25000x g for 1 hour at 4°C to harvest large size MVs. Subsequently, the collected supernatants were transferred to new tubes and ultra-centrifuged at 35000 rpm (corresponding to about 200000x g) for 2 hours at 4°C for the isolation of small size MVs [86]. The direct conversion from g to rpm is influenced by the type of rotor used, which it is possible to

calculate through a formula that uses the k-factors (clearing factors) [87]. Another problem is the isolation of EXs and MVs from biological fluids, both related to the starting volumes (for EXs isolation with density gradient separation) and the yield of the final sample; in fact, body fluids contain high amounts of non-EV particles (lipoproteins, viruses, and aggregate-forming biomolecules). EXs generally overlap in size with viruses and lipoproteins, whereas ectosomes overlap with the size range of bacteria. With regard to viruses, pure samples of EXs can be obtained by adding a separation step with iodixanol gradients [88]. Protein contamination is frequent with both EXs and MVs; the problem can be fixed by filtering the sample under pressure or specific purification protocols after ultracentrifugation, which not only affects EV purity and yield, but, often leads to protein loss in the preparation [89]. The use of EVs in diagnostic and clinical studies implies a standardization of isolation and quantification methods to ensure total sterility of the final product. *Sáenz-Cuesta* and coll. compared different isolation protocols of EVs from urine and blood and found that the final concentration of EVs are not be so pure and quantitatively sufficient to validate the product and guarantee quality controls [90]. To this aim, a number of reviews [46, 48, 50, 78] and websites (http://www.exocarta.org and http://www.journalofextracellularvesicles.net/ /index.php/jev) are currently available.

I.2.7. Patho-physiological roles of EVs and clinical application

In recent years, several works have shown that a variety of cell types are capable of releasing EVs and EXs into the extracellular space both *in vivo* and *in vitro*. Despite the increasing interest on EVs, their physiological role is still unclear, although it is widely accepted that their main role is to act as carrier of active biological molecules. The function played by EVs depends on the types above described: EVs/EXs carrying endosomal or cytosolic molecules; MVs carrying specific molecules and communicating with target cells; ABs

carrying and presenting cellular debris to complete the process of cell dismantlement.

To allow more accurate analysis, several diagnostic platforms have been developed, such as immunomagnetic exosome RNA (iMER) analysis, miniaturized micro–nuclear magnetic resonance (μNMR) microfluidic chip system, Exochip, and label-free high-throughput nano-plasmonic exosome assay (nPLEX) using surface plasmon resonance (SPR). High-throughput procedures are under development for harvesting EVs from peripheral blood to ensure that EV research is extended into routine diagnostic and therapeutic settings. Most of the studies regarding the possible patho-physiological roles of EVs and EXs have been based on indirect in vitro evidence, especially in the context of the immune system [91].

The immunosuppressive effects on T cells and NK cells can be mediated by EVs; also EVs can play a crucial role in the induction of regulatory T and myeloid cells to further inhibit the immune response [92-94].]. Conforti and colleagues in an in vitro study demonstrated that EVs in co-culture with peripheral blood mononuclear cells (PBMCs) inhibited B cell proliferation and immunoglobulin release, even if they had inferior inhibition ability as compared to their corresponding MSCs [95]. Placenta-derived EXs, purified from the blood of pregnant women, carry immunosuppressive molecules that induce tolerance toward the foetus. Placenta-derived EXs from at term pregnancies carries higher levels of these immunosuppressive molecules compared with EXs from preterm pregnancies. In this study, Fas ligand (FasL) was identified as the putative agent responsible for the inhibition of T-cell, suppressing the activity of maternal cytotoxic T and NK cells [96]. EVs may also stimulate the immune system, with the final effect depending on many factors, such as the identity of the donor and target cells, as well as the biological context in which this interaction takes place.

Van Niel and coll. showed that human intestinal epithelial cells, after IFN-γ treatment, secrete EXs bearing accessory molecules that may be involved in antigen presentation; this phenomenon did not depend on direct cellular contact with effector cells [97]. Two years later, the same group confirmed *in*

vivo the immunogenicity of the peptide-MHC-II associated with EXs, showing the migration of these complexes towards the gut lymph nodes with a pro-inflammatory effect in mouse [98]. EXs have a role also in viral infections, as recently described by *De Carvalho* et al. who showed that EXs from $CD4^+$ T cells inhibit HIV-1 infection *in vitro*, suggesting that EXs might act as decoy receptors for the virus, binding its surface proteins and preventing its interaction with target cells [99]. Thus, EVs play an active role in the mechanisms that involve the immune system, both physiological and pathological. The effects of EVs were observed and analyzed in various studies of different inflammatory diseases. In 2014 a compassionate case of therapy-refractory GvHD was treated with EXs derived from MSCs of four different BM-unrelated donors. The patient recovered within a few months after repeated treatments with allogenic MSC-derived EXs [100], concluding that the MSC-derived EXs may provide a potential new and safe tool to treat GvHD and potentially other inflammatory diseases.

II. RATIONAL HYPOTHESIS AND AIMS

MSCs derived from several tissues, through the release of soluble factors in the microenvironment, regulate proliferation and functions of immune effector cells (IECs) of both innate immunity (neutrophils, monocytes and NK cells) and adaptive immunity (T and B cells). During local inflammation, high levels of interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) make MSCs immunosuppressive towards IECs; then MSCs release several immunomodulatory and trophic molecules (transforming growth factor-β, indoleamine-2,3-dioxygenase, prostaglandin-E2, nitric oxide, and others), thus promoting the tissue regeneration and the modulation of immune response. Cellular communication, however, does not occur only through the release of soluble factors; in fact, cell-to-cell communication is also mediated by signal carriers, i.e. EVs. Recently the EV-mediated paracrine activity of MSCs has been proved. Several research groups have demonstrated that EVs play a key role in tissue repair [45] and immune regulation [101]. This would lead to the use of EVs instead of MSCs, their isolation being more feasible in non-pharmaceutical environment.

The aim of this work is to evaluate the immunomodulatory capability of bone marrow MSC (BM-MSC)-derived EVs. To achieve this goal we performed:

- Isolation, quantification and characterization of BM-MSC-derived EVs
- Assessment of EV uptake by IECs
- Assessment and quantification of the immunoregulation properties of BM-MSC-derived EVs in co-culture with both PBMCs and purified IECs (B cells in particular) by using the standardized immunological assays normally employed to characterize MSC functions.

III. MATERIALS AND METHODS

III.1. Isolation and expansion of human MSCs and IECs

PBMCs (Peripheral Blood Mononuclear Cells) were isolated from human blood using Lymphoprep™ (Stemcells Technologies), by density gradient centrifugation. Mononuclear cell ring was collected, PBMCs were counted and frozen or utilized for functional assays. Purified B cells were isolated from PBMCs in two-steps: magnetic labelling (with appropriate negative selection kits) and magnetic separation (using VarioMACS separator and columns, Miltenyi Biotec). IECs were counted and frozen or utilized for functional assays.

MSCs from 14 different donors were isolated from BM aspirates of healthy donors (informed consent, approved by Ethical Committee of Azienda Ospedaliera Universitaria Integrata Verona; N. 1828, May 12, 2010 "Institution of cell and tissue collection for biomedical research in Onco-Hematology"). BM aspirates were cultured in 225 cm^2 flasks at 1×10^5 nucleated cells/cm^2 concentration in alpha-minimal essential medium (α-MEM), 10% heat-inactivated foetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin and 2 mM L-Glutamine (all from Sigma-Aldrich). After 72 hours, non-adherent cells were removed and medium was replaced twice a week. Full characterization of MSCs has been already described by our group elsewhere [29, 33]. MSCs were detached (0.05% Tripsin-EDTA; Gibco) and harvested when 80% confluent, and then either reseeded at $1 \times 10^3/cm^2$ concentration or frozen until use. All experiments were performed between passages 2 and 7.

In all experiments, MSCs at 80% confluence were treated or not for 40–48 hours with 10 ng/mL IFN-γ and 15 ng/mL TNF-α (R&D Systems) to induce the inflammatory priming, as previously described by or group elsewhere [102].

III.2. Purification of MSC-EVs

Culture medium of MSCs at 80% confluence was aspirated, cells were washed with phosphate-buffered saline (PBS) to remove the residual FBS, and fresh culture medium supplemented with 10% EV-depleted FBS (obtained through 18 hour-centrifugation at 100.000g) was added. After 2 days of incubation, culture medium of MSCs previously treated or not with inflammatory cytokines was collected and underwent different steps of centrifugation, as previously described by other groups [80, 103]. Briefly, culture medium were centrifuged 10 minutes at 300g, 30 minutes at 4°C at 2000g to remove cell debris and ABs, and then centrifuged for 90 minutes at 4°C at 100.000g to collect EVs. The pellet were washed with PBS and underwent another step of ultracentrifugation for 90 minutes at 4°C at 100.000g to concentrate and purify EVs, which were then resuspended in PBS for immunological assays or stored at -80°C.

III.3. Characterization and quantification of EVs

The instrument calibration to detect EVs was performed by comparing them with different fluorescent latex beads of different size, 0.1μm, 0.2μm, 0.5μm and 1.0μm (Life Technologies) by flow cytometry on BD FACSCanto II. EVs were quantified by TruCount Tubes (BD Biosciences) to obtain absolute counts. Each tube contained a lyophilized pellet that once resuspended released a known number of 4.3 μm beads. The tubes were used according to manufacturer's recommendations and the absolute number was calculated by using the following formula: (number of events in the EV-containing gate/number of events in the bead-containing gate) × (number of beads per test/volume). To eliminate noise events, 0.22 μm-filtered PBS was analyzed under identical conditions and the number of events was subtracted from each analysis. The protein concentration of EVs was determined by Quantum Micro Protein method (EuroClone).

Immunophenotypic analysis: EVs were adsorbed to 3.9 μm latex beads (Life Technologies). Briefly, 5 μg of resting or primed EVs were mixed with 10 μl of latex beads for 15 minutes at room temperature. Then, 1 ml of PBS was added to each sample and incubated in a rotating wheel overnight. Next, 110 μl of glycine 1 M was added to the sample and mixed on the bench at room temperature for 30 minutes. Bead-bound EVs were centrifuged for 3 minutes at 4000 rpm, pellets were washed in PBS/0.5% BSA (bovine serum albumin) for three times and resuspended in 0.5 ml of PBS/0.5% BSA. Finally, 10 μl of bead-bound EVs were stained with specific antibodies for 30 minutes at 4 °C. For the staining, the following monoclonal antibodies against human markers were used: IgG1k-PE, CD73-PE, CD90-PE, CD105-PE, CD54-PE (ICAM-1), CD106-PE (VCAM-1), HLA-ABC-PE, HLA-DR-PE and CD63-PE all from BD Biosciences, IgG2b-PE and CD274-PE (programmed death-ligand 1 or PD-L1) from Biolegend. All tubes were washed and resuspended in 200 μl of PBS/BSA 0.5%. Data analysis was conducted using FlowJo software (TreeStar).

Western Blot: MSCs and MSC-EVs were solubilized in RIPA buffer (50mM Tris HCl pH 7.2, 1% v/v Sodium Deoxycholate, 1% v/v Triton X-100, 3% v/v SDS, 150mM NaCl, 1mM EDTA) with SIGMAFAST^{TM} (Sigma Aldrich) and Sodium orthovanadate (Sigma Aldrich). Successively, 35μg of MSC and MSC-EV proteins were subjected to 10% gradient SDS-PAGE and then blotted onto nitrocellulose membrane filters (GE Healthcare). The correct transfer was confirmed by staining of the membrane with Ponceau Red. The membrane were blocked with 5% not fat milk in TBST (20mM Tris-HCl pH 7.5, 150mM NaCl and 0.1% Tween), except for anti-HSP70, which was blocked with 2% BSA in TBST. Then, the blots were incubated at 4°C overnight with the following primary antibodies: rabbit polyclonal anti-Alix (Novusbio), rabbit monoclonal anti-CD9 (Novusbio), rabbit monoclonal anti-HSP70 (Santa Cruz), rabbit monoclonal anti-LAMP1 (Cell Signaling), rabbit polyclonal anti-GRP78 (Abcam), rabbit polyclonal anti-Giantin (Abcam). Next, the blots were washed with TBST and incubated for 1 hour at room

temperature with the peroxidase-conjugated secondary antibodies (Cell Signaling). After washing with TBST, the proteins were detected by ECL (Euroclone) and analyzed by LAS 4000 Image analyzer (GE Healthcare).

III.4. Immunological assays

To assess the BM-MSC-derived EV immunomodulatory capabilities on B cells, standardized protocol was carried out as previously described by our group [33]. In each well, resting and primed-MSCs were cultured with B cells at $2x10^4$ cell concentration (corresponding to a confluent monolayer) in 96 well plates. After MSC adhesion, $2x10^4$ B cells previously stained with 5μM carboxyfluorescein succinimidyl ester (CFSE) or Violet Cell Trace from Life Technologies were added.

PBMCs were stimulated with 5 μg/ml phytohemagglutinin (PHA) for 4 days in Roswell Park Memorial Institute (RPMI) supplemented with 10% heat-inactivated foetal bovine serum (FBS). B cells were activated with 5 μg/mL F(ab')2 anti-human IgM/IgA/IgG (Jackson Immunoresearch), 50 IU/mL rhIL-2 (Proleukin; Novartis), 50 ng/mL polyhistidine-tagged CD40 ligand, 5 μg/mL anti-polyhistidine antibody (R&D Systems), and 0.5 μg/mL CpG ODNs (Invitrogen) for 4 days in RPMI supplemented with 10% FBS. In all the experiments, cells were harvested at the end of co-culture and stained with PerCP-Vio700 or Vioblue mouse anti-human CD45 monoclonal antibody and TOPRO-3 Iodide (Life Technologies); the proliferation was assessed on viable TOPRO-3neg CD45pos cells by FlowJo software (TreeStar) as the percentage of cells undergoing at least one cell division. The proliferation rate was obtained according to the following formula: (percentage of $CD45^{pos}$ cell proliferation with MSCs)/(percentage of $CD45^{pos}$ cell proliferation without MSCs) × 100.

To test whether paracrine factors were involved in immunomodulatory mechanism, Transwell®-24 system with a 0.4μm pore size (BD Biosciences) was utilized keeping the same BM-MSC:B cell ratio.

In all the experiments, at the end of co-culture, cells were harvested and stained with PerCP-Vio700 or Vioblue mouse anti-human CD45 monoclonal antibody and TOPRO-3 Iodide (Life Technologies); proliferation was measured on viable TOPRO-3^{neg} CD45pos cells by FlowJo software (TreeStar) as the percentage of cells undergoing at least one cell division. The proliferation rate was obtained according to the following formula: (percentage of $CD45^{pos}$ cell proliferation with MSCs)/(percentage of $CD45^{pos}$ cell proliferation without MSCs)x100.

III.5. EV-uptake assay and immunofluorescence

To assess EV internalization by PBMCs, MSC membranes were stained with 2×10^{-6} M of PKH26 Red Fluorescent dye (Sigma-Aldrich) according to manufacturer's recommendations. Then, PKH26-labeled or -unlabeled MSCs were cultured in presence of IECs and EV uptake was assessed after 1, 2 or 4 days. At the end of co-culture, cells were detached by trypsin and stained with the following monoclonal antibodies: CD45-Vioblue (Miltenyi Biotec), CD3-V500 (BD Biosciences), CD14-FITC (Miltenyi Biotec), CD16-PercP-Cy5, CD19-PE-Cy7 (BD Biosciences) to identify the different IEC population, while TOPRO-3 was used to identify viable cells. The internalization of MSC-derived EVs by IECs was analyzed by FACS analysis. To further confirm the transfer of EVs into IECs, cells were analyzed at the end of co-culture by confocal microscopy. Briefly, cells were detached by trypsin and stained with Viobright-FITC anti-human CD45 monoclonal antibody (Miltenyi Biotec). Then, cells were fixed using Cytofix/Cytoperm kit (BD Biosciences) and TOPRO-3 (Invitrogen Life Technologies) was used to reveal nuclei. Finally, cells were loaded into the CytoSpin centrifuge's sample chamber and centrifuged 5 minutes at 400 rpm.
Images were obtained by LSM 710 confocal microscopy (Zeiss) at 63x magnification and elaborated with ZEN imaging software (Zeiss).

III.6. Statistical analysis

Statistical analysis was performed by Prism software (GraphPad) using the Wilcoxon test to compare differences between EVs from resting and primed-MSCs, while one-way ANOVA test was used to assess the differences in immunomodulatory effects. Data were expressed as mean ± SEM. $P<0.05$ was considered statistically significant.

IV. RESULTS

Different uptake of MSC-derived EVs by IECs. To assess whether the communication between MSCs and IECs could be driven by the exchange of EVs, MSCs, labeled or not with PKH26, were co-cultured with unlabelled IECs (Fig. 7).

When $PKH26^{pos}$ MSCs, either resting or inflammatory-primed, were co-cultured with unfractionated PBMCs, EVs were mostly internalized by monocytes and scarcely by lymphocytes after 24 hours up to day 4 (Fig. 7); in fact, at the end of co-culture the percentage of $PKH26^{pos}$ monocytes was 71.29 ± 5.042% in presence of resting $PKH26^{pos}$ MSCs and 60.93 ± 5.668% in the presence of inflammatory-primed $PKH26^{pos}$ MSCs. Among lymphocyte subsets, $CD19^{pos}$ B cells displayed the highest EV uptake (5.442 ± 1.243%) as compared to $CD56^{pos}$ NK cells (1.35 ± 0.46%) and $CD3^{pos}$ T cells (0.702 ± 0.30%) in presence of resting MSCs. EV internalization by unselected lymphocytes increased following inflammatory priming (8.147 ± 0.726% for B cells, 2.90 ± 0.38% for NK cells and 1.65 ± 0.27% for T cells).

Purified B cells maintained the degree of EV uptake, but showed only a slight increase of EV uptake by both resting (1.50-fold) and primed (0.10-fold) $PKH26^{pos}$ MSCs as compared to $PKH26^{pos}$/ $CD19^{pos}$ B cells from PBMCs in the same co-culture conditions (Fig. 8). Confocal microscopy confirmed the internalization of MSC-derived EVs, thus excluding their non-specific adhesion to cell membrane (Fig. 9).

We then assessed whether pre-activated B cells maintained the same capability of internalizing EVs (Fig. 10). Again, the levels in EV-uptake were maintained in purified B cells. Intriguingly, when resting and primed $PKH26^{pos}$ MSCs were co-cultured with activated CFSE-labelled B cells, we observed a significant distribution of $PKH26^{pos}$ EVs inside the early cell progeny, thus suggesting that EVs could affect B cell proliferation (Fig. 11).

When resting $PKH26^{pos}$ MSCs were co-cultured with B cells (control condition), the latter were not inhibited and $PKH26^{pos}$ B cells were equally

distributed in each cell generation. Conversely, B cells co-cultured with primed MSCs were significantly inhibited and became strongly positive for $PKH26^{pos}$ EVs.

Overall, our data show that the uptake of MSC-derived EVs occurs in both resting and, mostly, in activated IECs, thus highlighting a possible role for EVs in immunosuppression.

MSC-mediated immunomodulation is driven by paracrine factors. We assessed whether the immunomodulatory properties of MSCs in close contact with B cells were comparable to the effects exerted by their paracrine signals. To this aim, resting or primed MSCs were cultured in presence of B cells both in standard conditions and in Transwell® conditions, thus preventing cell-to-cell contact but not the exchange of soluble molecules (Fig. 12).

In both co-culture systems, resting MSCs exerted a suppressive effect on B cells. Accordingly, B cell division was not inhibited by resting-MSCs in both co-culture settings, due to their inability to induce MSC licensing [22]. However, following pre-treatment with IFN-γ and TNF-α, MSCs acquired a significant immunosuppressive effect, B cell proliferation by more than 80% in both co-culture approaches (Fig. 12).

These results are in agreement with the well-known concept that the immunosuppressive features of human MSCs are mostly cell-to-cell contact-independent [34], thus suggesting a possible role for EVs in intercellular signaling through active molecule delivery.

Immunosuppressive effect of EVs on B cell proliferation. $PKH26^{pos}$ EVs were cultured in presence of PBMCs to verify the ability of IECs to internalize purified EVs. We observed the highest EV internalization by B cells subpopulations (Fig. 7). Thereafter, to validate the immunomodulatory effects of MSC-derived EVs on B cell proliferation, purified EVs (obtained from resting or primed MSCs) were added to B cell cultures, 3×10^6 particles/1×10^4 B cell (Fig. 14). When assessing EV-dependent immunomodulation on PHA-

stimulated PBMCs, no inhibitory effect on $CD45^{pos}$ cell proliferation was observed (Fig. 13).

Then, we tested the immunosuppressive effect of EVs on purified B cells on the basis of the evidence that sorted B cells showed a higher EV uptake as compared to T and NK cells (Fig. 7). Resting EVs displayed a significant suppressive effect on B cell proliferation that became more evident following inflammatory priming (Fig. 14).

Characterization of MSC-derived EVs. Next, we performed a qualitative and quantitative analysis of MSC-derived EVs. Biochemical analysis revealed the presence of specific exosome markers, such as CD9, Alix, LAMP1 and HSP70, both in resting and primed EVs. Moreover, to exclude the presence of potential contaminants, we verified the absence of Giantin and GRP78, which are specific molecules of the Golgi apparatus and the endoplasmic reticulum, respectively (Fig. 15B). Furthermore, immunophenotyping revealed the expression of CD63, an exosome marker, both in resting and primed-EVs (Fig. 15A). As for mesenchymal molecules, EVs expressed CD90 and CD105, but not CD73; the expression of CD54 (ICAM-1), which is an adhesion molecule involved in the tethering of EVs to target cells [46], was weak in EVs from resting MSCs, but increased following inflammatory priming; by contrast, CD106 (VCAM-1) was undetectable in resting EVs and weakly expressed in primed EVs at the levels observed in MSCs [29]. Differently from MSCs, resting and primed EVs never expressed MHC class I (HLA-ABC) and II (HLA-DR).

As the quantity of released EVs depends on the initial MSC number, EVs were quantified as EV:MSC ratio to compare EVs obtained from resting and primed MSCs (data not shown). The number of EVs secreted by resting MSCs was significantly higher than that from primed MSCs (n = 35), without significant differences among different cell passages and donors.

V. FIGURES

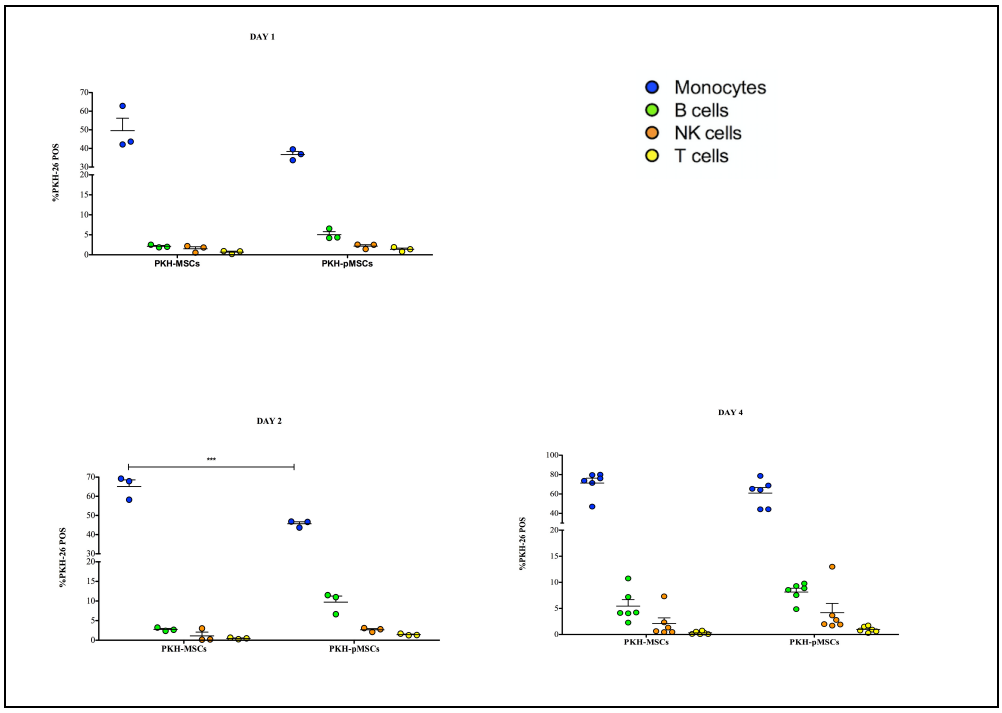

Fig. 7: Internalization of MSC-EVs by IECs

Resting and primed PKH26-MSCs were cultured in presence of unstimulated PBMCs in order to assess the transfer of MSC-EVs to IECs. After 4 days, the cells were harvested and labeled with anti-CD45, anti-CD3, anti-CD14, anti-CD16, anti-CD19 to identify the different IEC lineage inside unfractionated PBMCs (A); anti-CD45 was used for sorted-IECs (B). TOPRO-3 was added to detect viable cells. The EV-uptake by IECs was detected as percentage of lineage specific^{pos}/PKH26^{pos} IECs by FACS. Error bars represented mean ± SEM of three/six independent experiments. ***P< 0.001.

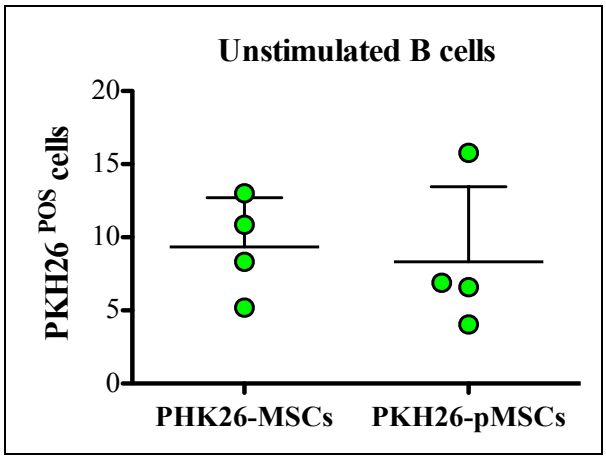

Fig. 8: Internalization of MSC-EVs by unstimulated B cells

Resting and primed PKH26-MSCs were cultured in presence of sorted unstimulated-B cells, which were labeled with CD45 and TOPRO-3 at the end of culture. The EV-uptake by B cells was analized after 4 days of co-cultures

and detected as percentage of lineage specific^{pos}/PKH26^{pos} B cells by FACS. Error bars represented mean ± SEM of four independent experiments.

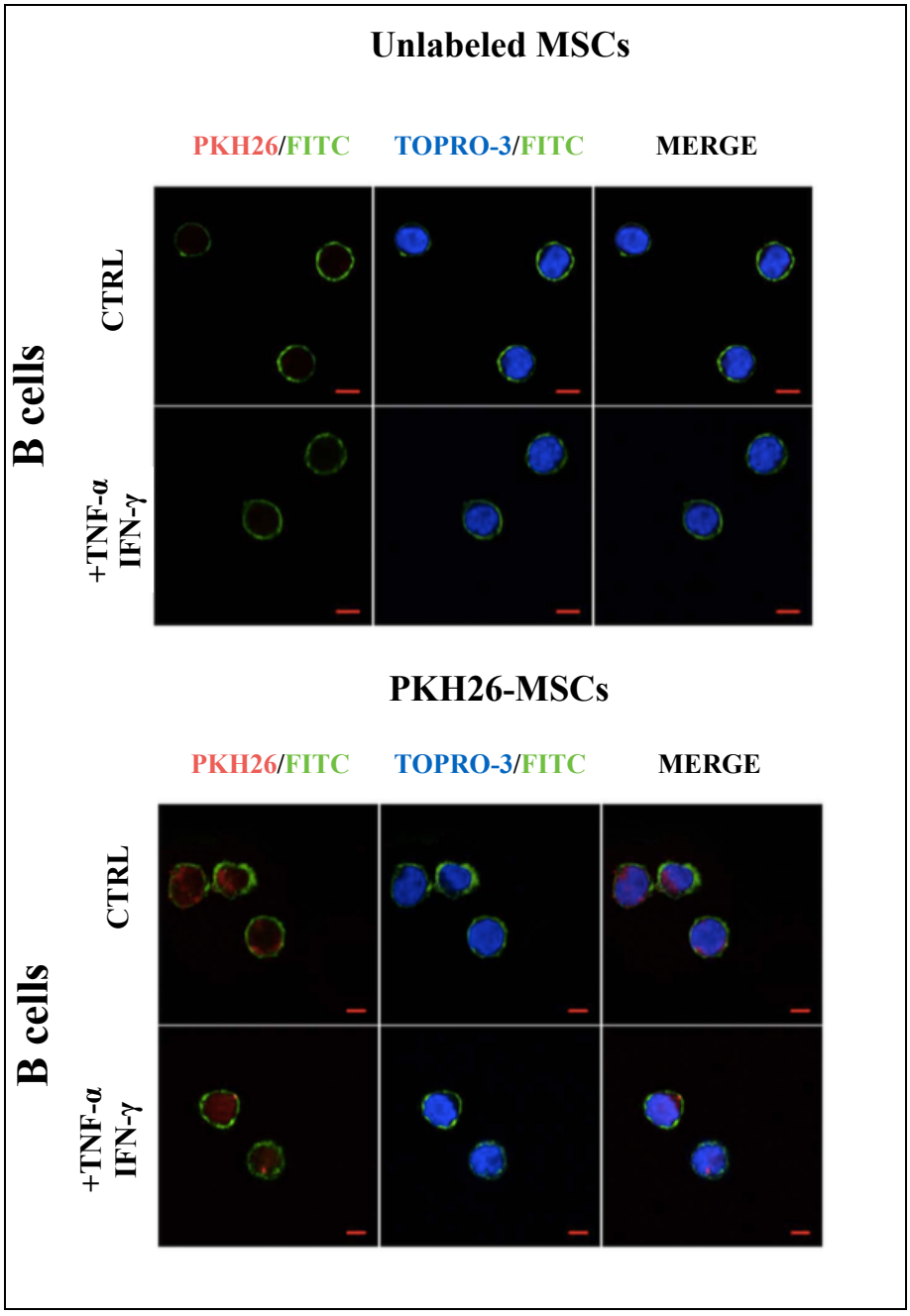

Fig. 9: Representative immunofluorescence staining of CD45pos/PKH26pos B cells

Resting and primed PKH26-MSCs and unlabeled-MSCs were cultured in presence of sorted unstimulated-B cells. At the end of co-cultures, cells were detached and labeled with anti-CD45 (green) and TOPRO-3 (blue nuclei) to assess the incorporation of PKH26-EVs (red). Scale bars: 5µm.

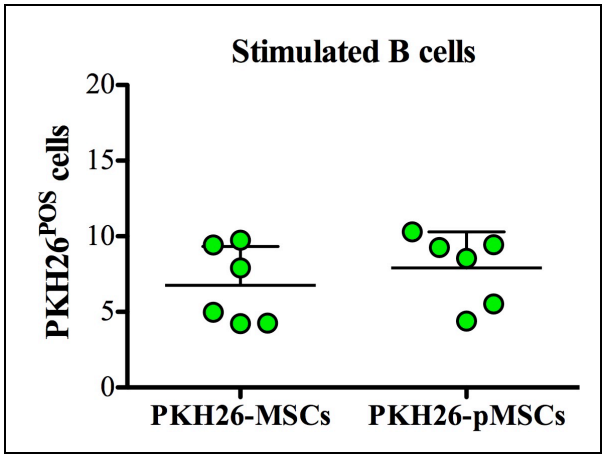

Fig. 10: Internalization of MSC-EVs by stimulated B cells

Resting and primed PKH26-MSCs were cultured in presence of sorted stimulated-B cells, which were labelled with CFSE before of co-culture. At the end of co-culture, B cells were labeled with CD45 and TOPRO-3. The EV-uptake by B cells was analized after 4 days of co-cultures and detected as percentage of lineage specific^{pos}/PKH26^{pos} B cells by FACS. Error bars represented mean ± SEM of six independent experiments.

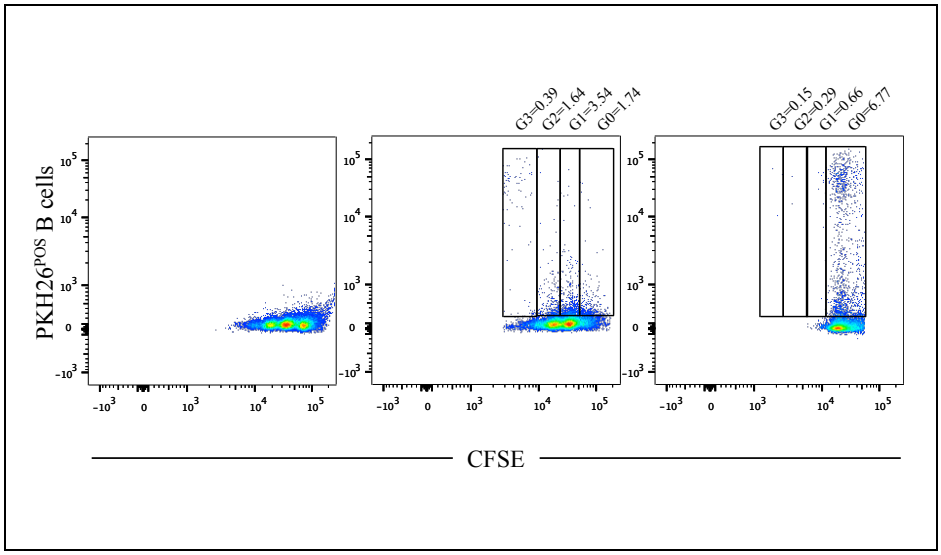

Fig. 11: Representative CFSE plot

Resting and primed PKH26-MSCs were cultured in presence of sorted stimulated-B cells, which were labeled with CFSE before of co-culture. EV-uptake by B cells was analized after 4 days of co-culture and showed the localization of EVs inside B cell generations as percentage of CFSE^{pos}/PKH26^{pos} B cells.

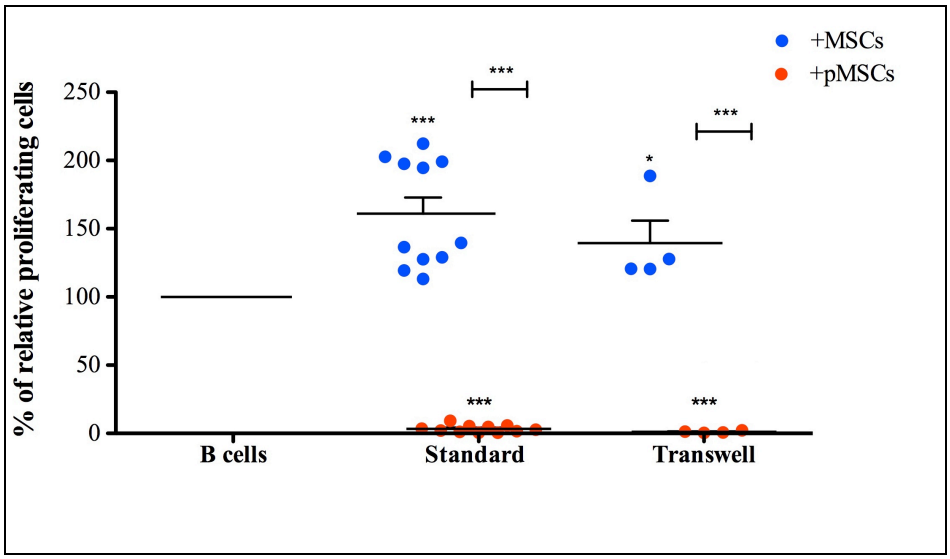

Fig. 12: Effect of resting and primed MSCs in standard or Transwell® condition

Transwell® system with MSCs in the bottom well and IECs in the top well.0.4µm-porous-membrane was used to prevent the cell-cell interaction and allow the exchange of soluble molecules. Ratio-dependent MSCs immunomodulatory effect on sorted-B cells. Sorted-B cells were cultured in presence of indicated amount of MSCs. CFSE fluorescence was analyzed after 4 days of co-culture. The results are expressed as relative proliferation percentage of B cells, normalized to B cells cultured alone (100%). *P< 0.05, ***P< 0.001.

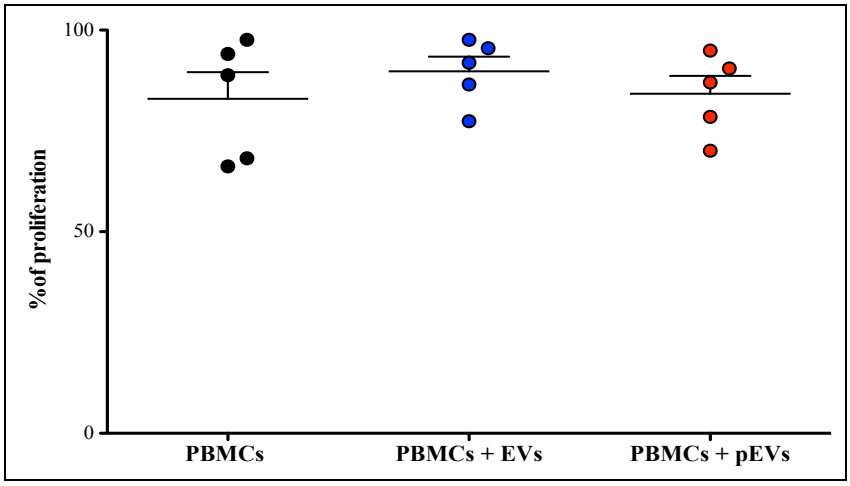

Fig. 13: Effect of resting and primed EVs on PBMC proliferation

EVs were added to stimulated and CFSE-labeled PBMCs ($1x10^4$:$3x10^6$ PBMC:EV ratio) and after 4 days the cells were harvested and analyzed by FACS analysis. Error bars represented mean ± SEM of five independent experiments.

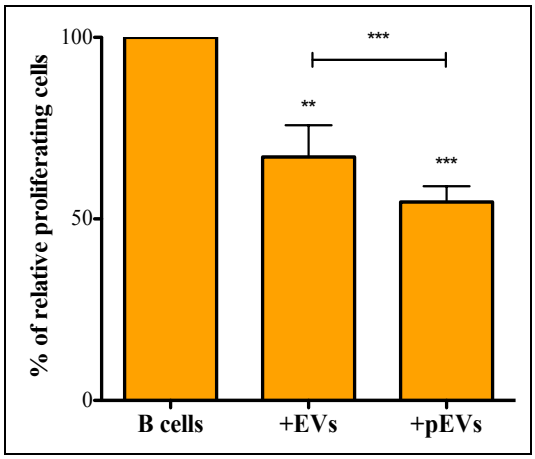

Fig. 14: Immunosuppressive effect of MSC-derived EVs on B cell proliferation

EVs were purified from resting and primed MSCs and added to sorted and CFSE-labeled B cells ($1x10^4$:$3x10^6$ B cell:EV ratio) that were activated by specific stimuli. After 4 days of co-cultures, the cells were harvested and B cell proliferation was assessed by FACS analysis. The results are expressed as relative proliferation percentage of B cells, normalized to B cells alone (100%). Error bars represented mean ± SEM of four independent experiments. **P< 0.01, ***P< 0.001.

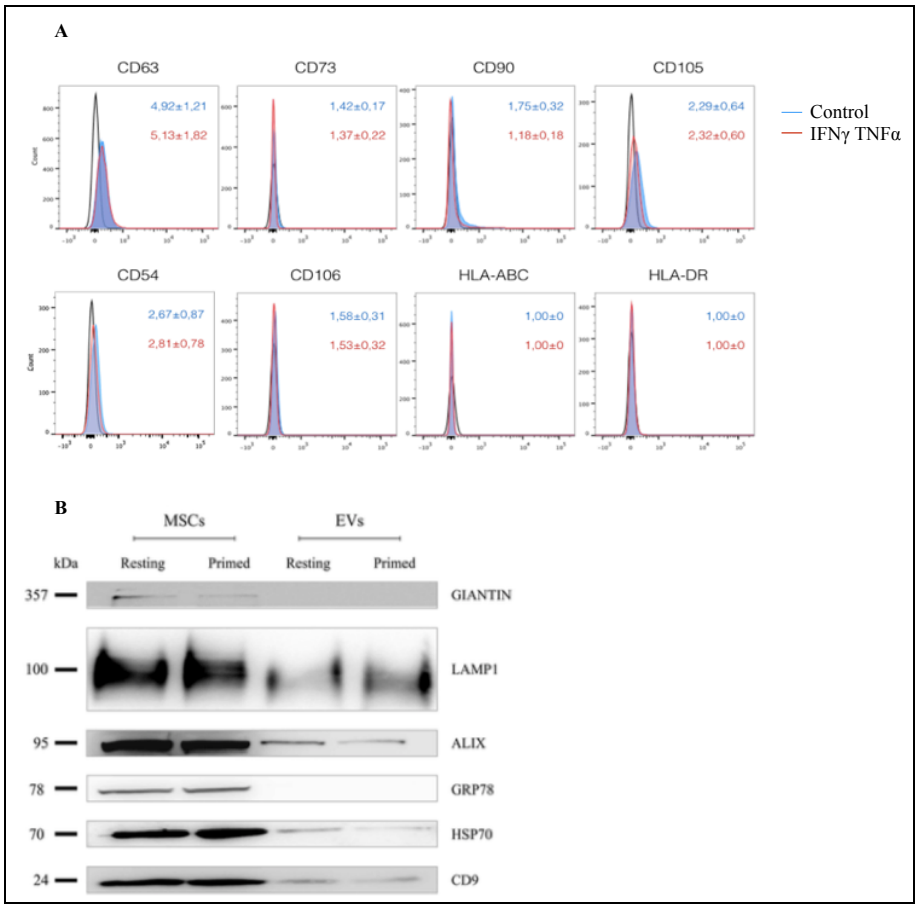

Fig. 15: Characterization of MSC-Evs

(A) **Representative plots** of immunophenotypic analysis of MSC-EVs showing the expression profile of a specific exosome marker (CD63), mesenchymal stromal cell markers (CD73, CD90 and CD105), adhesion molecules (ICAM-1 and VCAM-1) and MHC class I and II (HLA-ABC and HLA-DR, respectively). The histograms display the isotipic controls (black curve) and specific markers of resting (blue curve) and primed (red curve) EVs.

(B) **Immunoblot analysis** of CD9, Alix, HSP70, Giantin, and GRP78 expression in resting and primed MSCs and their purified EVs. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. This blot is representative of three independent experiments showing the same trends.

VI. DISCUSSION AND CONCLUSIONS

EVs are shuttles of bioactive molecules playing a role as information transporters through their internalization by target cells, thus eliciting different responses. EV effects are not limited to adjacent cells, but EVs may exert their activity in distant tissues; in fact, EVs may transfer receptors or proteins from the cell of origin to the target cells. EVs may convey genetic information by horizontal transfer of mRNA and microRNA (miRNA) inducing functional changes in the target cells. Most cell types produce EVs capable of transmitting multiple immunological signals. EVs may trigger the immune system depending on their molecular composition and cells of origin, not only by carrying peptide-loaded MHC molecules or tumor and pathogen antigens to antigen presenting cells [104, 105], but also by dampening inflammatory response through the exposition of pro-apoptotic molecules, such as CD95L, TRAIL or Galectin-9 [106, 107].

Different studies have shown the beneficial effects and the therapeutic potential of EVs produced by MSCs in various experimental models of degenerative and inflammatory diseases. Once injected into model systems, EVs are able to reach the site of inflammation, where they can improve the tissue regeneration by stimulating resident stem cells, and at the same time modulate the inflammatory response [108, 109]. As far as clinical application for cellular therapy is concerned, EVs have some advantages as compared to whole cell suspensions, due to the lack of risk of generating tumors, and could replace MSCs in immunoregulatory cell therapy for inflammatory and autoimmune diseases, such as GvHD, ARDS (Acute Respiratory Distress Syndrome), multiple sclerosis and lung injury [100, 101]. However, the mechanisms underlying these processes are topics being studied. In addition, many reports have shown variable effects of EVs on activated T and B cells [95, 110, 111], but the results of those studies are not easily comparable due to the different methodological approaches, related to both the protocols of purification and quantification of EVs and the experimental read-out to assess

their immunomodulatory effects on IECs. Immunological assays are essential to identify and measure the influence of cells or their products; however, there are several critical variables that can lead to different results, such as the use of purified IECs rather than unfractionated PBMCs, cell ratios in co-culture systems, type and duration of cell stimulation, cell viability and proliferation rate assessment [112]. For this reason, we carried out the characterization of EVs from BM-MSCs, in terms of protein content and immunomodulatory functions; by using purified IEC-based immunological assays, previously validated in cooperative studies [30, 33], we showed that the ability of MSCs to regulate IEC proliferation is almost fully driven by paracrine mechanisms, as MSCs and IECs can communicate through the exchange of EVs that possess a comparable immunosuppressive effect to their cells of origin. The degree of EV-mediated immunomodulation seemed to be proportional to the ability of B cells to take up EVs. Our data have shown that there is active communication between MSCs and immune cells mediated by EV exchange. Despite the high EV uptake by PBMCs after 4 days of co-culture, EV-mediated inhibition on proliferation induced by mitogen stimuli was not observed. This result may have at least two explanations. During the early phases of co-culture, most of MSC-derived EVs are internalized by monocytes, as they act as antigen-presenting cells. MSC effect on monocyte polarization towards anti-inflammatory macrophages (M2) has already been previously demonstrated by different research groups [113], but to this aim co-culture time needs to be longer. Another aspect is that the inhibitory effect was evaluated in PBMCs, in which the proliferative response induced by mitogenic stimuli was mainly mediated by T cells. Notably, by using unfractionated PBMCs EVs released by MSCs were almost entirely incorporated by monocytes, thus lowering the effect on lymphocyte populations; conversely, purified IECs exhibited a higher degree of EV uptake. However, T cells were less prone to internalize EVs as compared to B cells: in fact, a significant suppression of B-proliferation was observed in control conditions and significantly increased after TNF-α and IFN-γ treatment. These results confirm that the immunosuppressive effect is

mainly mediated by EVs, as previously demonstrated in Transwell® co-culture assays. In standard co-culture assays, in control conditions, a trophic effect on B cell-proliferation prevailed, clearly mediated by cell contact.

The quantification of EVs used for the immunological assays was fundamental; the determination of the absolute counts through FACSCanto and TruCount beads resulted in a reliable and accurate method not only to develop standardized immunological methods, but also in view of future clinical application. Inflammatory cytokines dramatically influence MSC immunosuppression by modifying their secretoma and interactions with IECs both in vitro and in vivo, thus affecting the clinical effects of MSCs through a functional shift from supportive to inhibitory behaviour [34]. Some authors previously reported that IFN-γ-treated MSCs display different protein content inside EVs compared to resting MSCs, and this phenomenon has an impact on their protective potential against ischemic acute kidney injury [114]. However, the effect of inflammatory priming on EV-mediated immunosuppression has been never reported so far. Here, we have better highlighted the role of IFN-γ and TNF-α on the release and effect of MSC-derived EVs. MSCs displayed a lower rate of EV release following inflammatory priming (data not shown); nevertheless, primed EVs were more internalized by IECs, probably because of their higher expression of ICAM-1, a molecule involved in the tethering between EVs and target cell [46].

Although EVs clearly lack the potential to directly form tumors following *in vivo* administration, this does not imply that MSC-EV administration to human subjects is without any risk of promoting neoplasia. For instance, multiple myeloma (MM) cell proliferation has been shown to be enhanced in presence of either autocrine or paracrine secretory factors released by BM-MSCs. For example, *Roccaro et al.* isolated EVs from BM-MSCs derived from both MM patients and healthy controls. In this study, MM BM-MSC-derived EVs promoted MM tumor/cell growth as compared to normal BM-MSC-derived EVs. MM BM-MSC-derived EVs may also induce cell dissemination and metastasis to distant BM niches [49]. *Lee et al.* reported contradictory results suggesting that MSC-EVs suppress angiogenesis *in vitro*

by down-regulating the mRNA and protein levels of VEGF in tumor cells in a concentration-dependent manner, but this inconsistency is due to tumor type or MSC heterogeneity [115].

The *in vitro* immunological features of MSC-derived EVs here described are consistent with their beneficial effects observed in vivo in different inflammatory diseases [101, 108, 114], including refractory GvHD [100]. Our group contributed recently to show that the clinical improvement following MSC administration in two patients with ARDS was mediated by the secretion of EVs containing several proteins known to be involved in the therapeutic effect in other disease models [116]. Here, we propose quantitative and reproducible immunological assays that can be useful to measure the potential beneficial effect of EV administration. These techniques can be applied to MSCs derived from different tissues to identify the most effective MSC sources for the development of novel therapeutic strategies and to release clinical-grade EVs.

VII. REFERENCES

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