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REVIEW

Crosstalk Between Extracellular Vesicles and Regulatory T Cells Across Cancers: From Interaction to Therapeutic Potential

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Abstract: Regulatory T cells (Treg cells) play a crucial role in maintaining immune tolerance and regulating immune responses, especially in cancer, where their immunosuppressive function is highly significant. Treg cells accumulate in the tumor microenvironment (TME), interact with tumor cells and other immune cells, and suppress anti-tumor immunity through various mechanisms, including secretion of immunosuppressive cytokines, direct contact with target cells, and depletion of key nutrients and signaling molecules. Regulating Treg cells has become a novel approach for enhancing cancer immunotherapy. Extracellular vesicles (EVs) are small vesicles with a lipid bilayer membrane secreted by all cells and play an important role in tumor biology as communication mediators by transmitting proteins, RNA, and other bioactive molecules in TME. In the past years, an increasing amount of research has uncovered the effects of EVs on Treg in TME, greatly enriching our understanding of Treg in tumor progression. Additionally, due to the potential of EVs as "natural nanoparticles" for drug and gene delivery, targeting Treg via an EV-delivery system has become a hotspot. Therefore, we comprehensively summarized the updates on the effects of EVs on Treg in TME and EV-related therapy for tumor treatment.

Keywords: regulatory T cells, extracellular vesicle, tumor microenvironment, immunotherapy

Introduction

Regulatory T cells (Tregs) play an essential role in the immune system and FoxP3 is considered a specific marker for them.[1] Tregs are a special subset of $CD4^+$ T cells and function in peripheral tissues.[2,3] In immune responses, Tregs can directly contact and inhibit effector T cells, suppress the activity of antigen-presenting cells (APCs), and exert their effects by secreting immunosuppressive cytokines. These functions make Treg cells of great significance in preventing autoimmune reactions, regulating immune responses, promoting tissue repair, and maintaining immune tolerance.[2] However, such functions may be utilized in certain pathological states, especially in tumor progression, to form adverse outcomes. In the tumor microenvironment (TME), the function of Treg cells becomes more complex and crucial.[3] Many tumor cells recruit and maintain Treg cells by releasing specific cytokines and chemical factors (such as CCL22, TGF-β, etc)., causing them to aggregate in tumor tissue.[4,5] These Treg cells form a suppressive tumor immune microenvironment that benefits tumor cells in immune evasion, thereby accelerating tumor progression. Due to the immunosuppressive effect of Treg cells in the TME, their function has become one of the crucial targets in cancer immunotherapy. Regulating Treg cells to enhance the efficiency of immunotherapy has become a novel treatment regimen for cancers. However, Treg cells have a double-edged sword role in cancers. They also contribute to good survival in cancer patients. A high rate of FOXP3+ tumour-infiltrating lymphocytes positively correlated with CD8+ T cells in oestrogen receptor-negative breast cancer.[6] Similar results were also found in colorectal cancer[7] and ovarian cancers,[8] which indicated Treg cells are

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associated with better prognosis. The reasons for these contradictory phenomena include the heterogeneous functions and phenotypes of Treg subpopulations and the complex cell communications in TME.[9]

Extracellular vesicles (EVs) are nanoscale particles secreted by all cells that play important roles in multiple biological processes, including intercellular communication, immune responses, and disease development.[10] EVs include multiple subtypes, mainly including exosomes, microvesicles, and apoptotic bodies, which vary in diameter, origin, and function. EVs usually carry various biological molecules, including RNA (both mRNA and non-coding RNAs, such as miRNAs, lncRNAs, and circRNAs), proteins, lipids, metabolites, etc. EVs are considered essential communication mediators due to their ability to transmit biomolecules across different cells.[10,11] The tremendous amount of EV research in tumors during the past years has demonstrated their wide range of effects on tumor progression, from tumor proliferation, metastasis, immune evasion, metabolism reprogramming, treatment resistance, angiogenesis, etc.[10,12] More importantly, EVs are considered ideal drug carriers because of their natural biocompatibility and low immunogenicity. Researchers can engineer EVs to carry specific drugs or gene therapy agents and deliver them precisely to both malignant and non-malignant cells.[11] Therefore, exploring the roles of EVs in tumor progression and taking advantage of EVs as drug delivers become a hotspot.

Recently, the effects of EVs on Tregs were uncovered rapidly, and repressing Treg-beneficial EVs seems helpful in tumor treatment. Studies on targeting Treg via EVs to improve antitumor immunity are merging gradually, especially the important roles of non-coding RNAs in EVs. For example, miR-196b-5p and miR-3200-3p from non-small-cell lung cancer (NSCLC) cells can induce the proliferation and senescence of Treg cells;[13,14] miR-320c, miR-27a-3p and miR-30a-5p from colon cancer cells can promote the immunosuppressive function of Treg.[15] To provide an overall understanding of these advances in the relationships between EVs and Tregs in tumors, we systematically reviewed the expanding functions of Tregs in TME, the effects of EVs on Treg in the context of various kinds of tumors, and the clinical potential of Treg-targeting EVs in cancer treatment.

Tregs in Tumor

Treg-Related Immunity in Tumor

Tregs are highly associated with various malignant behaviors in tumors, such as tumor growth and metastasis, immune evasion, metabolism reprogramming, therapy resistance, and so on. Among these, the ability of Tregs to reshape tumor immune microenvironment is outstanding. First, Tregs could repress immune function by direct intercellular contact via some well-known immune inhibitory receptors, including cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), Lymphocyte-activation gene 3 (LAG-3), etc. CTLA-4 is an essential immune inhibitory receptor on the Tregs surface. On the one hand, CTLA-4 on Tregs could bind to the CD80/CD86 on the surface of antigen-presenting cells (APCs), such as dendritic cells, to block their co-stimulatory signals for effector T cells and further inhibit the activation and proliferation of effector T cells.[16] On the other hand, CTLA-4 on Tregs could remove CD80/CD86 on the APC surface via trogocytosis to prevent T cell activation signaling and increase the release of PD-L1, another famous immune inhibitory protein.[17] Besides CD80/CD86, CTLA-4 also removes other immune-stimulating factors. For example, Tregs deletes B7 on APC in a similar way that trogocytosis and CTLA4-mediated cis-endocytosis function cooperatively to clear B7 on APCs.[18] The high-affinity binding of LAG-3 to MHC Class II, the main ligand of LAG-3, can directly inhibit the function of APCs, thereby indirectly inhibiting the activation and proliferation of effector T cells.[19] The interaction between LAG-3 and MHC II not only acts on APCs but also inhibits the ability of other immune cells (for example, macrophages).[20] Apart from direct interaction, Tregs can also exert immune suppression by releasing immunosuppressive cytokines including IL-10, TGF-β, and IL-35.[21,22] TGF-β has multiple immunosuppressive functions, including inhibiting the activation and biofunction of effector T cells, NK cells, macrophages, and other immune cells. Besides, TGF-β also enhances the differentiation and expansion of Tregs.[23] Therefore, TGF-β secretion by Tregs may further promote their differentiation and expansion while inhibiting other immune cells, thus forming a vicious cycle in the TME. IL-10 can induce the expression of PD-L1 in monocytes, thereby reducing T-cell infiltration.[24] IL-35 can exert inhibitory effects on anti-tumor immune responses by promoting the expression of various immunosuppressive molecules such as PD-1, TIM-3, and LAG-3, limiting T cell recruitment, and inhibiting anti-tumor immune memory. Tregs can also secrete EVs

containing IL-35, thereby exerting immunosuppressive effects.[25] The different factors secreted by Tregs can cooperate to jointly shape a tumor immune suppressive microenvironment. For instance, IL-10 and IL-35 are secreted by different subgroups of Tregs. Still, the inhibitory molecules IL-10 and IL-35 released by these Tregs work together to exert immunosuppressive function and promote the depletion of T cells in tumors.[22] Both IL-10, IL-35, and TGF-β can alter the polarity of macrophages, transforming them into M2-type immunosuppressive phenotypes, which have the effects of promoting tumor growth and immune suppression.[26–28] Interestingly, in addition to secreting immunosuppressive factors, IL-2 receptor CD25, a marker highly expressed on Treg, can also absorb the immunostimulatory molecule IL-2, thereby reducing the promoting effect of IL-2 on other immune cells, preventing them from fully proliferating and exerting their function of killing tumors.[29,30] In addition, Tregs can also alter the function and vitality of immune cells through metabolism. On the one hand, in solid tumors, as the tumor progresses rapidly, aerobic glycolysis (ie Warburg effect) often occurs, resulting in a large accumulation of lactate. In this microenvironment, tumor-killing immune cells such as cytotoxic T cells are significantly inhibited, while Tregs can adapt well to lactate. Even worse, studies have shown that lactate can further promote the survival and function of Tregs.[31,32] On the other hand, Tregs can consume a large amount of ATP through CD39 and CD73, metabolize ATP into the immunosuppressive adenosine, and further shape the tumor-suppressive immune microenvironment.[33]

Other Functions of Tregs in Tumor

Tregs can indirectly facilitate tumor growth, metastasis, and treatment tolerance by shaping the aforementioned immunosuppressive microenvironment. However, some studies suggest that Tregs can directly participate in the malignant biological behavior of these tumors. Tregs can promote angiogenesis. When recruited into tumors in a hypoxic microenvironment, Tregs can release vascular endothelial growth factor A (VEGFA), which is beneficial for cancer angiogenesis.[34] Tregs and vascular endothelial cells interact tightly with each other. Endothelial cells can promote the differentiation, expansion, migration, and aggregation of Tregs in various ways.[35–37] Endothelial cells exposed to prostaglandin E2 (PGE2), hypoxia, and Treg-induced VEGF and IL-10 express Fas ligand (FasL), which promotes T cell apoptosis. However, Tregs can evade FasL-mediated apoptosis by expressing the anti-apoptotic gene FADD like IL-1β-converting enzyme.[38] Considering the pro-angiogenic and immunosuppressive properties of Tregs, the interaction between endothelial cells and Tregs may form a feedback interaction, potentially creating a favorable microenvironment for angiogenesis and immunosuppression. In terms of promoting tumor metastasis, Tregs can promote metastasis not only via enhancing angiogenesis but also through directly affecting the invasion and metastasis ability of tumors. The pro-metastatic effect of TGF-β has been widely confirmed.[39–41] Meanwhile, Oh et al confirmed that TGF-β secreted by Tregs can induce EMT transformation in melanoma cells. Cells undergoing EMT transformation have strong mobility, which is a crucial step in the cascade of tumor invasion and metastasis.[42] The formation of novel blood vessels is beneficial for the further growth and proliferation of tumor cells.[43] In addition, considering the promoting effect of VEGF on tumor proliferation,[44–46] VEGF secreted by Tregs may have a direct promoting effect on the proliferation and growth of tumor cells, which is similar to the release of TGF-β by Tregs to enhance cancer cell invasion ability.

Based on the multifarious roles of Tregs in tumors mentioned above (Figure 1), Tregs are often closely associated with adverse clinical outcomes of tumors. The level of Tregs in peripheral blood increases with tumor staging, with the highest Tregs level observed in patients with metastases.[47] An increasing number of infiltrating Tregs in tumor tissues indicates a poorer prognosis and a higher risk of recurrence.[48–50] Tregs may lead to resistance to various treatments, including traditional radiotherapy, chemotherapy, target therapies, and immune-related therapies.[51–54] However, it should be noted that a higher number of Tregs does not always indicate a poor prognosis, and some studies even suggest that a higher number of Tregs may indicate a better tumor prognosis. Two independent studies from Salama and Frey et al showed that a higher number of $FOXP3^+$ Tregs in colorectal tumors suggests a better prognosis.[7,55] This may be related to the anti-inflammatory effect of Tregs. Inflammation is considered an important factor in promoting tumor progression, but the immunosuppressive environment formed by Tregs may counteract this effect.[56–58] The different prognostic roles of Tregs in tumors indicate that our understanding of the complex functions of Tregs in tumors is not yet perfect, and more research is needed to reveal them.

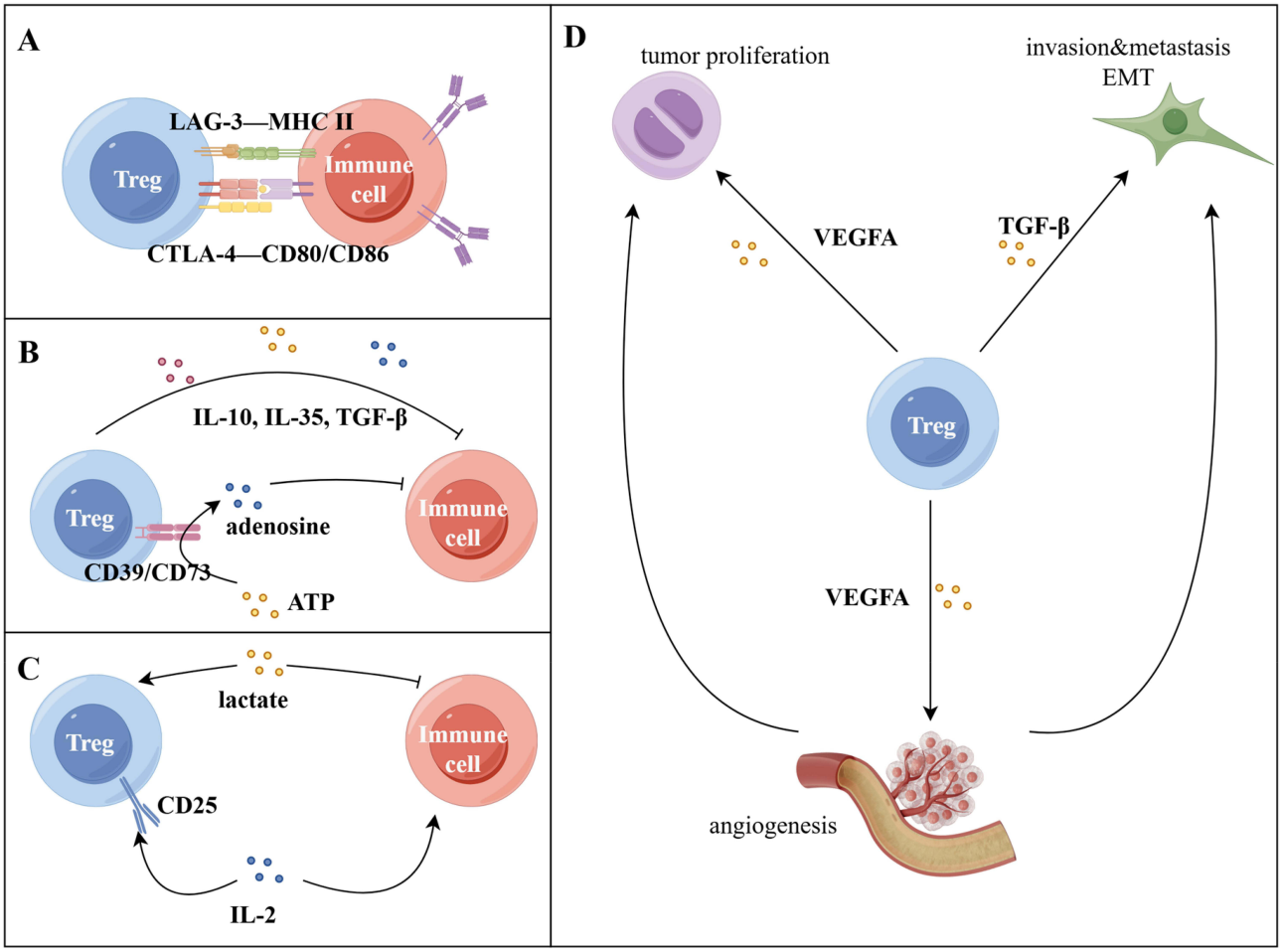

Figure 1 Roles of Treg in tumor. (**A**) Treg could directly inhibit other immune cells, especially antigen-presenting cells, via LAG-3 and CTLA-4. (**B**) On the one hand, Treg could secret immune inhibitory factors, such as IL-10, IL-35, and TGF-β, to inhibit other immune cells. On the other hand, Treg could inhibit immune cells via metabolism reprogramming, such as metabolizing ATP into adenosine via CD39 and CD73. (**C**) Tumor microenvironment (TME) benefits Treg but impairs tumor-inhibiting immune cells. Lactate in TME could promote Treg function but inhibit other immune cells, such as $CD8^+$ T cells. CD25 on the Treg surface could consume IL-2 to active Treg to avoid IL-2-mediated activation in other immune cells. (**D**) Treg could secret VEGFA, TGF-β, and other factors to induce angiogenesis, tumor proliferation, and metastasis.

The Types of EVs

The first category is exosomes, which originate from the intracellular endosomal system. Specifically, the plasma membrane invaginates to form early endosomes, which undergo further sorting and maturation into late endosomes (referred to as multivesicular bodies, MVBs). Intraluminal vesicles (ILVs) within MVBs then fuse with the plasma membrane and are released into the extracellular space, giving rise to exosomes.[59] As one of the most extensively studied EV subtypes, exosomes are characterized by a small diameter, typically ranging from 30 to 150 nm. Their surface is enriched with specific marker proteins, including members of the tetraspanin family (CD9, CD63, CD81), Alix, and TSG101.[60] Exosomes encapsulate bioactive molecules derived from their parental cells, such as nucleic acids (mRNA, miRNA, lncRNA) as well as proteins and lipids, enabling them to participate in long-range intercellular signaling.[61]

The second category is microvesicles (MVs), also referred to as shedding vesicles. Unlike exosomes, MVs are formed directly through budding and shedding from the plasma membrane without involving the endosomal pathway. Their biogenesis is closely linked to the disruption of the asymmetric distribution of phospholipids in the cell membrane. With a larger diameter than exosomes, MVs typically range from 100 to 1000 nm and exhibit a broad size distribution.[62] They retain surface markers characteristic of their parental cell membrane, including integrins, selectins, and adhesion molecules.[63] Internally, MVs carry nucleic acids and proteins, enabling them to participate in local intercellular signaling.

Additionally, they are involved in physiological and pathological processes such as coagulation and inflammatory responses.[64]

The third category is apoptotic bodies (ABs), which are exclusively generated during cellular apoptosis (programmed cell death). Specifically, the cell membrane of apoptotic cells undergoes shrinkage and budding (forming apoptotic blebs) before the cell ultimately fragments to release large vesicles containing nuclear fragments, organelles (eg, mitochondria, ribosomes), and other cytoplasmic contents—these are apoptotic bodies. As the largest subtype of EVs, ABs typically have a diameter ranging from 1 to 5 μm (with some reaching up to 10 μm).[65,66] They characteristically expose phosphatidylserine on their surface, which acts as a "phagocytic signal" to recruit phagocytes such as macrophages for their clearance. Internally, ABs contain apoptosis-related proteins (eg, caspase family members) and nuclear DNA fragments. Their primary function is to facilitate the clearance of apoptotic cellular debris, thereby preventing the leakage of cellular content that could induce inflammation. Additionally, ABs may modulate the functions of neighboring cells through the transmission of genetic material.[67]

In addition to these three mainstream types of EVs, there are also some rare types, such as oncosomes—large-sized vesicles (0.5 to 10 μm) released by tumor cells, rich in oncogenic proteins and nucleic acids, and closely associated with tumor invasion and metastasis—and exosome-like vesicles, which are vesicles of a size similar to exosomes that some cells (eg, platelets, nerve cells) may release through non-endosomal pathways, have functions overlapping with those of exosomes, and are temporarily classified as a special subtype.[68]

Effects of EVs on Treg

With the gradual deepening of research on EVs, it is now clear that various cells can influence the proliferation, differentiation, expansion, metabolism, and other biofunctions of Tregs by releasing EVs (Figure 2). Next, based on relevant research on different tumors, we will systematically summarize the effects of EVs from different cell sources on Tregs under different tumor backgrounds (Table 1).

Head and Neck Cancer

In the field of head and neck cancer (HNC), most research explored the effects of tumor-derived EVs or EVs from patient's plasma on Treg. In 2010, Whiteside's team reported that tumor-derived EVs containing TGF-β1 and IL-10 could induce the differentiation of $CD4^+CD25^+$ Tregs from $CD4^+CD25^-$ T cells, promote the proliferation of Treg, and augment the immunosuppression of Tregs.[69] In 2014, Whiteside's team showed that both exosomes from normal control or head and neck squamous cell carcinoma (HNSCC) patients carried enzymatically active CD39 and CD73 and could hydrolyze eATP to 5'-AMP and to adenosine.[70] When $CD4^+CD39^+$ Tregs contact with $CD73^+$ exosomes, they could produce immunosuppressive adenosine.[70] In 2016, Whiteside's team examined the response of different T cell subsets to tumor-derived EVs via mRNA profiles. They found that Tregs were more sensitive to exosomes from HNSCC cell line PCI-13 than other T cell subsets and the PCI-13-derived EVs downregulated the expression of the adenosine pathway gene and enhanced the production of adenosine in $CD4^+CD39^+$ Tregs.[71] In 2017, Whiteside's team demonstrated that PCI-13-derived EVs also enhanced inosine production in Tregs and the effects of tumor-derived EVs on Tregs depend on cell surface signaling rather than uptake mechanisms.[72] Besides, Whiteside's team re-emphasized exosomes from HNSCC patients' plasma induce the CD39 expression and adenosine production in $CD4^+CD39^+$ regulatory T cells and the effects of exosomes from patients with active disease were more effective than that from patients with active disease no evident disease after oncologic therapies.[73] In 2018, Whiteside's team noticed that $CD3^-$ exosomes from HNSCC patients' plasma were more effective than $CD3^+$ exosomes in reshaping the metabolism of Tregs that induces the generation of 5'-AMP and purines in Tregs and inducing the differentiation of $CD4^+CD39^+$ Tregs from $CD4^+$ T cells.[74,75] In 2020, Beccard et al, who are Whiteside's et al, discovered $CD45^-$ exosomes from high-stage HNSCC patients' plasma were more effective than other exosomes in inducing the differentiation of $CD4^+CD39^+$ Tregs from $CD4^+$ T cells.[76] Since this year, researchers from other institutes also reported the impacts of EVs on Tregs in the context of HNSCC. Lopatina et al found that exosomes from HNSCC endothelial cells also stimulated $CD4^+CD25^+FoxP3^+$ Tregs from peripheral blood mononuclear cells (PBMCs).[77] Wei et al showed that cancer cell-derived exosomal PD-L1 could induce the differentiation of activated Treg and M2 tumor-associated macrophages (TAM).[78] Besides, cancer cell-derived exosomal

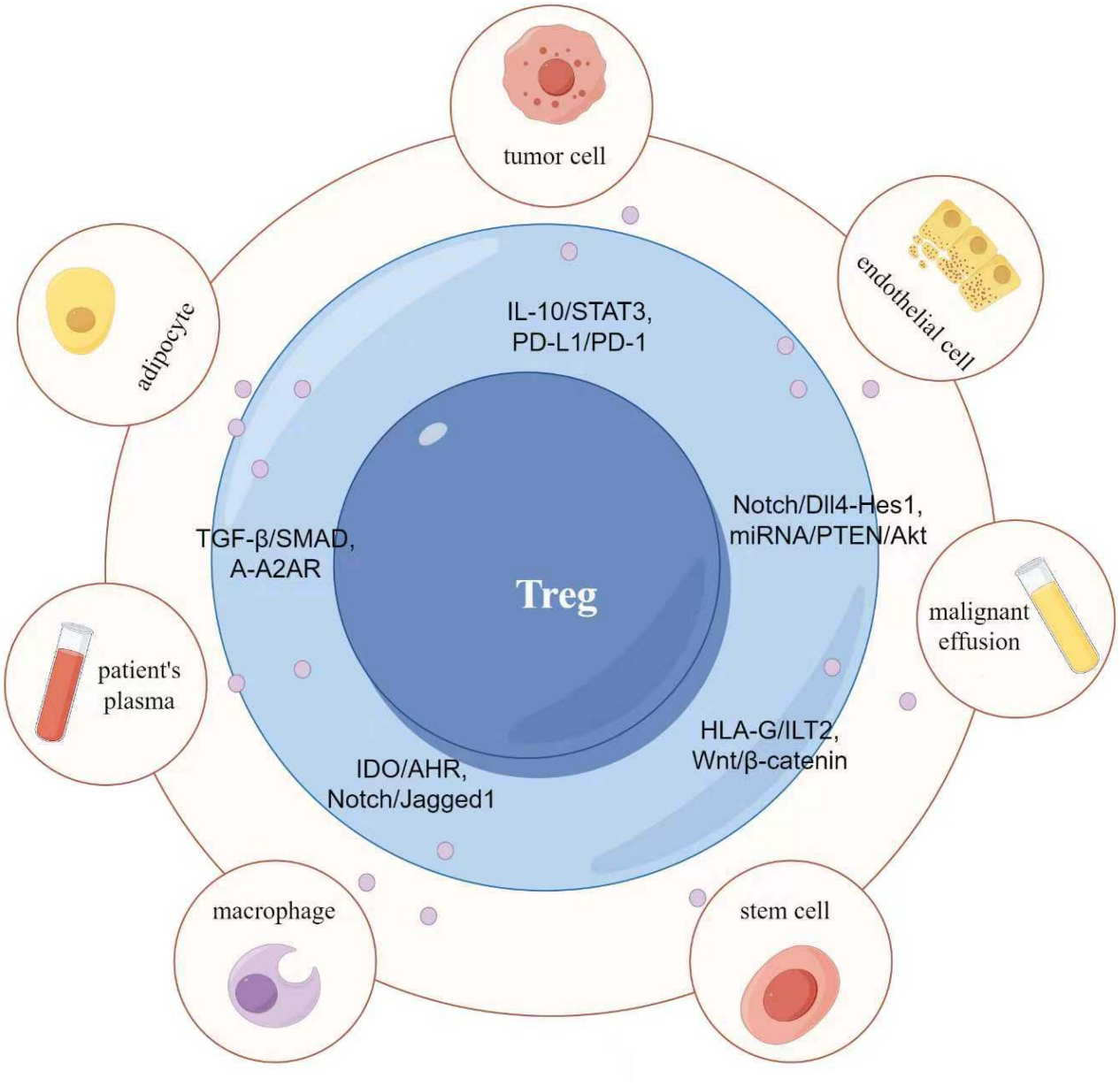

Figure 2 Effects of different EVs on Treg in tumor. In the context of cancer, EVs from various cells (including tumor cells, adipocytes, endothelial cells, macrophages, and stem cells) and patients' body liquid (including plasma and effusion) could promote the expansion and function of Treg in tumors.

PD-L1 also enhances the positive feedback loop between aTreg-M2 that aTregs promotes the M2 polarization of M0 macrophages and M2 macrophages also promote the differentiation of aTregs from $CD4^{+}CD25^{-}$ T cells.[78] In nasopharyngeal carcinoma (NPC), a subtype of HNC, exosomes from NPC cells reshape tumor immunity by inhibiting the proliferation and expansion of T cells, blocking the differentiation of Th1 and Th17, and boosting Tregs.[81] Mrizak et al discovered similar phenomena: NPC-derived exosomes recruited conventional $CD4^{+}CD25^{-}$ T cells and mediated their conversion into $CD4^{+}CD25^{+}$ Treg; NPC-derived exosomes enhanced Tregs expansion via inducing the expression of CD25 and FOXP3 in $Tim3^{-}$ Tregs.[79] Later, Ye et al further demonstrated that miR-24-3p in NPC-derived exosomes targets FGF11 to mediate inhibitory changes in tumor immunity above.[80] Two studies focused on oral squamous cell carcinomas (OSCC), another subtype of HNC. Chen et al found that OSCC cells could maintain the expression of Foxp3 in Tregs via inhibiting miR-325-3p-mediated Foxp3 degradation due to the transmission of exosomal has_circ_0069313

Table 1 Effects of EVs on Treg in Different Tumors

EV Origin	Cargoes	Tumor Type	Effects on Treg Cells	Reference
Head and neck cancer				
Cancer cell	TGF-β1 and/or IL-10	HNSCC	Induce the proliferation of Treg; induce the differentiation of $CD4^+CD25^+$ Treg from $CD4^+CD25^-$ T cells; enhance the immunosuppressive function of Treg	[69]
Plasma	CD39 and CD73	HNSCC	Induce adenosine production in $CD4^+CD39^+$ Treg	[70]
Cancer cell		HNSCC	Treg mRNA profiles are more sensitive to exosomes from cancer cells; induce adenosine production in $CD4^+CD39^+$ Treg	[71]
Cancer cell		HNSCC	Induce inosine production in Treg;	[72]
Patient's plasma		HNSCC	Induce the CD39 expression and adenosine production in $CD4^+CD39^+$ Treg cells	[73]
Patient's plasma		HNSCC	Induce the differentiation of $CD4^+CD39^+$ Treg from $CD4^+$ T cells	[74]
Patient's plasma		HNSCC	Induce the production of 5'-AMP and purines in Treg	[75]
Patient's plasma		HNSCC	Induce the differentiation of $CD4^+CD39^+$ Treg from $CD4^+$ T cells	[76]
Tumor endothelial cell		HNSCC	Stimulated $CD4^+CD25^+FoxP3^+$ Treg from peripheral blood mononuclear cells	[77]
Cancer cell	PD-L1	HNSCC	Induce Treg differentiation	[78]
Cancer cell		NPC	Recruit $CD4^+CD25^-$ T cells and mediate their conversion into $CD4^+CD25^+$ Treg	[79]
Cancer cell	miR-24-3p	NPC	Induce Treg differentiation	[80]
Cancer cell		NPC	Promote Treg differentiation	[81]
Cancer cell	has_circ_0069313	OSCC	Promote the Treg function through inhibiting mir-325-3p-induced Foxp3 degradation to maintain Foxp3 levels	[82]
Arecoline-treated cancer cell	mtDNA D-loop and PD-L1	OSCC	Promote immunosuppressive Treg cell numbers	[83]
Digestive system neoplasm				
Cancer cell	PD-L1	Esophageal cancer	Enhance the expansion and immunosuppressive functions of circulating $CTLA4^+$ follicular Treg	[84]
Peripheral blood	TGF-β1	GC	Induce the differentiation of Tregs from naïve T cells	[85]
Cancer cell	circGSE1	HCC	Induce Treg expansion	[86]
Cancer cell	14-3-3ζ	HCC	Induce Treg expansion	[87]
Cancer cell	miR-500a-3p	HCC	Induce Treg expansion	[88]
Chemoresistant cancer cell	miRNA-425-5p	HCC	Promote the expansion of Tregs from $CD4^+$ cells	[89]
Cancer cell		PDAC	Promote the regulatory phenotype of T cells	[90]
Cancer cell	miR-320c, miR-27a-3p and miR-30a-5p	Colon cancer	Promote the immunosuppressive function of Treg	[15]
Cancer cell		CRC	Reduce spleen Treg	[91]
Cancer cell	miR-208b	CRC	Induce Treg expansion	[92]
Cancer cell	TGF-β1.	CRC	Promote the regulatory phenotype of T cells	[93]
CRC cell		CRC lung metastasis	Inhibit the number of $CD4^+FoxP3^+$ Treg cells	[94]
Lung cancer				
Cancer cell		Lung cancer	Increase CCL1 secretion from fibroblasts to indirectly promote the differentiation of $CD4^+FoxP3^+$ Tregs from $CD4^+$ T cells	[95]

(*Continued*)

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Table 1 (Continued).

EV Origin	Cargoes	Tumor Type	Effects on Treg Cells	Reference
Cancer cell	EGFR	Lung cancer	Induce tolerogenic DCs to indirectly increase the differentiation of Treg from Th0 cells	[96]
Cancer cell	miR-196b-5p	NSCLC	Promote Treg proliferation	[13]
Cancer cell	miR-3200-3p	NSCLC	Promote Treg senescence	[14]
Cancer cell	CD39	NSCLC	Induce the differentiation of Treg from CD4+ T cells	[97]
Cancer cell	PD-L1	Lung carcinoma	Promote the immunosuppressive function of dendritic cells to indirectly induce the differentiation of $CD4^+Foxp3^+$ Treg from $CD4^+$ T cells	[98]
Female cancers				
ASC		Breast cancer	Induce the differentiation of Treg from $CD4^+$ T cells	[99]
Cancer cell		Breast cancer	Reduce $CD39^+$ Treg cells	[100]
Cancer stem cells	FOXP3	Breast cancer	Generate $FOXP3^+$ Tregs from $CD4^+$ T cells	[101]
Cancer cell	lncRNA SNHG16	Breast cancer	Promote the emergence of CD73+γδT1 Treg	[102]
AMSC; cancer cell		Breast cancer	Enhanced the expression of Foxp3 mRNA	[103]
Cancer cell	PD-L1	Breast cancer	Promote the immunosuppressive function of dendritic cells to indirectly induce the differentiation of $CD4^+Foxp3^+$ Treg from $CD4^+$ T cells	[98]
Cancer cell	TGF-β1	Breast cancer	Induce the differentiation of Treg from $CD4^+$ T cells	[104]
Cancer cell	TGF-β	Cervical cancer	Induce the differentiation of Treg from naïve $CD4^+$ T cells	[105]
M2 Macrophage	miR-29a-3p and miR-21-5p	Epithelial ovarian cancer	Increase T regulatory cell	[106]
CAA	SIRT1	Ovarian cancer	Increase the number of Treg cells in cancer tissue	[107]
Cancer cell	TGF-β1 and/or IL-10	Ovarian carcinomas	Induce the proliferation of Treg; induce the differentiation of $CD4^+CD25^+$ Treg from $CD4^+CD25^-$ T cells; enhance the immunosuppressive function of Treg	[69]
Hematological system tumors				
Tumor cell	miR-24-3p	Acute myeloid leukemia	Reduce the apoptosis of Treg and stimulate Treg proliferation	[108]
Tumor cell	CD19	B-cell lymphoma	Promote the Treg phenotype conversion of CAR-T cell	[109]
Tumor cell		Multiple myeloma	Reduce the apoptosis of healthy people-derived Treg and enhance the viability of healthy people-derived Treg; increase the apoptosis of multiple myeloma patient-derived Treg and reduce the viability of multiple myeloma patient-derived Treg	[110]
Tumor cell	4-1BBL/CD137L	Myeloid leukemia	Promoted suppressive phenotype of Tregs	[111]
Tumor cell	-	TP53-type acute leukemia	Increase Tregs and decrease $CD8^+$ T cells	[112]
Tumor cell		Paediatric pre-B acute lymphoblastic leukaemia	Altered the T cells profile into regulatory type by increasing the expression of FOXP3 and Tregs-related cytokines, including TGF-B and IL-10	[113]
Others				
Cancer cell	TGF-β1	Mesothelioma	Enhance the suppressive function of $CD4^+CD25^+$ Treg cells	[114]
Macrophage	miR-4443	Malignant pleural effusion	Promote the differentiation of naïve T cells into Treg	[115]
Malignant-effusion	TGF-β1	Malignant effusion	Maintain the number of Treg and the expression of FOXP3	[116]
Cancer cell		Melanoma	Increase Treg in lymph nodes	[117]

(*Continued*)

Table 1 (Continued).

EV Origin	Cargoes	Tumor Type	Effects on Treg Cells	Reference
Cancer cell		Melanoma	Function in the endosome of dendritic cells to induce IFN-β secretion from dendritic cells and indirectly induce Treg proliferation, which could be blocked by the co-incorporation of CD300a on the surface of dendritic cells	[118]
Cancer cell	PD-1	GBM	Increase the infiltration and expansion of Treg cells	[119]

Abbreviations: AMSC, adipose-derived mesenchymal stem cell; ASC, adipose-derived stem cell; CAA, cancer-associated adipocyte; CRC, colorectal cancer; GC, gastric cancer; GBM, glioblastoma multiforme; HCC, hepatocellular carcinoma; HNSCC, head and neck squamous cell carcinoma; NPC, nasopharyngeal carcinoma; NSCLC, non-small cell lung cancer; OSCC, oral squamous cell carcinomas; PDAC, pancreatic ductal adenocarcinoma.

from cancer cells to Tregs.[82] Ko et al reported that arecoline-induced cytosolic mtDNA D-loop leakage and PD-L1 expression in OSCC cells were packaged into EVs in arecoline-treated OSCC cells to promote the number of Tregs.[83]

Digestive System Neoplasms

In esophageal cancer, cancer-derived EV carried PD-L1 and promoted the expansion and immunosuppression of circulating follicular Tregs. In gastric cancer, exosomes from the peripheral blood of gastric cancer patients contain TGF-β1 to induce the differentiation of Tregs from naïve T cells and increase Tregs in lymph nodes. In hepatocellular carcinoma (HCC), 14-3-3 protein zeta (14-3-3ζ), miR-500a-3p, and circGSE1 in exosomes from HCC cells were reported to increase the expansion of Tregs.[86–88] Similarly, exosomal miRNA-425-5p from chemoresistant HCC cells can be absorbed by CD4+ T cells to promote the increase of Tregs through regulating the translation of PTEN.[89] In pancreatic cancer, EVs from BxPC-3, a pancreatic ductal adenocarcinoma cell line, promote the regulatory phenotype of T lymphocytes via inducing the upregulation of immune checkpoint proteins represented by PD-1, PD-L1, CTLA4, and Tim-3 and the expansion of $FOXP3^+$ Tregs.[90] The studies about the influences of EVs on Tregs in the context of colorectal cancer (CRC) were relatively more than other digestive system neoplasms. EVs from CRC carried TGF-β which induced the phenotype conversion of T cells into Treg-like cells via TGF-β/Smad signaling.[93] Wang et al demonstrated that exosomes from colon cancer cells transmit miR-27a-3p, miR-30a-5p, and miR-320c into Tregs to increase Tregs via inhibiting the expression of IRF4, which is an inhibitory factor for the number of Tregs in colon cancer tissue.[15] Another study also showed similar results. Exosomal miR-208b is related to oxaliplatin resistance in CRC patients, and exosomal miR-208b from CRC cells could induce the expansion of Tregs via targeting PDCD4.[92] However, some research showed the opposite effects of EVs on Treg. Ganji et al showed that CT26-derived exosomes can block cancer progression in vivo by decreasing Tregs and upregulating IFN-γ.[91] Kobayashi et al used another CRC cell line Colon-26 and found similar inhibitory impacts of CRC-derived EVs on Treg. Colon-26-derived EVs significantly inhibit the number of $CD4^+FoxP3^+$ Tregs in the mice model of CRC lung metastasis via upregulating PDCD1, GITR, and CD69.[94] The anti-Treg roles of cancer-derived EVs may be attributed to the heterogeneity properties of EVs. The heterogeneity of EVs does not exist randomly; instead, it is co-regulated by factors such as the type of source cell, cell activation state, and microenvironmental signals. It can be mainly categorized into the following four dimensions, each of which is closely coupled with function. Due to the presence of heterogeneity, the molecular composition of EVs also exhibits high heterogeneity. The molecular components of EVs (proteins, nucleic acids, lipids) are the direct executors of their functions—even EVs derived from the same source may exhibit completely different functions due to differences in their molecular cargo. Heterogeneities across different dimensions can act synergistically to co-regulate complex biological processes.[120,121]

Lung Cancer

In lung cancer, EVs from tumor cells could directly or indirectly induce the differentiation of Treg. Exosomal CD39 from non-small-cell lung cancer (NSCLC) cells also results in the insufficiency of ATP and the hyperactivation of AMPK to directly induce the mal-differentiation of T cells and increase the differentiation of Tregs from $CD4^+$

T cells.[97] Exosomal epidermal growth factor receptor (EGFR) could indirectly affect the differentiation of Tregs via inducing tolerogenic dendritic cells which can promote the differentiation of Tregs from Th0 cells. Similarly, Ning et al reported that PD-L1-containing exosomes released by lung LLC Lewis lung carcinoma inhibit the differentiation of $CD11c^+$ dendritic cells from myeloid precursor cells and promote the immunosuppressive function of dendritic cells, which indirectly induces the increase of Tregs.[98] Wang reported that lung cancer-derived exosomes indirectly increase $CD4^+$ $FoxP3^+$ Tregs in the lung by enhancing the contact between fibroblasts and Treg. In detail, lung cancer-derived exosomes increase the secretion of CCL1 from fibroblasts, which promotes Tregs differentiation by activating CCR8.[95] Besides, exosomal miR-196b-5p from lung cancer cells could promote pyroptosis in T cells and the proliferation of Tregs via regulating the expression of ING5.[13] Interestingly, exosomal miR-3200-3p from lung cancer cells could induce the senescence of Treg. However, VEGFR2, which usually are overexpressed in lung cancer cells, reduces miR-3200-3p in cancer-derived exosomes. As a result, the senescence of Tregs was inhibited in the lung cancer TME to accelerate disease progression.[14]

Female Cancers

Zhu et al explored the influence of EVs released from adipose-derived stem cells (ASCs) on tumor immune microenvironment in the background of breast cancer in vitro. They showed that EVs released from ASCs could not only block the M1 polarization and enhance the M2 polarization of $CD14^+$ monocytes but also promote $CD4^+$ T cells to differentiate into Tregs, thus forming the immunosuppressive microenvironment of breast cancer.[99] Fathollahi et al studied the effect of EVs released by either adipose-derived MSCs or breast cancer cells on the recall-antigen-specific immune responses. The results showed that EVs released by either adipose-derived MSCs or breast cancer cells can upregulate Foxp3 mRNA, the main regulatory factor of Tregs, in splenocytes, and reduce the expression of Tbx21 and Gata3 mRNA, the main mediators of T helper (TH) 1 and TH2 responses. However, only EVs from breast cancer cells can increase the secretion of IL-10 and TGF-βin splenocytes.[103] Exosomes from breast cancer stem cells carried Foxp3 to generate $Foxp3^+$ Tregs from anti-CD3/anti-CD28-treated (antigen-activated) $CD4^+$ T cells at an early time-point of 24 h.[101] These studies strongly suggest that EVs can increase the number and/or enhance the function of Tregs. Ni et al further explored the influence of breast cancer-derived EVs on Tregs. They found that exosomes secreted by breast cancer cells can transmit lncRNA SNHG16 which induces γδT1 cells to express CD73 by regulating the miR-16-5p/SMAD5 axis, ultimately promoting the emergence of $CD73^+$γδT1 Tregs and forming the immunosuppressive microenvironment of breast cancer.[102] Cancer-derived EVs also carry TGF-β1 to induce Tregs differentiation in breast cancer.[104] Interestingly, PD-L1 in EVs released from breast cancer cell line 4T1 induced DCs which can promote $CD4^+$ T cells to differentiate into $CD4^+Foxp3^+$ Tregs and inhibit the differentiation of $CD4^+IFN\text{-}\gamma^+$ Th1 cells.[98] This result indicates that tumor-derived EVs can indirectly affect the differentiation of Tregs, thereby forming an immunosuppressive microenvironment. However, a study showed that EVs from breast cancer cells reduce the number of Tregs. Santoro et al explored the influence of EVs released by breast cancer cell lines BT474 and HS578T under 2D and 3D conditions on various immune cells from peripheral blood through in vitro and found that EVs derived from BT474 significantly reduce $CD39^+$ Tregs.[100]

In cervical cancer, Ni et al demonstrated that exosomal TGF-β induces the expansion of Tregs from naïve $CD4^+$ T cells via activating STING signaling which induces FOXP3 transcription through TBK1-IRF3-mediated SMAD3 and STAT5 phosphorylation independent of interferon-β.[105] In ovarian cancer, three studies reported the effects of EVs from different origin cells on Treg. Szajnik proved that exosomal TGF-β could induce the proliferation of Treg, promote the differentiation of $CD4^+CD25^+$ Tregs from $CD4^+CD25^-$ T cells, and enhance the immunosuppressive function of Tregs.[69] Zheng et al reported that cancer-associated adipocyte-derived EVs (CAA-EVs) delivered SIRT1 to form a suppressive immune microenvironment via inhibiting tumor-inhibitory M1 macrophages and increasing tumor-promoting M2 macrophages and Tregs.[107] Zhou et al reported that compared to exosomes from untreated monocytes or M1 macrophages, exosomes from M2 macrophages significantly increased the ratio of Treg/Th17 when $CD4^+$ T cells were treated with these exosomes from different macrophages.[106]

Hematological System Tumors

As the effects of EVs on Tregs in the context of hematological system tumors, present most research, if not all, reported tumor-derived EVs benefit Treg. In paediatric pre-B acute lymphoblastic leukaemia, tumor-derived exosomes induce apoptosis of T cells and enhance the regulatory phenotype of T cells via increasing FOXP3 expression and the secretion of Tregs-related cytokines, such as TGF-β and IL-10.[113] In B-cell lymphoma, exosomal CD19 from tumor cells benefit the initial activation of CD19-CAR T-cells but subsequently induce the apoptosis of CAR-T cells and the differentiation into Tregs phenotype.[109] In myeloid leukemias, 4–1BBL/CD137L in EVs from leukemia cells enhance the immunosuppressive function Tregs via regulating the expression of CD30 and TNFR2[111] and exosomal miR-24-3p reduces the apoptosis of Tregs and stimulate Tregs proliferation via activating JAK3/STAT5 signaling and increasing the expression of p-NF-κB and p-ERK protein.[108] Exosomes from Kasumi-1, acute leukemia cell line expressing high TP53, increase Tregs and decrease $CD8^+$ T cells.[112] In multiple myeloma (MM), exosomes from MM cell lines OPM2 and U266B1 inhibited the apoptosis of Tregs from healthy people and elevated the viability of Tregs from healthy people.[110] However, U266B1-derived exosomes increase the apoptosis of Tregs from MM patients and both U266B1-derived and OPM2-derived exosomes could reduce the viability of Tregs from MM patients.[110]

Melanoma, Glioma, Mesothelioma and Malignant-Effusion

Zhu et al systematically explored the effects of melanoma-derived sEVs on systemic immunity and found that melanoma-derived sEVs increased Tregs in lymph nodes.[117] Nakazawa et al reported that B16 melanoma-derived EVs could indirectly promote Tregs proliferation via dendritic cells.[118] In detail, B16 melanoma-derived EVs were internalized into endosomes in dendritic cells to activate TLR3-TRIF signaling to generate IFN-β which induces the proliferation of Treg. However, when dendritic cells express CD300a, CD300a could be internalized into endosomes as well to prevent EV-mediated IFN-β secretion and following Tregs proliferation.[118] Cytokine-free and PD-1-containing exosomes from glioblastoma multiforme (GBM) cells could increase the infiltration and expansion of Tregs, which is inhibited by LRRC4, a tumor suppressor for GBM.[119] Another study on glioma reported that exosomes from glioma stem cells form an immunosuppressive microenvironment via myeloid-derived suppressor cells rather than Tregs.[122] Mesothelioma-derived exosomes augment the immunosuppressive function of $CD4^+CD25^+$ Tregs.[114] In the patients with malignant effusion, TGF-β1 in exosomes collected from cancer malignant effusions help maintain the number of Tregs and the expression of FOXP3.[116] Besides, macrophages in malignant effusion exhibit the M2 phenotype and secrete more exosomes than macrophages in the blood, and macrophage-derived exosomal miR-4443 also induces the differentiation of Tregs.[115]

In conclusion, within TME and physiological as well as pathological processes, the functions of EVs are highly dependent on their cellular sources. EVs secreted by different cells exhibit significant differences in the proportion of vesicle subtypes, the composition of molecular cargo, and the effects on target cells (eg, regulatory T cells, Treg).[123] Such differences are the core manifestation of EV heterogeneity and determine their diverse roles in tumor immune regulation.[124] From the perspective of regulatory effects on Treg, EVs from different cellular sources show functional differentiation into "pro-suppression" or "pro-activation": most tumor cell-derived EVs (eg, miR-196b-5p-containin EVs from NSCLC cells, TGF-β^+ EVs from ovarian cancer cells) focus on "enhancing Treg function".[13] They create a TME conducive to tumor escape by inducing Treg proliferation, inhibiting Treg senescence, and increasing the secretion of immunosuppressive factors (eg, IL-35, adenosine); a small number of tumor cell-derived EVs (eg, EVs from CT26 colon cancer cells and Colon-26 colon cancer cells), however, due to cargo heterogeneity (eg, high expression of PDCD4 and IFN-γ-related RNA), can instead reduce the number of Treg, downregulate the expression of FoxP3 in Treg, and exert anti-tumor immune effects; the regulatory effects of immune cell-derived EVs are strongly associated with the polarization state of the source cells.[125] EVs secreted by non-activated dendritic cells (DCs) can promote Treg differentiation by transmitting MHC-II/LAG-3 signals. In contrast, EVs secreted by DCs stimulated with tumor antigens (eg, DCs loaded with Hepa1-6 cell lysates) can reduce the number of CD25+FoxP3+ Treg and reverse immune suppression; mesenchymal stem cell-derived EVs generally exhibit the property of "promoting Treg generation", but the abundance of their cargo is regulated by the microenvironment.[77] For instance, EVs from adipose-derived stem cells (ASCs) can significantly

promote Treg differentiation in breast cancer, while in inflammatory models, they can alleviate tissue damage by regulating Treg metabolism.

Treg-Derived EVs

Tregs are well-known for their unique immunosuppressive properties, and the EVs they release may exhibit similar effects. Studies have shown that Treg-derived EVs carry the immunosuppressive molecule CD73, which contributes to the formation of a Treg cell-mediated immunosuppressive microenvironment.[126] Similarly, miRNAs carried by EVs from Tregs can also inhibit the functions of other immune cells, such as pathogenic T helper 1 cells and macrophages.[127,128] Consequently, in TME, Treg cell-derived EVs may be important contributors to the formation of the tumor immunosuppressive microenvironment. A study showed that in head and neck cancer patients, CD3+CD15s+ exosomes (from Tregs) increased in patients with cancer recurrence, which indicates a significant role of Treg-derived EVs in cancer progression.[129] Another two studies provided more direct evidence and showed exosomes from CD8+25+ Tregs contain CD8, CD25, GITR, Foxp3, pMHC-II and pMHC-I, and can inhibit dendritic cell-induced CD8+ T responses and immunity against B16 melanoma and lung cancer.[130,131] The immunosuppressive property of Treg cell-derived EVs provides a new approach for the treatment of various diseases characterized by excessive immune responses, such as transplantation rejection, tissue repair, acute myocardial infarction, inflammatory bowel disease, etc.[132,133] However, the application of natural Treg-EVs in cancers is limited mainly because re-activating immunity rather than inhibiting immunity is the common strategy to treat cancer.

EV-Related Therapy to Target Treg for Cancer Treatment

The therapeutic potential of EVs in cancer mainly includes targeting EVs and taking advantage of EVs for delivery tools, which are the most important clinical implications of EVs on Treg.[121] In this section, we discuss how to treat cancer via these two ways to interfere with Treg.

Targeting EVs

As mentioned above, the promoting effects of EVs on Tregs are one main reason for cancer progression. Therefore, reducing these Treg-beneficial EVs represents a helpful strategy for cancer treatment. Some popular methods include RAB27A knockdown, GW4869, as well as novel AH-D peptide.[121] These methods may also work well in targeting Treg-beneficial EVs. For example, the aforementioned 4–1BBL-containing EVs could result in the expansion of Treg, and these EVs are secreted from leukemic cells in a Rab27-dependent way.[111] Therefore, knockout Rab27 in leukemic cells reversed the immunosuppressive function of Tregs.[111] AH-D peptide could result in the rupture of EV membrane via sensing EV membrane from cancer cells with high curvature to reduce the number of EVs in vivo.[134] The usage of AH-D peptide greatly decreases the percentage of $CD4^+CD25^+$ Tregs in B16F10 tumor-bearing C57BL/6 mice.[134] Interestingly, the emergence of Treg-beneficial EVs is due to the function loss or down-expression of some tumor suppressor genes. The recovery of these genes also is an effective way to target Treg-beneficial EVs. The best example is LRRC4 mentioned above.[119] Besides these, other drugs or biomaterials also decrease Treg-beneficial EVs. In contrast to tumor suppressor genes, some tumor-promoting genes could increase Treg-beneficial EVs. For example, PKC-ζ is related to radiotherapy resistance. In detail, irradiation-induced the phosphorylation level of PKC-ζ in breast cancer 4T1 cells, which results in the secretion of TGF-β1-containing EVs to form immunosuppressive TME via promoting the differentiation of Tregs from $CD4^+$ T cells.[104] Inhibition of PKC-ζ via siRNA or naringenin, a natural flavonoid, could revere the secretion of TGF-β1-containing EVs and immune suppression to overcome radiotherapy resistance.[104] Some known drugs also repress tumors by reducing Treg-beneficial EVs. Macitentan, an oral drug approved by the FDA, can decrease the number of PD-L1-containing EVs. The combination of macitentan and anti-PD-L1 antibody can boost the number and activity of $CD8^+$ T cells but reduce Tregs number in tumors and lymph nodes in the tumor model of triple-negative breast cancer (TNBC), colon cancer, and lung cancer.[135] Similarly, the combination of botulinum neurotoxin type A1 and anti-PD-L1 therapy also reduces blood exosomes and reverses the increase of Tregs in the B16-F10 syngeneic mouse tumor model.[136] Estrogen, a well-known hormone therapy for some cancers, could reduce the level of TGF-β1 in EVs

Table 2 Targeting EVs to Inhibit Tregs for Cancer Treatment

Treatment	Effects on EVs	Effects on Treg	Reference
Rab27 knockout	Reduce the release of 4–1BBL-containing EVs	Reduce Treg numbers in the spleen and diminish the expression of suppressive CD39, and IL-10 on Tregs	[111]
AH-D peptide	Result in the rupture of cancer-derived EVs with high curvature	Reduce the percentage of $CD4^+CD25^+$ Treg cells	[134]
LRRC4 recovery	Reduce the release of cytokine-free and PD-1-containing exosomes	Inhibit the infiltration and expansion of Treg cells	[119]
Naringenin or si-PKC-ζ	Reduce the release of TGF-β1-containing EVs	Inhibit the differentiation of Treg from $CD4^+$ T cells	[104]
Combination of macitentan and anti-PD-L1	Reduce the release of PD-L1-containing EVs	Reduce Treg number	[135]
Combination of botulinum neurotoxin type A1 and anti-PD-L1 therapy	Reduce blood exosomes	Reduce Treg number	[136]
Estrogen	Reduce the TGF-β1 level in cancer-derived EVs	Reverse the ability of cancer-derived EVs to induce Treg	[137]

from colon cancer MC38 cells thus blocking the ability of MC38-derived EVs to induce Tregs.[137] These research proved the feasibility of targeting Treg-beneficial EVs for cancer treatment (Table 2).

EV Delivery

Harnessing EVs, including native EVs, pre-generation modified, post-generation modified, and hybrid EVs, as drugs or tools to deliver drugs/biomaterials is another strategy for cancer treatment (Figure 3 and Table 3).

In the field of drug delivery, EVs exhibit unique and irreplaceable advantages over traditional drug delivery (such as CTLA-4 antibody therapy, etc) and artificially synthesized nanoparticles (such as liposomes, polymeric nanoparticles, and metallic nanoparticles). The core of these advantages lies in the high alignment between their "natural biological properties" and "precise delivery capabilities".[150–152] First, EVs possess excellent biocompatibility and low immunogenicity. As naturally released vesicles from cells, their membrane structure is fully compatible with the components of biological membranes (eg, phospholipids, cholesterol, membrane proteins), which allows them to avoid foreign body rejection reactions commonly induced by traditional drug or artificial nanoparticles (such as complement system activation and excessive phagocytosis by macrophages).[152,153] Autologous EVs can achieve long-term circulation in the body, significantly reducing toxic and side effects. In contrast, even after surface modification (eg, PEGylation), traditional drug or artificial nanoparticles still struggle to completely eliminate the risk of immune recognition.[154,155] Second, EVs have precisely targeted delivery capabilities. The surface of their membrane carries cell-specific molecules from their source cells (eg, integrins, adhesion proteins, glycosylation modifications), which can act like a "biological navigator" to recognize specific receptors on the surface of target cells (eg, EGFR highly expressed on tumor cells, CD31 on vascular endothelial cells), enabling active targeted accumulation of drugs at lesion sites.[153,156] By comparison, the targeting ability of artificial nanoparticles or traditional drug mostly relies on the chemical conjugation of exogenous ligands (eg, antibodies, peptides).[151] This modification process is not only complex and prone to affecting particle stability but may also lead to reduced targeting efficiency due to ligand detachment. Furthermore, EVs feature efficient cargo loading and release mechanisms. Their natural compartmental structure is compatible with various types of drugs (eg, small-molecule chemotherapeutic drugs, nucleic acid drugs, protein drugs). Moreover, they can directly deliver drugs into the cytoplasm of target cells through membrane fusion or endocytosis, preventing drugs from being degraded in lysosomes.[157,158] On the other hand, traditional drug or artificial nanoparticles often face issues such as low drug encapsulation efficiency, easy leakage, or drug inactivation due to insufficient lysosomal escape efficiency after entering cells.[159] Finally, EVs also demonstrate excellent biological barrier penetration. Thanks to their nanoscale size (30–1000

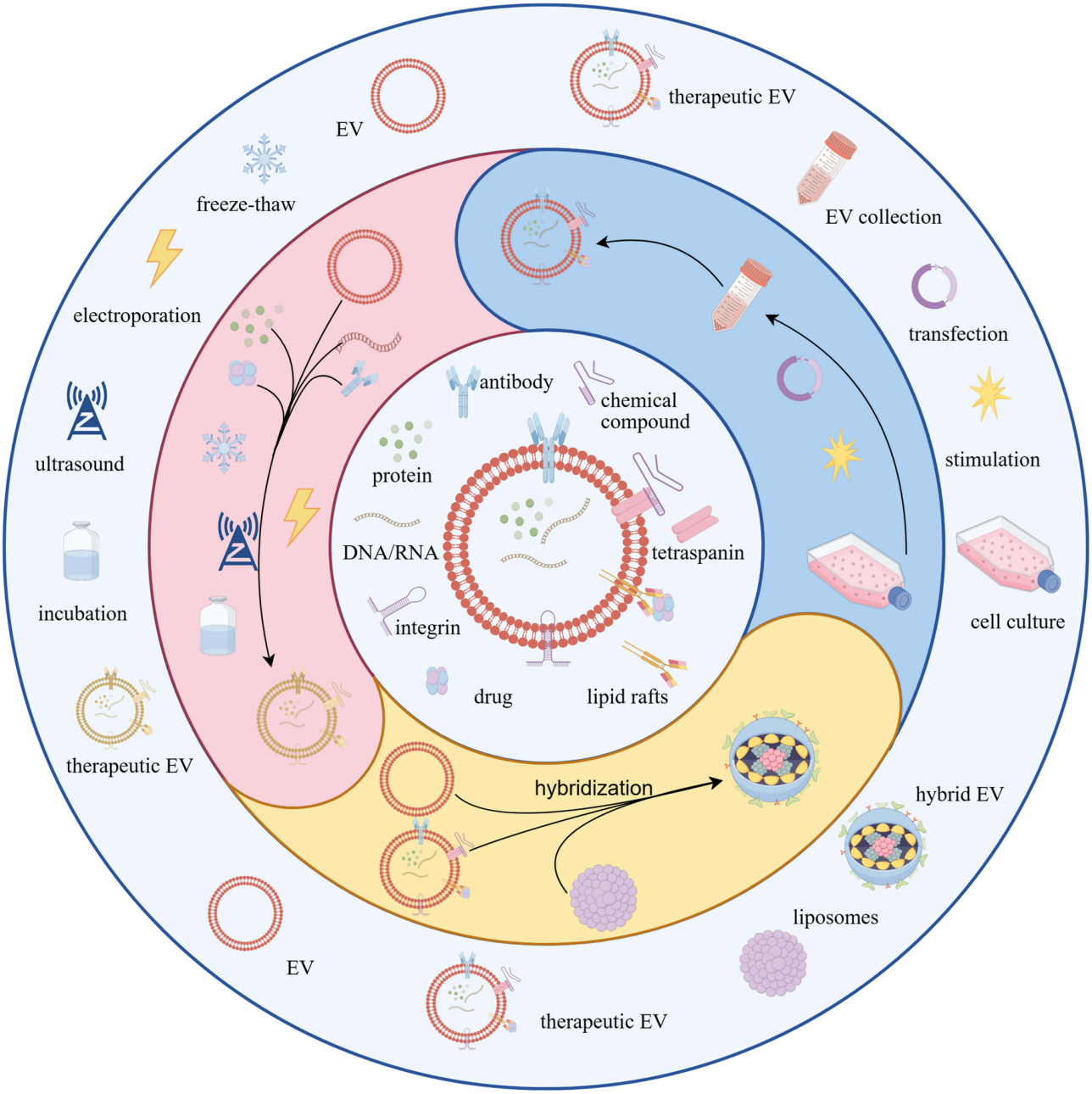

Figure 3 Therapeutic EVs for targeting Treg in cancer treatment. Therapeutic EVs could be generated via pre-generation modification, post-generation modification, and hybridization.

nm) and natural membrane structure, EVs can penetrate physiological barriers that are difficult for artificial nanoparticles to cross, such as the blood-brain barrier and blood-ocular barrier. This provides a new pathway for drug delivery in refractory diseases like central nervous system diseases and ocular diseases.[153,156] These natural advantages make EVs a core candidate for the next generation of drug delivery systems. Especially in terms of precise therapy and reducing drug toxic and side effects, their application potential significantly surpasses that of traditional drug or artificially synthesized nanoparticles.

Native EVs with inhibitory effects on Tregs have been mentioned above[91,94,100] (Figure 4). Pre-generation modified EVs include EVs acquired via endogenous loading and EVs from cells with/without certain stimulation. The endogenous loading mainly involves gene editing. Gao et al acquired Treg-targeting EVs from miR-124-3p transfected bone marrow

Table 3 Treg-Targeting EVs in Cancer Treatment

Therapeutic EVs	Origin Cells	Engineering Model	Drug	Effects on Treg	Combined Treatment	Reference
miR-124-3p-expressing exosomes	Bone marrow mesenchymal stromal cells overexpressing miR-124-3p	Endogenous loading/ genetically engineering	miR-124-3p	Reduce lactate uptake in Tregs to repress the immunosuppressive function of Tregs	Anti-PD-1 therapy	[138]
DEX_{AFP}	Dendritic cell line DC2.4 cells overexpressing AFP	Endogenous loading/ genetically engineering	α-fetoprotein (AFP)	Decrease $CD25^+Foxp3^+$ Tregs		[139]
Exo^{smart}	Dendritic cell line DC2.4 cells overexpressing CD62L and OX40L	Endogenous loading/ genetically engineering	CD62L and OX40L	Inhibit Tregs		[140]
mDC-derived exosomes	Dendritic cells treated with Hepa1-6 cell lysates	Cell stimulation		Decrease the number of $CD25^+Foxp3^+$ Tregs	Microwave ablation	[141]
HSP70-containing exosomes	Heat-stressed MC38 colon cancer cells	Cell stimulation	HSP70	Induce the conversion of Tregs into Th17 cells		[142]
OX40-EVs	B16F10 melanoma cells overexpressing OX40L	Endogenous loading/ genetically engineering	OX40L	Inhibit the expression of FoxP3 to block the immunosuppressive phenotype of Tregs		[143]
miR-124-3p-expressing exosomes	CT-26 cancer cells	Modified calcium chloride method	miR-124-3p	Inhibit $CD4^+CD25^+Foxp3^+$ Tregs		[125]
RSL3-loading exosomes	M1 macrophage	Cell stimulation plus incubation	RSL3	Reduce the number of $CD4^+CD3^+FOXP3^+$ Tregs		[144]
EXO-OVA-mAb	Bone marrow cells	Cell stimulation plus incubation	OVA and anti-CTLA -4 antibody	Increase the ratio of CTLs/Tregs		[145]
spMEXO	PANC-02 cells	Cell stimulation plus electroporation plus incubation	CCL22 siRNA, CP05 peptide and MART-1 peptide	Suppress Tregs via inhibiting the CCR4/CCL22 axis between dendritic cells and Tregs	Chemotherapy	[146]
SN/Mn@gHE	Genetically engineered CD47-overexpressed cancer cells and M1 macrophages	Genetically engineering plus cell stimulation plus hybrid exosomes plus sonication plus extrusion	SN38 and MnO2	Decrease the number of $CD4^+Foxp3^+$ Tregs		[147]
HE-THD	M1 macrophages	Hybrid exosomes plus membrane extrusion plus physical encapsulation technique	Thalidomide	Inhibit $CD4^+Foxp3^+$ Tregs expansion and proliferation		[148]
ELP	Mesenchymal stem cells	Hybrid exosomes plus freeze-thaw cycles	Paclitaxel	Decrease the number of Tregs		[149]

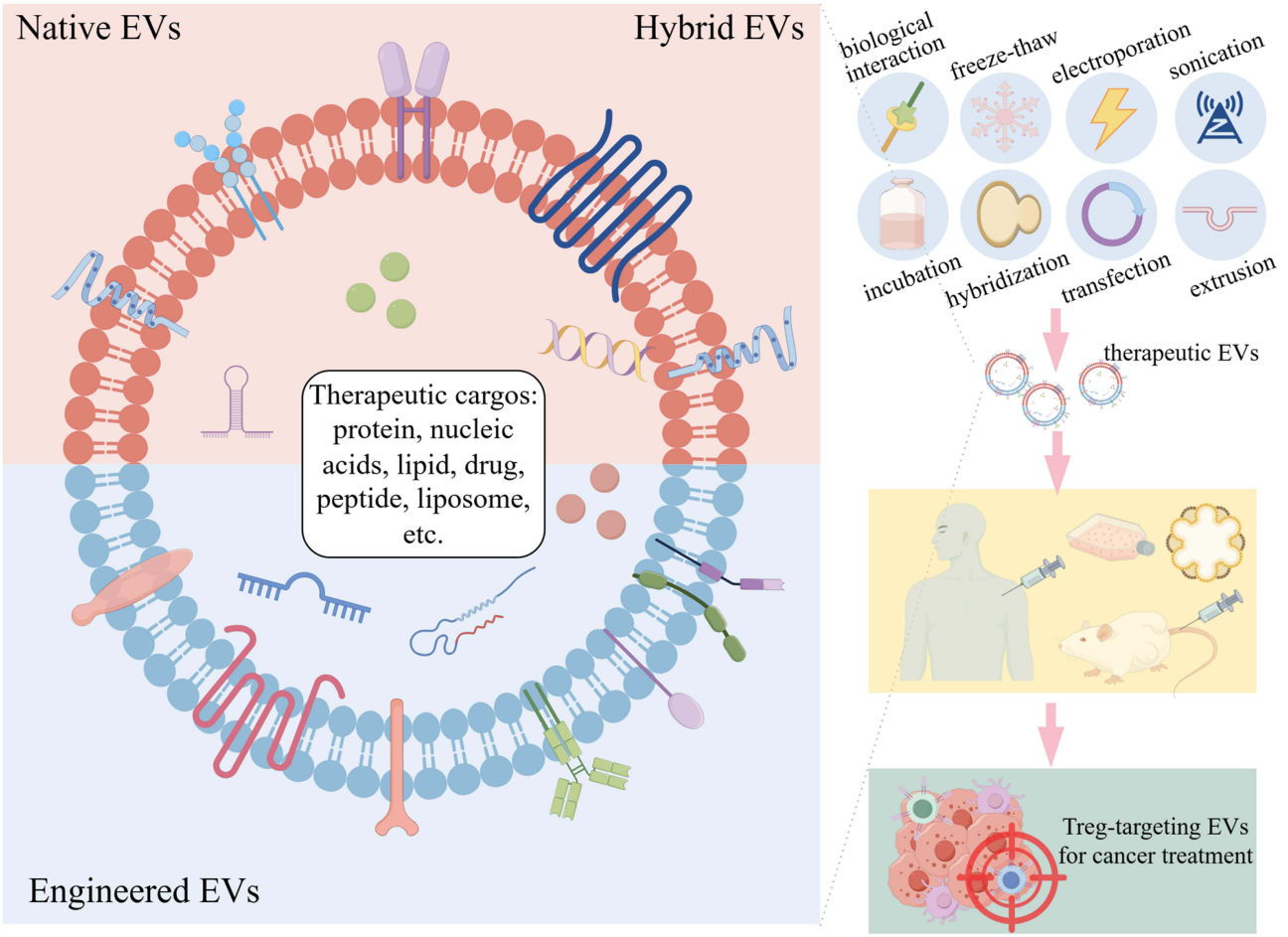

Figure 4 Schematic illustration of native, engineered, and hybrid extracellular vesicles (EVs) for therapeutic applications. Native EVs can be engineered to load various therapeutic cargos—including proteins, nucleic acids, lipids, drugs, peptides, and liposomes—through multiple modification strategies such as biological interaction, freeze–thaw cycles, electroporation, sonication, incubation, hybridization, transfection, and extrusion. These hybrid or engineered EVs can be tailored to deliver specific therapeutic molecules in vivo, enabling precision applications such as regulatory T cell (Treg)-targeted EV therapy for cancer treatment.

mesenchymal stromal cells (BM-MSCs).[138] Exosomes from genetically engineered BM-MSCs carried large amounts of miR-124-3p, which directly targets monocarboxylate transporter 1 (MCT1) to reduce the lactate uptake in Treg, finally repressing the immunosuppressive function of Tregs.[138] These genetically engineered exosomes greatly improve the efficiency of anti-PD-1 therapy in ovarian cancer-bearing mice via reversing Treg-mediated immunosuppression.[138] Lu et al used genetically engineered DC2.4 cells to obtain therapeutic EVs.[139] They transfected DC2.4 cells with lentivirus expressing α-fetoprotein (AFP) gene and then collected DEX_{AFP} (dendritic cell-derived exosomes expressing AFP) from these modified DC2.4 cells.[139] DEX_{AFP} stimulated more IFN-γ-expressing $CD8^+$ T cells and caused a decrease in $CD25^+Foxp3^+$ Tregs, which induced effective tumor suppression in vivo.[139] Ji et al also developed Exo^{smart} (exosomes with high expression of CD62L and OX40L) via infecting donor DC2.4 cells with lentivirus overexpressing CD62L and OX40L.[140] CD62L on the surface of Exo^{smart} provides target T cells with a high affinity to lymph nodes while OX40L on the surface of Exo^{smart} promotes the expansion of T cells and inhibits Tregs.[140] In 4T1 breast tumor-bearing mice, Exo^{smart} greatly inhibits tumor growth and metastasis in lymph nodes via activating immunity in tumor-draining lymph nodes.[140] However, some research also demonstrated the validity of EVs from donor cells treated with certain stimulation for cancer treatment via targeting Treg. Zhong et al treated dendritic cells with Hepa1-6 cell lysates and then collected exosomes (mDC-derived exosomes).[141] Compared with microwave ablation (MWA) monotherapy, the combination of MWA and mDC-derived exosomes greatly decreased the number of $CD25^+Foxp3^+$ Tregs to improve the immune microenvironment and inhibit tumor growth.[141] More interestingly, besides these non-malignant cells, tumor-derived

EVs also gained much attention in the field of therapeutic EVs. Guo et al discovered that heat-stressed MC38 colon cancer-derived exosomes contain HSP70 and could induce the conversion of Tregs into Th17 cells in MC38-bearing mice.[142] Semionatto et al generated genetically engineered tumor cells via transfecting B16F10 melanoma cells with retroviral vectors encoding OX40L and then harvested OX40-EVs for cancer treatment.[143] OX40-EVs exhibited a great ability to inhibit the expression of FoxP3 to block the immunosuppressive phenotype of Tregs.[143] The exploration of the application of post-generation modified EVs is another hotspot in cancer treatment and the way to load drugs or biomaterials into EVs could be divided into endogenous and exogenous loading,[160] thus some drugs or biomaterials could be loaded into EVs after generation. Coincidentally, Rezaei et al also acquired miR-124-3p-expressing exosomes, but they adapted post-generation modification rather than genetically engineering as Gao et al did.[125,138] Rezaei et al cocultured exosomes from CT-26 cancer cells with miR-124-3p via a modified calcium chloride method.[125] These post-generation modified miR-124-3p-expressing exosomes also function well in inhibiting $CD4^+CD25^+Foxp3^+$ Tregs.[125] Wang et al designed RSL3-loading exosomes via coculturing RSL3 with exosomes but they treated primary born-derived macrophage with lipopolysaccharide in advance before collecting exosomes.[144] These RSL3-loading exosomes stimulated anti-cancer immunity, including reducing the number of $CD4^+CD3^+FOXP3^+$ Tregs in tumor-beating mice.[144] RSL-loading exosomes are the product of the combination of pre-generation and post-generation modification in essence. Phung et al also applied a similar combination to acquire EXO-OVA-mAb, which increased the ratio of CTLs/Tregs (cytotoxic T lymphocytes/Treg) in tumor sites. They cocultured exosomes from bone marrow cells, which are treated with OVA in advance, with anti-CTLA-4 antibody.[145] The combination is not limited to this. Zhou et al combined cell stimulation, electroporation, and incubation.[146] They treated PANC-02 cells with mitoxantrone to acquire exosomes (MEXO), then loaded CCL22 siRNA into MEXO via electroporation, and finally obtained spMEXO via incubating CCL22 siRNA-loading MEXO with CM peptide, the conjugation of CP05 peptide and MART-1 peptide.[146] spMEXO can indirectly suppress Tregs via inhibiting the CCR4/CCL22 axis between dendritic cells and Tregs in vivo, and the combination of spMEXO with chemotherapy achieved a synergistic effect, including boosting anti-tumor immunity, suppressing tumor growth, and improving overall survival rate.[146] Hybrid exosomes include mixing exosomes with nano-biomaterials and mixing exosomes from different cells. GT-exos (exosomes from genetically engineered CD47-overexpressed cancer cells) and M1-exos (exosomes from M1 macrophages) were hybridized to generate genetically engineered hybrid exosomes (gHE). Then, SN38 and MnO_2 were successively added into gHE via sonication and extrusion respectively to acquire SN/Mn@gHE.[147] SN/Mn@gHE decreased $CD4^+Foxp3^+$ Tregs and myeloid-derived suppression cells but increased central $CD44^+CD62L^-$ and effector memory T cells.[147] Liposome is another popular drug delivery nanoparticle and also is a good partner for exosomes. Yang et al designed hybrid exosomes (HE) based on exosomes from M1 macrophages and liposomes via simple thin film hydration followed by a membrane extrusion.[148] Thalidomide (THD) was loaded into HE to form HE-THD via a physical encapsulation technique. In vivo, HE-THD inhibited $CD4^+Foxp3^+$ Tregs expansion and proliferation for anti-cancer immunity.[148] Another study generated hybrid exosomes loaded with paclitaxel (ELP) via fusing exosomes from mesenchymal stem cells and folate-targeted liposomes based on repeated freeze-thaw cycles with the existence of paclitaxel.[149] In CT26 tumor-bearing mice, ELP significantly decreased Tregs and raised the proportion of activated $CD8^+$ cells and $CD4^+$ T cells.[149]

Conclusion and Perspective

A great deal of research emphasized the fundamental effects of EVs on Tregs. In TME, EVs form a communication network thus EVs from various cells including tumor cells, macrophages, adipocytes, etc., patients' plasma, and effusion could regulate the expansion, proliferation, differentiation, and death of Tregs. The function molecules in Treg-regulating EVs include PD-L1, PD-1, TGF-β, non-coding RNA, CD19, CD39, CD73, IL-10, SIRT1, 4–1BBL, CD137L, EGFR, and other biological molecules, most of which are well-known immunosuppressive factors. Generally speaking, most studies proved the promoting effects of EVs on Treg in the context of tumors which reflects the fact that tumors often harness EVs to benefit tumor-promoting cells again.[121] More interestingly, the emergence of Treg-beneficial EVs is associated with the downregulation of tumor-inhibiting genes or upregulation of tumor-promoting genes in some research, which is consistent with the natural tumor progression. However, a few reports discovered the inhibitory effects of EVs on Treg, which reflect EV heterogeneity and our imperfect understanding of the interaction between EVs and Treg in cancer.

The wide promoting effects emphasized the importance of targeting tumor-promoting EVs again. Some methods function well in preventing the release of Treg-promoting EVs or deleting them in cell experiments or tumor-bearing models, which showed clinical potential in cancer treatment. Besides, the engineering EVs inhibiting Tregs also provide a novel selection for tumor treatment. However, there remain some challenges before EV-related therapy enters clinical practice. Which kinds of cells are best for EV origin? Tumor-derived EVs provide a high affinity to tumor cells but could inherit some tumor-promoting properties; immune cell-derived EVs could activate anti-tumor immunity, but their target ability and yield may not be perfect. The isolation method and EV generation also need further improvement. The batch effect is another problem in generating therapeutic EVs. In addition, present research usually uses cell lines or mouse models, which indicates the development of EV-related therapy still is in the laboratory stage. Clinical trials and other 3D models will upgrade the current understanding of them. Anyway, the emerging work focusing on the influences of EVs on Tregs and EV-related therapy for targeting Treg shows our deepening comprehension of tumors and promises a better treatment strategy in the future.

Consent for Publication

The authors have consented to publish this article.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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