T Lymphocyte and CAR-T Cell-Derived Extracellular Vesicles

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Extracellular vesicles (EV) are a very diverse group of cell-derived vesicles released by almost all kind of living cells. EV are involved in intercellular exchange, both nearby and systemically, since they induce signals and transmit their cargo (proteins, lipids, miRNAs) to other cells, which subsequently trigger a wide variety of biological responses in the target cells. However, cell surface receptor-induced EV release is limited to cells from the immune system, including T lymphocytes. T cell receptor activation of T lymphocytes induces secretion of EV containing T cell receptors for antigen and several bioactive molecules, including proapoptotic proteins. These EV are specific for antigen-bearing cells, which make them ideal candidates for a cell-free, EV-dependent cancer therapy.

exosomes | T lymphocytes | immune synapse | secretory granules | multivesicular bodies | cytotoxic activity | cell death | CAR T lymphocytes

1. Introduction

1.1 Extracellular Vesicle Types

Extracellular vesicles (EV) are a very diverse group of cell-derived, lipid bilayer enclosed vesicles released by almost all kind of living cells, and this process has been highly conserved throughout evolution [1]. There are several subtypes of EV that have distinctive structural, biochemical properties and composition depending of their intracellular origin that, in turn, affect their function [2]. EV are highly heterogeneous, which in great part is responsible for hindering the characterization and description of their properties and functions [1], and are often classified in terms of their generation mechanism. The first type includes the EV released by dying apoptotic cells, which are called apoptotic bodies. Apoptotic bodies have a wide range of sizes and exhibit different compositions and features from EV derived from living cells, and are not discussed in this entry. The second type of EV are directly formed by outward budding of the plasma membrane and are called microvesicles, ectosomes, ectovesicles or, generically, shedding vesicles [3] (**Figure 1**). The third type, exosomes, are secreted via the fusion of multivesicular bodies (MVB) with the plasma membrane [4][5] and have diameters ranging 30–150 nm (depending of the estimation technique [6]), smaller than shedding vesicles (100 nm to >1 μm) (**Figure 1**). MVB are subcellular organelles containing intralumenal vesicles (ILV) [7][8] that are components of the endolysosomal system, which also comprises early endosomes, late endosomes and lysosomes [9][10]. MVB are formed by inward budding from the external membrane of late endosomes and successive pinching off of budding vesicles into the

lumenal space of late endosomes (**Figure 1**). ILV present in MVB are called exosomes when they are released into the extracellular medium [11].

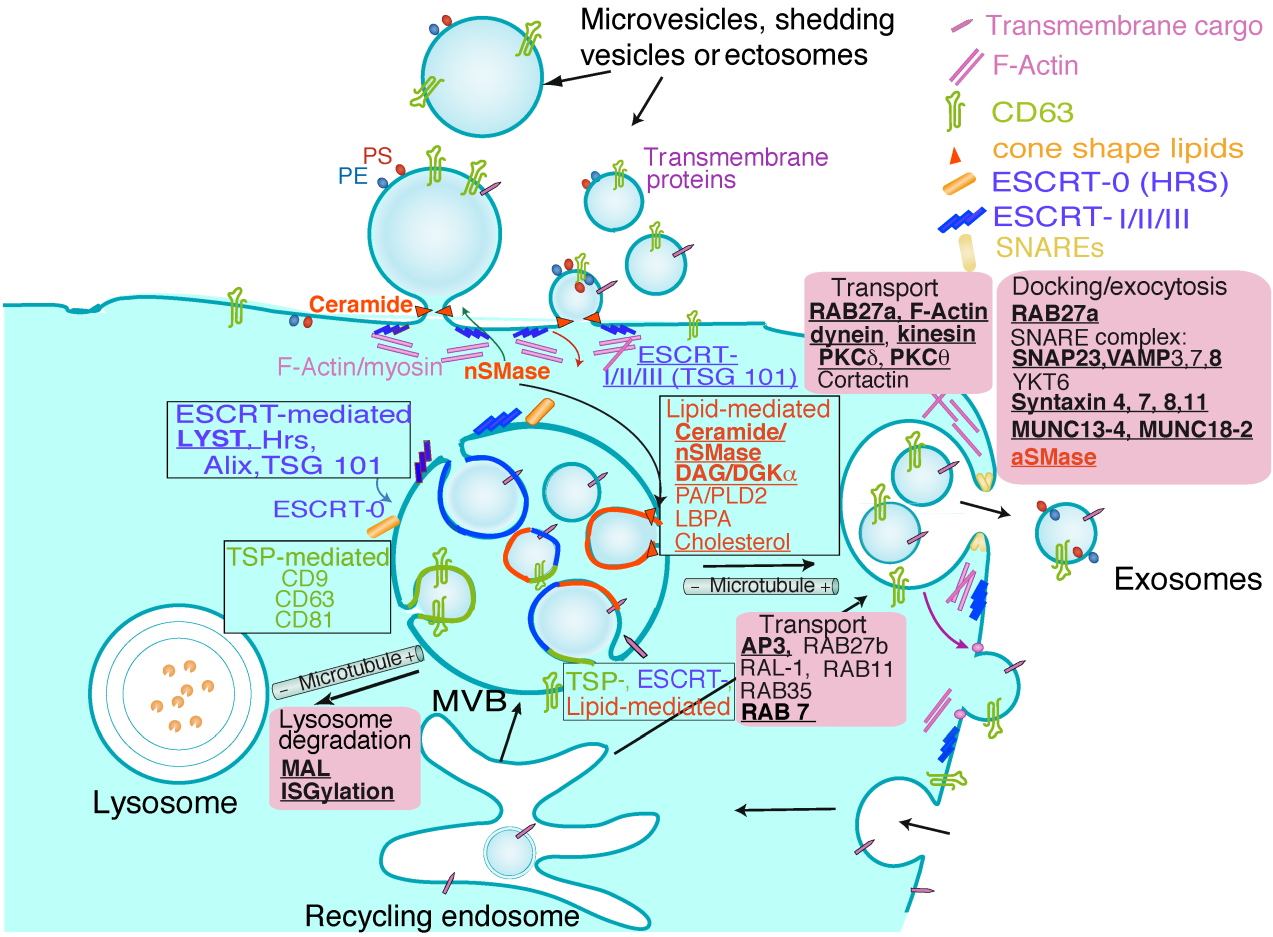

Figure 1. Extracellular vesicles. EV of different intracellular origins can be secreted by eukaryotic cells. The figure represents the different types of vesicles released, either by direct budding from the plasma membrane or by generation of ILV inside MVB, that subsequently fuse with the plasma membrane releasing exosomes. Apoptotic bodies released by dying cells have been excluded. For clarity's sake, only the constitutive secretion of EV and exosomes is represented, although in certain immune cells such as T and B lymphocytes the traffic of MVB and the secretion of exosomes can be induced via T cell receptor (TCR) and B cell receptor (BCR) stimulation [12]. Traffic of MVB comprises three general phases: ILV biogenesis during the maturation of MVB, transport of MVB to the plasma membrane and docking and fusion of MVB to the plasma membrane, whereas EV secretion involves a single step. Transport and fusion of MVB to the lysosomes may lead to MVB degradation. For more details, please refer to [1][6][13]. General and T lymphocyte-specific mechanisms of shedding vesicles and exosome biogenesis and MVB traffic are represented. The inward, intraluminal budding of specific membrane nanodomains from the MVB limiting membrane produces ILV. The invagination of ILV and the sorting of specific cargoes can be produced by the action of three mechanisms that are enclosed in black line boxes: Endosomal Sorting Complex Required for Transport (ESCRT)-0-I-II-III machinery (blue), tetraspanins (TSP) (green) or certain lipids as cholesterol, ceramide, diacylglycerol (DAG) and lysobisphosphatidic acid (LBPA) (red). In addition, multiple machineries (represented as

mixed colors) can collaborate in ILV biogenesis. It is unclear whether the three mechanisms act simultaneously on the same MVB or each one acts on different MVB, although all mechanisms are shown operating in the same MVB for clarity's sake. Black line rectangles enclose the general mechanisms involved in exosome biogenesis, whereas the regulators of MVB traffic (including transport to lysosomes for degradation, transport to the plasma membrane, docking and fusion with the membrane) are enclosed in magenta boxes. ESCRT-0 components (hepatocyte growth factor-regulated tyrosine kinase substrate -Hrs-, STAM) are generally not observed in plasma membrane budding leading to shedding vesicles, whereas ESCRT-I-II-III are involved in these processes (reviewed in [13]). However, both ESCRT-0 and ESCRT-I-II-III are involved in ILV formation inside MVB [1][13]. Actin cytoskeleton depolymerization is required for secretion of shedding vesicles and exosomes. In addition, externalization of phosphatidylethanolamine (PE) and phosphatidylserine (PS), that binds Annexin V, occurs in plasma membrane-derived EV and, to a lower extent, in exosomes. Bold, underlined characters identify those molecular components or processes that regulate MVB secretory traffic in T lymphocytes: lysosomal trafficking regulator (LYST) [14], neutral sphingomyelinase 2 (nSMase2) [15], DAG [16], diacylglycerol kinase α (DGKα) [17][18][19][20], acidic sphingomyelinase (aSMase) [21], MAL [22][23], ISGylation [24], Adaptor protein 3 (AP3) [25], Rab27a [26], Rab11, Rab7 [27], dynein [28], kinesin-1 [29], cortical F-actin [30][31], centrosomal area F-actin [32][33], protein kinase C δ (PKCδ) [31][34], protein kinase C θ (PKCθ) [35][36], vesicle-associated membrane protein 8 (VAMP-8) [37], syntaxin 4 (STX4) [38], syntaxin 7 (STX7) [39], syntaxin 8 (STX8) [40], syntaxin 11 (STX11) [41], SNAP23 [38]. Underlined characters identify molecules involved in shedding vesicles generation in T lymphocytes: tumor susceptibility gene 101 (TSG101) and vacuolar protein sorting 4 (VPS4) [42]. LYST has Hrs (an ESCRT-associated protein) as binding partner, which supports that LYST participates in MVB biogenesis [43][44].

The term exosomes (initially used to define shedding vesicles with diameters ranging from 40 to 1000 nm [45]) was later adapted to define nanovesicles of endosomal source that are liberated by fusion of MVB with the plasma membrane, as a means to discard specific out of date constituents during red cell maturation [4], and since then this proposal has been widely accepted by the scientific community, although not fully standardized yet. However, growing evidence supports that EV in general, and exosomes in particular, have much wider biological functions than removal of certain unwanted proteins, and all these EV are involved in intercellular communication, both locally and systemically, since they may transfer their cargo (proteins, lipids, miRNAs) between cells, and also may trigger new cues in recipient or target cells [1][10][12][46]. Thus, EV have been shown to affect the physiology of neighbouring target cells in diverse ways, from inducing cell signaling upon cell surface receptor triggering, to generating new properties in the target cells after acquisition of novel receptors, enzymes or genetic material contained into the EV [2][47].

2. Extracellular Vesicles from T Lymphocytes

IS formation by T lymphocytes subsequent to TCR binding to antigen bound to MHC on the APC surface is a very dynamic, plastic and critical outcome involved in antigen-specific, cellular and humoral immune responses [48][49]. IS establishment integrates signals and combines molecular interactions leading to a proper and antigen-specific immune response [50]. There are two main groups of IS built by T lymphocytes leading to quite different, although

essential, immune effector functions [48][50][51]. The interaction of helper T lymphocytes (Th), generally $CD4^+$ cells, with MHC-II-bearing APC causes T lymphocyte activation (cytokine secretion, proliferation, etc.). In contrast, naïve CTL, usually $CD8^+$ cells, recognize antigen-associated MHC-I on APC and become activated or "primed" to proliferate in the first phase, and kill target cells bearing the antigen in the second, effector phase. In the effector phase, primed CTL similarly form IS with target cells (virus-infected cells or tumor cells) leading to specific killing. Thus, the functional outcomes produced by the formation of an effective, mature IS include activation (naïve CTL and Th lymphocytes), killing (primed CTL), and functional anergy or apoptosis induction [52] once the effector phase is finished. IS formation induces the convergence of T lymphocyte secretion granules towards the MTOC and, almost simultaneously, MTOC polarization and secretion granules move towards the central supramolecular activation cluster (cSMAC) at the IS [49][53]. T lymphocyte secretion granules include cytokine-containing secretion granules in Th lymphocytes, cytolytic granules/secretory lysosomes in CTL, and MVB in Th lymphocytes and CTL. This dedicated mechanism appears to specifically endow the immune system with a superbly tuned tactic to enhance the efficiency of decisive secretory effector roles of T lymphocytes, while diminishing nonspecific, cytokine-controlled stimulation, target cell killing and activation induced cell death (AICD) of bystander cells [54].

CTL cytolytic granules are secretory lysosomes that have an MVB structure, and their degranulation causes ILV secretion as nanosize "extracellular vesicles" at the synaptic cleft made at the CTL-target cell interface, formerly described by Peters et al. [55]. Although CTL-secreted vesicles were not referred in this study as canonic exosomes, their creation and mode of exocytosis warrants this classification [10] (see above). Cytolytic granules contain perforin and granzymes that are located in a lumenal, electron dense core characterized by EM and also accumulate in the ILV [56]. In addition, it was shown that ILV and their derived exosomes contained, apart from the proapoptotic proteins perforin and granzymes, the exosome marker CD63 and molecules relevant for CTL-target cell interaction, such as TCR and CD8 [56][55], demonstrating that most of the cytotoxic factors exocytosed into the cleft between CTL and the target cell are membrane-enveloped or exosome-associated. However, release of perforin and granzymes in soluble form coming from the lumenal core cannot be excluded [56][57]. Perforin, which is inactive in the acidic environment of secretory lysosomes, is activated by neutral pH and Ca^{2+} at the synapse and polymerizes and forms a transmembrane pore that allows the entry of granzymes into the target cell; granzymes trigger caspase-dependent and independent cell death [44]. It was hypothesized that the presence of the TCR complex, CD8, and possibly other relevant molecules on these nanovesicles displaying their extracellular portions facing outwards, may ensure unidirectional delivery of lethal factors to target cells, since it was proposed that ILV released into the synaptic cleft bind specifically to the relevant antigen-MHC-I complex on the target cell membrane, and not to the CTL itself or to bystander cells [58]. This model would explain not only why a CTL does not kill itself, but also why bystander cells, which are in close proximity but do not bear the proper antigen, are not killed [58]. Subsequently, it was demonstrated that newly synthesized Fas ligand (FasL) is also stored in the limiting membrane of CTL secretory lysosomes and that polarized degranulation controls FasL delivery to the T cell surface, which is consistent with the role of a FasL-dependent pathway in CTL-mediated cytotoxicity [59].

In addition, it was shown that FasL can also be sorted from the MVB limiting membrane to ILV and hence to exosomes upon T lymphocyte activation, since T lymphocyte activation induced 100–200 nm "microvesicles" secretion including proapoptotic FasL and Apo2L [60] (**Figure 2** and **Figure 3**). These microvesicles were

subsequently characterized as canonic exosomes, since they arose from $FasL^{+}Apo2L^{+}$ ILV after MVB fusion with the plasma membrane [61]. Exosomal FasL and Apo2L, with the same topology as cell surface FasL and Apo2L, can bind to their respective death receptors on the surface of target cells, or effector T lymphocytes themselves, inducing caspase-dependent apoptosis [62][63] (**Figure 3**). Proapoptotic exosomes are thus involved in AICD of effector T lymphocytes, which constitutes an important suicide or fratricide mechanism participating in the downregulation of T cell-dependent immune responses [61][60][64]. Another major contribution was to demonstrate that inducible, polarized exosome secretion occurred at the IS formed by living Th lymphocytes and APC [18], as occurred in the IS formed by CTL [56][55] (**Figure 3**).

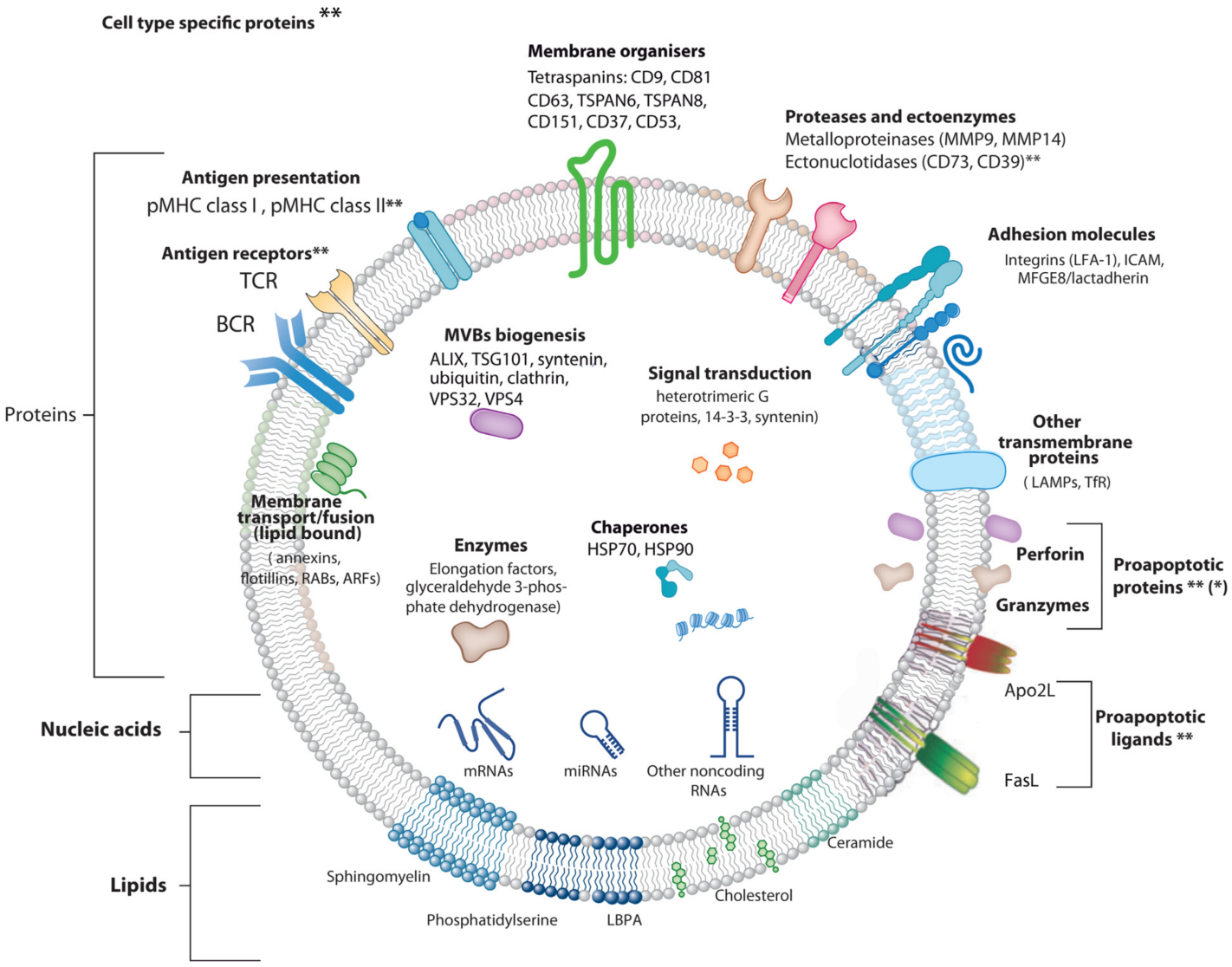

Figure 2. Exosome structure and molecular composition. Exosomes are surrounded by a phospholipid bilayer and contain nucleic acids and proteins (grouped by biological function), lipids, and nucleic acids. Exosomal proteins include annexins, important for transport; tetraspanins and integrins important for cell targeting and binding, and Alix and TSG101, involved in exosomal biogenesis from endosomes. Abbreviations: FLOT1, flotillin1; HSP, heat shock protein; MHC, major histocompatibility complex; RabGDI, RabGDP-dissociation inhibitor; RAP1B, Ras-related protein1B; TSG101, tumor susceptibility gene 101. (**) labels those proteins that are specifically found in

exosomes produced by T or B lymphocytes, whereas the rest of the indicated proteins are mostly found with high frequency (>30%) in exosomes produced by different cell types [2]. (*) indicates it is not clear whether perforin/granzymes are located or not inside exosomes. For more details regarding exosome composition visit http://www.exocarta.org, accessed on 24 January 2022.

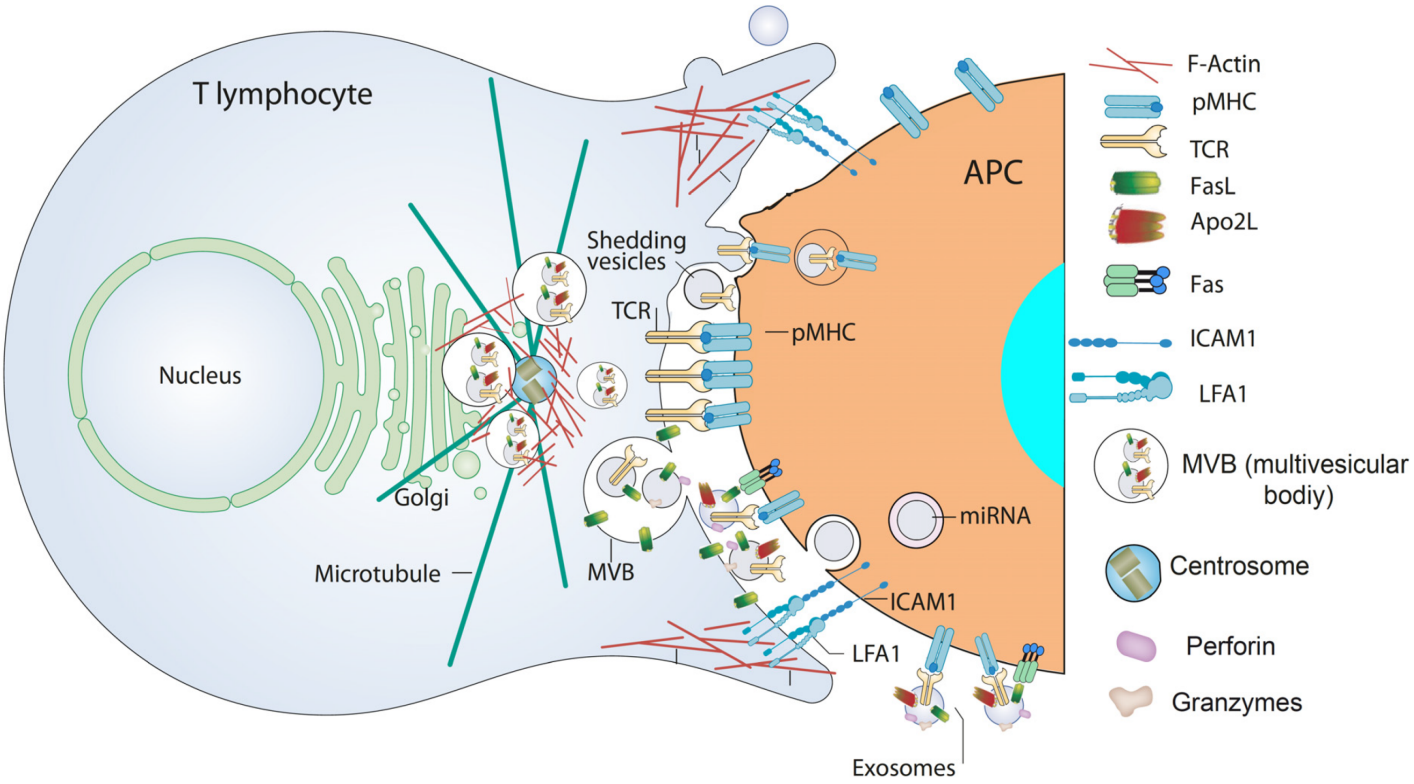

Figure 3. EV in the immune synapse. In a mature IS produced by TCR stimulation via the peptide-MHC complex (pMHC) on the APC and the interaction of accessory molecules (such as Intercellular Adhesion Molecule 1—ICAM1—with Lymphocyte function-associated antigen 1—LFA-1) F-actin is reduced at the cSMAC, the central region of the IS. F-actin accumulates at the distal SMAC (dSMAC), and F-actin around the centrosome depolymerizes. These F-actin reorganization processes, acting in a coordinated manner, may assist centrosome traffic towards the IS and the simultaneous convergence of MVB towards the F-actin depleted area in the cSMAC, facilitating MVB fusion at the cSMAC, and the subsequent exosome secretion carrying TCR and proapoptotic molecules in the synaptic cleft. In addition, shedding vesicles emerging from the plasma membrane and containing TCR are represented at the synaptic cleft. Both exosomes containing miRNA [15] and shedding vesicles [65][66] are engulfed by APC and provide biological responses in APC. For more details please refer to [12][67][65][68].

The hypothesis derived from all these publications that TCR activation of the effector T cell may induce the release of $CD63^+$ exosomes bearing TCR was formally demonstrated by using TCR agonists to activate Jurkat Th, CTL and $CD4^+$ lymphocytes [19][69]. Taken together, these reports constitute a major milestone in the exosome field since they demonstrate that TCR stimulation triggers inducible exosome secretion by T lymphocytes (both in CTL and Th cells) [2][12]. However, it is remarkable that, depending on the stimulation regime (absence or presence of co-stimulation signals), $CD4^+$ T cell activation promotes the differential release of distinct EV subpopulations [70].

Adding more complexity to the EV field, CD63-enriched shedding vesicles or ectosomes directly budding from the Th lymphocyte plasma membrane and accumulating at the IS formed with a B lymphocytes acting as APC have been described [42] (**Figure 3**). These synapse-induced shedding vesicles were enriched in TCR and, upon endocytosis by APC, were capable of signaling via pMHC-II stimulation [42][71] (**Figure 3**), suggesting these synaptic ectosomes may facilitate the activation of B cells and other APC presenting the cognate pMHC-II. In this report, centrally accumulated TCR were located on the surface of extracellular microvesicles that bud at the IS centre. Members of the ESCRT-I family such as TSG101 sort TCR for inclusion in shedding vesicles, whereas VPS4 mediates microvesicle scission from the T-cell plasma membrane. TSG101 interference reduced EV production, whereas VPS4 function disruption rendered budding vesicles unable to undergo fission from the plasma membrane [42] (**Figure 3**). However, neither the existence of T cell-derived shedding vesicles in the CTL IS, nor a proapoptotic role for these EV, has been demonstrated yet. In addition, although nSMase has been shown to be involved in plasma membrane budding in several cell types [13][72], its participation in EV release from T lymphocytes has not been reported yet, in contrast with its role in ILV biogenesis [73].

3. Chimeric Antigen Receptor (CAR) T Cells and CAR T Cell-Derived EV

3.1 Cancer Therapeutic Approaches

The role of EV from immune cells, including T lymphocytes, in anti-tumor immunity has been recently and exhaustively reviewed [74][75]; therefore, researcher focus on potential therapeutic uses of CAR T cell-derived EV. CAR T lymphocyte-based immunotherapy has proven to be a promising treatment of patients suffering several refractory cancer diseases [76]. CAR consists of an extracellular domain that confers antigen-recognition specificity, a transmembrane domain, and an intracellular signaling domain (CD3ζ) that provides activation signals to T lymphocytes [76]. However, several challenges preclude the use of adoptively-transferred CAR T cells, including their low efficacy against solid tumors, immunosuppression by tumor microenvironment, poor T cell persistence, T cell dysfunction or exhaustion, cytokine release syndrome (CRS) [76][77] and immune effector cell-associated neurotoxicity syndrome (ICANS). CRS is associated with supraphysiologic cytokine production and massive in vivo T cell expansion [78], whereas the cause of ICANS remains poorly understood, although appears to be related with direct central nervous system toxicity by the CR T cells, diffusion of inflammatory cytokines through the blood-brain barrier and the disfunction of this barrier caused by CAR T cells and/or cytokines [79]. It is out of the scope of this entry to summarize the preclinical and clinical uses of CAR T cells for cancer therapy (there are more than 250 clinical trials testing CAR T cells; please refer to recent and superb reviews on this topic [76][77]), thus researcher will focus on trials using CAR T cell-derived EV.

Considering the early hypothesis that the presence of both TCR and proapoptotic molecules (FasL, Apo2L, perforin, granzymes A and B) on T cell-derived exosomes would confer on them both antigenic specificity and guiding cytotoxicity [56][58][55], making them potent vectors to deliver proapoptotic cues to target cells bearing the cognate antigen, several strategies have been developed to test the use of CAR T cell-derived exosomes for cancer therapy. The fact that TCR activation boosts the secretion of CTL-produced exosomes, and the presence of

the TCR/CD3ζ complex in these exosomes (see above) [69] would reinforce this approach. TCR/CD3ζ complexes endocytosed after recognition of the pMHC-II complexes are targeted to MVB, then to ILV and hence exosomes [69]. A crucial prerequisite for using CAR T cell-derived exosomes to specifically induce tumor cell death is the presence on exosomes of the CAR molecule, since its antibody-derived, antigen-binding variable fragment endows them with tumor cell specificity, as occurs with CAR T cells [76][80]. Since CAR is an artificial molecule containing the CD3ζ intracellular signaling and localization domain, it was unknown whether CAR was present in exosomes and shedding vesicles, and whether CAR conferred a specific cytotoxic effect, until recent reports characterized CAR expression and function in exosomes [81] and shedding vesicles [82]. This prerequisite has been endorsed in several preclinical studies by using exosomes and/or EV produced by CAR T cells [81][83][84] (**Table 1**).

Table 1. CAR T cell and cell-derived EV: preclinical studies.

Target Molecule	EV-Producing Cell	EV Types	EV Phenotype	Anti-Tumor Mechanism	Target Cell
EGFR, HER2 [81]	Human CAR T cells (?) [1]	Exosomes	CAR^+, $CD3^+$, $CD63^+$, $perforin^+$, granzyme B^+, $CD45^-$, $CD28^-$	Perforin/ granzyme B [2]	$EGFR^+$, $HER2^+$ human breast cancer cells
HER2 [84]	Human CAR T cells $CD4^+$ (46%) $CD8^+$ (49%)	EV (small EV, probably exosomes plus larger EV) [3]	CAR^+, $CD3^+$, $CD63^+$, granzyme B^+	Granzyme B [2]	$HER2^+$ human breast cancer cells, ovarian cancer cells
Mesothelin [83]	Human CAR T cells $CD4^+$ (58%) $CD8^+$ (31%)	Probably exosomes [4]	CAR^+, $CD3^+$, $CD63^+$, $perforin^+$, granzyme B^+	Perforin/ granzyme B [2]	Triple negative human breast cancer cells
CD19 [85]	Human CAR HEK293 cells	Probably exosomes [4]	CAR^+, $CD63^+$, $CD81^+$	Indirect induction of proapoptotic genes in target cells	$CD19^+$ human B cell leukemia
CD19 [82]	Human CAR HEK293 cells	Probably shedding vesicles [4]	CAR^+, annexin V binding (PS exposure)	MYC Gene disruption mediated by CRISPR/Cas9	$CD19^+$ human B cell leukemia cell lines
Mesothelin CD19 [86]	Human and mouse CAR T Cells (?) [1]	EV [4]	Unknown [5] Contain RN7SL1	Recruitment of endogenous anti-tumor	Mouse melanoma expressing human CD19

CAR T cell-derived exosomes exhibit excellent capability for use as direct aggressors in immunotherapy, since ex vivo-produced human exosomes transporting human EGFR and HER2-specific CAR have powerful in vivo activity

Target Molecule	EV-Producing Cell	EV Types	EV Phenotype	Anti-Tumor Mechanism	Target Cell
			[81]	immunity byRN7SL1	

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derived exosomes, showing that only when exosome entry into cells is mediated via binding to the CD19 antigen on the surface of CD19+ B cells does pro-apoptotic signaling occur, resulting in selective cytotoxicity [83][84]. Certainly, the evoked apoptotic mechanism does not depend on Fas, FasL, perforin and granzymes, as for CAR T cell-derived exosomes, since HEK293 cells do not express these molecules. More recently, an interesting paper demonstrated an in vivo role of the RNA component of signal recognition particle 7SL1 (RN7SL1, a non-coding RNA that activates interferon-IFN-stimulated genes) contained in EV from CAR T cells in tumor rejection [86]. In this approach, the CAR construct contains downstream of the CAR sequence a U6 promoter that drives the transcription of human RN7SL1 and, optionally, a 5′LTR promoter driving the transcription of a peptide antigen. The authors demonstrate that RN7LS1-containing CAR T cells deliver RN7SL1 in EV in vivo, that orchestrates endogenous immune activation to improve responses against the tumor. EV released by CAR T cells upon CAR engagement transfer RN7SL1 to endogenous immune cells (myeloid cells, DC and T cells), but not to the tumor cells, and EV release and RN7LS1 transfer is inhibited by a nSMase inhibitor. RN7LS1 inside target cells activates a RIG-I-dependent, IFN-mediated inflammatory response and induces transcription of IFN-stimulated genes. RN7LS1 delivery in EV to immune cells improves immunostimulatory properties of myeloid and DC cells that, in turn, effectively activate the function of endogenous CD8 T cells against the tumor. All these immune cells, acting together, may trigger solid tumor rejection even in case of CAR-recognized antigen loss by the tumor. Although in this research neither EV nature or composition, nor the presence and contribution of CAR and proapoptotic proteins in the EV to tumor rejection, have been established, this strategy opens new venues based on improved endogenous immunity against tumor cells conferred by EV, since CAR T cells can now co-deploy antigenic peptides with RN7SL1 released by EV to enhance their efficacy against tumor cells, even when tumors lack adequate neoantigens [86]. **Table 1** summarizes the most relevant features of the preclinical trials involving CAR T cell and HEK293-derived EV. Most of these trials have been performed ex vivo or in vivo using xenograft models in mice.

The advantages of CAR T cell-derived exosomes as cell-free immunotherapy are their independence of CAR T cell life span and division, their stability, some obvious logistic issues, the low risk of collateral toxicity (i.e., CRS incidence) when contrasted to CAR T cells, and the fact that exosomes lacking PD-1 (in contrast to PD-1-expressing T cells) are refractory to PD-L1 immunosupression by the tumor [81] (**Table 2**). In this context, it is remarkable that CAR T cell-induced CRS is one of the most harmful complications that follow infusion of the CAR T cells and occurs in approximately two thirds of CAR T cell recipients, generally within 10 days after cell infusion. This life-threatening complication is generally ascribed to uncontrolled release of cytokines from CAR T cells [80]. Moreover, exosomes could be distributed through the blood circulation and other body fluids as supported by the abundance of exosomes found in most body fluids. In addition, exosomes have the ability to cross certain biological barriers, such as the blood–brain barrier and blood-tumor barrier, as documented by the presence of tumor cell-originated exosomes in body fluids [80] (**Table 2**).

Table 2. CAR T cell and CAR T cell-derived EV. A comparison.

Event	CAR T Cells	CAR T Cell-Derived EV
Cytokine releasing syndrome	++	−
Neurotoxicity	++	−
Cross the blood barrier	−	++
Efficiency against solid tumors	+/−	++
Immunosuppression by tumoral PD-L1	+	−
Immunological memory	+ [1]	(?) [2]

[1] Depends on the use of central memory or effector memory CAR T cells. [2] Not formally established.

The fact that such a cell-free "exosome therapy" involves the on-bench stimulation and expansion of the effector CAR T cells [81] may allow the genetic modification and/or pharmacologic treatment of the cultured cells to increase T cell activation, and/or exosome biogenesis, to enhance EV production. In addition, exosome collection and purification ex vivo involve the elimination of effector CAR T cells, and also bystander or contaminating cells, before exosome infusion [81]. Thus, this strategy would circumvent the undesirable possibility of transducing, for instance, CAR to residual tumoral cells during T cell manufacturing that may lead to provocation of resistance to CAR T cell therapy by unintentional transduction of a single leukemic B cell, as reported in [87]. These findings illustrate the need for purging residual contaminating tumor cells from engineered CAR T cells or using alternatives such as cell-free, exosome-based therapies.

Taking into account that it is not known whether CAR signaling or the involvement of any of the exosomal proapoptotic molecules (perforin, granzymes, FasL, etc.) mediate the therapeutic effects described, these findings require further confirmation, and to formally establish the contribution of these molecules, although they convincingly support the use of exosomes as biomimetic nanovesicles in antitumor therapy [81]. Recent reviews have dealt with the proapoptotic mechanisms evoked by the proapoptotic molecules present in EV on target cells; please refer to these for further details [74][88].

It is remarkable that most of the strategies directed to unveil the role of the molecular components involved in exosome biogenesis/degradation and/or release in T cells (ESCRT, tetraspanins, Rabs, MAL, ISGylation, SNAREs, etc.) are mainly based on either the expression of dominant negative mutants or RNA interference, which leads, in a vast majority of the approaches, to EV secretion inhibition [6][89]. Thus, although these approaches have been useful in establishing the necessity of diverse components for exosome secretion, very few of these approaches have led to an increase of exosome secretion [17][18][19][89][90]. In principle, the positive modulation of exosome secretion ex vivo could be a useful strategy to enhance the effectiveness of exosomes for subsequent in vivo applications. Among all the potential approaches designed to enhance on bench exosome secretion, perhaps the regulation of the lipid pathways and their metabolites (DAG, ceramide) involved in exosome biogenesis appears the most feasible approach, due the existence of quite specific pharmacologic agents suitable for exosome induction ex vivo. Thus, increasing DAG levels using DGK inhibitors (i.e., R59949) [19], or ceramide levels using

sphingomyelin synthase inhibitors (i.e., D609) [91], which have been shown to increase exosome secretion, may constitute useful tools for this strategy.

Although the timing and modes of activation and maturation are different, both CTL and Natural Killer cells (NK) utilize an overlapping arsenal consisting of cytotoxic effector proteins including FasL [59], perforins, granzymes and granulysin contained in their secretory lysosomes [92][93] (recently reviewed in [88]). The molecular mechanisms controlling the maturation and traffic of the secretory lysosomes and the polarized secretion of exosomes towards the synapse are, in great part, common to both cell types [44][59][94]. Thus, it is conceivable that approaches directed to increase exosome secretion in CTL ex vivo can also be useful to boost exosome secretion by CAR NK cells. However, although TCR activation enhances the constitutively low secretion of exosomes by T lymphocytes, resting NK cells secrete pro-apoptotic exosomes with no differences in the amounts of exosomes or marker expression relative to activated NK cells via NK activating and inhibition receptors [92][95]. NK cell expansion ex vivo increases exosome secretion [95], as occurs in T lymphocytes, which constitutes an useful strategy to produce on a large scale exosomes that may lead to new preclinical and clinical applications [95]. Different to T lymphocytes, NK cell recognition of their targets is not controlled by antigen specificity but rather through the integration of signals evoked by activating and inhibitory receptors, activated by a myriad of ligands in the target cells, which requires a deeper knowledge of the complex NK biology and signaling before any experimental design. This fact probably has led to CAR NK therapy being less developed than CAR T cell therapy in general [96], and therefore there are less pre-clinical trials using CAR NK-derived EV in particular [95]. However, the use of CAR NK cells has some advantages (and disadvantages) when compared with CAR T cells [96]. Although the use of CAR NK cells in clinical trials has been less extended in comparison to CAR T cells, in principle it is conceivable the use of NK-derived EV in future anti-tumor therapies.

References

1. van Niel, G.; D'Angelo, G.; Raposo, G. Shedding light on the cell biology of extracellular vesicles. Nat. Rev. Mol. Cell Biol. 2018, 19, 213–228.
2. Thery, C.; Ostrowski, M.; Segura, E. Membrane vesicles as conveyors of immune responses. Nat. Rev. Immunol. 2009, 9, 581–593.
3. Lindenbergh, M.F.S.; Stoorvogel, W. Antigen presentation by extracellular vesicles from professional antigen-presenting cells. Annu. Rev. Immunol. 2018, 36, 435–459.
4. Johnstone, R.M.; Adam, M.; Hammond, J.R.; Orr, L.; Turbide, C. Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). J. Biol. Chem. 1987, 262, 9412–9420.
5. Johnstone, R.M. The jeanne manery-fisher memorial lecture 1991. Maturation of reticulocytes: Formation of exosomes as a mechanism for shedding membrane proteins. Biochem. Cell Biol. 1992, 70, 179–190.

6. Colombo, M.; Raposo, G.; Théry, C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. Annu. Rev. Cell Dev. Biol. 2014, 30, 255–289.
7. Babst, M. Mvb vesicle formation: Escrt-dependent, escrt-independent and everything in between. Curr. Opin. Cell Biol. 2011, 23, 452–457.
8. Scott, C.C.; Vacca, F.; Gruenberg, J. Endosome maturation, transport and functions. Semin. Cell Dev. Biol. 2014, 31, 2–10.
9. Geuze, H.J. The role of endosomes and lysosomes in mhc class ii functioning. Immunol. Today 1998, 19, 282–287.
10. Denzer, K.; Kleijmeer, M.J.; Heijnen, H.F.; Stoorvogel, W.; Geuze, H.J. Exosome: From internal vesicle of the multivesicular body to intercellular signaling device. J. Cell Sci. 2000, 113 Pt 19, 3365–3374.
11. Pan, B.T.; Teng, K.; Wu, C.; Adam, M.; Johnstone, R.M. Electron microscopic evidence for externalization of the transferrin receptor in vesicular form in sheep reticulocytes. J. Cell Biol. 1985, 101, 942–948.
12. Calvo, V.; Izquierdo, M. Inducible polarized secretion of exosomes in t and b lymphocytes. Int. J. Mol. Sci. 2020, 21, 2631.
13. Mathieu, M.; Martin-Jaular, L.; Lavieu, G.; Thery, C. Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication. Nat. Cell Biol. 2019, 21, 9–17.
14. Nagle, D.L.; Karim, M.A.; Woolf, E.A.; Holmgren, L.; Bork, P.; Misumi, D.J.; McGrail, S.H.; Dussault, B.J., Jr.; Perou, C.M.; Boissy, R.E.; et al. Identification and mutation analysis of the complete gene for chediak-higashi syndrome. Nat. Genet. 1996, 14, 307–311.
15. Mittelbrunn, M.; Gutierrez-Vazquez, C.; Villarroya-Beltri, C.; Gonzalez, S.; Sanchez-Cabo, F.; Gonzalez, M.A.; Bernad, A.; Sanchez-Madrid, F. Unidirectional transfer of microrna-loaded exosomes from T cells to antigen-presenting cells. Nat. Commun. 2011, 2, 282.
16. Quann, E.J.; Merino, E.; Furuta, T.; Huse, M. Localized diacylglycerol drives the polarization of the microtubule-organizing center in T cells. Nat. Immunol. 2009, 10, 627–635.
17. Alonso, R.; Mazzeo, C.; Merida, I.; Izquierdo, M. A new role of diacylglycerol kinase alpha on the secretion of lethal exosomes bearing fas ligand during activation-induced cell death of t lymphocytes. Biochimie 2007, 89, 213–221.
18. Alonso, R.; Mazzeo, C.; Rodriguez, M.C.; Marsh, M.; Fraile-Ramos, A.; Calvo, V.; Avila-Flores, A.; Merida, I.; Izquierdo, M. Diacylglycerol kinase alpha regulates the formation and polarisation of mature multivesicular bodies involved in the secretion of fas ligand-containing exosomes in t lymphocytes. Cell Death Differ. 2011, 18, 1161–1173.

19. Alonso, R.; Rodriguez, M.C.; Pindado, J.; Merino, E.; Merida, I.; Izquierdo, M. Diacylglycerol kinase alpha regulates the secretion of lethal exosomes bearing fas ligand during activation-induced cell death of t lymphocytes. J. Biol. Chem. 2005, 280, 28439–28450.
20. Chauveau, A.; Le Floc'h, A.; Bantilan, N.S.; Koretzky, G.A.; Huse, M. Diacylglycerol kinase alpha establishes t cell polarity by shaping diacylglycerol accumulation at the immunological synapse. Sci. Signal. 2014, 7, ra82.
21. Herz, J.; Pardo, J.; Kashkar, H.; Schramm, M.; Kuzmenkina, E.; Bos, E.; Wiegmann, K.; Wallich, R.; Peters, P.J.; Herzig, S.; et al. Acid sphingomyelinase is a key regulator of cytotoxic granule secretion by primary t lymphocytes. Nat. Immunol. 2009, 10, 761–768.
22. Ventimiglia, L.N.; Alonso, M.A. Biogenesis and function of t cell-derived exosomes. Front. Cell Dev. Biol. 2016, 4, 84.
23. Ventimiglia, L.N.; Fernandez-Martin, L.; Martinez-Alonso, E.; Anton, O.M.; Guerra, M.; Martinez-Menarguez, J.A.; Andres, G.; Alonso, M.A. Cutting edge: Regulation of exosome secretion by the integral mal protein in T cells. J. Immunol. 2015, 195, 810–814.
24. Villarroya-Beltri, C.; Baixauli, F.; Mittelbrunn, M.; Fernández-Delgado, I.; Torralba, D.; Moreno-Gonzalo, O.; Baldanta, S.; Enrich, C.; Guerra, S.; Sánchez-Madrid, F. Isgylation controls exosome secretion by promoting lysosomal degradation of mvb proteins. Nat. Commun. 2016, 7, 13588.
25. Clark, R.H.; Stinchcombe, J.C.; Day, A.; Blott, E.; Booth, S.; Bossi, G.; Hamblin, T.; Davies, E.G.; Griffiths, G.M. Adaptor protein 3-dependent microtubule-mediated movement of lytic granules to the immunological synapse. Nat. Immunol. 2003, 4, 1111–1120.
26. Stinchcombe, J.C.; Barral, D.C.; Mules, E.H.; Booth, S.; Hume, A.N.; Machesky, L.M.; Seabra, M.C.; Griffiths, G.M. Rab27a is required for regulated secretion in cytotoxic t lymphocytes. J. Cell Biol. 2001, 152, 825–834.
27. Daniele, T.; Hackmann, Y.; Ritter, A.T.; Wenham, M.; Booth, S.; Bossi, G.; Schintler, M.; Auer-Grumbach, M.; Griffiths, G.M. A role for rab7 in the movement of secretory granules in cytotoxic t lymphocytes. Traffic 2011, 12, 902–911.
28. Combs, J.; Kim, S.J.; Tan, S.; Ligon, L.A.; Holzbaur, E.L.; Kuhn, J.; Poenie, M. Recruitment of dynein to the jurkat immunological synapse. Proc. Natl. Acad. Sci. USA 2006, 103, 14883–14888.
29. Kurowska, M.; Goudin, N.; Nehme, N.T.; Court, M.; Garin, J.; Fischer, A.; de Saint Basile, G.; Ménasché, G. Terminal transport of lytic granules to the immune synapse is mediated by the kinesin-1/slp3/rab27a complex. Blood 2012, 119, 3879–3889.
30. Ritter, A.T.; Asano, Y.; Stinchcombe, J.C.; Dieckmann, N.M.; Chen, B.C.; Gawden-Bone, C.; van Engelenburg, S.; Legant, W.; Gao, L.; Davidson, M.W.; et al. Actin depletion initiates events leading to granule secretion at the immunological synapse. Immunity 2015, 42, 864–876.

31. Herranz, G.; Aguilera, P.; Davila, S.; Sanchez, A.; Stancu, B.; Gomez, J.; Fernandez-Moreno, D.; de Martin, R.; Quintanilla, M.; Fernandez, T.; et al. Protein kinase c delta regulates the depletion of actin at the immunological synapse required for polarized exosome secretion by T cells. Front. Immunol. 2019, 10, 851.
32. Bello-Gamboa, A.; Velasco, M.; Moreno, S.; Herranz, G.; Ilie, R.; Huetos, S.; Dávila, S.; Sánchez, A.; Bernardino De La Serna, J.; Calvo, V.; et al. Actin reorganization at the centrosomal area and the immune synapse regulates polarized secretory traffic of multivesicular bodies in t lymphocytes. J. Extracell. Vesicles 2020, 9, 1759926.
33. Fernández-Hermira, S.; Sanz-Fernández, I.; Botas, M.; Calvo, V.; Izquierdo, M. Analysis of centrosomal area actin reorganization and centrosome polarization upon lymphocyte activation at the immunological synapse. In Methods in Cell Biology; Academic Press: Cambridge, MA, USA, 2021.
34. Ma, J.S.; Monu, N.; Shen, D.T.; Mecklenbrauker, I.; Radoja, N.; Haydar, T.F.; Leitges, M.; Frey, A.B.; Vukmanovic, S.; Radoja, S. Protein kinase cdelta regulates antigen receptor-induced lytic granule polarization in mouse cd8+ ctl. J. Immunol. 2007, 178, 7814–7821.
35. Quann, E.J.; Liu, X.; Altan-Bonnet, G.; Huse, M. A cascade of protein kinase c isozymes promotes cytoskeletal polarization in T cells. Nat. Immunol. 2011, 12, 647–654.
36. Liu, X.; Kapoor, T.M.; Chen, J.K.; Huse, M. Diacylglycerol promotes centrosome polarization in T cells via reciprocal localization of dynein and myosin ii. Proc. Natl. Acad. Sci. USA 2013, 110, 11976–11981.
37. Dressel, R.; Elsner, L.; Novota, P.; Kanwar, N.; Fischer von Mollard, G. The exocytosis of lytic granules is impaired in vti1b- or vamp8-deficient ctl leading to a reduced cytotoxic activity following antigen-specific activation. J. Immunol. 2010, 185, 1005–1014.
38. Spessott, W.A.; Sanmillan, M.L.; Kulkarni, V.V.; McCormick, M.E.; Giraudo, C.G. Syntaxin 4 mediates endosome recycling for lytic granule exocytosis in cytotoxic t-lymphocytes. Traffic 2017, 18, 442–452.
39. Pattu, V.; Qu, B.; Marshall, M.; Becherer, U.; Junker, C.; Matti, U.; Schwarz, E.C.; Krause, E.; Hoth, M.; Rettig, J. Syntaxin7 is required for lytic granule release from cytotoxic t lymphocytes. Traffic 2011, 12, 890–901.
40. Bhat, S.S.; Friedmann, K.S.; Knörck, A.; Hoxha, C.; Leidinger, P.; Backes, C.; Meese, E.; Keller, A.; Rettig, J.; Hoth, M.; et al. Syntaxin 8 is required for efficient lytic granule trafficking in cytotoxic t lymphocytes. Biochim. Biophys. Acta 2016, 1863, 1653–1664.
41. zur Stadt, U.; Schmidt, S.; Kasper, B.; Beutel, K.; Diler, A.S.; Henter, J.I.; Kabisch, H.; Schneppenheim, R.; Nürnberg, P.; Janka, G.; et al. Linkage of familial hemophagocytic

lymphohistiocytosis (fhl) type-4 to chromosome 6q24 and identification of mutations in syntaxin 11. Hum. Mol. Genet. 2005, 14, 827–834.

42. Choudhuri, K.; Llodra, J.; Roth, E.W.; Tsai, J.; Gordo, S.; Wucherpfennig, K.W.; Kam, L.C.; Stokes, D.L.; Dustin, M.L. Polarized release of t-cell-receptor-enriched microvesicles at the immunological synapse. Nature 2014, 507, 118–123.
43. Tchernev, V.T.; Mansfield, T.A.; Giot, L.; Kumar, A.M.; Nandabalan, K.; Li, Y.; Mishra, V.S.; Detter, J.C.; Rothberg, J.M.; Wallace, M.R.; et al. The chediak-higashi protein interacts with snare complex and signal transduction proteins. Mol. Med. 2002, 8, 56–64.
44. de Saint Basile, G.; Menasche, G.; Fischer, A. Molecular mechanisms of biogenesis and exocytosis of cytotoxic granules. Nat. Rev. Immunol. 2010, 10, 568–579.
45. Trams, E.G.; Lauter, C.J.; Salem, N., Jr.; Heine, U. Exfoliation of membrane ecto-enzymes in the form of micro-vesicles. Biochim. Biophys. Acta 1981, 645, 63–70.
46. Valadi, H.; Ekstrom, K.; Bossios, A.; Sjostrand, M.; Lee, J.J.; Lotvall, J.O. Exosome-mediated transfer of mrnas and micrornas is a novel mechanism of genetic exchange between cells. Nat. Cell Biol. 2007, 9, 654–659.
47. Robbins, P.D.; Morelli, A.E. Regulation of immune responses by extracellular vesicles. Nat. Rev. Immunol. 2014, 14, 195–208.
48. Fooksman, D.R.; Vardhana, S.; Vasiliver-Shamis, G.; Liese, J.; Blair, D.A.; Waite, J.; Sacristan, C.; Victora, G.D.; Zanin-Zhorov, A.; Dustin, M.L. Functional anatomy of t cell activation and synapse formation. Annu. Rev. Immunol. 2010, 28, 79–105.
49. de la Roche, M.; Asano, Y.; Griffiths, G.M. Origins of the cytolytic synapse. Nat. Rev. Immunol. 2016, 16, 421–432.
50. Xie, J.; Tato, C.M.; Davis, M.M. How the immune system talks to itself: The varied role of synapses. Immunol. Rev. 2013, 251, 65–79.
51. Huse, M.; Quann, E.J.; Davis, M.M. Shouts, whispers and the kiss of death: Directional secretion in T cells. Nat. Immunol. 2008, 9, 1105–1111.
52. Friedl, P.; den Boer, A.T.; Gunzer, M. Tuning immune responses: Diversity and adaptation of the immunological synapse. Nat. Rev. Immunol. 2005, 5, 532–545.
53. Huse, M. Microtubule-organizing center polarity and the immunological synapse: Protein kinase c and beyond. Front. Immunol. 2012, 3, 235.
54. Calvo, V.; Izquierdo, M. Imaging polarized secretory traffic at the immune synapse in living t lymphocytes. Front. Immunol. 2018, 9, 684.

55. Peters, P.J.; Geuze, H.J.; Van der Donk, H.A.; Slot, J.W.; Griffith, J.M.; Stam, N.J.; Clevers, H.C.; Borst, J. Molecules relevant for t cell-target cell interaction are present in cytolytic granules of human t lymphocytes. Eur. J. Immunol. 1989, 19, 1469–1475.
56. Peters, P.J.; Borst, J.; Oorschot, V.; Fukuda, M.; Krahenbuhl, O.; Tschopp, J.; Slot, J.W.; Geuze, H.J. Cytotoxic t lymphocyte granules are secretory lysosomes, containing both perforin and granzymes. J. Exp. Med. 1991, 173, 1099–1109.
57. Golstein, P.; Griffiths, G.M. An early history of t cell-mediated cytotoxicity. Nat. Rev. Immunol. 2018, 18, 527–535.
58. Peters, P.J.; Geuze, H.J.; van der Donk, H.A.; Borst, J. A new model for lethal hit delivery by cytotoxic t lymphocytes. Immunol. Today 1990, 11, 28–32.
59. Bossi, G.; Griffiths, G.M. Degranulation plays an essential part in regulating cell surface expression of fas ligand in T cells and natural killer cells. Nat. Med. 1999, 5, 90–96.
60. Martinez-Lorenzo, M.J.; Anel, A.; Gamen, S.; Monle n, I.; Lasierra, P.; Larrad, L.; Pineiro, A.; Alava, M.A.; Naval, J. Activated human T cells release bioactive fas ligand and apo2 ligand in microvesicles. J. Immunol. 1999, 163, 1274–1281.
61. Monleon, I.; Martinez-Lorenzo, M.J.; Monteagudo, L.; Lasierra, P.; Taules, M.; Iturralde, M.; Pineiro, A.; Larrad, L.; Alava, M.A.; Naval, J.; et al. Differential secretion of fas ligand- or apo2 ligand/tnf-related apoptosis-inducing ligand-carrying microvesicles during activation-induced death of human T cells. J. Immunol. 2001, 167, 6736–6744.
62. Nagata, S. Apoptosis by death factor. Cell 1997, 88, 355–365.
63. Krammer, P.H.; Arnold, R.; Lavrik, I.N. Life and death in peripheral T cells. Nat. Rev. Immunol. 2007, 7, 532–542.
64. Nagata, S.; Suda, T. Fas and fas ligand: Lpr and gld mutations. Immunol. Today 1995, 16, 39–43.
65. Dustin, M.L. What counts in the immunological synapse? Mol. Cell 2014, 54, 255–262.
66. Dustin, M.L.; Choudhuri, K. Signaling and polarized communication across the t cell immunological synapse. Annu. Rev. Cell Dev. Biol. 2016, 32, 303–325.
67. Calvo, V.; Izquierdo, M. Role of actin cytoskeleton reorganization in polarized secretory traffic at the immunological synapse. Front. Cell Dev. Biol. 2021, 9, 109.
68. Mittelbrunn, M.; Sanchez-Madrid, F. Intercellular communication: Diverse structures for exchange of genetic information. Nat. Rev. Mol. Cell Biol. 2012, 13, 328–335.
69. Blanchard, N.; Lankar, D.; Faure, F.; Regnault, A.; Dumont, C.; Raposo, G.; Hivroz, C. Tcr activation of human T cells induces the production of exosomes bearing the tcr/cd3/zeta complex. J. Immunol. 2002, 168, 3235–3241.

70. van der Vlist, E.J.; Arkesteijn, G.J.; van de Lest, C.H.; Stoorvogel, W.; Nolte-’t Hoen, E.N.; Wauben, M.H. Cd4(+) t cell activation promotes the differential release of distinct populations of nanosized vesicles. J. Extracell. Vesicles 2012, 1, 18364.

71. Saliba, D.G.; Cespedes-Donoso, P.F.; Balint, S.; Compeer, E.B.; Korobchevskaya, K.; Valvo, S.; Mayya, V.; Kvalvaag, A.; Peng, Y.; Dong, T.; et al. Composition and structure of synaptic ectosomes exporting antigen receptor linked to functional cd40 ligand from helper T cells. eLife 2019, 8, e47528.

72. Menck, K.; Sönmezer, C.; Worst, T.S.; Schulz, M.; Dihazi, G.H.; Streit, F.; Erdmann, G.; Kling, S.; Boutros, M.; Binder, C.; et al. Neutral sphingomyelinases control extracellular vesicles budding from the plasma membrane. J. Extracell. Vesicles 2017, 6, 1378056.

73. Trajkovic, K.; Hsu, C.; Chiantia, S.; Rajendran, L.; Wenzel, D.; Wieland, F.; Schwille, P.; Brugger, B.; Simons, M. Ceramide triggers budding of exosome vesicles into multivesicular endosomes. Science 2008, 319, 1244–1247.

74. Wen, C.; Seeger, R.C.; Fabbri, M.; Wang, L.; Wayne, A.S.; Jong, A.Y. Biological roles and potential applications of immune cell-derived extracellular vesicles. J. Extracell. Vesicles 2017, 6, 1400370.

75. Vergani, E.; Daveri, E.; Vallacchi, V.; Bergamaschi, L.; Lalli, L.; Castelli, C.; Rodolfo, M.; Rivoltini, L.; Huber, V. Extracellular vesicles in anti-tumor immunity. Semin. Cancer Biol. 2021, in press.

76. June, C.H.; O’Connor, R.S.; Kawalekar, O.U.; Ghassemi, S.; Milone, M.C. Car t cell immunotherapy for human cancer. Science 2018, 359, 1361–1365.

77. Chan, J.D.; Lai, J.; Slaney, C.Y.; Kallies, A.; Beavis, P.A.; Darcy, P.K. Cellular networks controlling t cell persistence in adoptive cell therapy. Nat. Rev. Immunol. 2021. 21, 769–784.

78. Sterner, R.C.; Sterner, R.M. Car-t cell therapy: Current limitations and potential strategies. Blood Cancer J. 2021, 11, 69.

79. Dong, Y.; Wan, Z.; Gao, X.; Yang, G.; Liu, L. Reprogramming immune cells for enhanced cancer immunotherapy: Targets and strategies. Front. Immunol. 2021, 12, 609762.

80. Tang, X.J.; Sun, X.Y.; Huang, K.M.; Zhang, L.; Yang, Z.S.; Zou, D.D.; Wang, B.; Warnock, G.L.; Dai, L.J.; Luo, J. Therapeutic potential of car-t cell-derived exosomes: A cell-free modality for targeted cancer therapy. Oncotarget 2015, 6, 44179–44190.

81. Fu, W.; Lei, C.; Liu, S.; Cui, Y.; Wang, C.; Qian, K.; Li, T.; Shen, Y.; Fan, X.; Lin, F.; et al. Car exosomes derived from effector car-T cells have potent antitumour effects and low toxicity. Nat. Commun. 2019, 10, 4355.

82. Xu, Q.; Zhang, Z.; Zhao, L.; Qin, Y.; Cai, H.; Geng, Z.; Zhu, X.; Zhang, W.; Zhang, Y.; Tan, J.; et al. Tropism-facilitated delivery of crispr/cas9 system with chimeric antigen receptor-extracellular

vesicles against b-cell malignancies. J. Control. Release 2020, 326, 455–467.

83. Yang, P.; Cao, X.; Cai, H.; Feng, P.; Chen, X.; Zhu, Y.; Yang, Y.; An, W.; Yang, Y.; Jie, J. The exosomes derived from car-t cell efficiently target mesothelin and reduce triple-negative breast cancer growth. Cell. Immunol. 2021, 360, 104262.
84. Aharon, A.; Horn, G.; Bar-Lev, T.H.; Zagagi Yohay, E.; Waks, T.; Levin, M.; Deshet Unger, N.; Avivi, I.; Globerson Levin, A. Extracellular vesicles derived from chimeric antigen receptor-t cells: A potential therapy for cancer. Hum. Gene Ther. 2021, 32, 1224–1241.
85. Haque, S.; Vaiselbuh, S.R. Cd19 chimeric antigen receptor-exosome targets cd19 positive b-lineage acute lymphocytic leukemia and induces cytotoxicity. Cancers 2021, 13, 1401.
86. Johnson, L.R.; Lee, D.Y.; Eacret, J.S.; Ye, D.; June, C.H.; Minn, A.J. The immunostimulatory rna rn7sl1 enables car-t cells to enhance autonomous and endogenous immune function. Cell 2021, 184, 4981–4995.e14.
87. Ruella, M.; Xu, J.; Barrett, D.M.; Fraietta, J.A.; Reich, T.J.; Ambrose, D.E.; Klichinsky, M.; Shestova, O.; Patel, P.R.; Kulikovskaya, I.; et al. Induction of resistance to chimeric antigen receptor t cell therapy by transduction of a single leukemic b cell. Nat. Med. 2018, 24, 1499–1503.
88. Del Vecchio, F.; Martinez-Rodriguez, V.; Schukking, M.; Cocks, A.; Broseghini, E.; Fabbri, M. Professional killers: The role of extracellular vesicles in the reciprocal interactions between natural killer, cd8+ cytotoxic t-cells and tumour cells. J. Extracell. Vesicles 2021, 10, e12075.
89. Kowal, J.; Tkach, M.; Théry, C. Biogenesis and secretion of exosomes. Curr. Opin. Cell Biol. 2014, 29, 116–125.
90. Sinha, S.; Hoshino, D.; Hong, N.H.; Kirkbride, K.C.; Grega-Larson, N.E.; Seiki, M.; Tyska, M.J.; Weaver, A.M. Cortactin promotes exosome secretion by controlling branched actin dynamics. J. Cell Biol. 2016, 214, 197–213.
91. Tsutsumi, R.; Hori, Y.; Seki, T.; Kurauchi, Y.; Sato, M.; Oshima, M.; Hisatsune, A.; Katsuki, H. Involvement of exosomes in dopaminergic neurodegeneration by microglial activation in midbrain slice cultures. Biochem. Biophys. Res. Commun. 2019, 511, 427–433.
92. Lugini, L.; Cecchetti, S.; Huber, V.; Luciani, F.; Macchia, G.; Spadaro, F.; Paris, L.; Abalsamo, L.; Colone, M.; Molinari, A.; et al. Immune surveillance properties of human nk cell-derived exosomes. J. Immunol. 2012, 189, 2833–2842.
93. Wu, C.H.; Li, J.; Li, L.; Sun, J.; Fabbri, M.; Wayne, A.S.; Seeger, R.C.; Jong, A.Y. Extracellular vesicles derived from natural killer cells use multiple cytotoxic proteins and killing mechanisms to target cancer cells. J. Extracell. Vesicles 2019, 8, 1588538.
94. Lettau, M.; Janssen, O. Intra- and extracellular effector vesicles from human t and nk cells: Same-same, but different? Front. Immunol. 2021, 12, 804895.

95. Wu, F.; Xie, M.; Hun, M.; She, Z.; Li, C.; Luo, S.; Chen, X.; Wan, W.; Wen, C.; Tian, J. Natural killer cell-derived extracellular vesicles: Novel players in cancer immunotherapy. Front. Immunol. 2021, 12, 1970.
96. Hu, W.; Wang, G.; Huang, D.; Sui, M.; Xu, Y. Cancer immunotherapy based on natural killer cells: Current progress and new opportunities. Front. Immunol. 2019, 10, 1205.