

Chapter 19

Exosome and Secretion: Action On?



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Abstract Originally treated as part of a cellular waste, extracellular vesicles (EVs) are being shown to possess a vast variety of functions, of which exosome is the most studied one. Most cells, such as tumor cells, immunocytes, and fibroblasts can secrete exosomes, especially under certain stresses the amount is much higher, and the contents of exosome represent the status of the donor cells and the tumor microenvironment. As crucial transporters for cells' content exchange, much attention has been raised in the utilities of exosomes to suppress immune response, and to modify a microenvironment favorable for cancer progression. Exosomal immune checkpoints, such as programmed cell death ligand 1 (PD-L1), contribute to immunosuppression and are associated with anti-PD-1 response. Many forms of soluble immune checkpoint receptors have also been shown to influence efficacy mediated by their therapeutic antibodies. Therefore, targeting pro-tumorous exosomes may achieve antitumor effect supplementary to existing therapies. Exosome, itself natural liposome-like structure, allows it to be a potential drug delivery tool.

Keywords Tumor-derived exosome · Exosomal PD-L1 · Soluble immune checkpoint receptors · miRNA

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455

19.1 The Introduction of Exosome in Cancer Immunology

Mammalian cells synthesize and release heterogeneous EVs which can be generally subclassified as exosomes (30–120 nm in diameter), microvesicles (MVs, or ectosomes or microparticles, 0.1–1.0 μm), and apoptotic bodies (0.8–5.0 μm), each differing in their biogenesis, composition, and biological functions from others (Han et al. 2019) (Fig. 19.1). Exosomes are considered to be endosomally derived, while MVs bud from the surface of plasma membrane. EVs are circular pieces of membranes that incorporate various bioactive molecules including membrane receptors, proteins, mRNAs, microRNAs (miRNAs), and organelles, and thus potentially affect target cells by transferring their cargo or by receptor-to-ligand interaction. Endosomally-derived vesicles was first reported in transferrin recycling Rat reticulocyte endocytoses and then releases transferrin through multivesicular endosomes or multivesicular bodies (MVBs), which were described as often in 250–300 nm diameter but range from 120 to 800 nm diameter and may be irregularly shaped, into the surrounding microenvironment via fusion with plasma membrane of the parental cell. The MVBs intracellular passage does not enter into lysosome (Pan and Johnstone 1983; Harding et al. 1983).

The biogenesis of exosomes includes three distinct steps, begin with the development of endocytic vesicles by inward budding of the plasma membrane, then the generation of MVBs by invagination of the endosomal membrane, and at last, the fusion of plasma membrane with MVBs and subsequent release of the vesicular components, named exosomes (Batista et al. 2011) (Fig. 19.1). Exosomes and other EVs differ in size, density, morphology, marker expression, and rely on specific enzymes for their biogenesis. Vital enzymes in their generation include budding of intravesicular vesicles promoter—NSMASE2 (aka SMPD3), and fusion of the MVB

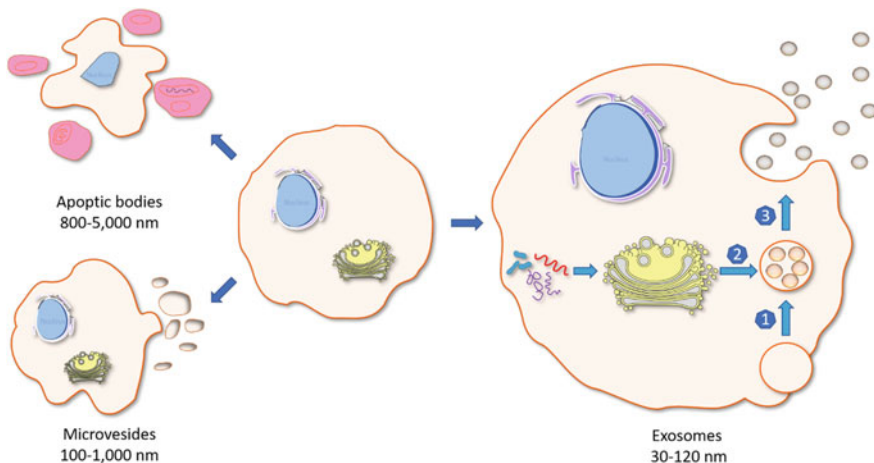


Fig. 19.1 The biogenesis of exosomes

to the plasma membrane catalyzer—RAB27A (Ostrowski et al. 2010; Kosaka et al. 2010). Genetic manipulation of these enzymes provides an opportunity to dissect the role of exosomes in vivo. Various exosome biomarkers have been identified, for example, TSG101, Ras-related protein Rab-11B (RAB11B), CD63, charged multivesicular body protein 2a (CHMP2A), and CD81 proteins, as well as lipids, including cholesterol, ceramide, sphingomyelin, and phosphatidylserine (Dickens et al. 2017; Colombo et al. 2014; Raposo and Stoorvogel 2013).

The isolation of pure exosomes is vital to uncover their action mechanisms and for further application in biomedical sciences. Since they are tiny and the isolation is challenging, a variety of techniques have been developed to facilitate the isolation. Successful isolation of exosomes can be achieved in a variety of ways like ultracentrifugation, ultrafiltration, chromatography, affinity capture on antibody-coupled magnetic beads, and polymer-based precipitation (Peterson et al. 2015). Practically, the size difference between cells, subpopulations of EVs, and proteins allow them to be separated and isolated by centrifugation. Ultrafiltration is rapid but hard to get rid of contaminating proteins, which can be improved by combining with ultracentrifugation. Membranes are used to filtrate cells and large EVs, and then ultracentrifugation can further separate exosomes from proteins (Alvarez et al. 2012). Aiming to purify exosome isolations, immuno-affinity purification (IP) techniques have been adopted that can selectively capture desired exosomes from a complex population basing on certain surface markers. IP techniques are rapid, convenient, and compatible with regular laboratory equipment, during which process anti-CD63, anti-CD9, and anti-CD81 antibodies are typically used (Tauro et al. 2012).

Exosomes released from tumor cells actively lead to tumor progression and metastasis (Chiodoni et al. 2019). It has been shown by studies that exosome released from cancer cells can be stimulated under different stresses, which also cause the alteration of exosome's content. Oxidative and heat stresses have been shown to induce the exosome generation from leukemia/lymphoma T and B cell lines (Hedlund et al. 2011) and hypoxic environment has been reported to effectively augment tumor-derived exosomes (TEX) shedding from breast cancer cells (Wang et al. 2014). Moreover, sublethal doses of a number of chemotherapeutic drugs, such as proteasome inhibitors and genotoxic drugs, enhance exosome release in various cancer models. As such, multiple myeloma (MM) cells can enhance the release of nanovesicles receiving bortezomib or melphalan treatment (Lehmann et al. 2008; Yu et al. 2006).

Tumor-suppressor gene p53 has been described in stress-induced exosome secretion. In this regard, irradiation of prostate cancer cells triggered an amplified secretion of exosome-like vesicles by p53 activation (Lehmann et al. 2008). Furthermore, a p53-inducible gene product, the tumor-suppressor-activated pathway 6 (TSAP6), was found to regulate exosome trafficking and secretion in cells undergoing DNA damage (Yu et al. 2006; Amzallag et al. 2004). The fact that exosome secretion is reported to be severely restrained in TSAP6-deficient mice can further support these observations (Lespagnol et al. 2008). Moreover, increased exosome secretion in tumor cells can be observed in a senescent phenotype (Borrelli et al. 2018), which can pose pro-tumorigenic effects in pre-malignant recipient cells. Senescence-associated secretory

phenotype (SASP) can be enhanced either by inactivation of p53 or gain of oncogenic RAS (Coppe et al. 2008).

EVs are implicated in multiply processes, such as tumor cells proliferation, chemoresistance, immune escape, metabolic reprogramming, metastatic enhancement, and angiogenesis. Herein, we will mostly focus on how exosomes affect cancer immunology.

19.2 The Role of TEX in Cancer Immunology (Detailed in Fig. 19.2)

By generating an immunosuppressive microenvironment, TEX is beneficial for tumor growth and distant dissemination. Studies have been showing the mechanisms and targets of immune suppression by TEX are diverse (Graner et al. 2018), while some reporting TEX is immune activator. Both success of immune-based therapies and active endogenous antitumor immune responses rely on effector cells that directly target and kill cancer cells, such as cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells. We will herein discuss how TEX contributes to those cancer-related immunocytes' function.

19.2.1 TEX Suppresses T Cell Functions

Tumor cells actively release immunosuppressive MVs into the microenvironment, disrupting T cell immunosurveillance. Tumor-derived MVs harvested in suspension

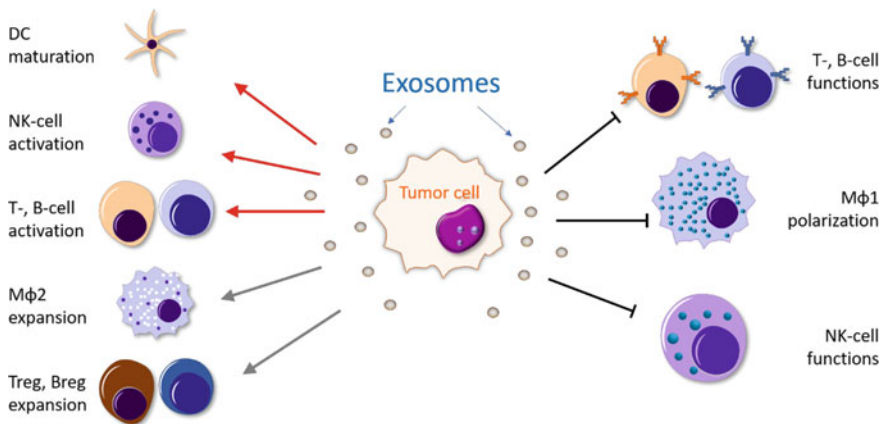


Fig. 19.2 The roles of tumor-derived exosomes in cancer immunology

of head and neck squamous cell carcinoma (SCCHN) cell line, PCI-13, with combination of size-exclusion chromatography and ultracentrifugation methods, were variably enriched in major histocompatibility complex (MHC) class I. And those MVs suppressed signaling and expansion, and induced apoptosis of activated CTLs, while enhancing the proliferation and suppressor activity of CD4⁺ CD25⁺ FOXP3⁺ Treg cells (Wieckowski et al. 2009), leading to immune suppression to allow tumor escape. Melanoma cell line-derived exosomes are able to alter the metabolic function of CTL cell line, CTLL-2, by transferring mRNA/miRNA contents. Notably, the delivery of mRNA/miRNA loaded exosome happened within a short time frame. Four of the top 20 mRNAs found within B16F0 exosomes (such as Cmtm4, Wsb2, Ptpn14, and Fam168b) were detected in CTLL-2 cells in 30 min. Another cluster of genes was upregulated at 4 h upon exosome treatment, which contained several gene targets involved with cellular metabolism. CMTM4 mRNA is upregulated in TEX exposed CTLL-2 cells, and this is potentially significant given its role in PD-L1 trafficking (Mezzadra et al. 2017).

Exosomes isolated from the ascites of ovarian cancer (OVCA) patients are identified, which can be internalized rapidly by T cells, and inhibited various T cell response endpoints like translocation of NFAT and NF κ B into the nucleus, upregulation of CD69 and CD107a, release of cytokines, and elevated cell proliferation. However, T cell viability was not affected and T cell arrest was transient. After removal of the immunosuppressive exosomes, T cell regained their activation potential within 24 h (Shenoy et al. 2018).

However, TEX can activate CTL clones after processing by antigen-presenting cell (APC) expressing the correct MHC haplotype (Andre et al. 2002). TEX comprises cancer-related antigens that may initiate an immune response using dendritic cell (DC) as intermediaries. Melanoma-associated antigen (MAGE) was enriched in TEX from malignant ascites of patients with melanoma, and it can be recognized by T cells (MART-1). These TEX, once delivered to DCs, facilitated in vitro cross-presentation of the antigen, and response of a CTL clone, leading to an efficient in vitro antitumor cellular activation, as monitored by the quantity of interferon (IFN)- γ produced, and by the induction of specific cancer cell lysis (Andre et al. 2002; Wolfers et al. 2001).

19.2.2 Mechanisms of TEX Influencing NK Cells and DCs

TEX can regulate the functions of NK cells, by affecting the differentiation of precursors to mature antigen-presenting cells. NKG2D (activating natural killer group 2 member D) ligand on tumor cells triggers cytotoxic activity in NK cells through interacting with NKG2D (Bauer et al. 1999; Rincon-Orozco et al. 2005). TEX NKG2D is loaded with many other functional molecules including death receptor ligands, MHC class I/II, adhesion molecules, etc. (Whiteside 2013). Therefore, the alteration of the immune response by NKG2DL containing TEX is also impacted by other co-existing molecules within the exosome (Clayton et al. 2008). NKG2D interacts with several kinds of ligands. For instance, the NKG2DL in humans includes MICA/B and

UL16-binding proteins 1–6 (ULBP1–6), exosomal MICA*008 decreased NKG2D expression on NK cell and suppressed its cytotoxicity (Ashiru et al. 2010). Exosomal ULBP3 was also reported to bring down NKG2D on primary NK cells and restrain NK cell-mediated elimination of MICA-expressing target cells (Fernandez-Messina et al. 2010). Nevertheless, human DCs can secrete exosomal NKG2DL to directly enhance NK cells' function *ex vivo* (Viaud et al. 2009).

The other extracellular presence of NKG2DL is soluble form, which can down-regulate NKG2D on NK cells or T cells, leading to suppressed cytotoxicity. Soluble MICA (sMICA) downregulated NKG2D by enhancing its endocytosis and then degradation, leading to a reduced NKG2D expression on tumor-infiltrating T cells (Groh et al. 2002). Similarly, sULBP was found to downregulate NKG2D on NK cells (Fernandez-Messina et al. 2010).

Moreover, heat shock protein 70 (Hsp70) on tumor cell surfaces is a recognized ligand for the NK cell receptor CD94 and can enhance the cytotoxicity of NK cells against Hsp70-positive tumor target cells (Gross et al. 2003). By contrast, TEX surface Hsp70 will cause reduction of NK CD94, and in this case TEX functions as systemic decoy for NK cells (Hedlund et al. 2011). In addition, transforming growth factor β (TGF- β), acting as a component of TEX (Graner et al. 2009), has also been shown to be an immune suppressor of NK cells (Szczepanski et al. 2011). Fetal liver mesenchymal stem cell (mSC)-derived exosomes contain several immunomodulatory molecules—latency-associated peptide (LAP), TGF β , and thrombospondin 1 (TSP1), through stimulating the downstream TGF- β /Smad2/3 signaling, inhibiting proliferation, activation, and cytotoxicity of NK cells (Fan et al. 2019).

In normal human cells, inhibition of exosome secretion reduces removal of harmful nuclear DNA, causes the stacking of nuclear DNA, and thus activates cGAS-STING pathway and causes type I IFN production from those cells (Takahashi et al. 2017), indicating exosome secretion is vital for maintaining parental cell's homeostasis. Exosomal double-stranded (Ds) DNA secreted from irradiated mouse breast cancer cells could be transferred to DCs and upregulate DCs' surface costimulatory molecules, leading to the STING-dependent activation of type I IFNs (Diamond et al. 2018). The same phenomenon has been observed under the antitumor agent topotecan, an inhibitor of topoisomerase I treatment (Kitai et al. 2017). Collectively, these findings indicate that dsDNA associated with TEXs induces type I IFN production directly from cancer cells or indirectly through the DCs stimulation. Type I IFNs are known to play a crucial role in cancer progression through the promotion of anti-cancer immune responses, (Medrano et al. 2017; Zitvogel et al. 2015) such as directly activate NK cell-mediated functions, increase perforin-dependent cytotoxicity (Nguyen et al. 2002), and induce TNF-related apoptosis-inducing ligand (TRAIL) expression. In addition, with the coordinated action of IL-12, type I IFNs greatly promote NK cell-mediated IFN- γ production.

19.2.3 Mechanisms of TEX Influencing Macrophages

Preclinical and clinical studies indicate that tumor-associated macrophages (TAMs) provide important pro-tumorigenic and survival factors (Noy and Pollard 2014). TEX stimulates the macrophage infiltration and polarization in remote site for establishment of premetastatic niche. After injection of breast TEXs into mice, the amount of macrophage is shown to be increased in axillary lymph nodes, with CD206 positive M2 macrophages much more detected than NOS2 positive M1 macrophages (Piao et al. 2018). Regarding macrophages, toll-like receptor (TLR) signaling is vital for pro-inflammatory cytokines, miRNAs, and other components secretion, greatly enhancing inflammation and favoring cancer development. Through TLR on macrophages, breast TEXs activate NF- κ B and induce secretion of G-CSF, TNF- α , IL-6, and CCL2, while genetic depletion of TLR2 or MyD88, a vital signaling adaptor of the NF- κ B pathway, completely abrogates this effect (Chow et al. 2014). TEX can also induce monocytes to release immune-modifying factors. TEX in chronic lymphocytic leukemia (CLL) can enhance monocytes to secrete cytokines IL-6, CCL2, and CCL4, and express PD-L1 (Haderk et al. 2017). HY4, a noncoding Y RNA enriched in exosomes of CLL patient plasma can achieve the same effects on monocytes as TEX, resulting in cancer-associated inflammation and potential immune evasion via PD-L1 upregulation.

Under hypoxic stress, TEX enhances oxidative phosphorylation in bone marrow-derived macrophages through transfer of let-7a miRNA and subsequent suppression of the insulin-Akt-mTOR signaling pathway, leading to M2 polarization (Park et al. 2019).

However, macrophage receiving TEX miRNAs also have antitumor effects, as in the case where murine breast cancer was treated with EGCG (epigallocatechin gallate), a component of green tea extract with known anti-cancer properties (Jang et al. 2013). EGCG enhances TEX-derived miR-16, which is an important regulator of CHUK/IKK α (inhibitor of nuclear factor kappa-B kinase subunit alpha) complex. Delivery of these TEX to macrophage is favorable for M1 phenotype cytokines secretion. MiR-16 causes reduction of CHUK/IKK α complex and subsequent accumulation of I- κ B, thus preventing NF- κ B activation and M2 phenotype cytokines production (Hagemann et al. 2009; Lawrence 2009).

19.2.4 Other Immune Cell Lineages

Other immune cells are also subject to the impact of EVs produced by tumor cells. B cells primed by hepatocellular carcinoma (HCC) cell line-derived exosome strongly expressed T cell immunoglobulin-1 (TIM-1) protein and were endowed with suppressive activity against CD8+ T cells. A major portion of TIM-1 is expressed by B cells and serves as a marker for Breg cells (Jang et al. 2013). HCC-exosome has high level of HMGB1, which activates B cells and promotes TIM-1⁺Breg cell expansion

through the TLR2/4 and mitogen-activated protein kinase (MAPK) signaling pathways (Yan et al. 2012, 2018). TIM-1+ Breg cells secrete the highest proportion of IL-10 among all types of B cells.

19.3 Exosome and Cancer Immune Checkpoints (Fig. 19.3)

19.3.1 Exosomal PD-L1

Tumor cells evade the immune surveillance through increasing surface expression of PD-L1, which interacts with PD-1 on T cells (Dong et al. 2002; Chen and Han 2015), contributing to dephosphorylation of the T cell receptor as well as its co-receptor CD28 through Shp2 phosphatase, thus inhibiting antigen-driven activation of T cells (Graner et al. 2018; Hui et al. 2017). Therapeutic antibodies of PD-L1 and PD-1 block this interaction, and therefore can reactivate the antitumor immune effect (Chen and Mellman 2017). Both anti-PD-1 and PD-L1 antibodies have shown remarkable promise in curing tumors, such as renal cell carcinoma and metastatic melanoma (Chen and Han 2015; Ribas et al. 2016; Topalian et al. 2016). Unfortunately, a major portion of patients have low response rate (Ribas et al. 2016; Zaretsky et al. 2016), requiring deep understanding of PD-L1-mediated immune evasion to predict patient response and promote treatment efficacy.

PD-L1 is found on the surface of EVs, and more interestingly EV PD-L1 levels have been related with cancer progression and response to immunotherapy (Yang et al. 2018; Theodoraki et al. 2018; Ricklefs et al. 2018; Chen et al. 2018). Chen et al. reported that metastatic melanomas released EVs, with exosomes being the major form, bearing PD-L1 on their surface. Treatment with IFN- γ can enhance PD-L1 level on these vesicles, leading to CTLs dysfunction and tumor growth acceleration.

The evolvement of systemic exosomal PD-L1 along the course of anti-PD-1 therapy has predictive value for cancer prognosis. The amplitudes of the increase in systemic exosomal PD-L1 during early stages of pembrolizumab treatment, indicating the adaptive response of the tumor cells to T cell re-invigoration, can stratify clinical responders from non-responders. However, circulating exosomal PD-L1 before and on treatment may reflect different states of antitumor immunity. High pretreatment level may signify the “exhaustion” of patient T cells to a turning point, by which they are unable to be re-invigorated by the anti-PD-1 treatment. For the on-treatment patients, on the contrary, a rise in the level of exosomal PD-L1, correlating proportionally with the T cell re-invigoration, would represent a strong antitumor immunity, thereby a favorable prognosis. Therefore, TEX PD-L1 may serve as a predictor for anti-PD-1 therapy efficacy (Chen et al. 2018).

PD-L1 from TEX imposes systemic immunosuppression through inhibiting T cell activation in the draining lymph node. Wild-type tumor cells grow slower with exposure to exosomal PD-L1-deficient tumor cells, which are injected simultaneously at a distant site. Inhibition of exosomal PD-L1 induces systemic antitumor immunity,

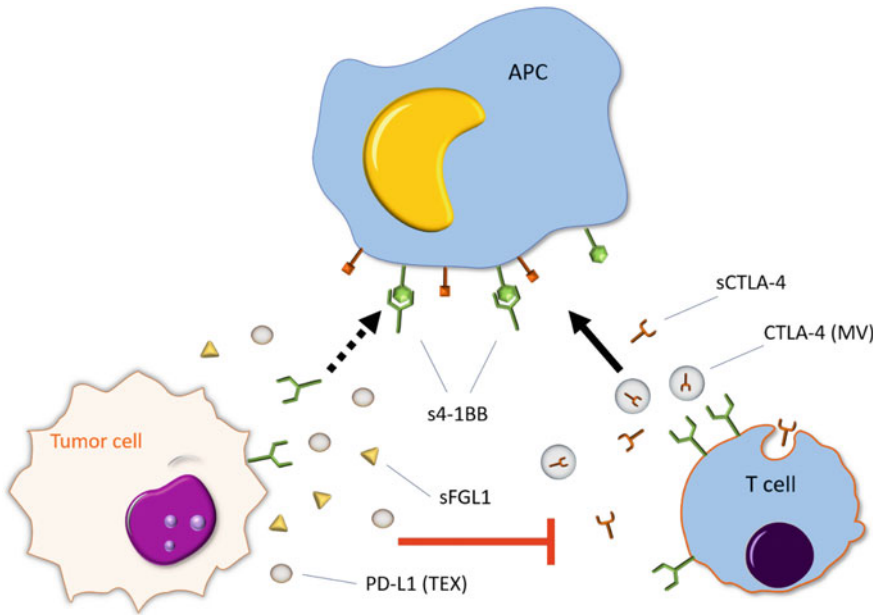


Fig. 19.3 Cross talk among tumor cells, APCs and T cells

even in models resistant to anti-PD-L1 antibodies (Poggio et al. 2019). Systemically introduced exosomal PD-L1 rescues growth of tumors unable to secrete their own. Anti-PD-L1 antibodies work additively, not redundantly, with exosomal PD-L1 blockade to suppress tumor growth.

Not only PD-L1 proteins, but also PD-L1 mRNA can be detected in exosomes. Researchers have found that PD-L1 mRNA contained exosome was more enriched in periodontitis patients than control subjects. Exosomal PD-L1 mRNA level in saliva correlates with the severity/stage of periodontitis, and can potentially be used to distinguish periodontitis from the healthy (Yu et al. 2019).

Yang et al. revealed in cancer cell lines, exosomal PD-L1 significantly suppressed CD3/CD28-driven ERK phosphorylation and NF- κ B stimulation of T cells in a dose-dependent manner, as well as PHA-induced interleukin-2 (IL-2) secretion, leading to T cell dysfunction. Exosomes are capable of transferring functional PD-L1 to other cells. Exosomal PD-L1 detected on the surface of target cells is able to bind to PD-1 and mount an immunosuppressive effect. Suppression of exosome secretion from 4T1 tumor cells by either pharmacological inhibitor GW4869 treatment or Rab27a knocks down remarkably restrained tumor growth, and hence the antitumor effect is relatively superior than anti-PD-L1 therapeutic treatment. Combination of inhibition of exosome secretion and anti-PD-L1 antibody treatment achieved much stronger tumor suppression.

Researchers also reported exosomal release of PD-L1 occurs at the expense of surface PD-L1 levels. ALIX, as endosomal sorting complexes required for transporting (ESCRT)-associated protein, is the regulator of both PD-L1 surface presentation and EGFR activity in basal-like breast cancer (BLBC) cells. Besides locating on cell surface, PD-L1 also exits in the limiting membrane as well as intraluminal vesicle (ILVs) of CD63-positive MVBs in HCC1954 cells after IFN γ treatment. ILVs are the intracellular precursors of extracellular vesicles. Failure of PD-L1 incorporation in ILV leads to defective exosomal packaging, and following MVB-PM (plasma membrane) fusion thereby enhances cell surface PD-L1. In ALIX^{KD} cells, a higher ratio of PD-L1 was observed at the limiting membrane of MVBs, compared within the endosomal lumen, resulting in prolonged and enhanced stimulation-induced EGFR activity as well as defective PD-L1 exosomal release, and its promoted redistribution to the cell surface, which implies an enhanced immunosuppressive phenotype (Monypenny et al. 2018).

19.3.2 Soluble Immune Checkpoint Receptors

The immunomodulatory interactions of cytotoxic T lymphocyte antigen-4 (CTLA-4), 4-1BB and PD-1 on T cells with their corresponding ligands on APCs or tumor cells have been extensively studied. Antibodies such as anti-CTLA-4 (ipilimumab) and anti-PD-1 (nivolumab) that counter these ligand–receptor interactions have shown clinical improvements in patients with solid tumors or autoimmune diseases. Still, a large portion of patient is unresponsive to these therapies and the full immunomodulatory mechanisms of these ligand–receptor interactions have not been resolved. More researchers are trying to uncover alternatively spliced soluble isoforms of these receptors to amplify efficacy mediated by their therapeutic antibodies.

19.3.2.1 Soluble PD-L1 as a Biomarker in Patients Treated with Checkpoint Inhibitors

Soluble PD-L1 (sPD-L1) may serve as a putative predictive biomarker for disease outcome and patient stratification under some circumstances. For instance, sPD-L1 may be a marker of systemic inflammation in pancreatic cancer (Kruger et al. 2017). sPD-L1 level in sera correlates with aggressiveness of renal cell carcinoma (RCC) and predicts survival in patients with MM or diffuse large B cell lymphoma (Wang et al. 2015; Rossille et al. 2014; Frigola et al. 2011).

sPD-L1 detected in the sera of RCC patients may cause systemic immunosuppression, facilitating tumor progression and resulting in poor prognosis (Frigola et al. 2011). Higher preoperative sPD-L1 levels were associated with poor clinical characteristics, including larger tumor volume, later stage, higher grade, and more tumor necrosis. Twice of sPD-L1 levels was related with a 41% increased risk of death. SPD-L1 was also detected in the cell supernatants of some PD-L1-positive RCC cell

lines. Those sPD-L1s retained receptor-binding domain was indicated by protein sequencing and was able to trigger pro-apoptotic signals in T cells (Frigola et al. 2011).

Multiple splice variants of PD-L1 have been identified and elevated sPD-L1 was observed in sera of patients with metastatic melanoma compared with healthy. High pretreatment sPD-L1 levels are correlated with rapid deterioration under anti-CTLA-4 or anti-PD1-based therapy, perhaps due to enhancing aberrant splicing activities in tumor cells, large tumor burden, or a diminishing antitumor immune effect, which are not easy to treat with a checkpoint blockade. Rise in sPD-L1 after 5 months of treatment rather than early alterations of sPD-L1 levels correlated with partial responses when receiving anti-CTLA-4 or anti-PD-1 therapy (Zhou et al. 2017).

Soluble PD-L1 harbors potential utility for antitumor therapy by blocking PD-1/B7-H1 pathway in murine model. In preclinical models of hepatocarcinoma (HCC), intramuscular injection of a plasmid encoding sPD-1 was reported to enhance lysis of tumor cells and extend overall survival of tumor-bearing mice (He et al. 2005).

19.3.2.2 Soluble CTLA4

The transmembrane isoform of CTLA-4 (Tm-CTLA-4) receptor plays a critical role in downregulating the immune response and sustaining the immune homeostasis. Alternatively spliced mRNA of the CTLA-4 gene that lacks exon 3 is found in human, mouse, and rat immune cells (Magistrelli et al. 1999; Oaks et al. 2000). When sCTLA-4 was first described, it was considered as a product of resting T cells, with its manufacture being cut down following T cell activation (Oaks et al. 2000). However, recently researchers have shown human T cells release more sCTLA-4 under physiological stimuli such as peptide immunogens or other recall antigens, indicating that sCTLA-4 may have functions relevant to ongoing immune responses (Ward et al. 2013). Antagonism of sCTLA-4 by isoform-specific monoclonal antibody (mAb) could remarkably stimulate antigen-dependent immune effects in a range of experimental systems *in vitro* and *in vivo*, as is shown in enhanced cell proliferation and pro-inflammatory cytokine levels like IFN- γ . Furthermore, in a melanoma-bearing B16F10 mouse model, isoform-selective anti-sCTLA-4 mAb treatment can achieve similar effect as panCTLA-4 mAb on the reduction of lung metastases, demonstrating soluble isoform is capable of modulating the overall outcome (Ward et al. 2013).

The extracellular domain of sCTLA-4, similar to that of the integral membrane isoform, has the MYPPY motif that can bind to the CD28-shared CD80/CD86 ligands on APCs. In a mixed lymphocyte response, recombinant sCTLA-4 showed immunomodulatory effect on inhibiting cell proliferation in a dose-dependent manner (Oaks et al. 2000).

19.3.2.3 Other Soluble Immune Checkpoints

Soluble 4-1BB can restrain over-zealous immune responses by acting in a negative feedback loop, as shown in animal models to suppress development of type I diabetes (Kachapati et al. 2013). Moreover, human renal, lung, melanoma, and hepatocellular tumor cell lines can generate s4-1BB under hypoxic stress, causing 4-1BB ligand engagement, thus blocking its costimulatory effect (Labiano et al. 2016).

Lymphocyte-activation gene 3 (LAG-3) is an immune suppressive receptor, with major MHC-II as a canonical ligand. Wang et al. demonstrated that fibrinogen-like protein 1 (FGL1), secreted by liver, acted as the major LAG-3 ligand independent of MHC-II. Inhibition of antigen-specific T cell activation was observed by FGL1-LAG-3 interaction, ablation of which enhances T cell response in mice. Tumor cells generated excessive FGL1, and increased plasma FGL1 was correlated with stronger resistance to anti-PD-1/PD-L1 therapy and a poor outcome in patients (Wang et al. 2019).

19.4 Immunocytes-Derived Exosomes in Cancer Immunology

Not only tumor cells, but also a variety of immune cells are able to release exosomes, such as T cells, DCs, macrophages, B cells, and mast cells (Skokos et al. 2003). Immunocytes-derived exosomes are shown to modulate tumor microenvironment and affect cancer outcome.

19.4.1 T Cell-Derived Exosomes

T cell-derived exosomes contain heterogeneous components, targeting divergent cells, and perform distinct types of function. Using proteomic approach, researchers were able to show activated T cells can secrete exosomes that contain signaling components associated with RAS, such as ZAP70, RAP1, RASGRP1, and AKT, and these vesicles can lead to ERK phosphorylation in mast cells (Azoulay-Alfaguter and Mor 2018). Activated T cells can release EVs which promote proliferation of autologous resting CD8 T cells (Wahlgren et al. 2012). T cells were activated and released exosomes after interaction with antigen-bearing DCs. In return, T cells could enhance the protective roles of DCs via transfer of exosomal DNA, and may modulate the immune system when encountering threats (Torralba et al. 2018). On the other hand, T cell-derived exosomes can prevent autoimmune damage through bioactive FasL and TRAIL, which eliminated activated T cells (Monleon et al. 2001).

Fibroblastic tumor stroma consisting of mesenchymal stem cells (MSCs) and cancer-associated fibroblasts (CAFs) promotes the invasion and metastasis of cancer

cells. EVs derived from activated CD8⁺ T cell could disrupt fibroblastic stroma-mediated tumor growth, as evidenced from activated CD8⁺ T cells in healthy mice transiently secreting cytotoxic EVs which causes significant inhibition of invasive and metastatic properties of tumor through apoptotic elimination of mesenchymal tumor stromal cells. EV-releasing CD8⁺ T cells infiltrate in neovascular areas with high mesenchymal cell density, and tumor MSCs preferentially engulf CD8⁺ T cell-derived EVs than other cell populations like tumor cells. Thereby, CD8⁺ T cells can prevent cancer progression via EV-mediated depletion of mesenchymal tumor stromal cells besides their conventional direct cytotoxicity (Seo et al. 2018).

Follicular helper T cells (Tfh cells) secreted exosomes correlate with occurrence and progression of antibody-mediated rejection (AMR) in renal transplantation. Tfh cell-derived exosomes could promote the proliferation and differentiation of B cells and may play a critical role in the development of AMR after renal transplantation. Analysis of the peripheral blood from 42 kidney transplant patients indicated that CTLA-4 level of CD4⁺ CXCR5⁺ exosomes was significantly lower in AMR group than that in non-AMR group (Yang et al. 2019).

19.4.2 NK Cell-Derived Exosomes

NK cells in tumor microenvironment could attenuate cancer progression through their exosomes. NK cell-derived exosomes performed cell-killing activity targeting cancer cells through the cytotoxicity factors, including perforin, granulysin, and granzymes A and B, which were able to activate caspase pathways in tumor cells, as well as blocking caspase inhibitors (Jong et al. 2017). NK cell-derived exosomes contain tumor-suppressive miR-186, which downregulates certain oncogenic proteins, for example, the mitotic kinase aurora kinase A (AUKRA) and N-myc proto-oncogene protein. TGF- β 1 could inhibit the levels of miR-186 in NK cells, rendering NK cells inactive. Restoration of miR-186 levels increases the cell-killing capabilities of NK cells, resulting in decreased tumor burden and prolonged survival in neuroblastoma (Schmittgen 2019).

19.4.3 DC-Derived Exosomes

DC is the key player in antigen-presenting process, its EVs are in charge of inter-cellular communicators in adaptive immunity. DCs and DC-derived exosomes have several similarities. Like DCs, their exosomes express functional MHC-peptide complexes, T cell stimulatory factors, and other components that interact with other immune cells. After activated by T cells, DCs exhibited a capacity for antigen-specific T cell activation through exosomes (Lindenbergh et al. 2019). Accumulated evidence has shown that DC-derived exosomes could facilitate immune cell-dependent cancer therapy (Pitt et al. 2016). Given the component of DCs in modulating immune

responses, a majority of studies focused on the effect of DC-derived exosomes against tumor progression, the potential immune-modifying function, and feasibility and safety of application (Chen et al. 2018a, b; Pitt et al. 2014). DC-derived exosomes can limit cancer cells via activation of naïve T cells and NK cells (Gao and Jiang 2018).

19.4.4 Macrophage-Derived Exosomes (MDE)

Clinical and experimental evidence has shown that tumor-associated macrophages induce cancer initiation and progression. MDE has been shown to accelerate colorectal cancer (CRC) cells' migration and invasion through its miRNA contents including miR-21-5p and miR-155-5p. Both miR-21-5p and miR-155-5p downregulate expression of BRG1, which is crucial for the CRC metastasis (Lan et al. 2019). After activated by IL-4, macrophage can enhance invasiveness of breast cancer cells via the Mef2c-b-catenin pathway through transferring miR-233. MiR-223 antisense oligonucleotide reduced the expression of miR-223 in macrophages, thus depressing the invasiveness of the co-cultured breast cancer cells (Yang et al. 2011).

Hypoxic epithelial ovarian cancer (EOC) cells induced macrophages into a TAM-like phenotype, which then deliver exosomes to the co-cultivated EOC cells, enhancing the malignant phenotype and drug resistant of EOC cells via the PTEN-PI3K/AKT pathway (Zhu et al. 2019).

19.4.5 Other Immunocytes-Derived Exosomes

B cell-derived exosomes mediate part of B cell's functions, including antigen-presenting capacities, MHC-restricted antigen recognition, and induction of different types of immune responses as well. In lymph nodes, B cell-derived exosomes were found to act reciprocally with CD169+ macrophages and further interrupted the spread of viruses or tumor cells (Saito et al. 2015). B cell-derived exosomes promoted T cell response, and those effects were independent of B cell presence or B cell-secreted antibody (Saunderson and McLellan 2017).

Myeloid-derived suppressor cells (MDSCs), known as an immune suppressor, are generated from immature myeloid cells under certain conditions. MDSC-derived exosomes contained different cargos in accordance with the immunosuppressive activity (Geis-Asteggiane et al. 2018). It was also demonstrated that MDSCs are present in cancer patients, inhibiting antitumor immunity and scrambling anti-cancer immunotherapies. MDSC-derived exosomes, which were primed by tumor milieu, could also promote oncogenesis (Burke et al. 2014).

19.5 Exosome as Biomarker and Vaccine for Cancer Progression

19.5.1 *Liquid Biopsy*

TEX delivers malignant signals in a variety of forms, including nucleic acids, such as messenger RNA (mRNA) and miRNAs, or proteins like chemokines, cytokines, growth factors, or angiogenic and immunomodulatory molecules (Chiodoni et al. 2019). Measuring exosomal contents would be noninvasive, however, a promising way to detect cancer occurrence and monitor tumor progression. The problem is exosomal-specific proteins are in very low abundance, and thus a large amount of serum or culture medium is needed to enrich sufficient exosome to conduct the proteomics or western blot measurement. Thereby, exosome protein profile is still in the starting stage. Genomic profile of the exosome, including miRNA, mRNA, long noncoding RNA (lncRNA), and mitochondrial RNA, being amplified by PCR to increase quantity seems to be excellent candidate biomarkers to subclassify tumor types.

19.5.2 *Exosomal miRNA (Detailed in Table 19.1)*

In recent years, much of the research on cancer blood biomarkers has shifted from protein-based to nucleic acid-based molecules (Chiodoni et al. 2019). For example, Fang et al. reported that hepatoma cells produced high levels of miR-103 and release it in exosomes to induce tumor metastasis, indicating exosome miR-103 can be used as a predictive marker for cancer progression (Fang et al. 2018). Shi et al. found exosome-derived miR-638 was significantly decreased in serum of HCC patients with advanced disease, such as at later TNM stage (III/IV) or with larger tumor size (>5 cm) (Shi et al. 2018). Moreover, several lncRNAs, like lnc-h19, lnc-sox2ot, and lncRNA-ARSR, have been investigated in circulating exosomes and closely related with tumor stage and overall survival of patients (Fang et al. 2018; Shi et al. 2018; Qu et al. 2016; Zhao et al. 2018; Lin et al. 2018; Conigliaro et al. 2015). Several studies have implicated exosome-derived miRNAs as potential biomarkers for detection of CRC occurrence and monitor recurrence. The serum levels of exosomal miRNAs, such as miR-1224-5p, miR-1229, miR-21, miR-223, miR-150, and let-7a, are much higher in CRC patients than healthy, dropping after tumor resection (Ruiz-Lopez et al. 2018). Exosomal miRNAs such as miR-19a, miR-18a, and miR-100 may be useful to detect the recurrence of CRC (Komatsu et al. 2014; Matsumura et al. 2015; Cha et al. 2015). These findings suggest that exosomal RNA molecules detected from circulation or other sources can serve as biomarkers to evaluate cancer occurrence and progression, with potentially high sensitivity and specificity.

Table 19.1 TEX miRNAs regulate immune cells' response

miRNA name	Target immune cell	Activation/suppression
miR-23a (Berchem et al. 2016)	NK cell	Suppression
miR-362-5p (Yang et al. 2015; Wu et al. 2015; Ni et al. 2016)	NK cell	Suppression
miR-24-3p (Ye et al. 2014)	T cell	Suppression
miR-891a (Ye et al. 2014)	T cell	Suppression
miR-106a-5p (Ye et al. 2014)	T cell	Suppression
miR-20a-5p (Ye et al. 2014)	T cell	Suppression
miR-1908 (Ye et al. 2014)	T cell	Suppression
miR-21 (Fabbri et al. 2012; Hsieh et al. 2018)	Macrophage	Activation/suppression
miR-214 (Yang et al. 2015)	T cell	Suppression
miR-146a-5p (Cheng et al. 2019)	T cell	Suppression
miR-212-3p (Ding et al. 2015)	Dendritic cells	Suppression
miR-203 (Zhou et al. 2014)	Dendritic cells	Suppression
miR-29a (Fabbri et al. 2012)	Macrophage/Dendritic cells	Suppression
miR-16 (Jang et al. 2013)	Macrophage (M2)	Activation
miR-222-3p (Ying et al. 2016)	Macrophage (M2)	Activation

19.5.2.1 TEX miRNAs and T Cells

Bland et al. found out that the tumor cell line B16F0 can deliver mRNA/miRNA loaded exosomes to CTLs and alter their metabolic function and IFN- γ production. TEX from nasopharyngeal carcinoma was reported to contain high levels of miR-106a-5p, miR-1908, miR-24-3p, miR-891a, and miR-20a-5p, yielding almost 20 targets linked to the MAPK1 pathway for potential downregulation. The net effect on T cells was a shift from Th1 and Th17 phenotypes to Th2 and Treg phenotypes, through suppression of ERK/STAT1/STAT3 phosphorylation (Ye et al. 2014). Evaluating overexpressed tumor miRNAs from patients with non-small cell lung cancer, breast cancer, pancreatic cancer or HCC, the ubiquitous miR-21 and miR-214 were shown to be consistently upregulated in tissue and in plasma exosomes/microvesicles. The same phenomenon was observed in murine sarcoma and lung cancer models, where miR-214 was enriched in MVs and downregulated PTEN in T cells, favoring Treg cell's expansion (Yin et al. 2014; Walsh et al. 2006), but runs somewhat contradictory to other reports concerning the role of PTEN in Treg maintenance (Shrestha et al. 2015; Sharma et al. 2015).

19.5.2.2 TEX miRNAs and NK Cells

In lung cancer and leukemia, TEX-derived miR-23a was found to decrease the level of LAMP1 (lysosome-associated membrane glycoprotein 1)/CD107a (Berchem et al. 2016), which is an NK cell activation marker signifying lymphocytes degranulation (Cohnen et al. 2013). As the TEX also deliver TGF β that inhibits NKG2D expression, they were generally considered as NK cell inhibitors. TEX-derived miR-362-5p has distinct effects depending on tumor cells themselves (Yang et al. 2015; Wu et al. 2015; Ni et al. 2016), but seems to be crucial in enhancing NK cell response via downregulation of CYLD, a suppressor of NF- κ B signaling (Ni et al. 2015). However, the overabundance of the miRNA could lead to overstimulation resulting in hypo-responsiveness (Shifrin et al. 2014).

19.5.2.3 TEX MiRNAs and Monocytes

It was shown TEX-pulsed DCs could supply antigens to T cells and promote effector T cell's response. The context in the immune system is likely a critical factor to determine TEX involvement of immune stimulation versus immune suppression (Kunigelis and Graner 2015). Researchers revealed an inhibition of stimulatory capacity in immature DCs when exposed to the human pancreatic cancer cell line-Panc-1 TEX (Zhou et al. 2014), which can deliver miR-203 to DCs and decrease TLR4 expression and downstream cytokines like TNF α and IL12. The changes of the cytokines in the tumor microenvironment can influence both T cells and B cells interacting with DCs.

A few years ago, Fabbri et al. revealed that TEX from non-small cell lung carcinoma (NSCLC) transfers miR-29a and miR-21 to macrophage existing in the tumor microenvironment (Fabbri et al. 2012). These miRNAs bound and enhanced TLR8 (murine TLR7) as ligands, activating the NF- κ B pathway and resulting in production of IL6 and TFN α , creating a prometastatic inflammatory microenvironment. TLR7/8 belongs to intracellular TLRs subset, existing in endosomal and other vesicular membranes, and mediates innate immune reactions against multiple pathogens (Cervantes et al. 2012; Challagundla et al. 2015). MiR-21 from neuroblastoma TEX could also trigger TLR8 in monocytes, which led to upregulation of miR-155 in those cells, and the latter miRNA could then be sent back to the cancer cells via exosomes, resulting in downregulation of telomeric repeat binding factor 1 (TERF1). TERF1 is a telomerase inhibitor, downregulation of which increases cisplatin resistance in neuroblastoma cancer cells (Guo et al. 2009). Thus, the cross-interaction through exosomal miRNAs in the microenvironment is generally beneficial for the tumor.

19.5.3 *Clinical Potential of Exosomes*

The major role of TEX is to create an adaptive microenvironment for cancer cells to grow; however, several studies showed that EVs can inhibit cancer progression,

either by direct effect of the EV-transported protein and nucleic acid contents or through antigen presentation to immunocytes. Tumor cells can deliver some of the same antigens through exosomes as the ones presenting on the surface (Chiodoni et al. 2018, 2019). For instance, DCs under the influence of rat glioblastoma cell-derived exosomes can trigger a strong antitumor reaction and dramatically prolong median survival in glioblastoma-bearing rats when used in combination with α -galactosylceramide (Liu et al. 2017). Given the relative longevity of EVs within the circulation, modification of those antitumor ones creates the potential to design new tools for cancer therapy.

The exosome liposome-like structure allows them to be loaded with various drugs. Exosomes are considered as a new generation of a natural nanoscale delivery system. Hemopurifier[®] is currently being accessed for its efficacy on seizing exosomes released by cancer cell lines or released in biofluids from cancer patients (Marleau et al. 2012). Researchers are studying a refined biomimetic nanostructure to deliver doxorubicin to breast cancer patient, by re-engineering immuno-exosome with a synthetic liposome (Rayamajhi et al. 2019).

Indeed, exosomes derived from different types of cells present different signaling molecules, and thereby have a great potential for targeted drug therapy (Xu et al. 2016). In a mouse breast cancer model, treatment with human-specific anti-CD9 or anti-CD63 antibodies inhibited metastasis to the lungs, lymph nodes, and thoracic cavity via the depletion of circulating EVs. EVs incubated with the targeted antibodies were preferentially internalized by macrophages and might be further eliminated by macrophages (Nishida-Aoki et al. 2017). Phase I clinical trial for advanced CRC has been performed using ascites-derived exosomes and granulocyte-macrophage colony-stimulating factor (GM-CSF). Combination of those two components efficiently enhances antitumor cytotoxic T cell response as a safe and feasible immunotherapy of advanced CRC (Dai et al. 2008).

19.6 CAF-Derived Exosome

The tumor microenvironment comprises tumor cells, nontumor cellular, and noncellular components such as fibroblasts, inflammatory cells, lymphocytes extracellular matrix, blood vessels, and signaling pathways. This dynamic context contributes to tumorigenesis through complex interactions of these elements. One of the main components of tumor microenvironment is CAFs. The interaction of tumor cells and CAFs has been reported to promote cancer progression (Alguacil-Nunez et al. 2018). Exosomes can induce normal fibroblast differentiation into CAFs through TGF β signaling (Ringuette Goulet et al. 2018). CAFs then pose pro-tumor feedback to induce epithelial–mesenchymal transition of bladder cancer cells via paracrine IL-6 signaling (Goulet et al. 2019).

Next-generation sequencing and bioinformatics study on primary human normal and CAFs from nine paired normal colorectal mucosa and cancer tissues displayed

significant differences between the ncRNA component and enrichment within exosomes of the normal and CAFs. NcRNA regulatory factors are specifically detected in CAF-generated exosomes, indicating a specific interaction between CAFs and CRC cells (Herrera et al. 2018).

Exosomal miRNAs were profiled from paired patient-derived normal fibroblasts and CAFs, from an ongoing prospective biomarker study. In vitro CAFs exosomes are delivered to CRC cells, with a subsequent increase in cellular miRNA levels, influencing tumor cell proliferation and chemoresistance. An exosomal CAF signature composed of miRNAs 21, 215, 181a, 329, 199b, and 382 was identified. Of these, miR-21 showed highest abundance in CAF exosomes. In an orthotopic CRC murine model, co-injection with miR-21-overexpressing fibroblasts led to increased liver metastases than with control fibroblasts (Bhome et al. 2017).

CAFs constitute the majority of the tumor bulk of pancreatic ductal adenocarcinomas (PDACs). CAFs exposed to chemotherapy have an active role in regulating the survival and proliferation of cancer cells through exosome secretion. Gemcitabine increases the secretion of both miR-146a and Snail in pancreatic CAF exosomes. Blocking CAF exosome secretion inhibited PDAC tumor cell survival (Richards et al. 2017). Exosomal miR-196a derived from CAFs confers cisplatin resistance in head and neck cancer through targeting CDKN1B and ING5 (Qin et al. 2019). In addition, loss of exosomal miR-3188 in CAFs leads to HNC progression (Wang et al. 2019).

Fibroblast growth factor 2(FGF2)-FGFR1 signaling regulates generation of leukemia-protective exosomes from bone marrow stromal cells. It was demonstrated that bone marrow stromal cells deliver exosome FGF2 to leukemia cells, protecting leukemia cells from tyrosine kinase inhibitors (TKIs). Expression of FGF2 and its receptor, FGFR1, are both enhanced in a subgroup of stromal cell lines and primary AML stroma. Activated FGF2/FGFR1 signaling can further enhance exosome secretion. Inhibiting FGFR cuts off stromal autocrine growth and remarkably suppresses secretion of FGF2-containing exosomes, contributing to compromised stromal guard of leukemia cells. In addition, *Fgf2* $-/-$ mice transplanted with retroviral BCR-ABL acute leukemia had prolonged survival compared with *Fgf2* $+/+$ mice given TKI. Therefore, suppression of FGFR can downregulate stromal function, inhibit exosome secretion, and serve as a therapeutic target to conquer TKIs resistance (Javidi-Sharifi et al. 2019).

19.7 Conclusions

Tumor cells can develop a variety of mechanisms, including transferring TEX to evade and subvert the immune system for their survival. EVs represent a diverse category of cellular releasing products present in multiple types of biofluids and cell culture media. Exosomal contents directly reflect the metabolic state of the cells from which they originate. PD-L1 can be transferred to multiple cell types including tumor cells, macrophages, and DCs through PD-L1-containing exosomes

in the tumor microenvironment, indicating a systemic regulatory role of exosomal PD-L1. Exosomal PD-L1 represents an unexplored therapeutic target, which could overcome resistance to current immune checkpoint inhibitors. The general pattern is that TEX deliver miRNAs to immune cells that ultimately lead to situations that benefit the cancer. Administration of therapeutic antibody effectively inhibits EV-induced tumor metastasis and that the removal of EVs could be a novel cancer treatment (Nishida-Aoki et al. 2017).

Though our understanding of EVs continues to grow, it is far from complete. Experimental data accumulated since decades ago evidently suggests that EVs play pivotal roles for some, if not all, cancer hallmarks. Until now, the field of EV research has drawn mounting interest from scientists and physicians, with growing number of investigators dissecting on the critical role of EVs in cancer biology, and thereby requires more transparent reporting and documenting to streamline interpretation and enable replication of experiments. EV-TRACK, a crowdsourcing knowledge-base (<http://evtrack.org>), is recently built to improve centralization of EV biology and relevant methodology to help reviewers, authors, editors, and funders to fulfill experimental guidelines and increase research reproducibility (Van Deun et al. 2017; Consortium et al. 2017). Vesiclepedia (<http://www.microvesicles.org>) is an established web-based compendium of components including RNA, lipids, proteins, and metabolites transported by EVs from both published and unpublished researches, with the input currently from 1254 EV investigations, consisting of 38,146 RNA entries, 349,988 protein entries, and 639 lipid/metabolite entries (Pathan et al. 2019). There are also alternative or supplementary initiatives to characterize EVs, for example, ExoCarta and EVpedia, two typical web domains that help researchers to promptly upload proteomic lists of identified proteins of the EVs being investigated (Mathivanan and Simpson 2009; Kim et al. 2015). Widespread implementation of those knowledgebases by the EV scientific community is believed to facilitate the success of the exosome research in the long run.

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