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Extracellular vesicles in anti-tumor immunity

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Abstract

To what extent extracellular vesicles (EVs) can impact anti-tumor immune responses has only started to get unraveled. Their nanometer dimensions, their growing number of subtypes together with the difficulties in defining their origin hampers their investigation. The existence of tumor cell lines facilitated advance in cancer EV understanding, while capturing information about phenotypes and functions of immune cell EVs in this context is more complex. The advent of immunotherapy with immune checkpoint inhibitors has further deepened the need to dissect the impact of EVs during immune activation and response, not least to contribute unraveling and preventing the generation of resistance occurring in the majority of patients. Here we discuss the factors that influence/drive the immune response in cancer patients in the context of cancer therapeutics and the roles or possible functions EVs can have in this scenario. With immune cell-derived EVs as leitmotiv we will journey from EV discovery and subtypes through their physiological and pathological non-cancer functions to their similarities with cancer EVs and on how to revert their detrimental consequences on immune responses to cancer.

Keywords: Extracellular vesicles; exosomes; anti-tumor immunity; biomarker

1. Introduction

Extracellular vesicles (EVs) are key players in intercellular crosstalk. Released by every type of cell, these nanometer-sized vesicles can travel long distance or deliver their messages in the neighborhood. Their phenotype and function depend on the status of their cell of origin, thereby determining their purpose. Ten years ago, with the general consensus of the dedicated scientific community, all the different vesicle subtypes have been joined together under the name of 'extracellular vesicles'. This decision based mainly on the impossibility of allocating markers or other characteristics like size to specific vesicle types. In fact, hallmarks defining exosomes, deriving from endosomal compartments, could be detected also on EVs deriving from the cell membrane. Analogous overlaps were found upon comparing the sizes: despite exosomes are defined as 30-150 nm EVs, they cannot be definitely distinguished from membrane EVs defined as 100-1000 nm and not even from apoptotic bodies, sized 50-5000 nm. Due to this complexity the International Society for Extracellular Vesicles (ISEV) uniting and representing the majority of EV investigators decided to divide EVs into small, i.e. sEVs < 200 nm, and large, i.e. lEVs > 200 nm, EVs. The discovery of EVs deriving from additional cellular compartments such as mitovesicles from mitochondria or oversize EVs like oncosomes, produced only by tumor cells, further highlights the complexity of vesicle subtypes. The rapid development of dedicated technologies

enabled dissection of EV composing proteins, nucleic acids and lipids in vesicle families and generated knowledge of unexpected functions. In the context of immune responses, EVs can impact them in multiple ways functioning as extended contactless arms of the releasing immune cells. In fact, based on their composition they may not only reflect but also affect the actual immune status of the host. Pioneering studies investigating the cancer EV-immune cell outcome evidenced how detrimental these small vesicles could be, leading to the discovery of a continuously growing number of EV-mediated mechanisms involved in tumor promotion and immune evasion prevailing over immune activation and tumor attack. Nonetheless, studies downsizing their impact as bystander effect with little to none or even positive consequences have also emerged, indicating that effects may not only depend on the EV or target cell surface composition but also on the condition of the surrounding milieu and on the fate of the EVs upon interaction with the receiving cells. EVs may in fact act with or without being internalized once docked on the plasma membrane, depending on the type of cell or its status and environment during the interaction. Macrophages for instance appear to uptake EVs in most cases, while T cell-EV interactions appear to be predominantly of the surface-surface type, but also T cells can internalize EVs upon activation. Of note, the 'content' of EVs can also function at the intracellular level, further emphasizing the complexity of this fascinating scientific field. Deciphering their composition and functions will contribute enabling their control and in a visionary future induced modulations of EV composition might help exploiting them as anti-tumor allies in cancer therapy.

2. Discovery, biogenesis and composition of EV subtypes

The generation and release of nanometer-sized vesicles was first noticed in platelets in the 1940s [1], while the so-called 'exosomes', small 30-150 nm sized EVs, were observed as released during maturation of reticulocytes into erythrocytes to dispose of obsolete proteins, such as the transferrin receptor (TfR, CD71) [2-4]. In the coming years the intensive investigation of EV biogenesis gave rise to the discovery of diverse vesicle subtypes and compositions [5]. Viable cells release EVs via different molecular pathways, involving intracellular compartments and plasma membrane shedding. A set of conserved proteins acting in lysosomal and exosomal trafficking, the endosomal sorting complex for transport (ESCRT), regulates ESCRT-dependent biogenesis and release, while in ESCRT-independent pathways EVs are produced via alternative mechanisms involving lipid raft segregation in a ceramide and sphingomyelinase dependent manner. Intraluminal vesicles (ILVs) originating from early endosomes accumulate inside the late endosome

part of multivesicular bodies (MVBs), migrate along the microtubules by means of a dynamic process regulated by cholesterol to reach and fuse with the plasma membrane releasing their content in EVs, the formerly called 'exosomes'. ILV biogenesis involves the ESCRT action as protein sorting machinery of the MVBs and the interaction of lipid molecules [6]. During this process vesicles from other cellular compartments like the trans-Golgi network (TGN) can fuse with endosomes ongoing maturation. However, not all vesicles produced by this machinery reach the extracellular milieu. Endosomes and MVBs can be subject to back-fusion, disintegration or degradation by fusion with lysosomes/autophagosomes [7]. MVBs formed in the absence of ESCRT complex result in EV enrichment of cholesterol, ceramide and other sphingolipids [8]. ESCRT-independent biogenesis of ILVs requires the clustering of cholesterol and sphingomyelin into lipid rafts, its cleavage resulting in ceramide accumulation in microdomains of the endosomal membrane resulting in inward budding and ILV formation. Specific lipids and lipid-modifying enzymes such as neutral sphingomyelinase 2 [8] and phospholipase D2 [9] participate in the budding of ILVs and impact exosome production. Alix associates to ESCRT complex and is recruited on the endosomal membrane by binding BMP lipid participating to the sorting of the EV cargo content [10]. The docking of MVB to the plasma membrane followed by fusion and release of EVs into the extracellular milieu is regulated by Rab27a and Rab27b, as elegantly shown by the group of Clotilde Thery. By RNA interference they demonstrated two different roles for the isoforms: inhibition of Rab27a led to massive increase of MVB, while silencing of Rab27b induced the redistribution of MVBs towards perinuclear regions, indicating that both isolforms are necessary for exosome, sEV, secretion [11]. In contrast, membrane-derived EVs, the former microvesicles, microparticles or ectosomes and the oversized oncosomes, originating from highly migratory amoeboid tumor cells, are generated from the plasma membrane by outward budding and fission [12,13]. Their budding is promoted by the translocation of acid sphingomyelinase on the outer leaflet of the plasma membrane. Membrane-derived EV biogenesis is also activated after modification of the plasma membrane by aminophospholipid translocase or by membrane modifications caused by sphingomyelin and cholesterol binding proteins. EVs exposing phosphatidylserine (PS), after losing the plasma membrane phospholipid asymmetry can be recognized by their binding to annexin V [14]. General identification and "proof" of EV presence in a sample is determined by the detection of EV markers of which the most classical are the tetraspannins CD63, CD81 and CD9, abundantly expressed especially on the sEV subtypes exosomes and membrane-derived microvesicles, hsp70, Alix and TSG101, these latter both

belonging to the ESCRT [15,16]. A different origin possibly occurs for exomers, which are non-membranous nanoparticles [17] and for the recently described mitovesicles, both being small-sized EV populations with a yet undefined biogenesis. Mitovesicles are double-membrane EVs highly enriched in mitochondrial proteins that were isolated from brain tissues in Down syndrome [18]. They seem associated to mitochondrial dysfunction, a setting not only involved in neurologic disorders but largely associated with cancer, although their potential release by tumor cells remains to be explored. Exomers are nanoparticles lacking components of ESCRT complexes particularly enriched in enzymes and metabolic proteins involved in glycolysis and mTORC1 signaling, and containing nucleic acids and lipids [17,19]. Comprehensive proteomic and nucleic acid analysis showed substantial differences in these non-membranous nanoparticles. Exomers carry proteins associated to endoplasmic reticulum (ER), mitochondria and microtubules; they can carry galectin-3-binding protein (LGALS3BP), a modulator of cell communication and immune responses, and high levels of triglycerides and ceramides [17]. The characterization of EVs isolated by high-resolution density gradient fractionation and direct immunoaffinity capture demostrated that non-membranous EVs cargo dsDNA, miRNAs (miRs), Ago2 and HDL [20]. Lipids are abundant EV constituents that exert potent regulatory functions upon EV uptake by recipient cells. The lipid composition of EVs explored by MS spectrometry technology showed that the levels of lipid classes, encompassing cholesterol, sphingomyelin, phospholipids glycosphingolipid and gangliosides, differ consistently from those of their originating cells [21]. The comparative analysis of lipidomic profiles of plasma and plasma EVs isolated by PEG precipitation highlighted the enrichment of particular lipid classes including saturated fatty acids (FAs) in EVs [22]. The accumulation of disaturated phospholipids asscoaites with EV increased rigidity compared to parental cells, potentially aimed at protecting their functional cargo from degradation and for circulation in biological fluids with prolonged lifespan. The FA content of EVs can be released via phospholipases and is a source of eicosanoids, such as prostaglandins (PG) and leukotrienes with potent effects in immunity and cancer [23]. For example, EV carried PGE2 can induce the expansion of myeloid-derived suppressor cells (MDSCs) fostering tumor growth [24] and the recruitment of Th17 cells mediating tumorigenesis in the intestine [25]. Among others, examples of the regulatory effects of EV lipid components on immune responses include their impacting eosinophil activation [26] and the accumulation of cholesterol in cells of the atherosclerotic plaque, induced via endogenous PS receptor-mediated uptake of T cell EVs [27]. Infection by SARS-CoV-2 regulates plasma EV composition in terms of GM3 ganglioside, sphingomyelin and DAG

content and the ability of EVs to modulate inflammation and immune responses [28,29]. EVs produced by skin adipocytes can transfer FA and fatty acid oxidation (FAO) enzymes to melanoma providing energy and promoting tumor cell migration [17,30], the differentiation of macrophages [31] and the expression of immunomodulatory molecules [32]. The prevailing local and systemic coditions are involved in the biogenesis mechanisms of EVs. Further developments of selective isolation methods will further increase knowledge about EV populations [33].

3. Interaction of EVs with target cells

The selective incorporation of molecules determined by their cells of origin defines how and where EVs will exert their functions [34]. The binding to their target cells can be of different natures with consequently different outcomes. Thus, the dissection of these mechanisms will generate knowledge EV functional roles. In 2000 first evidence indicated that the EV targeting of cells was not casual. B cell exosomes almost exclusively bound to follicular dendritic cells (DCs) but not to lymphocytes, plasma cells, macrophages, other DCs, erythrocytes, mast cells and basophils present in the culture [35]. EVs can dock on the cell membrane via protein-protein interaction with potential evolution into internalization by fusion and/or endocytosis. The 'lipid interdigitation' occurring during fusion relies on fusogenic lipids abundantly present in EVs, and this internalization type can be fostered by tumor microenvironment (TME) characteristics, such as acidity [36]. Thus, microenvironmental conditions including pH might dictate the type and rate of EV internalization, an aspect that could have great impact on mounting effective anti-tumor immune responses [37]. Some unique and common features characterize the types of EV endocytosis, which include clathrin, caveolin- and lipid-raft dependent as well as independent endocytosis, macropinocytosis and phagocytosis [38]. Uptake via macropinocytosis is not selective, deriving from proximity of the EVs to the cells. However, some EVs can naturally induce macropinocytosis or promote it through manipulation of EV composition [39,40]. Phagocytosis is the common method shared by specialized immune phagocytes like the monocyte-macrophage-DC system, as the process depends on specific mechanisms and receptors characteristically expressed by this cell type, including Phosphatidylinositol-3-kinase (PI3K) and the actin cytoskeleton. From the EV side the exposure of PS induces their phagocytosis through binding to receptors of the T cell immunolgobulins (TIM) family [38], some of which are acknowledged targets of ICI immunotherapy. During phagocytosis the EVs enveloped by membrane deformations form phagosomes, which can be eventually directed to lysosomes [41,42]. Since the different

endocytosis types can co-exist, EVs are internalized via different simultaneously occurring mechanisms, potentially impacting the fate of the EV cargo and on the interaction outcome of the the cell. In clathrin-dependent endocytosis dynamin-2 forms a collar-like structure in the neck of the EV-containing invagination leading to its scission with the consequent transport of the EV content to the endosomal compartment. Cancer cells predominantly exploit clathrin-mediated EV uptake facilitated by the high expression of CD71, the transferrin receptor, indicating that the EV composition can influence its internalization [43]. Dynamin-2 also regulates caveolin-dependent uptake and caveolins and clathrins generate specific pits aimed at internalizing particles. Caveolae are smaller than clathrin pits and caveolin-mediated uptake is involved in the transport of cholesterol, albumin and intracellular signaling pathways and plays a role in EV internalization by B cells [44]. While the endocytosed material of caveolae is generally smaller than 60 nm, clathrin pits can endocytose up to 120 nm particles [45]. The preferential exploitation of a particular internalization mechanism might shed light on the cell targeting of EVs. Lipid raft uptake depends on raft forming sterol and sphingolipid portion of the plasma membrane [46]. The expression of proteins on the outer EV membrane determines receptor-mediated endocytosis (RME), starting with a surface-surface interaction through receptor-ligand binding followed by complete internalization. Main participating proteins include lectins, adhesion molecules, heparin sulfate proteoglycans (HSPG), TIMs and mucins. Lectins like CD33, CD169 and C-type lectins expressed by DCs, lymph node (LN) macrophages and antigen presenting cells (APC) internalize EVs from macrophage, B cell and mesenchymal stem cell (MSC) based on EV exposure of the respective interacting lectins [47-50]. Tumor or non-tumor EVs interacting with leukocytes are facilitated by adhesion molecules like CD44, CD11, CD54, CD49d, lactadherin (Milk fat globule-EGF factor 8 protein, MFGE8), the tetraspanins CD9 and CD81 [51], and integrins. Indeed, the expression of $\alpha_6\beta_4$ and $\alpha_v\beta_5$ integrins by breast and pancreatic cancer EVs determined their exclusive uptake by lung fibroblasts or liver macrophages, contributing to metastasis development [52]. Similarly, $\alpha_v\beta_6$ integrin expressed on prostate cancer EVs induce monocyte-M2 polarization, in line $\alpha_v\beta_6$ expressing monocytes in peripheral blood of prostate cancer patients but not in healthy donors [53]. Other molecules used to internalize immune EVs, potentially underlying a more regulated release, interaction and internalization than tumor EVs, include ICAM-1 and its ligand LFA-1. The activity of this system has been corroborated by different groups investigating the interaction of EVs from DCs or macrophages with DCs, T cells and brain endothelial cells [54,55]. Less is known about the roles of HSPG in this context. So far, evidence derives from tumor EVs since heparanase

and syntenin are upregulated in cancer histotypes and heparanase also regulates the EV generation [56]. Additionally, glioblastoma multiforme (GBM)-derived EVs can bind via fibronectin to HSPGs on the recipient cell's surface. After internalization, EVs that co-localize with HSPGs of the syndican and glypican type, lead to the activation of ERK1/2 [57,58]. Functional involvement of specific molecules could be demonstrated thanks to inhibitors such as Dynasore, a blocker of dynamin, mediating clathrin- and caveolin-dependent EV endocytosis [59]. Lectin- and adhesion molecule-mediated interactions generally induce signaling and inflammation, but also migration, as shown by immune, endothelial, stromal cells and leukocytes interaction with EVs deriving from platelets and reticulocytes, tumor cells and DCs [38]. In contrast, immune responses are evoked by the EV-immune cell interaction through immunoglobulin-adhesion molecule binding (IgG/ICAM-1), similarly to TIM family-PS, which regulate T, B cell, DC, mast and natural killer (NK) and endothelial cell functions, including immune responses, phagocytosis, antigen presentation and elimination of apoptotic cells [60-62]. Due to their heterogeneity and variable composition, the interaction and uptake of EVs is characterized by a complex mechanical behavior, whose dissection could fill some gaps between biological functions and physical properties of EVs [63]. Atomic force microscopy (ATM) revealed that different types of EVs from various origins and presenting different lipid and protein compositions share similar mechanical properties. Additionally, vesicle stiffness decreases upon increase in their protein/lipid ratio. Thus, depending on the condition of the EV producing cells, soft and protein-rich as well as stiff protein-poor EVs can be generated, impacting their uptake [64]. Both cancerous and non-cancerous cells preferentially internalize softer rather than less elastic EVs. Additionally, softer EVs may uniformly disperse in the cytoplasm, whereas EVs with reduced softness are internalized at a lower rate and accumulate in the perinuclear region, trapped in the endosomal/lysosomal compartmen as a result of endocytosis. This indicates that softer EVs might be uptaken via fusion, with a different outcome of the internalized molecules composing the EVs. *In vivo,* soft vesicles accumulate in tumors, potentially explaining at least in part the often-observed 'natural' tumor delivery of injected EVs [65,66]. Furthermore, malignant EVs displayed a reduced stiffness and trafficked more readily across the HUVEC monolayer than non-malignant EVs, indicating an increased extravasation potential of cancer-derived EVs [67].

4. Relevance of EVs in anti-tumor immune responses

4.1. Anti-tumor immune responses

Cancer is characterized by a progressive accumulation of genetic alterations along an abnormal gene regulation. The altered genotypic features confer specific biological traits to tumor cells known as 'cancer hallmarks' that, among others, include the capacity of avoiding immune destruction [68]. This implies that cancer cells can be target of immunological recognition and elimination, but also that a clinically manifest cancer indeed escapes the immunological control by developing strategies to actively suppress the immune anti-tumor response. The generation of an anti-cancer immune response entails stepwise events and involves different immune cells subsets. This set of ordinate events is called 'cancer-immunity cycle' [69], which initiates by the release at the tumor site of tumor-associated antigens (TAAs) including the highly immunogenic neo-antigens generated by mutated proteins or, more in general, by the genomic instability. Tumor specific antigens are released upon immunogenic tumor cell death, likely induced by natural immunity cells, such as NK cells. Through their NKG2D receptor, NK cells recognize the MICA, MICB and ULBPs proteins induced on tumor cells by genotoxic stress, DNA damage and oxidative stress. Additionally, NK cells produce cytokines and chemokines such as CXCL1, which triggers the recruitment of conventional (c)DC1 DCs [70]. This DC subset is specialized in taking up antigens, migrates to the draining LNs and cross-presents the tumor antigen on MHC-I and MHC-II molecules to T cells, leading to priming and activation of anti-tumor T cells at the immunological synapses (IS). In a virtuous 'cancer-immunity cycle' clonally expanded tumor-specific CD8 and CD4 T cells expressing different TCRs are generated, which exit the LNs and traffic to the tumor bed. Infiltrating tumor-specific T cells then restart the virtuous immunity cycle, by the recognition via TCR of their nominal tumor antigen bound to the MHC-I and killing the cancer cells. A new wave of antigen release is generated and new specific T cells can be expanded by the completion of new cycles. Apart from primed DCs that travel from tissues and naïve T cells, encountering principally in peripheral LNs to form the IS, also EVs have defined roles in this scenario. Upon antigen-dependent contacts, T cell MVBs translocate towards the IS and $CD63^+$ EVs are delivered unidirectionally from T cells to APCs to boost inflammatory responses of DCs. This antigen-specific EV transfer occurs first by EV attachment to the APC cell membrane continuing thereafter as a fusion or internalization process to deliver miRs involved in the regulation of gene expression of the receiving APC [71]. Controlled by protein kinase C δ (PKCδ) that is activated by diacylglycerol (DAG), the secretion of EVs is a consequence of the convergence of MVB towards the microtubule-organizing centre (MTOC) and the polarization of the MTOC to the IS, a process coordinated by two distinct pathways involved in the F-actin reorganization at the IS, FMNL1 and paxillin

phosphorylation [72,73]. Recently, it has been elegantly shown how during the IS formation genomic and mitochondrial DNA exposed on the EV membrane and unidirectionally transferred from T cells to APCs leads to the activation of the cGAS/STING cytosolic DNA-sensing pathway and to the expression of IRF3-dependent interferon regulated genes, including IFN-stimulated genes (ISGs) Ifit1, Ifit2, Ifit3, Isg15, Usp18, andCxcl10, and the antiviral signaling factors Gbp5 and Gbp6 [74]. Of note, the activation of the cGAS/STING pathway can occur by tumor DNA internalized via EVs by DCs and macrophages and cross-prime $CD8^+$ T cells via tumor-antigen presentation. Tumors can also secrete cGAMP either as soluble molecule or incorporated in EVs that is uptaken by DCs, other immune cells or even fibroblasts, activates STING-IRF3 and induces NK-mediated tumor killing [75,76]. Many innate and adaptive immune cells of the different cell lineages exploit polarized secretion of lytic granule EVs, specialized secretory lysosomes rapidly synthesized upon target cell recognition [77], to guarantee the antigen specificity of the final response. These include NK cells [78], cytotoxic T lymphocytes (CTLs) [79], $CD4^+$ T cells [80] and B lymphocytes [81]. Crosstalk via EVs appears as a complementary method of immune cell functional regulation, amplification of the immune response, elimination of harmful immune cells and fine-tuning of immune activity. Despite the enormous growth of uncovered mechanisms better defining the exact functional roles of EVs in the context of immune responses, many queries remain regarding the potential redundancy of EVs with soluble factors, the 'reasons' why and when cells communicate via EVs, direct cell-to-cell contact or soluble factors. In other words, in the daily discovery run of a myriad of EV functions a definitive and potentially exclusive 'purpose' of EVs in this context is still lacking. Could it be a matter of distance, in that EVs could represent the vehicle of choice to 'send' long-distance messages to other districts of the body? Or may EVs, harboring the combined presence of diverse molecules, represent the means of choice in case different cellular responses must be elicited at the same time [82], or because their membrane-bound full-length proteins may result more efficient, as in case of TRAIL and HSP70 [83,84], or resistant to inhibition, as shown for neutrophil elastase [85], than the soluble counterparts?

4.2. EV types sustaining anti-tumor immune responses

Dendritic cell EVs

From the beginning of EV research, among immune cell types DCs appeared as abundant producers of exosomes, and thanks to the early uncovering of their immune stimulatory roles and antigen presentation DC-derived EVs have been thoroughly characterized and, given their immune

derivation, tested in preclinical and clinical settings in cancer [86]. Stimulation of T cells by DC and B cell EVs was one of the first discovered functions of immune EVs. In fact, B cell EVs exposing HLA-DR generated MHC Class II-restricted T cell responses, demonstrating that exosomes carry MHC class II-peptide complexes. This led to the hypothesis that EVs may contribute to the maintenance of long-term T cell memory or T cell tolerance and that they could be exploited as vehicles for immunotherapeutic purposes [87]. Their effective anti-tumor activity in mouse cancer models based on the release of antigen-presenting EVs (dexosomes, Dex) after pulsing their cells of origin with tumor peptides, and such vesicles induced strong enough anti-tumor T cell responses to eradicate tumors [88]. The abundant expression of components of the antigen presenting machinery (MHC-I and MHC-II) and costimulatory molecules (CD40, CD86, CD80), the expression of adhesion proteins like lactadherin and ICAM-1, together with the C-type lectin receptor DC-SIGN, decorating their surface, render DC EVs suitable stimulators of T cell-mediated immune responses [89,90]. The protein composition of DC EVs reflects the maturation status of the releasing cell. Exosomes secreted by immature and mature murine DCs differ by the gradual acquisition of MHC class II-peptide complexes, costimulatory molecules such as B7.2/CD86 and adhesion molecule ICAM-1, along with increased $CD4^+$ proliferation potential in functional T cell stimulation tests. Therefore, mature DC EVs play an active role in inducing T cell stimulation and specific immune responses. [90]. In human setting, DC EVs, loaded with viral or tumoral antigens, induce $CD8^+$ T cell activation and suppression of tumor growth [91,92]. EVs can, depending on their size, polarize T cell stimulation toward effectors involved in cell-mediated immunity or antibody-mediated humoral immune responses. Indeed, the importance of EV size has been tackled by Tkach *et al.,* who showed that different EV subtypes promote the release of specific cytokines by $CD4^+$ T cells and determine the orientation of T helper cell responses. Large EVs promote the secretion of Th2 cytokines, including IL-13, IL-5 and IL-4, by contrast, small EVs bearing MHC class II promote IFNγ secretion, belonging to Th1 responses. Differential functions of large and small EVs in their specific $CD4^+$ Th activation is due to the enrichment of specific molecules involved in IS stabilization, including CD80 on the surface of large EVs and CD40 and DC-SIGN on the surface of small EVs. However, their differential activity is abolished when DCs were treated with maturation-inducing stimuli. In this case, all EVs are able to induce IFNγ and promote Th1 responses [93]. Besides DC-derived EV direct antigen presentation to T cells, EVs carrying exogenous MHC-peptide complexes on their surface can also coat other DCs and be presented to T cells without any antigen processing [94]. MHC-cross dressed DCs can retain or internalize

exogenous EVs, depending on their stage of maturation. Indeed, coating EVs remain on the surface of mature DCs, while they will be further processed in case of immature DCs before being presented to T cells [95]. In this process, DCs provide costimulatory molecules and the functional exchange of peptide-MHC complexes enhance the number of presenting cells to amplify the primary T cell immune response, explaining their key role in the MHC-cross dressing process [96]. It has been demonstrated that EV transfer between DCs requires the presence of the leukocyte function-associated antigen-1 (LFA-1) on their cell surface. Indeed, $CD8^+$ DCs, expressing high levels of LFA-1, preferentially capture EVs compared to $CD8^-$ DCs [54,97]. LFA-1 is also found to be crucial in mediating EV recruitment by activated T cells [98]. The molecular transfer between DCs and T cells can also occur in the opposite direction and modulation of DC functional properties can become a mechanism of T cell regulation. Indeed, incubation of anergic T cell-derived EVs with DCs abolished T cell activation during subsequent antigen exposure [99]. Further, after antigen specific interaction, T cells can modulate DC activation by transferring CD3/TCR complex to DC surface, reducing the stimulation of $CD4^+$ T cells but retaining their ability to activate cytotoxic $CD8^+$ activation. TCR complex transferred to DCs masks the antigen-loaded MHC II molecules and enters the mechanism of regulation of the adaptive $CD4^+$ T cell response [100]. Functional regulation of DCs is also subjected to EVs released from particular T cell subset. For instance, Treg-derived EVs induce a tolerogenic phenotype in DCs via the transfer of miR-150-5p and miR-142-3p. Upon the acquisition of these miRs, DCs decrease IL-10 and IL-6 cytokine production, as detected in response to lipopolysaccharide (LPS) stimulation [101]. Recently, it has been shown that EV cargo also consists of miRs, which reflect the physiological status of EV-producing cells and which can regulate gene expression and function of the recipient cell [102]. Subsequently, the functional transfer of EV-delivered miRs plays a key role in immune regulation [103]. Endogenous miR-155 and miR-146a can be secreted within exosomes and transferred within primary bone marrow-derived DCs. In this context, miR-155 promoted and miR-146a repressed inflammation in response to LPS stimuli, thus contributing to a mechanism of regulation of inflammatory responses [104]. Mittelbrunn M. et al demonstrated that miRs are transferred via EVs during T cell-DC immune interaction and can be functional in the recipient cells [71]. The contribution to anti-tumor immunity by DC-derived EVs is also triggered via activation of NK cells, which occurs via NKG2D ligand-receptor interaction [105], and TNF-mediated IFNγ production by activated NK cells. Additionally, members of the TNF superfamily like FasL and TRAIL are able to induce apoptotic cell death and directly kill cancer cells [106]. Moreover, NK cell activation can be mediated by the

expression of BAT3 on the DC-derived EV surface and its binding to the ligand NKp30 mediates NK cell cytotoxicity and cytokine release, revealing a new model of NK/DC crosstalk [107].

T cell EVs

T cell-derived EVs play major roles in relation to TCR-triggered immune responses. This includes T cell-mediated cytotoxicity, $CD4^+$ T cell activation-induced cell death (AICD), antigen presentation, intercellular exchange of miRs [108-111, 71] and thymic development [112]. First evidence that also T cells could release EVs came in 2002, when Blanchard and coworkers activated T cells by TCR triggering and found CD3/TCR molecule-exposing vesicles in the supernatants. Additionally, T cell derived EVs contained CD2 and LFA-1, MHC class I and class II, and the chemokine receptor CXCR4, potentially involved in interaction processes with the target cells [113]. The effective release of EVs was then visualized with FM1-43 dye to occur principally during activation and EVs were shown to contain raft-associated CD3 proteins, GM1 glycosphingolipids, and PS at the outer membrane leaflet [114]. The production and release of EVs from T cells appeared to differ from the machinery of other cell types in that it depended on the expression of MAL (Myelin and lymphocyte protein), a T cell tetraspannin localized in the endoplasmic reticulum of T cells and involved in EV biogenesis and sorting [115]. Other studies showed that during target cell killing Rab7 containing EVs are released through MVB fusion with the plasma membrane by activated T cells in a Rab27-dependent manner [116, 117]. Rab27-dependent release of EVs seems a fundamental EV production mechanism of functional immune cells since Rab27 deficiency interferes with inflammatory responses and inhibits chronic inflammation, as shown in animal models [118, 119]. Upon activation, T cells release massive amounts of EVs containing small RNAs of which T cell-associated miRs are increased after immunization, as shown in mice and humans. The extracellular increase of T cell miRs correlates with a general downregulation of cellular miRs [122], indicating that activation leads to a potentially specific expulsion of specific small RNAs by T cells. Recently, Chiou et al have shown that T cell activation released EVs contain specific tRNA fragments (tRFs), which, if remaining within the T cells inhibit their activation and cytokine production. T cells may thus exploit EVs to selectively secrete tRFs that can repress T cell activation [123]. tRNA fragments are small RNAs generated by tRNA fragmentation that can have a variable sequence and size and that regulate cell homeostasis and adaptations to stress processes [124]. EVs carrying tRFs may be found at systemic level in plasma or serum of patients and give indication about ongoing immune activation during anti-tumor immune responses. An attempt to isolate T cell EVs was made by immunocapture with anti-CD3 antibodies from plasma of HNSCC patients.

The authors captured $CD3^+$ EVs from non CD3-EVs and found that in contrast to donors, whose samples contained 20-30% of T cell EVs, those from patients contained more than 50% of $CD3^+$ EVs. Additionally, phenotype studies performed via bead-EV flow cytometry showed and enrichment of immunoregulatory molecules, especially CD15s expressed by highly immune suppressive Treg, indicating that the increase in $CD3^+$ T cell EVs may be related to expansion of this immune suppressive cell subset [125]. T cell EVs bearing the pro-apoptotic molecule membrane FasL appeared to play a role in controlling DGKalpha mediated AICD of T cells [110]. The dissection of T cell EV subtypes revealed that during activation T cells produce larger > 200 nm (lEVs) as well as small (sEVs) < 200 nm. In contrast, apoptotic stimuli led to a massive release only of lEVs displaying a different protein composition with respect to those lEVs released during activation and containing signaling and proteins of the actin-myosin cytoskeleton. Small EVs, apparently produced only by activated T cells carried cytoplasmic/endosomal proteins like heat shock protein 70 (HSP70) or tumor susceptibility gene 101 (TSG101) protein, microtubule-associated proteins, and ubiquitinated proteins [126]. EVs from activated T cells can carry CCL5 (RANTES), reflecting the activation status of their cells of origin, and in the presence of IL-2 could induce proliferation of the resting counterpart, which displayed an increased proportion in $CD8^+$ T cells [127]. The concept that an antigen-specific CTL response might be amplified through CTL-derived EVs, which influence bystander CD8 T cells, was further corroborated by Li and coworkers, who found that CTL EVs are tunable in that their function and morphology can be influenced by the stimulation of their originating cells. EVs produced by IL-12 stimulated CTLs following antigen stimulation were able to induce activation and production of IFNγ and granzyme B (GZB) by $CD8^+$ bystander cells in the absence of antigens [128]. On the other hand also negative affects, such as promotion of tumor immune escape mediated by T cell EVs have been described: in a cancer mouse model $CD8^+FasL^+$ T cell EVs from tumor-bearing animals, but not healthy mice, induced metastatization of B16 melanoma and 3LL lung cancer via FasL-induced MMP9 expression [129]. These results may find explanation in the shaping of the immune system by the presence of cancer cells in the host's body, similarly to the promotion of MDSCs and regulatory T cells. In line with this hypothesis is the demonstration that EVs from activated $CD8^+$ T cells from non-cancer bearing mice could interrupt the fibroblast stroma-mediated progression. CTL EVs migrated to neovascular areas with high mesenchymal cell density, where they induced tumour MSC depletion by apoptosis [130]. In an *in vivo* study of uterine corpus endometrial cancer (UCEC) Zhou et al. showed that the most downregulated miR in human UCEC was miR-765, a negative regulator of proteolipid protein 2

(PLP2) thereby protmoting progression and epithelial mesenchymal transition (EMT). Mir-765 was instead highly expressed in $CD45RO^{-}CD8^{+}$ T cells and their EVs. Treatment with these EVs limited estrogen-driven ERβ/miR-765/PLP2/Notch signaling axis UCEC development via regulation of the miR-765/PLP2 axis [131]. In contrast, EVs from exhausted T cells are only starting to be elucidated, as demonstrated by Wang and coworkers, who isolated CD8 T cells from lesions surgically removed from HCC patients and divided the exhausted $CD8^{+}PD\text{-}1^{+}TIM3^{+}$ from the non-exhausted $CD8^{+}PD\text{-}1^{-}TIM3^{-}$ cells. Exhausted T cell EVs were uptaken by non-exhausted CD8 T cells and were able to induce exhaustion together with reduced IFNγ and IL-2 cytokine production on non-exhausted CD8 T cells. Additionally, exhausted T cell EVs displayed a different lncRNA profile [132]. LncRNAs can function as decoys, scaffolds and enhancer RNAS and are part of the big ncRNA population comprising also miR and piwi-interacting RNAs, which are involved in various mechanisms including transcriptional and post-transcriptional regulation and chromatin remodeling, through chromatin-based mechanisms and cross-talk with other RNA species [133]. Another evidence comes from the autoimmunity field; in the RA joint investigators have detected T cell EVs, which contained exhaustion markers and T cell inhibition-associated miR and which could be functionally involved in driving T cell exhaustion [134].

NK cell EVs

NK cells play important roles in the immune surveillance of tumors as they function as first-line defense in the control of tumor growth and metastasis diffusion. Just like T cells also NK cells release 'granules', which are EVs containing cytotoxic molecules like granzymes and perforins. NK cell EVs were first described in 2012, produced by resting as well as activated cells and also detectable in plasma of healthy donors. NK EVs presented exosomal characteristics including dimensions and surface molecule repertoire. Additionally, they carried the typical NK cell markers CD56 and NKG2D and pro-apoptotic molecules such as FasL. Functional experiments revealed their cytotoxic potential against tumor cells and activated immune cells, suggesting a role of NK EVs in immunosurveillance and homeostasis [135]. Characterization of NK EVs produced on a large scale confirmed previous results, further showing that cytotoxic killing of target cells was caspase-dependent nd mediated by NK EV content of perforin, granulysin and granzymes A and B [136]. Apart from *in vitro* experiments, NK EVs derived from NK-92MI (IL-2 dependent) cell line displayed anti-tumor activity against mouse melanoma B16F10 cells. The cytotoxic content of NK-92 EVs resembled peripheral blood derived NK EVs and, despite the species difference, NK92-EVs

displayed strong anti-tumor effects also in solid tumors [137]. NK cells could be 'educated' by neuroblastoma-derived EVs to release NK EVs with enhaced cytotoxic activity, probably depending on the activation of NK cells exposed to tumor EVs [138]. In this line, hypoxia appeared to activate NK-92 cell line to produce EVs with increased FasL, perforin and granzyme expression, compared to its normoxia counterpart [139]. The actual content of NK EVs deriving from expanded and activated NK-cell-enriched lymphocytes (NKLs) was confirmed by proteomics. NK EVs contained NK activating molecules, including NKG2D, DNAM1, NKp44, NKp46, molecules involved in apoptosis such as TRAIL and its death receptors DR4 and DR5, Fas and FasL, cytokines TNFalfa, IFNgamma and IL-6 and various adhesion molecules, apart from the EV markers [140]. NK EVs can exert their cytotoxic activity also via miRs. Neviani et al showed that NK EVs carry tumor suppressor miR-186, which was downregulated in MYCN-amplified neuroblastoma cell lines and in lesions from high-risk with respect to low-risk patients. The expression of miR-186 positively correlated with the expression of NK cell activating markers NKG2D and DNAM-1, indicating that the activation of NK cells in the tumor microenvironment plays a fundamental role in this type of cancer. The immune suppressive cytokine TGFβ produced by tumor cells, Tregs, macrophages and other components of the tumor microenvironment can inhibit miR-186 expression by NK cells. Restoring miR-186 expression by NK cells led to its release via NK EVs and their cytotoxic activity towards neuroblastoma cells was partially mediated by miR-186 delivery [141]. TGFβ promotes liver fibrosis by activating hepatic stellate cells (HSCs) [142] and NK EVs can attenuate this effect by transferring miR-223, a miR highly expressed by NK cells and their EVs, to HSCs leading to inhibition of autophagy via ATG7 targeting [143]. The role of DNAM-1 in mediating NK EV-driven cytotoxicity was further corroborated using antibodies against the molecule or its ligands. Of note, characterization studies also revelaed the presence of PD-1 and IFNγ at the intravesicluar level, in fact their detection was only possible after permeabilization [144]. Other miRs contained in NK EVs such as miR-3607-3p have been investigated in the context of cancer therapeutics. This miR was downregulated in plasma EVs of patients affected by pancreatic cancer with respect to healthy donors and additionally the authors could detect a significant decrease of miR-3607-3p in plasma EVs deriving from patients displaying LN invasion with respect to those who were negative. Functional experiments showed that the delivery via EVs to pancreatic cancer cells inhibited progression via targeting of IL-26, increased in pancreatic cancer as compared to normal tissue [145]. The exploitation of NK EVs for therapeutic purposes is still hampered by lack of thourough knowledge of their content and effects, as well as by technical hurdles regarding their preparation

for clinical adiministration. Kang et al proposed an on-chip EV biogenesis microfluidic system with a graphene oxide chip, which was coated with NK cell antibodies to capture NK cells from peripheral blood of cancer patients. After 12 h incubation NK EVs were isolated, characterized and tested for their cytotoxic activities. According to the authors this technique allows obtaining NK EVs for immunotherapies at clinical level [146]. Together with the group of Stefano Fais we contributed to investigate the relevance and potential applications of NK EVs. Additionally, our recent work evidenced that NK EVs, enriched by lower speed centrifugation for large EVs or microvesicles and by ulracentrifuation for small EVs or exosomes and derived from ex vivo expanded human NK cells, stimulate PBMCs even in the presence of immune suppressive cytokines IL-10 and TGFβ. Proteomics and cytokine analysis revealed their content of NKG2D and CD94, perforin, granzymes, CD40L, and other molecules involved in cytotoxicity, homing, cell adhesion, and immune activation, together with EV markers tsg101, CD81, CD63, and CD9 in both subtypes of NK EVs. Finally, the specific quantification via $tsg101^{+}CD56^{+}$ capture of NK EVs evidenced lower levels in plasma of melanoma patients compared to healthy donors, reflecting the lower frequencies of NK cells measured in PBMCs of these patients [147].

4.3 EVs in tumor immune evasion

In cancer patients the positive evolution of the 'cancer-immunity cycle' can be counteracted in each of its steps generating an immune suppressive environment, ultimately leading to cancer immune evasion. This relies on specific modifications of the immunogenic profile of cancer cells, thereby escaping recognition by T cells due to the expansion of variants characterized by antigen loss or by defects in antigen processing and presentation. Tumor cells can modify their phenotype by acquiring the expression of immunosuppressive molecules to directly kill T cells (i.e. pro-apoptotic ligands FasL, TRAIL) [148] or of molecules with 'don't eat me' function (i.e. CD47), which inhibit their phagocytosis by macrophages [149]. In response to cytokines released by activated T cells, e.g. IFNy, cancer cells upregulate immune checkpoint proteins (ICs), such as PD-L1, actively limiting the activity of antigen activated CD8 T cells and involved in induction/maintainance of T cell exhaustion [150]. Release of immune suppressive molecules via EVs enables them to enter systemic circulation and exert immune suppressive functions also at distant sites from the originating TME [151]. 'Immunity cell cycles' are also hampered by immune suppressive specialized cellular subsets, namely regulatory T cells (Tregs) and MDSCs, acting at the tumor site and systemically in the draining LNs, where Treg compete for the generation of antigen specific

CD8 T cells. In fact, in solid cancer patients the accumulation of Treg in tumor-draining LNs has a negative prognostic value. Tregs express receptors for chemokines, such as CCR4, CXCR4 and CCR10 that can induce their migration towards the tumor. In cancer patients, increased Treg/Tconv (conventional T cells) and Treg/CD8 T cell ratios are often observed also at tumor site. MDSCs are a heterogeneous population of variably immature myeloid cells (IMCs) with suppressive activity, containing myeloid progenitor cells and granulocyte precursors, macrophages and DCs. Elevated MDSC levels in the peripheral blood of cancer patients translates into inhibition of autologous T cell proliferation and IFNγ production. Agreement in the scientific community indicates three main subsets of MDSCs: early (e)MDSC, defined as Lin^- $HLADR^{-/low}CD33^+CD11b^+$; polymorphonuclear (PMN)-MDSCs, defined as $CD14^-$ $CD15^+CD11b^+$ and monocytic (m)MDSC defined as $CD14^+HLADR^{low/-}$ [152,153]. mMDSC resemble monocytes in size and light scatter characteristics and also express the monocytic marker CD14. Their peripheral blood frequency correlates with tumor burden and is inversely associated with response to ICI immunotherapy [154]. Both, Treg and MDSCs can be induced by cancer EVs, selective carriers of tumor derived conditioning molecules that can lead to their generation and expansion in cancer patients [155,156].

4.3.1. Tumor EVs restrain anti-tumor immune responses

Ever since the release of intact vesicles from reticulocytes was first noticed scientists began to investigate if also transformed cells could exteriorate vesicles especially in view of their potential purposes and consequences for the host. The conditioned medium of tumor cells became the focus of the dedicated scientific community who started purifying and characterizing the vesicles that visible only by electron microscopy. Our group also contributed to these pioneering findings in that we identified an EV-mediated tumor immune escape mechanism consisting in the release by melanoma cells of what we at that time called ‘microvesicles’, exposing the pro-apoptotic molecule FasL. Unlike its soluble counterpart, FasL was full-length and thus embedded in the membrane surrounding each vesicle and displayed cytotoxic activity of Jurkat T cell line [157]. In the following years we found that tumor EVs could kill also tumor-specific $CD8^+$ T lymphocytes from CRC patients [148] and could interfere with monocyte differentiation into DCs, inducing a new phenotype associated with acquisition of immune suppressive functions [158]. The detection of such formerly unknown monocytes, which we named myeloid-derived suppressor cells, in the peripheral blood of advanced melanoma patients represented a milestone in the history of our

laboratory [153]. Research on tumor EVs remained a focus of our group throughout the years and we continue dedicating ourselves to dissect not only immune escape mechanisms, but also their potential role as therapeutic devices and biomarkers of resistance to therapies, especially targeted and immunotherapy in melanoma patients. Hereafter, we present a collection of the most important and recent findings related to the principally detrimental effects of EVs released by cancer cells.

Major breakthrough in dissecting the mechanisms of tumor EVs on angiogenesis, EMT, pro metastatic functions (pre metastatic niche formation), the immune system, restraining of anti-cancer agents such as sequestering of antibodies, drug elimination by cancer cells via EVs has been reported. It is interesting to note that the great majority of identified mechanisms appear to be shared by cancer EVs of different histotypes as detailed in the following paragraphs. A key role of EVs in promoting tumor angiogenesis has been shown for GBM EVs, which are enriched in hypoxia-regulated effector molecules that promote angiogenesis *in vitro* and *in vivo* through the proliferation of endothelial cells and the stimulation of pericyte PI3K/AKT signaling pathway activation and migration [159,160]. Similarly, EVs derived from nasopharyngeal, breast and pancreatic cancers are angiogenic and induce metastasis [161- 164]. Mesothelioma cells release tumorigenic EVs containing angiogenic proteins that promote the migration rate of angiogenic cells and increase tumor development through vascular reorganization [165]. Besides solid tumors, also EVs from chronic myelogenous leukemia cells can promote angiogenesis via direct interaction with endothelial cells [166,167]. EVs secreted by cancer cells of different origin actively contribute to the generation of a metastatic niche by influencing multiple cell types and cellular processes. Murine multiple myeloma derived-EVs induce the formation of the metastatic niche in bone marrow and promote angiogenesis *in vivo* [168]. In a melanoma mouse model BM education by tumor-derived EVs supports tumor vasculogenesis, invasion and metastasis [169]. Breast cancer cells secrete EVs, which manipulate human primary mammary epithelial cells (HMECs) to induce reactive oxygen species (ROS) formation, autophagy, and production of tumor factors. These responses may reprogram host microenvironment to new tumor cells for metastatic niche formation [170]. Inhibition of EVs uptake via targeting the exosomal integrins α6β4 and αvβ5 decreased lung and liver metastasis, respectively [171]. It has been recently reported that microenvironmental cytokines, particularly CCL2, can bind to proteoglycans exposed by EV surface and cause EV accumulation in specific organs, resulting in immune changes and higher metastatic burden [172]. Molecular insight into EV cargo has disclosed the pivotal roles of miR in cancer

metastasis by reprogramming the target cell transcrtiptome. In addition, tumor EVs actively participate to miR biogenesis. Indeed, the RISC Loading Complex, including Dicer, AGO2 and TRBP, and required to process precursor miRs (pre-miRs) into mature effective miRs, could be detected in EVs released by breast cancer cells, together with pre-miRs. These findings suggest that tumor EVs increase tumorigenic potential of recipient target cells in a DICER-dependent manner [173]. Astrocyte-derived EVs transfer PTEN-targeting miR to metastatic tumor cells favoring brain metastasis outgrowth, in addition to increased CCL2 secretion, which recruits IBA1-expressing myeloid cells, enhancing proliferation and survival of brain metastatic tumor cells [174]. Exosomal miR-23a from hypoxic lung cancer cells promotes angiogenesis through targeting tight junction protein ZO-1 and prolyl hydroxylase, thereby increasing vascular permeability and cancer transendothelial migration [175]. Breast cancer cells produce EVs transferring miR-122 that block glucose uptake via pre-metastatic niche cells and disrupt energy metabolism, promoting cancer progression [176]. Similarly, MDA-MB-231 triple negative breats cancer EVs transfer miR-10b that promotes tumorigenesis and cell invasion [177]. Also horizontal propagation of oncoproteins carried by tumor EVs may increase oncogenic activity of target cells. In gliomas, the extracellular and systemic release of membrane EVs containing the oncogenic receptor EGFRvIII actively contributes to glioma aggressiveness through activation of MAPK/AKT pathways, EGFRvIII-regulated genes (VEGF, BCL-XL, p27) and increase in anchorage-independent growth capacity [178]. Transfer of the MET oncoprotein via melanoma EVs to bone marrow progenitor cells promotes metastatic progression [169]. Several studies reported that nucleic acids carried by tumor EVs reflect the genetic status of the tumor, for example, amplification of cMyc oncogene was detected in EVs from medulloblastoma cells, while EGFRvIII mutant/variant mRNA was detected in EVs from GBM patients [179]. This indicates that nucleic acids isolated from tumor EVs can be considered as tumor biomarkers to be used in cancer blood-based diagnostics. As the RISC Loading Complex is selectively present only in tumor and not in normal cells, also DICER detection in EVs could be considered a biomarker for cancer diagnosis [173]. In general the encounter of tumor EVs with immune cells has a negative outcome for the host. In fact, these interactions can activate immunosuppressive events by inducing phenotypic changes in different immune cell populations [180,181]. We reported that a set of specific miR (miR-146a, miR-146b, miR-155, miR-125b, miR-100, let-7e, miR-125a, and miR-99b) carried by EVs derived from melanoma cell cultures confer immunosuppressive properties to healthy donors' monocytes. Of note, we also showed that baseline levels of these miRs in plasma from melanoma patients clustered with the

clinical efficacy of CTLA-4 or programmed cell death protein 1 (PD-1) blockade, thus representing a predictive peripheral blood biomarker of resistance to ICIs [156]. Similarly, EVs from Ret mouse melanoma cells induced the upregulation of PD-L1 on immature myeloid cells (IMCs) via inducible HSP86 expressed by EVs, which triggered TLR4 and NFkB activation on IMCs, generating PD-L1^{+}CD11b^{+}Gr1^{+} MDSCs that suppressed T cell functionality [182]. Major contribution in this context has been provided by the group of Theresa Whiteside advancing the field of tumor EV-mediated immunosuppression [155]. In her recent work she and her coworkers set up a strategy to separate tumor-derived from non tumor-derived EVs in plasma of patients to investigate their composition by high-throughput approaches not only to study their composition but also to evaluate their potential application as disease biomarkers. Importantly, the recovery of the autologous non-tumor plasma EV fraction of each patient allowed getting insight into the still elusive changes in EV composition occurring during tumor development and progression at systemic level. Of interest is also the possibility to compare the non-tumor fraction of patients with the one extracted from plasma of healthy donors. At functional level these authors found an enrichment of immunostimulatory proteins in non-tumor EVs of melanoma patients' plasma together with a weak immunosuppressive potential compared to tumor EVs, which inhibited T cell activation, proliferation and reduced NKG2D expression by NK cells. Surprisingly, just as tumor EVs also non-tumor EVs induced apoptosis in CD8^{+} T cells, suggesting the presence of alterations of the circulating 'normal' EV counterpart induced by melanoma [183]. Studies in tumor animal models have highlighted different phenotypes between EVs released at the tumor site and present in the tumor microenvironment and those detectable at distance in the periphery [184]. The adoption of 3D cultures, obtained by gel-based cultures, microfluidic systems and bioreactors, has enabled the study of tumor EVs in a more similar manner to *in vivo* conditions compared to 2D cultures. EVs released by 3D cultures are functional, as they can activate signaling pathways in recipient cells. Their cargo is responsive to both tissue architecture composition and asymmetry and orientation of tumor cells in 3D. Indeed, a recent study reported that the miR cargo of EVs from 3D cultures of cervical cancer cells is different from the miR cargo of EVs from 2D model, and exhibits high similarity (~96%) to *in vivo* circulating EVs from plasma of cervical cancer patients [185]. These results also demonstrate that NGS technique can be applied to investigate and characterize their content. This supports their use as non-invasive approach for investigating tumor biomarkers, drug screening and understanding of tumor progression and metastasis at the molecular precision level [186]. Obviously, with the advent of immunotherapy targeting ICs, the detection, characterization

of functional and clinical impact of these molecules not only as soluble factors but also carried by EVs has become a major focus in EV science. Generally speaking all ICs detected on tumor and non-tumor cell surface could be found in EVs and the EV-incorporated ICs displayed functional activities upon binding to their ligand/receptor expressed by the EV receving cell. Binding of GBM cell-derived exosomal LGALS9 (Galectin 9) to the TIM3 receptor of DCs inhibits antigen recognition, processing and presentation, thus leading to failure of the cytotoxic T cell-mediated antitumor immune responses [187]. CD8 T cells dysfunction is mediated by EVs carrying PD-L1 in breast cancer, and arginase-1 in ovarian cancer, respectively [188-190]. Melanoma EVs carrying PD-L1 were reported to suppress the function of CD8 T cells and predict response to anti-PD-1 therapy [191]. The discovery of IC expression by EVs further determined not only their exploitation as biomarkers of disease but also as indicators of response and resistance to cancer therapy, including ICIs, in the liquid biopsy.

4.3.2 Immune EV types counteract anti-tumor immune responses

Information about composition, phenotype and functions of EVs deriving from immune suppressive cells, namely monocytic-(M) and granulocytic or PMN-MDSC together with regulatory T cells, has received little attention so far, given that a thorough analysis of such EV subpopulations especially those deriving from human immune suppressive cells is hampered by the lack of appropriate models and the necessity of using patient-derived material. Nonetheless, if we consider the enormous potential of EVs in amplifying or restricting immune responses, elucidating the role of immune suppressive cell-derived EVs appears fundamental.

Regulatory T cell EVs

Regulatory T cells use a large variety of different tools to exert their suppressive functions and to induce and maintain peripheral tolerance. Mechanisms used by Tregs to prevent immune cell activation are cell-contact dependent and include the engagement of inhibitory receptors (e.g. CTLA-4) expressed on the membranes of the target cells, as well as direct cytotoxic killing by granzyme and perforin containing EVs. Other Treg-mediated inhibitory mechanisms are more wide-ranging and rely on the secretion of suppressive cytokines, such as IL-10, TGFβ, IL-35, or on IL-2 deprivation. Likely, immune inhibition mediated by a combination of contact- and secretion-mediated mechanisms is more successful. EVs are a quite recent addition to the arsenal of Treg functional activities. Similarly to other EVs also those deriving from Treg appear to transfer

"protected" suppressive signals to target cells, thus ensuring enhanced efficiency in suppression. Similarly to conventional T cells, $CD4^+CD25^+Foxp3^+$ Tregs produce EVs upon TCR triggering and their vesicles display the morphologic and phenotypic features of 'classical' sEVs with exosomal features, displaying a dimension of 50-100 nm and a positivity for CD81 and LAMP-1/CD63 and CD9, typical EV markers [192]. These 'classical' EVs are also produced by $CD8^+CD256^+Foxp3^+$ Treg expanded *in vitro* from naïve CD8+ T cells of C57/Balbc mice [193]. A detailed analysis of the mechanisms regulating EV release by $CD4^+$ Treg defined that it occurs with the same modality as in other T cells. It requires Rab27a and Rab27b, is hypoxia-sensitive and is regulated by changes in intracellular calcium and sphingolipid ceramide synthesis. Treg-derived EVs suppress immune responses by transferring immunomodulating proteins. CD73 and IL-35 have been detected in Treg EVs, demonstrating an active involvement in suppressing T cell activation. The ecto-5-nucleotide enzyme CD73 converts AMP into adenosine. Detected on EVs by immunofluorescence, this protein was functionally active since Treg EVs determined the conversion of AMP into adenosine *in vitro* [192]. The cytokine IL-35 has two subunits, one corresponding to the p35 chain of IL-12 and the second, Ebi3, to the Epstein-Barr-induced gene 3 protein. Treg secrete IL-35 in response to TCR triggering and $IL\text{-}35^+$ Treg cells, which have enhanced suppressive activity, are enriched in tumors. IL-35 limits antigen-specific T cell infiltration, effector function and memory. Moreover, Treg-derived IL-35 promotes exhaustion of T cells within the tumor microenvironment. IL-35 can be secreted as component of $CD81^+$ EVs and these vesicles are very efficient in targeting T and B cells and in inducing peripheral tolerance [194]. Treg EV can also exert immune suppression transfer of several miR, which showed suppressive functions toward T cells [118], DCs [195] and more in general function as mediators of tolerance also in peripheral tissues [196]. Transcriptional analysis and miR studies demonstrated that transfer of Treg EV containing let-7d to T helper cells suppress Th1 cell proliferation and cytokine secretion and in a mouse model let-7d EVs are essential in suppression and prevention of systemic inflammatory disease [118]. Treg derived exsosomes are also involved in mediating crosstalk between Tregs and DCs and also here miR are the main players. Indeed, the transfer of miR-150-5p and miR-142-3p via Treg EVs induced a tolerogenic phenotype in DCs, characterized by an increased IL-10 production [195]. Interestingly, miR-142-3p also has a broad activity in myeloid cells, where it interferes with the antigen processing machinery, decreases phagocytosis and induces an increased PD-L1 expression [197,198]. Treg vesicles and their transfer of specific miRNA might also target cells not belonging to the immune system, but directly involved in chronic inflammation, as occurring in inflammatory bowel disease

(IBD). By *in vitro* experiments in a mouse model it was clearly demonstrated that Treg EVs could be transferred to a colonic epithelial cell line treated with TNFa and that Treg EV administration alleviates IBD induced by dextran sulfate exposure. This therapeutic effect was dependent on EV enrichment in miR-195a-3p. Most likely, the pro-apoptotic Caspase 12 was the direct target of mi-195a-3p in the epithelial cells [196]. Major evidence has been progressively accumulated deciphering the nature and the role of Treg-derived EVs in immune suppression and more in general in the regulation of peripheral tolerance. However, the majority of these findings have been obtained in mice and a deeper exploration in the human setting is still lacking. This is mainly due to practical difficulties, since the frequency of natural occurring Tregs in human setting is low. However, taking advantage of the possibility of Treg cytokine-driven generation *in vitro* [199] some important key issues could be proved in the human setting.

Myeloid-derived suppressor cell EVs

Major contribution to the current knowledge about MDSC-derived EVs has been achieved by Sue Ostrand-Rosenberg and her group: by proteomic analysis of MDSC induced in BALB/c mice by the 4T1 mammary carcinoma, they identified 412 proteins among which S100A8 and S100A9, typically secreted by and chemotactic to MDSCs. In functional assays MDSC-EVs displayed tumor-promoting activities, indicating that just like other immune cell EVs, also those deriving from MDSC could contribute amplifying the effects of their cells of origin [200]. Building on this evidence, treatment with doxorubicin of 4T1 bearing mice induced activation of MDSCs, characterized by the expression of IL-13R and miR-126. This latter was released via EVs and rescued MDSCs from doxorubicin-induced myelotoxicity in a S100A8 and S100A9 manner, further corroborating previous findings [201]. On a more recent study in mice Rashid et al. compared MDSC EVs deriving from bone marrow (BM), spleen and tumor. Tumor-resident MDSCs produced more EVs than those from BM or spleen. The authors also observed that just like their cells of origin, EVs limited cytotoxic T cell and M1 macrophage functionality especially in the tumor microenvironment [202], indicating that EVs can amplify the action of MDSCs and that most probably EVs from potentially activated MDSCs, such as those residing at tumor site, might possess stronger immune inhibitory features than conventional MDSCs. A more in depth study comparing ‘conventional’ and ‘inflammatory’ MDSC-EVs proposes that inflammatory EVs harbor increased MDSC activities, such as S100A8 and S100A9, which are chemotactic for MDSCs and a stronger ability to polarize

macrophages towards a tumor-promoting phenotype via reduced IL-2 production by macrophages. In this interesting study, Fenselau and Ostrand-Rosenberg prepared conventional MDSC EVs from mice bearing 4T1 mammary carcinoma and inflammatory MDSC EVs from 4T1 producing IL-1beta inflammatory cytokine [203]. This comparison corroborates the findings described above and is of major interest for the human setting, where concrete evidence about MDSC-derived EVs especially in the context of cancer is still lacking. Studies investigating a specific MDSC subset, namely G-MDSCs in mice and PMN-MDSCs in humans have provided an insight of the suppressive potential of these EVs. MDSCs were isolated as HLA-DR^{-}CD11b^{+}CD33^{+} MDSCs from patients' CRC lesions and their EVs displayed expression of S100A9, a pro-inflammatory protein typically expressed by MDSC EVs with MDSC chemotactic properties [200,204]. The presence of S100A9 was responsible for tumor sphere formation in nude mice bearing human CRC SW480 tumors and which received MDSC-EVs deriving from human CRC lesion extracted MDSCs. Additionally, the authors demonstrated the tumor-promoting functions of MDSC-EVs by inhibition of Rab27a in EV producing MDSCs, which were co-transferred with SW480 CRC cells to nude mice. In case of Rab27a siRNA, the MDSCs displayed no tumor promoting functions and tumor growth was reduced with respect to untreated MDSCs [205]. Finally, strong evidence of functional suppressive activity also derives from studies investigating maternal-fetal tolerance. During pregnancy PMN-MDSCs are expanded and accumulate in peripheral blood, placenta and uterus [206]. In a recent work, Dietz et al. characterized the suppressive activities of EVs produced by CD66b^{+} PMN^{-}MDSCs, enriched from PBMCs of pregnant women. Their experiments show that PMN-MDSC-derived EVs contain Arginase I and inducible NO-synthase (iNOS), they can inhibit T cell proliferation and cytototxicity and induce Treg generation [207].

EVs from suppressive immune cells appear to generate enthusiasm, although EVs from monocytic MDSCs have not been investigated, leaving a fundamental gap of knowledge given the importance of this MDSC subset in restraining immune responses to immunotherapy with immune checkpoint inhibitors (ICIs) [208].

5. Perspectives and conclusions

Already in the mid 1980s the comparison of membrane shed EVs deriving from a non-metastasizing and its metastasizing variant highlighted differences in protein content, protein/lipid ratio and spontaneously shed plasma membrane EVs displayed T inhibitory functions in a T cell mediated tumor specific cytotoxicity assay [209,210]. Thereafter, almost all evidence on the

interactions and consequent fate of immune cells encountering EVs released by tumor cells is of detrimental nature for the cancer-bearing host. The evolvement of experimental settings, especially regarding the tracking of EVs *in vivo*, has corroborated most findings previously discovered principally in *in vitro* tumor EV-immune cells co-culture systems. Additional information about EV transcriptome, proteome and lipidome obtained with high-throughput approaches adapted to reliable detection even in the presence of very low amount of material, such as in case of isolated EVs, has hugely increased our possibilities in investigating the mechanisms governing the multifaceted activities mediated by EVs in immune responses (Figure 1). Very early evidence indicates that, similarly to DC EVs, also tumor EVs might contribute to stimulate immune responses due to their $CD8^+$ T cell cross-priming activity, leading to tumor rejection [211]. In fact, tumor EVs appeared as natural source of neoantigens and their detection in malignant effusions of cancer patients, together with their DC-mediated ability to expand tumor-specific T lymphocytes, further increased enthusiasm to exploit them as immunotherapeutic devices [92]. Clinical trials performed on this basis did not hold promise, neither as tumor peptide presenting DC EVs nor as tumor EVs, in eliciting measurable benefits [212-214]. In the following years the accumulating evidence of detrimental effects mediated by tumor EVs, ranging from immune suppression to EMT, and including angiogenesis, metastatic niche formation, malignant transformation through transfer of oncogenic molecules as well as generation of therapy resistance by expulsion of anti-cancer drugs or therapeutic antibody sequestration, led to the almost complete dissociation of the scientific community from exploiting tumor EVs as anti-cancer therapy in patients [215]. In contrast, the manipulation, inhibition or even elimination of cancer EVs from the body are being considered as potential [216], but at least in case of inhibition and elimination, unlikely, strategies to control their effects [217]. On the other hand, tumor EVs have found wide application as biomarkers of disease and response/resistance to therapy and, especially in the era of ICIs, appear as attractive constituents of the liquid biopsy in reflecting the actual immune status, not least due to their expression of ICs, such as PD-L1 [191]. The discovery of functional IC by EVs was demonstrated principally for vesicles deriving from tumor cells, but recently also immune cell EVs were evaluated for their expression. The presence of activatory and/or inhibitory IC on EVs adds another milestone to their potential as regulators of immune responses. Tumor EVs can impact the outcome of tumor-immune crosstalk in many ways and the groundbreaking discoveries highlight how technical advance has allowed achieving results with wide scale application, as shown by the increasing number of clinical trials that include EV

testing as part of the clinical endpoints and the FDA approval of a urine EV-based diagnostic test for prostate cancer [218]. Despite the utility of EVs is still confined to their application as biomarkers, this still marks their definitive entrance into clinical practice. Nonetheless, it can be envisaged that in near future successful cancer therapies may rely on EV-mediated amplification of desired effects on the immune system, potentially contributing thereby to disease control and elimination. Based on the limited effects induced by DC EVs observed in initial clinical trials, EVs deriving from DCs or other stimulatory immune cells and subjected or not to manipulations, have been proposed as adjuvants in combination with cancer therapeutics, such as ICIs [219]. Despite its feasibility only at preclinical level, manipulation of EVs may induce a reprogramming of the tumor microenvironment contributing to revert the prevailing immunosuppressive conditions, as shown for macrophage-derived EVs loaded with metformin and modified to express CD206 mannose receptor for targeting to M2-line TAM, present in the tumor microenvironment, leading to M1 reprogramming of these tumor-promoting macrophages [220]. This study is only one of the multiple promising strategies enabled by technological advances that may direct natural EVs or EV-membrane coated synthetic nanoparticles generated from different materials into clinical practice [221]. The exploitation of tumor EVs as natural source of tumor antigens could instead find application in the EVIR (EV-internalizing receptor) platform, where chimeric receptors enable specific and efficient uptake of cancer EVs by antigen presenting cells. Squadrito et al propose to potentiate DC presentation of EV-associated tumor antigens to $CD8^+$ T cells via MHCI recycling and cross-dressing [222].

An aspect not to be underestimated is the changing landscape of immune and/or tumor EVs elicited by the different cancer therapies, which may generate new predictive markers as well as therapeutic targets. Especially plasma EVs collected at baseline and at different time points during therapy with ICIs have generated enthusiasm among the EV and non-EV scientific community, including our group [156,223]. Numerous studies performed in search of biomarkers of response and/or resistance to ICIs have evaluated the transcriptome [224] and miRnome [225] and the proteome [226] by high-throughput analysis of whole plasma EVs, isolated according to different protocols. Apart from generating signatures potentially applicable to predict patients' outcome, these studies generated huge amounts of information, which may be accessed by the general scientific community to investigate the mechanisms underlying specific observations or validate results obtained with small-scale approaches. EV analysis is still complicated by the impossibility of thorough identification of EV cellular origins, and capturing methods based on the selection of

vesicles expressing specific EV markers, such as CD63, CD81 or CD9, enable refining the EV population of interest, but still cannot distinguish EVs deriving from particular cells. This may be overcome by capturing via cell surface proteins expressed also on EV surface, as shown for tumor EVs. In fact, quantification and isolation of tumor EVs based on the expression of tumor antigens has attracted interest soon after their discovery, as shown by the myriad of findings. To mention only some examples, in pancreatic cancer levels of glypican-1 in circulating EVs associate with tumor burden and patients survival [227], while the amount of PD-L1 expressing plasma EVs account for response/non response to PD-1 antibodies of melanoma patients [191]. In breast cancer patients proteomic analysis of EVs represents a promising approach for early detection and therapeutic monitoring of disease and high-risk relapse predictors. Of note, the authors reported stage-specific protein signatures, protein-based distinct clusters of healthy controls, chemotherapy-treated and untreated postsurgery samples [228]. Other studies distinguished cancer from non-cancer EVs in the same samples, as recently shown by the group of Theresa Whiteside who separated melanoma-derived EVs, based on their chondroitin sulfate peptidoglycan 4 (CSPG4) melanoma antigen expression, and both fractions were subjected to proteomic profiling [229]. Similar studies were performed by the same group in head and neck cancer patients, this time capturing T cell EVs expressing CD3 and tumor EVs to measure response to therapies, including targeted and immunotherapies with ICIs and radiotherapy [230]. Despite the number of patients evaluated in these studies is generally small, the investigation of a specific EV immune population, as described for $CD3^+$ T cell-deriving EVs, appears of groundbreaking interest, as this may represent the future development to capture all the nuances of the changing landscape of immune EVs composing vesicle populations.

Knowledge about EVs is rapidly evolving and every day milestones are reached in uncovering new aspects and corroborating ‘old’ findings at large scale. The technological advance enables an EV science that was a dream only a few years ago enlightening more and more the many still hidden secrets of EV heterogeneity, purposes and exploitation. We can envisage that just like changes of the immune cell population dictate the fate of cancer, EVs may represent a breakthrough in reflecting alterations of immunity.

Declarations

Seminars in Cancer Biology requires that all authors sign a declaration of conflicting interests. If you have nothing to declare in any of these categories then this should be stated.

Conflict of Interest

A conflicting interest exists when professional judgment concerning a primary interest (such as patient's welfare or the validity of research) may be influenced by a secondary interest (such as financial gain or personal rivalry). It may arise for the authors when they have financial interest that may influence their interpretation of their results or those of others. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

competing interests

I have no conflict of interest to declare.

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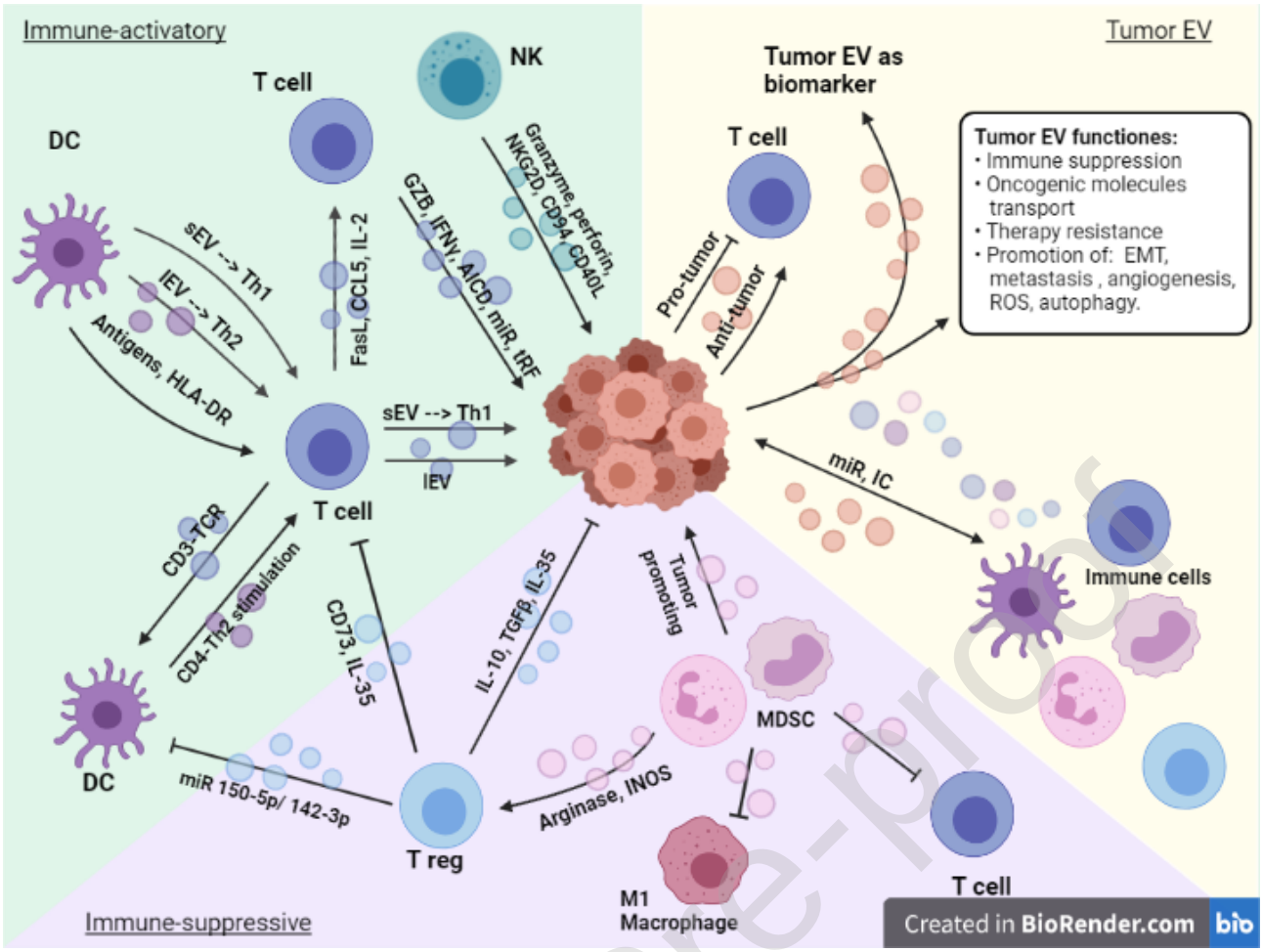

Figure 1. Overview of the molecules carried by tumor and immune-derived EVs and their effects on target cells. DC: dendritic cells; NK: natural killer cells; MDSC: myeloid-derived suppressor cells; Treg: T regulatory cells ; TCR: T cell receptor; sEV: small extracellular vesicles; lEV: large extracellular vesicles; tRF: tRNA fragments; IC: immune checkpoints; EMT: epithelial mesenchymal transition; ROS: reactive oxygen species; AICD: activation-induced cell death; iNOS: inducible NO-synthase; miR: microRNA. Created in Biorender.com.