REVIEW ARTICLE

The link between intracellular calcium signaling and exosomal PD-L1 in cancer progression and immunotherapy

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Abstract   Exosomes are small membrane vesicles containing microRNA, RNA, DNA fragments, and proteins that are transferred from donor cells to recipient cells. Tumor cells release exosomes to reprogram the factors associated with the tumor microenvironment (TME) causing tumor metastasis and immune escape. Emerging evidence revealed that cancer cell-derived exosomes carry immune inhibitory molecule program death ligand 1 (PD-L1) that binds with receptor program death protein 1 (PD-1) and promote tumor progression by escaping immune response. Currently, some FDA-approved monoclonal antibodies are clinically used for cancer treatment by blocking PD-1/PD-L1 interaction. Despite notable treatment outcomes, some patients show poor drug response. Exosomal PD-L1 plays a vital role in lowering the treatment response, showing resistance to PD-1/PD-L1 blockage therapy through recapitulating the effect of cell surface PD-L1. To enhance therapeutic response, inhibition of exosomal PD-L1 is required. Calcium signaling is the central regulator of tumorigenesis and can regulate exosome biogenesis and secretion by modulating Rab GTPase family and membrane fusion factors. Immune checkpoints are also connected with calcium signaling and calcium channel blockers like amlodipine, nifedipine, lercanidipine, diltiazem, and verapamil were also reported to suppress cellular PD-L1 expression. Therefore, to enhance the PD-1/PD-L1 blockage therapy response, the reduction of exosomal PD-L1 secretion from cancer cells is in our therapeutic consideration. In this review, we proposed a therapeutic strategy by targeting calcium signaling to inhibit the expression of PD-L1-containing exosome levels that could reduce the anti-PD-1/PD-L1 therapy resistance and increase the patient's drug response rate.

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Abbreviations

ALIX	ASG-2 interacting protein 2
B7–H1	B7 homolog 1
CA IX	carbonic anhydrase IX
CAFs	cancer-associated fibroblasts
CCBs	calcium channel blockers
CD274	cluster of differentiation 274
CDE	caveolin-dependent endocytosis
CME	clathrin-mediated endocytosis
COX2	cyclooxygenase-2
DCs	dendritic cells
DMA	dimethyl amiloride
DNA	deoxyribonucleic acid
EpCAM	epithelial tumor cells express the epithelial cell adhesion molecule
EGFR	epidermal growth factor receptor
EMT	epithelial–mesenchymal transition
ERK	extracellular signal-regulated kinases
ESCRT	Endosomal Sorting Complex Required for Transport
FDA	Food and Drug Administration
GPCRs	G-protein couple receptors
HER	human epidermal receptor
HIF	hypoxia inducible factors
HRS	hepatocyte growth factor-regulated tyrosine kinase substrate
Hsp	heat shock protein
IFN-γ	interferon-γ
IL-6	interleukin-6
ILV	intra luminal vesicle
IP3	inositol tri-phosphate
IRF-1	interferon regulatory factor-1
ITIM	immunoreceptor tyrosine-based inhibitory motif
ITSM	immunoreceptor tyrosine-based switch motif
iTregs	inducible Tregs
JAK	Janus kinase
LAG3	lymphocyte activation gene 3
mAbs	monoclonal antibodies
MAPKs	mitogen-activated protein kinases
MCU	mitochondrial Ca^{2+} uniporter
MHC-I	major histocompatibility complex I
MMP	matrix metalloproteinase
MVBs	multivesicular bodies
MYO10	myosin 10
NK cells	natural killer cells
nSMase 2	sphingomyelin phosphodiesterase 2
PD-1	program death protein 1
PD-L1	program death ligand 1
RNA	ribonucleic acid
ROC	receptor-operated calcium channel
RyR	ryanodine receptor
SNARE	soluble N-ethylmaleimide-sensitive factor-attachment protein (SNAP) receptor
STAT	signal transducer and activator of transcription
TME	tumor microenvironment
TNF-α	tumor necrosis factor-α
TSG101	tumor susceptibility gene 101
VEGF	vascular endothelial growth factor
VGCC	voltage-gated Ca^{2+} channels
VPS4	vacuolar protein sorting-associated protein 4

Introduction

Almost all cells can release a variety of membrane microvesicles and nanovesicles with diversified functional consequences. Microvesicles mainly differ from nanovesicles based on their size and mechanism of generation.[1,2] Microvesicles release from the plasma membrane through a shedding or budding process. They are generally larger than 0.2 μm in size and denote microparticles or ectosomes. On the other hand, nanovesicles, including exosomes, are between 30 and 100 nm in size, generate through reverse budding of the peripheral membrane of multivesicular bodies (MVBs) or late endosomes, and are categorized based on endocytic origin3. Exosomes are well-known for transporting a variety of signaling molecules, including nucleic acids, microRNA, functional proteins, and lipids. Due to their cell–cell communication characteristics, numerous studies have explored the role of exosomes in the physiological and pathophysiological processes including tumor metastasis,[4] immune modulation,[5] and neurodegenerative diseases.[6]

Emerging evidence reveals that cancer-derived exosomes are the carrier of immunosuppressive proteins like PD-L1 and its receptor PD-1, which are significant immune checkpoint molecules and can stimulate tumor progression via negative regulation of cellular immune responses.[7,8] PD-L1 is a type I transmembrane protein of 290 amino acids encoded by the CD274 gene and known as cluster of differentiation 274 (CD274) or B7 homolog 1 (B7-H1) and containing of immunoglobulin V-like and C-like extracellular domains.[9] However, PD-L1 is extensively expressed in numerous cell types, mostly in tumor cells, monocytes, macrophages, NK cells, DCs, and activated $CD8^+$ T cells and in immune-privileged sites such as the brain, cornea, and retina.[10] In normal physiological conditions, the activation of the PD-1/PDL1 signaling is closely associated with the initiation of peripheral tolerance, maintenance of $CD8^+$ T cells immune homeostasis, escaping hyperactivation as well as shielding against tissue damage.[11] In disease states, PD-L1 transmits a negative signal by interacting with its receptor PD-1 to regulate a series of processes of $CD8^+$ T cell-mediated cellular immune responses, including growth,

proliferation, and apoptosis, as well as functional maturation.[12] Recent studies have depicted that the activation of the PD-1/PD-L1 signaling pathway can arrest the $CD8^+$ T cell cycle at the G1 phase rather than causing direct apoptosis.[13] Furthermore, considering the $CD8^+$ T cells mediating inhibitory effects, the PD-1/PD-L1 signaling pathway can facilitate the down-regulation of $CD8^+$ T cell responses by inducible Tregs (iTregs).[14] Metastatic melanoma-derived exosomes contain PD-L1 on their surface and stimulation with interferon-γ (IFN-γ) enhances the amount of PD-L1 on these vesicles and down-regulates anti-tumor response to induce tumor growth.[7]

In normal cellular homeostasis, cytosolic Ca^{2+} level control several signaling processes, however disruption of normal Ca^{2+} is hypothesized to be a reason for enriched proliferation and metastasis induced in numerous cancer.[15] Ca^{2+}-dependent proliferation is facilitated by MAPK/calmodulin-dependent signaling, whereas invasion and migration are stimulated via Ca^{2+}-dependent cytoskeleton rearrangement and focal adhesion disassembly.[15,16] Although recent studies have identified Ca^{2+} channels that are amplified with pathophysiological consequences, the influences of Ca^{2+}-dependent effectors are less understood.

Several "Endosomal Sorting complex required for Transport (ESCRT)" and related proteins including HRS, TSG101,[17] ALIX, and VPS4[18] have been associated with exosome release. However, the ESCRTs playing a role in ILV formation on MVBs to modulate exosome release is still unclear.[19] Additionally, members of the Rab GTPase family such as Rab5, 7, 11, 27a, 27b, and 35 have also been identified as a regulator of exosome release.[20-26] Recent findings demonstrate that Ca^{2+}-dependent Rab binding protein Munc 13-4 is a crucial factor for Ca^{2+}-dependent membrane fusion. Moreover, they have identified Ca^{2+}, Munc13-4, Rab 11 dependent molecular pathway that underlines increased exosome secretion from cancer cell.[27,28] The activation of G-protein coupled receptors (GPCRs) resulting in exosome formation, release, and uptake also represent a potential role of GPCRs in the modulation of exosome biogenesis and function. Activated GPCRs promote the discharge of Ca^{2+} from the endoplasmic reticulum by the second messenger inositol triphosphate (IP3) and increased intracellular Ca^{2+} level initiates exosome formation and release.[29,30] H^+/Na^+ and Na^+/Ca^{2+} channels control the intracellular Ca^{2+} concentration and likely regulate the exosome secretion. Preclinical studies with CT26 tumor-bearing mice have mentioned that blocking these channels using dimethyl amiloride (DMA) inhibits exosome release.[31,32] These results suggest that blocking the secretion of exosomes from cancer cells by CCBs might constitute a potential target for cancer therapy. Additionally, L-type Ca^{2+} channels are expressed and frequently altered in many human cancers and contribute to cancer cell invasion and migration. As a result, controlling the invasion and migration of cancer cells and drastically reducing the number of MYO10 is accomplished by controlling L-type Ca2+ channels through CCBs.[33] In this review, we emphasize a novel approach of cancer therapy that involves limiting the release of exosomes from cancer cells that express PD-L1, which has implications on immunotherapeutic methods of cancer treatment.

Biogenesis and functional relevance of exosomes in cancer

The biogenesis of exosomes is regulated by the endosomal system. Early endosomes mature into the late endosomes or MVBs, necessary for the endosomal membrane to invigilate to produce ILVs in the lumen of the organelles.[3] The ESCRT complex plays a crucial role in this processing by recognizing ubiquitinated membrane proteins and promoting their internalization into the MVBs. Finally, the MVBs fuse with the cellular membrane to release exosomes into the extracellular space.[31,34] Exosome release can be triggered by hypoxia, which is frequently present in malignancies and is positively regulated by the Rab GTPases 27a and 27b.[31] (Fig. 1).

Exosomes contain a fundamental set of identical proteins which are released from different kinds of cells including members of the ESCRT complex (TSG101, Alix), members of the tetraspanin family (CD9, CD63, CD81, and CD82), and heat shock proteins (Hsp 60, Hsp 70, Hsp 90).[35] Apart from these, exosomes also bring some specific proteins. Melanoma cells secrete exosomes carrying the tumor-associate antigen Mart-1.[36] Exosomes from epithelial tumor cells express the epithelial cell adhesion molecule (EpCAM).[37] Gastric cancer, breast cancer, or pancreatic cancer cell-derived exosomes contain the members of the human epidermal receptor (HER) family.[31,38]

The uptake of exosomes in the recipient cell takes place in a non-random process in association with transmembrane proteins. Recent evidence describes that the tetraspanin-integrin complex plays a role noticeably in pursuing the binding of exosomes to recipient cells.[39] Moreover, a pro-inflammatory environment causes the expression of ICAM-1 as a receptor molecule on the membrane surface, which enhances the adhesion of exosomes to the recipient cells.[40] Exosomes can directly release their cargo to the cytoplasm of recipient cells through the fusion with the cell membrane. Cellular internalization of exosomes can occur by following several mechanisms such as phagocytosis, macropinocytosis, Clathrin-mediated endocytosis (CME), Caveolin-dependent endocytosis (CDE), and plasma membrane fusion.[41] Additional studies are required to elucidate how exosomes are heading for the target cells in cancer and whether the cell-specific composition of exosomes results in organotropism connected with metastatic cancer.

Exosome-mediated transfer of proteins between cancer cells can lead to drug resistance. Exosomes from two docetaxel-resistant prostate cancer cell lines can deliberate chemoresistance to non-resistant prostate cancer cell lines via exosome-mediated transfer of drug transporter, MDR-1.[42] Acquired drug resistance by exosome-mediated transfer of proteins can be recognized to the decreased intracellular concentration of chemotherapeutic agents, epithelial—mesenchymal transition (EMT), altered expression of oncogenes or tumor suppressor genes, enriched DNA damage repair, autophagy, and highly acidic microenvironment of tumors.[43,44] These observations characterize a new paradigm of how malignant cells may raise their tumorigenic potential and develop drug resistance via exosomes.

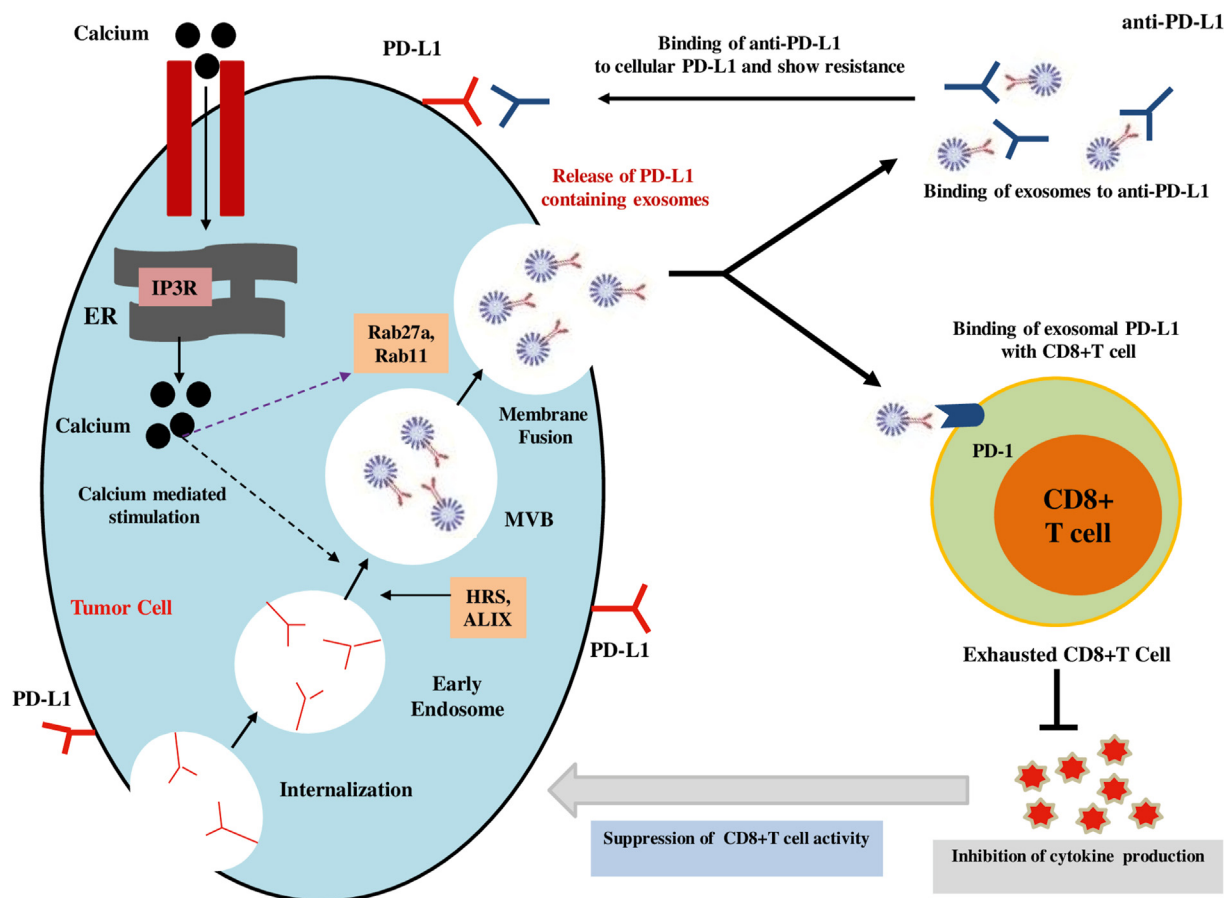Prostate and mesothelioma cancer cell-derived exosomes carry TGF-β1 protein, which is transmitted to

Figure 1 Role of calcium signaling in exosomal PD-L1 secretion and cancer progression. Intracellular calcium stimulates biogenesis factors and Rab family proteins to increase the secretion of PD-L1-positive exosomes from cancer cells. Exosomes carrying PD-L1 are responsible for limiting the effectiveness of anti-PD-1/PD-L1 therapy through binding to antibodies. Additionally, exosomes containing PD-L1 play a role in the suppression of immunity by reducing CD8^{+} T cell-mediated activities through inhibiting cytokine production.

recipient cells in a biologically active form and TGF-β1 containing exosomes can cause the differentiation of fibroblasts to myofibroblasts as a key source of tumor angiogenesis and matrix remodeling proteins.[45,46] Moreover, cancer cells expressing exosomes contain membrane-bound EGFR to endothelial cells that activate the autocrine VEGF/VEGFR-2 pathway to lead to tumor angiogenesis.[47,48] Additionally, CD105-positive exosomes contribute to forming a pre-metastatic niche by enhancing MMP2, MMP9, and VEGFR1.[48] Similarly, exosomes from fibroblasts stimulate an autocrine Wnt-signaling pathway in breast cancer cells to lead to migration, invasion, and metastasis.[49] Collectively, these studies have indicated that tumor-derived exosomes play an influential role in operating the tumor microenvironment (TME) for the benefit of cancer cells.

Cancer cell-derived exosomes control fundamental functional features of the lymphoid components of the TME and exert direct influence in immunosuppression to lead to tumor progression. Exosomes from gastric cancer cells (especially MKN-45 and MKN-28) changed gene expression and cytokine secretion patterns of CD8^{+} T cells to lead to an immunosuppressive condition for metastatic niche formation in the lung.[50] Moreover, exosomes from other

cancer cell lines facilitate IL-6 production in MDSCs through the activation of the Toll-like receptor 2 by the Hsp 72 which elicits an immunosuppressive effect.[32] It has been demonstrated that the exosomes secreted from hepatocellular carcinoma promoted the expansion of the TIM-1^{+} regulatory B cell (Breg) population by expressing IL-10, and inhibiting CD8^{+} T cells proliferation, TNF-α, and IFN-γ production to suppress anti-tumor immunity.[51] The expansion of regulatory T (Treg) cells is stimulated by the tumor cell-derived exosomes that cause immunosuppression by impairing the function of anti-tumorigenic CD8^{+} T cells.[51,52]

Elsner et al[53] demonstrated that exosomes from hepatocellular carcinoma cell lines and pancreatic cancer cells carry Hsp 70 that can directly up-regulate active natural killer (NK) cells, and consequently, NK cells induce apoptosis in tumors through granzyme B. Moreover, exosomes from tumor cells can transmit antigens to antigen-presenting dendritic cells through MHC-I molecules, and dendritic cells activate cytotoxic T lymphocytes and evoke an anti-tumor response, and suppress tumor growth *in vivo*.[54] Nonetheless, there is still an ongoing debate about whether cancer cells containing exosomes are an adequate mode of vaccination against tumor cells.

Biology of exosomal PD-L1

One of the mechanisms by which tumor cells induce immune evasion is the stimulation of surface expression of PD-L1 that interacts with PD-1 on effector T cells, suppressing their activation. To interfere with this pathological basis, anti-PD-1/PD-L1 mAbs have been developed; however, the overall response is limited.[7,55] Accumulating evidence recommends that exosomes are an important source of extratumoral PD-L1 and may be one molecular process contributing to PD-1 antibody treatment resistance.

Biogenesis and expression of exosomal PD-L1

Exosomal PD-L1 performs as the crossroads of inflammation and tumor progression, therefore illuminating the responsible factors as well as mechanisms that lead to its biogenesis and release is needed to intensely understand the inflammatory response of tumor cells during malignant evolution. Poggio et al[56] reported that exosomal PD-L1 is generated from the plasma membrane, rather than from the endoplasmic reticulum or Golgi apparatus. Similarly, PD-L1 internalized through the plasma membrane might be a source of PD-L1 in exosomes (Fig. 1). Evidence recommends that PD-L1 is distributed amongst diverse cellular compartments, which have been deeply connected with immunotherapy failure.[57] However, the precise molecular mechanisms that dictate exosomal PD-L1 biogenesis and PD-L1 distribution are not completely elucidated.

Exosomal marker HRS and PD-L1 co-localize with CD63, representing that PD-L1 is enveloped in exosomes and the pre-exosomal PD-L1 is localized in the subcellular structure.[56,58] Therefore, the knockdown of HRS in the WM9 cell line also led to a decrease in PD-L1 expression in exosomes but retained expression in the parent cell apparently due to shrank exosome formation.[7] The ESCRT-associated protein ALIX has been identified to directly regulate the loading of PD-L1 onto exosomes from the endosomal lumen in basal-like breast cancer cells. Deficiency of ALIX in HCC1954 cells resulted in retained tumor cell-surface PD-L1 expression but reduced exosomal PD-L1 level.[59] Furthermore, knockout of the Rab family protein Rab27a and nSMase 2 in PC3 cells inhibit exosome secretion and dramatically down-regulate the coexpression of CD63 and PD-L1 in sucrose fractionation.[56] PD-L1 may be sorted into exosomes from the plasma membrane, yet additional points remain to be clarified including how other mechanisms of endosome maturation and exosome release stimulate exosomal PD-L1 biogenesis.

Poggio et al[56] and Monypenny et al[59] noted that experimental deletion of members of the ESCRT complex and accessory proteins down-regulates exosomal PD-L1 secretion and up-regulates PD-L1 expression at the cell surface. Kim et al[60] found that the amount of exosomal PD-L1 in the culture supernatant of lung cancer cell lines characterized the amount of PD-L1 expression on the cell surface, while the plenty of exosomal PD-L1 isolated from plasma of non-small-cell lung cancer patients interrelated with tumor PD-L1 positivity. Undoubtedly, one of the major complications of immunotherapy is differential PD-L1 expression among tumor types, and there may have an association between the exosomal membrane and the amount of PD-L1 expressed at the cell surface.[59,61] Understanding the nature of the association between the exosomal membrane and the amount of PD-L1 expressed at the cell surface and the responsible factors that may lead to a PD-L1 alteration from the cell surface to the exosome membrane is required in further study.

Numerous human melanoma cell lines revealed that the expression level of exosomal PD-L1 is higher in metastatic tumors compared to primary tumors indicating that the PD-L1 distribution at the cellular level and the release of exosomal PD-L1 may be linked with the range of metastatic capacity of tumor cells.[7] In circulation, other forms of extracellular PD-L1 are also found; circulating PD-L1 microvesicles or its membrane-free soluble forms have been identified.[62,63] Further investigation is required to identify the biological consequence of other forms of soluble PD-L1 and the molecular mechanisms that lead to their biogenesis and secretion to advance immunotherapy efficacy.

Regulation of exosomal PD-L1

Moon et al[64] revealed that when $CD8^+$ T lymphocytes release IFN-γ, PD-L1 is triggered by attaching to the interferon-gamma receptor. This activates JAK/STAT signaling, which then activates IRF-1, further increasing PD-L1 production on tumor cells. The promotional effect of IFN-γ worked similarly for exosomal PD-L1. IFN-γ has been proven not to enhance the number of vesicles from release but exhibit higher binding affinity to PD-1, and thus exosomal PD-L1 is stimulated by IFN-γ.[7,56] Tumor cells not only contain PD-L1 molecules directly on the cell membrane but also impede the $CD8^+$ T cell activation to accomplish immune escape. Most importantly, tumor cells with high malignancy inhibit anti-tumor immunity by secreting PD-L1 enriched exosomes directly to the TME or through draining lymph nodes to distant places, targeting diverse effector T cells. However, once the exosomal PD-L1 is inhibited, the body may develop sustained robust anti-tumor immunity. Recent findings support the statement that PD-L1 release is partly facilitated by cytokine induction, likewise growth of exosomal PD-L1 release by IFN-α, IFN-γ, and TNF-α in melanoma and glioblastoma cells.[56,62] Additional cytokines, such as TGF-β1 and IL-17, have also been linked with PD-L1 expression; however, their impact on exosomal PD-L1 is not fully clarified.[65]

Tumor immune escape partly results from adaptive tumor responses by the influence of a variety of factors. Microenvironmental factors including alterations in pH, nutrients, and oxygen concentration are closely associated with immunosuppression. Concerning these issues, tumor hypoxia has been recognized as an influential driving force of immune escape and tumor progression.[66] The response to hypoxia is mediated by hypoxia-inducible factors (HIF) in low oxygen concentrations, which encourages the stabilization as well as nuclear translocation of HIF-1α to form the HIF1α/β complex which associates with STAT3 to stimulate PD-L1 expression.[66,67] Hypoxia plays a crucial role in increasing exosome secretion in a HIF-1α-dependent manner.[68] Moreover, hypoxia-mediated exosome release has been operated by Rab27a, which has also been associated with exosomal PD-L1 biogenesis.[21,68] Consequently, hypoxia can promote exosomal PD-L1 release, and inhibiting the response to hypoxia

by targeting HIF-1α and STAT3 can be a promising approach in both PD-L1 and exosome inhibition.

Along with TME factors, therapeutic interventions can also stimulate exosomal PD-L1 biogenesis and secretion. Zhang et al[69] describe that the chemotherapeutic agent 5-fluorouracil (5-FU) enhances the expression of exosomal PD-L1 via the miR-940/Cbl-b/STAT5a axis in patients with advanced gastric cancer. Dosset et al[70] highlighted that immunocyte-derived cytokines may rise PD-L1 expression after chemotherapy in colon cancer. Similarly, in a syngeneic model of prostate cancer, p300/CBP inhibition by a small molecule p300/CBP inhibitor intensely increased the efficacy of PD-L1 blockade treatment by hindering both the intrinsic and IFN-γ induced PD-L1 expression.[71]

Exosomal PD-L1 on CD8$^+$ T cell dysfunction

Considering the tumor immune escape, CD8$^+$ T cells have built up an exhausted phenotype owing to continuous antigen-mediated activation, inhibitory receptor signaling, metabolic dysfunction, and other microenvironmental factors.[72] The application of immune checkpoint inhibitors is a promising approach to treatment for advanced tumors, mainly because inhibiting the PD-L1/PD-1 axis strengthens CD8$^+$ T cell-mediated response which includes direct cytotoxic activity against tumor cells.[73] Upon binding to PD-L1, PD-1 goes through a conformational change that leads to the phosphorylation of the immunoreceptor tyrosine-based inhibitory motif (ITIM) and the immunoreceptor tyrosine-based switch motif (ITSM), enhancing the enrollment of cytoplasmatic SHP-1 and SHP-2 protein tyrosine phosphatases.[61] Afterward, SHP-1/2 inhibits the phosphorylation of intracellular mediators of the PI3K/AKT/mTOR and MAPK signaling pathways and consequently terminates CD8$^+$ T cell activation[61,74] (Fig. 1). However, CD8$^+$ T cells target and induce tumor cell apoptosis through cytotoxic activity. Tumor cell-derived exosomes with high levels of PD-L1, COX-2, CTLA-4, CD15s, or CD44v3, can induce apoptosis in activated CD8$^+$ T cells.[75] Chen et al and Poggio et al[7,56] depict that cancer cell secreting exosomes with PD-L1 inhibit CD8$^+$ T cells to facilitate the progression of melanoma *in vitro* and *in vivo*, and additionally down-regulate CD8$^+$ T cells activity in draining lymph nodes in prostate cancer. Moreover, exosomal PD-L1 from other cancer cell lines, such as colon (RKO) and lung (HCC827), has parallel inhibitory functions in CD8$^+$ T cell activation.[58]

To describe the molecular basis of exosomal PD-L1 operated functional inhibition of CD8$^+$ T cells, recent findings demonstrate that glioblastoma-derived exosomal PD-L1 blocks TCR-mediated T cell activation.[76] Throughout the CD8$^+$ T cell maturation, any failure in the TCR-CD3 complex may lead the defects in CD8$^+$ T cell function and immunosuppressive clinical symptoms. The CD3-ζ chain in CD8$^+$ T cells stimulates the signal transduction of the TCR-CD3 complex,[77] as well as down-regulation of the CD3-ζ chain in CD8$^+$ T cells, affects the cytotoxic functions.[78] Theodoraki et al[79] revealed that exosomal PD-L1 suppresses the CD8$^+$ effector T cell-mediated activity. The amounts of PD-L1 in circulating exosomes were positively associated with the knack of exosomal PD-L1 to suppress the CD8$^+$ effector T cell activation. Furthermore, the contribution of

the PD-L1-mediated pathway in the negative regulation of surface expression of CD69 (a T cell activation marker) in activated CD8$^+$ T cells after coincubation with exosomal PD-L1 has been confirmed as well. Similarly, exosomal PD-L1 suppresses CD3/CD28-operated CD8$^+$ T cell activation signaling cascades. Researchers have found that exosomal PD-L1 significantly down-regulates CD3/CD28-induced ERK phosphorylation and NF-κB activation.[58] In addition, melanoma cells release exosomal PD-L1 that can inhibit cytokine production and CD8$^+$ T cell-mediated cytotoxicity by inhibiting the expression of GzmB and the production of IL-2 and TNF. IL-2 can augment CTL activation and survival through the Janus kinase 1 (JAK1)/JAK3-STAT5 pathway.[7] These findings suggest that PD-L1 in exosomes effectively terminates CD8$^+$ T cell activation, inhibits CD8$^+$ T cell effector functions, down-regulates the number of CD8$^+$ T cells, and induces immunosuppression in the TME. Hence, further research is required to elucidate the contribution of additional inhibitory ligands in extracellular vesicles and how they associate with PD-L1 function.

Exosomal PD-L1 in acquired resistance to anti-PD-1/PD-L1 therapy

The clinical significance of plasma circulating exosomal PD-L1 has been shown in patients with a wide variety of malignancies. There is outsized evidence that indicates the significant role of exosomal PD-L1 in the low patient response rate of anti-PD-L1/PD-1 therapy (Fig. 1). Most importantly, the abundance of circulating exosomal PD-L1 before treatment is responsible for resulting in lesser clinical response.[7,79,80] Several preclinical studies indicated that the inhibition of exosomal PD-L1 secretion with GW4869, a selective inhibitor of nSMase 2, and GW4869 with ferroptosis inducer (Fe^{3+}), or knocking down Rab27a and Smpd3 in tumor cells enhanced the efficiency of anti-PD-1 therapy.[56,58,81] Likewise, FDA-approved drugs sulfisoxazole and macitentan are capable of reducing exosomal PD-L1 levels from breast cancer cells and xenograft models by targeting endothelin receptor A (ETA), and effectively reinvigorate exhausted CD8$^+$ T cells and thus elicit robust antitumor effects in combination with anti-PD-1 antibody.[82,83] Additionally, by decreasing the binding of PD-1 with exosomal PD-L1, macitentan enhanced CD8$^+$ T cell-mediated killing activity.[83] In another study, inhibition of exosomal PD-L1 level from the colorectal MC38 model can negatively regulate tumor growth and enhance the survival rate by PD-L1 blockage treatment.[56] These findings indicated that the exosomal PD-L1 mediated resistance to current anti-PD-L1/PD-1 therapy, and several pharmaceutical agents have the capability to regulate the therapeutic resistance through improving antitumor efficacy. However, the detailed molecular mechanism underlying the therapeutic resistance of exosomal PD-L1 is still unknown.

Intracellular calcium in exosome formation and secretion

Calcium is a ubiquitous intracellular messenger in eukaryotic cells and plays a regulatory role from the origin of cells to cell death. The constricted regulation of Ca^{2+} homeostasis is a

very sophisticated process, which is crucial for diverse cellular processes such as energy metabolism, cell signaling, and cell motility.[84] From the perspective of the evolutionary process, some approaches had been augmented to control the Ca^{2+} homeostasis that includes the compartmentalization in organelles, such as endoplasmic reticulum, mitochondria, nucleus, and a number of ion channels and pumps playing crucial roles in this complex network.[85–87] Mainly membrane-associated proteins are directly involved in calcium homeostasis: IP3R, the Sarco/endoplasmic reticulum and plasma membrane Ca^{2+}-ATPases, ryanodine receptor (RyR), the store- and receptor-operated calcium channels (SOC and ROC), likewise ORAI and transient receptor potential channel (TRP), calcium release-activated calcium channel (CRAC), voltage-gated Ca^{2+} channels (VGCC), mitochondrial calcium channels, purinergic ionotropic receptors, the exchangers, such as the Na^+/Ca^{2+} exchanger (NCX), and the mitochondrial Ca^{2+} uniporter (MCU).[16,87–90] The well-established issue is that the interruption at the cellular Ca^{2+} homeostasis is directly associated with the augmented free radical production, mitochondrial permeabilization, and numerous types of cell death,[15,91] which may lead to tissue toxicity and various diseases including cancer.

In almost all cell types, exosome release is likely a regulated process where the intracellular Ca^{2+} concentration is essential to persuade this regulated secretion process. Savina et al[29] showed that via binding with the receptor, transferrin increases exosome release in a calcium-dependent manner, which organizes a physiological stimulus for exosome secretion in K562 cells. The Rab family of small GTPases functions in the regulation of vesicle trafficking among diverse compartments along endo-lysosomal as well as secretory pathways. This report demonstrated that K562 cells have comparatively more massive extents of Rab 11 than the other 60 members of this protein family.[92] Therefore, Rab11 plays a key role in exosome secretion by regulating the linking between endocytic, recycling, and secretory pathways of exosome release.[22] Similarly, another group reported that treatment of transfected cells with a cytosolic calcium concentration increasing agent like monensin produced a marked widening of the MVBs and regulated exosome secretion in Rab11 overexpressing cells.[23] This result suggests convergent action of Rab11 and calcium is responsible for generating larger MVBs.

Munc 13-4 is a significant Ca^{2+}-dependent Rab binding protein expressed in numerous cells and tissues with secretory functions to regulate the trafficking of Rab 11-positive vesicles. It is categorized by the existence of two C2-domains at the N and C terminus and two central Munc-homology domains (MHC).[27,93] The significance of these domains in the collaboration of Munc13-4 with SNARE proteins for stimulating membrane fusion has been described recently.[94] Additionally, Munc13-4 is considered a key regulator of vesicle tethering and membrane fusion via promoting the Ca^{2+}-stimulated fusion of VAMP8-containing liposomes with liposome-positive exocytic or endosomal Q-SNAREs and straightly correlated with late endosomal SNARE complexes.[95] Messenger et al[28] indicate that the acute raise of Ca^{2+} concentration in cancer cells stimulated a fivefold enhancement of $CD63^+$, $CD9^+$, and $ALIX^+$ exosome secretion that was reduced by Munc13-4 knockdown.

It was worth noting that Munc13-4 controlled MVB maturation and produced MVBs competent for exosome release by a Rab11-positive trafficking pathway.

Extracellular vesicles like exosomes and microvesicles are released by almost all cells into the extracellular space in both normal physiological and disease conditions. Exosomes are significantly responsible for cellular communications through the trafficking of cytosolic components and membrane proteins, from donor cells to recipient cells. However, the exosome-mediated transmission of such factors is associated with cancer development and progression.[96] During cancer conditions, cells secrete extensive amounts of exosomes, and higher intracellular Ca^{2+} is responsible for tumor progression and metastasis, but the principle of this mechanism is unknown. Most importantly, elevated level of Ca^{2+} positively regulates exosome biogenesis and secretion machinery and finally lead to an excess amount of exosome release from cells (Fig. 1). Therefore, the discovery of approaches aiming at suppressing exosome biogenesis and secretion from tumor cells by targeting intracellular Ca^{2+} level may have important therapeutic implications.[97]

Pharmacological perspectives for inhibiting exosome biogenesis and secretion

Inhibition of the generation and secretion of tumor-derived exosomes has risen as a novel and remarkable therapeutic approach for the advancement of anticancer drugs.[2] Many findings have already depicted that the suppression of exosome secretion can be accomplished by antibodies,[98] chemical inhibitors,[99] or genetic manipulation[100] that can enhance therapeutic efficacy in the management of metastatic cancer. The effective approaches to reducing exosome secretion are targeting molecules by numerous pharmacological agents which are responsible for their formation, packaging, and release of them (Fig. 2). Identification and development of a new inhibitor of exosome secretion as a novel anti-cancer therapeutic agent by using drug repositioning approach, a process of finding new indication of existing FDA-approved drugs, can reduce risk of cytotoxicity and safety because these drugs have already passed toxicity and safety tests in humans.[101,102] For instance, the FDA-approved drug dimethyl amiloride (DMA) has been found to suppress exosome secretion by targeting H^+/Na^+ and Na^+/Ca^{2+} channels and eliminate the exosome-mediated immune suppressive effects, and enhance anti-tumor immunity as a chemotherapeutic agent.[29,32] It is obvious that ESCRT machinery is pivotal to exosome biogenesis, cargo sorting, and release. Other ESCRT-independent pathways, such as lipid-mediated and tetraspanin-mediated, also play a crucial role. However, several pharmacologically active compounds tipifarnib, neticonazole, climbazole, ketoconazole, triademenol, manumycin A, and nexinhibs showed exosome inhibitory effects by decreasing the protein expression involved in both ESCRT-dependent exosome biogenesis and transport (Rab27A).[55,99,101,103] Additionally, exosome inhibitors targeting ESCTR-independent N-sphingomyelinase (nSMase), GW4869,[104] and spiroepoxide[105] have the ability to block the secretion of the exosomes.
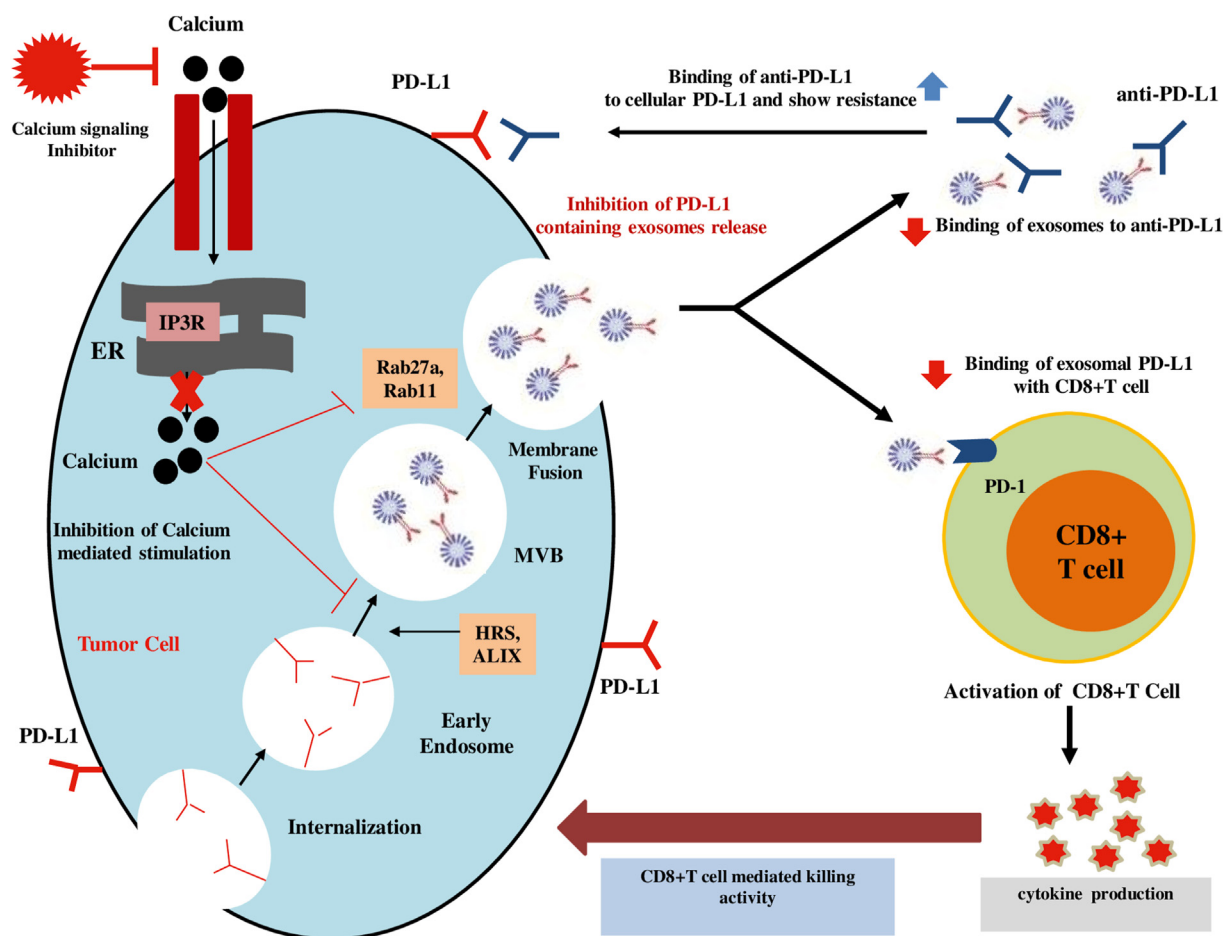
Figure 2   Proposed model of calcium signaling inhibitor-mediated enhancement of cancer immunotherapy. Calcium signaling inhibitors can inhibits the biogenesis and secretion of PD-L1-positive exosomes from cancer cells. Suppression of exosome secretion leads to the reduction of the interaction of exosomal PD-L1 with anti-PD-L1 antibodies and PD-1 on CD8$^+$ T cells. Consequently, anti-PD-L1 get a chance to bind the PD-L1 on tumor cell and augments the activation of CD8$^+$ T cells. Finally, calcium signaling inhibitor-mediated reinvigoration of exhausted CD8$^+$ T cells can lead to tumor cell killing and improve the overall anti-PDL1-related immune checkpoint blockage.

Further possible options for suppressing exosome secretion are the use of store-operated calcium channel blocking agent ketotifen,[106] proton pump inhibitors (PPIs),[107] and simvastatin.[108] Ketotifen was also presented to reduce the expression of several proteins which are associated with increased invasiveness of cancer cells and could decrease exosome release by controlling intracellular calcium levels.[109] PPIs are extensively prescribed to mitigate gastric acid that impairs the secretion of acidic vesicles and vesicle-like structures from tumor cells through inhibiting vacuolar H$^+$-ATPase-driven efflux pumps, and finally enhance the efficacy of chemotherapy.[110]

FDA-approved oral drugs sulfisoxazole and macitentan are capable of inhibiting the biogenesis and secretion of exosomes from breast cancer cells through interference with endothelin receptor A, a member of the G-protein coupled receptor (GPCR) family.[83,102] Most importantly, both drugs have shown significant anti-tumor and anti-metastatic effects in breast cancer xenograft mouse model, and the reduced expression of proteins contributed to exosome formation and release and induced lysosomal degradation of multivesicular endosomes.[55,83,102] Acute myeloblastic leukemia patients develop anti-cancer drug resistance due to altered drug targets, drug inactivation, reduced drug accumulation in the cancer cells, or alteration in the drug metabolic pathway and thus do not respond to treatment and experience cancer relapse. U937 cells showed resistance against the cytotoxic effect of the PEGylated liposomal doxorubicin drug through with PEGylated liposomal doxorubicin-containing chemotherapy regimens and found that the combination therapy significantly lowered the required concentrations of PEGylated liposomal doxorubicin as well as the drug side effects. Recently, one report showed that protein exchanger carbonic anhydrase IX (CA IX) plays a role in tumor pH regulation and overexpresses many cancer types. CA IX was found to be high in exosomes isolated from the plasma of prostate cancer patients suggesting that CA IX overexpression is also involved in exosome biogenesis and secretion. Therefore, CA IX inhibitor SLC-0111 is in a clinical trial. Cannabidiol, a phytocannabinoid extracted from *Cannabis sativa*, not only has analgesic and anti-

inflammatory actions but also showed chemo-preventive actions. 5 μM cannabidiol can also block exosome secretion by 50% from prostate cancer cell PC3, hepatocellular carcinoma HEPG2, and breast adenocarcinoma MDA-MB-231.[111,112] Leblanc et al[113] characterized a small pharmacological inhibitor targeting the PDZ2 domain of syntenin. The chemical compound is non-toxic to MCF-7 breast carcinoma cells and can impair cell proliferation, migration, and primary sphere formation and decreased the amount of exosomal syntenin, ALIX, and syndecan 4 (PDZ partner of syntenin), and thus affected exosome composition and activity.

The risk of cytotoxicity can be reduced by employing the drug repositioning technique to identify and develop new inhibitors of exosome secretion as novel anti-cancer therapeutic agents because these medications have already passed toxicity and safety tests in humans.[101,102] Therefore, the efficacy of the drug-repositioning strategy for preventing exosome secretion could provide a remarkable and safe approach for developing the immune inhibitory functions of exosomal-PD-L1 and therapeutic indications of metastatic cancer. Finally, designing appropriate clinical trials for the inhibition of exosomes to target cancers that have developed drug resistance is demanded.

Regulation of tumor microenvironment by calcium signaling

During tumor progression, cancer cells form a specific evolutionary process, that in conjugation with immune cells, extracellular matrix, and vascular endothelial cells, adapt to these changes. Together these components lead to tumor niche formation, which is called TME. As a crucial part of the ion channel, calcium channels play a significant role in tumorigenesis through the modification of the physic—chemical properties of TME.[114] Immune responses are significant components in TME, which are inhibited during tumor formation, and calcium signaling is one of the responsible factors. Calcium has been designated as a significant messenger for T lymphocytes, which forms a complex with calmodulin called CaM. CaM's affinity for CaM's targets, such as the CaMK (Ca^{2+}/CaM-dependent Ser-Thr kinase) family, is what activates calcium. Functional modulations of these kinases have been proven to be interconnected in the immune-suppressive nature in TME.[115] Recent studies demonstrated that the regulation of calcium signaling on $CD8^+$ T cells was mediated by CRAC channel STIM1 and STIM2,[116] and IP3.[117] Moreover, calcium signaling has been pointed out to regulate macrophage recruitment[118] and NK cells.[119] A detailed study on the molecular basis of the remodeling of immune response by calcium signaling could help improve the efficacy of immunotherapies as well as provide new insight into disease progression.

Excessive vascular growth is a vital sign of cancer, which support cancer cells to survive in TME. Many studies have described that calcium channels have a regulatory role in angiogenesis in different types of cancer. For instance, TRPV4 was released in excess amounts on tumor-derived endothelial cells and enhanced the angiogenic properties in breast cancer. However, TTRPV4-mediated calcium entry was responsible for the migration of endothelial cells, thus playing a regulatory role in angiogenesis.[120] Hypoxia is an essential phenomenon in TME for the continual division and proliferation of cancer cells. In this process, calcium signaling has reported a broad interconnection with hypoxia-inducible factor (HIF). Several studies have described that HIF-1 is controlled by calcium signaling in different approaches, likewise (a) transcription of HIF-1α,[121] (b) steadiness of HIF-1α (such as STIM1 in hepatocarcinoma,[122] TRPM8 in prostate cancer,[123] TRPC6 in glioma[124]), and (c) nuclear translocation of HIF-1α (such as TRPC5 in breast cancer[125]). Therefore, future research direction should focus on the modulation of both calcium signaling and HIF-1 pathways to control cancer progression.

Cancer-associated fibroblasts (CAFs) are the major element of the TME and take part in complex bidirectional association with tumor cells. Considering the broad contribution of CAFs towards cancer progression, proliferation, migration, genetic instability, and chemoresistance are associated with calcium signaling in CAFs and cancer.[126] For example, an *in vitro* study aiming at the interaction between prostate cancer cells and CAFs pointed out that resveratrol activates the TRPA1 calcium channel in CAFs and leads to calcium influx and release of VEGF and HGF. Therefore, TRPA1 activation significantly down-regulates prostate cancer cell death. However, the use of TRPA1 antagonist expressively inhibited the calcium influx, VEGF, and HGF emission and obstructed the protective effects on prostate cancer.[127] Plenty of studies have revealed the diverse role of calcium signaling in promoting tumorigenesis, as well as in the development of CAF features. There are still many unexplored issues of calcium signaling in CAF cancer progression.

Regulation of immune cells and immune checkpoints by calcium signaling

The transport of Ca^{2+} to the cytosol has paramount importance for immunoreceptor signaling, regulation of differentiation and activation of lymphocyte, antibody and cytokine secretion, and cytotoxicity.[128] Several well-characterized and inter-connected mechanisms are elucidated to increase the cytosolic free Ca^{2+} concentrations: release of ER Ca^{2+} stores and store-operated Ca^{2+} entry via plasma membrane channel.[128,129] Previously, Revy et al[130] noted that naïve T cell TCR recognition of MHC molecules on dendritic cells triggers small Ca^{2+} responses which are crucial for their survival. Surh et al[131] mentioned that Ca_v1.4 is a significant homeostasis regulator for naïve $CD4^+$ and $CD8^+$ T cells which represents that this channel modulates the signal required for their survival through connecting TCR signaling with self-peptide MHC molecules and IL-7R signaling after IL-7 exposure. Consequently, Omilusik et al[132] hypothesized that the interactions of TCR with self-antigen induce naïve T cells to open Ca_v1.4 channel. They found that Ca^{2+} influx from outside the cell through Ca_v1.4 channel is responsible for inducing a signaling cascade and contributes to the filling of intracellular Ca^{2+} stores critical for TCR survival. Moreover, the effect of Ca^{2+} on facilitating CD3 phosphorylation is mostly due to the charge of ion, as highlighted by replacing Ca^{2+} with non-physiological

ion that showed the same feedback effect. The regulatory pathway of Ca^{2+} has a positive feedback effect on amplifying CD3 phosphorylation that eventually enhances T-cell sensitivity to foreign antigens.[133] Collectively, these studies describe the role of the Ca^{2+} channel in lymphocyte physiology through the regulation of intracellular Ca^{2+} storage, antigen receptor signal transduction, and effector function.

Over the past decade, immune checkpoint blockade therapies have made a remarkable promise in the management of metastatic cancers. Antibody drugs, like anti-PD-1 and anti-PD-L1, and antagonists targeting immune checkpoints, exhibit advantages such as extensive applicability through cancer types and long-lasting clinical response when treatment is effective.[134] Remarkably, immune checkpoints are also connected with calcium signaling. Voltage-gated calcium ion trans-membrane channel subunits CACNA1E and CACNA1A are highly connected with tumor immunity and immune checkpoint expression regulation during tumorigenesis.[135,136] Reduced cell GSH levels caused an increase in PD-L1 expression when glutamine was scarce by reducing SERCA activity, which triggers the calcium/NF-kB signaling cascade. As a result, T cell antitumor activity was reduced when glutamine metabolism was inhibited in immunocompetent mouse tumors.[137] Additionally, suppression of proprotein convertases blocked proteolytic maturation of the Notch precursor, impaired calcium/NFAT and NF-κB signaling, and up-regulated ERK activation that lead to inhibition of PD-L1 expression.[138] Transcription factor NFATc1 is activated by calcium signaling and TCR. NFATc1 is stimulated by targeting PD-1 in $CD4^+$ and $CD8^+$ T cells in tumor-bearing mice and connected with increased antitumor cytotoxic responses.[139] Likewise, calcium fluctuations of $CD8^+$ T cells are augmented by targeting the immune checkpoint LAG3 with a novel antibody TSR-033.[140]

CaMK1D-mediated treatment showed resistance to anti-PD-1/PD-L1. In those cancer types of anti-PD-1/PD-L1 treatment, CaMK1D is up-regulated by CTL and then down-regulated by the activation and functions of caspase-3, caspase-6, and caspase-7 and finally leads to treatment failure.[141] Furthermore, the deficiency of TRAF3 hinders the auto-reactive B cell energy by enhancing calcium influx in response to BCR stimulation, which is responsible for autoimmune exhibitions and lymphoid organ disorders.[142] The expression of CCR5 stimulates calcium signaling and thus elevates regulatory T cell differentiation and migration to inflammation sites and recent studies suggested that there has profound coaction between CCR5 and immune checkpoints function.[143,144]

Therapeutic approaches targeting calcium signaling and immune checkpoints

Immune checkpoint blockade therapy using antibodies to block receptor—ligand interaction has become a promising anti-cancer therapy for numerous cancer types. For example, the efficacy and survival rates of patients who are treated with nivolumab are recovered more than the patients cured with conventional chemotherapy.[145,146] However, like other anti-cancer therapies immune checkpoint blockade therapy also faces great challenges, such as a low

overall response rate. Therefore, recent findings have described some reasons behind the low patient response rate to immunotherapy. Firstly, there may be other factors or mechanisms responsible for immunosuppression in tumors. Secondly, the PD-1 antagonists showed signs of immunotherapy resistance and did not provide the elongated immune response, but reduced the efficacy of treatment.[58,147] The existing strategy of immunotherapy can only object and block the PD-1/PD-L1 on the surface of tumor cells, which releases the exosomal PD-L1 to escape the immune response through binding to PD-1 on T cells to obstruct $CD8^+$ T cell activation.[58] Therefore, anti-exosomal PD-L1 therapy by using exosomal PD-L1 inhibitors may create systemic and robust anti-tumor immunity as well as may overcome the resistance of existing anti-PD-1/PD-L1 therapy.

Throughout this review, it has been presented that enhanced calcium signaling is largely connected with tumorigenesis by promoting malignant behaviors of cancer cells, contributes to numerous oncogenes and oncogenic signaling, exosome biogenesis and release, and suppression of immune response by regulating immune checkpoints. These observations altogether recommend that inhibition of calcium signaling can suppress the exosomal PD-L1 expression by impairing exosome secretion from cancer cells and that may be a promising strategy for cancer treatment by using immune checkpoint blockade therapy (Fig. 2). Therefore, all kinds of calcium-permeable ion channels or pumps seem to be natural targets. The application of calcium channel blockers in clinical practice has a long background. For instance, nifedipine is a first-line agent for hypertension.[148] Subsequently, nifedipine can down-regulate colorectal cancer proliferation and metastasis by inhibiting the NFAT2 activation and nuclear translocation by reducing the calcium influx. Additionally, nifedipine can activate tumor immune response by repressing the expression of PD-L1 and PD-1.[149] Another calcium channel blocker amlodipine can deplete PD-L1 expression and calpain-dependent stabilization of PD-L1 protein by impairing calcium flux and finally enhance $CD8^+$ T cell-mediated immunity.[150] Dihydropyridine calcium channel blockers (lercanidipine and nicardipine) and non-dihydropyridine calcium channel blockers (diltiazem and verapamil) suppress PD-L1 expression from the cytoplasm and cell surface by inhibiting the phosphorylation of STAT1, which is considered a key transcription factor of PD-L1.[151,152] When cancer cells are infiltrated by $CD8^+$ T cells, the PD-L1 expression is stimulated by IFN-γ, released by $CD8^+$ T cells. However, lercanidipine can reduce the IFN-γ-induced PD-L1 transcription and finally enhance the $CD8^+$ T cell-mediated killing activity.

Tumor cell surface PD-L1 and exosomal PD-L1 have the same topological structure and biological functions, which suggests that to regulate tumorigenesis, targeting exosomal PD-L1 would be an effective approach.[7] Therefore, the leading strategy for targeting exosomal PD-L1 is to eliminate exosomes, including inhibition of exosome biogenesis and secretion. There are many methods regarding the suppression of exosome formation and secretion. Among them, targeting the calcium signaling or calcium channel by using calcium channel blockers will be the effective approach, because intracellular calcium level and calcium

signaling are intensely connected with exosome biology, immune checkpoint regulation, and cancer progression. For example, ketotifen and simvastatin have been found to impair the secretion of exosomes.[106,108] However, we need more research to investigate the use of other calcium channel blockers (amlodipine, nifedipine, lercanidipine, diltiazem, and verapamil) in the regulation of exosome secretion and exosomal PD-L1 expression as these pharmacological agents have already shown the inhibitory functions on cellular PD-L1 expression. Attention should be paid to observing the cytotoxicity of exosome inhibitors to normal cells. The combination of inhibition of exosome secretion and anti-PD-L1 antibody therapy could be more effective because exosomal PD-L1 characterizes a striking therapeutic target in immunotherapy resistance.

Author contributions

Conflict of interests

Funding

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