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Extracellular vesicles derived from B7-H3 CAR-T cells: a promising strategy for Pancreatic cancer treatment

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1. Introduction to CAR-T Cells

1.1 Definition, Structure and Mechanism of Action of CAR-T Cells

Chimeric Antigen Receptor (CAR) T cells are genetically engineered T cells designed to recognize and eliminate malignant cells by incorporating a synthetic receptor that combines antigen specificity and T cell activation functions into a single molecule. The antigen-recognition domain of the CAR is specifically engineered to bind tumor-associated or tumor-specific antigens expressed on the surface of cancer cells, enabling targeted immune responses largely independent of major histocompatibility complex (MHC) presentation (June *et al.*, 2018). The CAR structure is flexible and generally consists of four primary components: (i) an extracellular antigen-binding domain, typically a single-chain variable fragment (scFv) derived from a monoclonal antibody, which directs antigen specificity independent of MHC presentation; (ii) a hinge or spacer region, which confers flexibility and appropriate spatial orientation for antigen binding; (iii) a transmembrane domain anchoring the receptor in the T cell membrane, often derived from molecules such as CD28, CD8 α or CD4, and ensuring proper receptor stability and assembly; and (iv) an intracellular signaling domain, often composed of CD3 ζ along with one or more co-stimulatory signaling domains (e.g., CD28, 4-1BB), which together trigger downstream signaling cascades upon antigen engagement (Fig.1) (Yoo *et al.*, 2021).

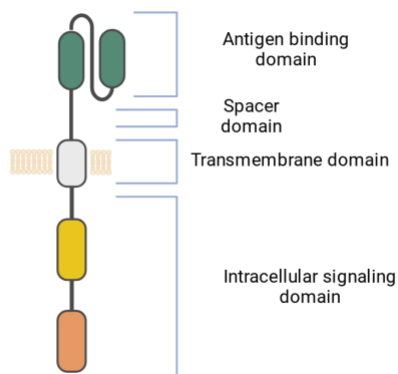


Figure 1 Structure of chimeric antigen receptor (CAR). The CAR includes antigen-binding domain, spacer domain, transmembrane domain and intracellular signaling domain.

Once CAR T cells are infused into the patient, they diffuse systemically and use tissue monitoring to identify and bind their target antigens that are present on malignant cells. The antigen binding by the scFv domain induces clustering and cross-linking of CAR molecules, which leads to activation of intracellular signaling via tyrosine phosphorylation of immune receptor tyrosine-based activation motifs (ITAMs), downstream activation of transcription factors, release of cytotoxic granules

containing perforin and granzymes, cytokine production, proliferation, and in some cases engagement of death receptor pathways like Fas/FasL for inducing apoptosis in target cells (Larson *et al.*, 2021) (Upadhyay *et al.*, 2021). The co-stimulatory domains incorporated have different kinetics and durability of response, which affects the phenotypes and persistence in vivo of central memory and effector memory (Honikel *et al.*, 2022).

1.2 Different Generations of CAR-T Cells

The development of Chimeric Antigen Receptor (CAR) T-cell therapy has undergone multiple generations of refinement aimed at improving T-cell activation, persistence, and anti-tumor efficacy. As mentioned above, the CAR receptor is composed of three main domains: extracellular, transmembrane, and intracellular. Over the years, structural modifications have been primarily focused on the intracellular domain to improve the therapeutic efficacy of CAR-T cells (June *et al.*, 2018). The intracellular component is different between different CAR generations, which defines the evolution between the first and fifth generation of CAR-T cells. While the extracellular and transmembrane domains are largely conserved across different CAR generations, it remains variable. The first-generation CARs are characterized by a single intracellular signaling domain, CD3 ζ , which provides a primary activation signal to the T cells. However, this design has shown limitations, particularly in the ability of CAR-T cells to produce sufficient levels of cytokines, such as interleukin-2 (IL-2). Consequently, early therapeutic approaches that utilized first-generation CAR-T cells often required exogenous IL-2 to promote T-cell activation and proliferation. Due to these limitations, first-generation CAR-T constructs were eventually phased out (Zhang *et al.*, 2017). A co-stimulatory domain was added to the intracellular region of the second generation of CAR-T cells, making it possible to deliver a dual activation signal. The first signal is triggered upon antigen recognition via the CAR binding to tumor-associated antigens presented by the major histocompatibility complex (MHC) on the surface of tumor cells. The second signal is mediated by co-stimulatory molecules such as CD28 or CD134 (OX40), leading to enhanced IL-2 production, full T-cell activation, and resistance to apoptosis. Without this co-stimulatory domain, CAR-T cells fail to exert their full cytotoxic potential. Thus, its inclusion not only improves cytokine production and antitumor efficacy but also prolongs CAR-T cell persistence in vivo (Zhang *et al.*, 2017). Third-generation CAR-T cells further build upon this framework by incorporating multiple co-stimulatory domains such as CD3 ζ -CD28-OX40 or CD3 ζ -CD28-4-1BB. These constructs aim to further enhance T-cell proliferation and cytotoxicity through increased cytokine production. In clinical settings, third-generation CAR-T therapies have demonstrated promising outcomes, particularly in the treatment of specific lymphomas and solid tumors, including colorectal cancer (Zhang *et al.*, 2017). Fourth-generation CAR-T cells,

also known as TRUCKs (T-cells Redirected for Universal Cytokine-mediated Killing), are derived from second-generation constructs but are engineered to include an inducible expression cassette for IL-12. By using this design, CAR-T cells can recruit and activate innate immune cells in the tumor microenvironment, which extends their cytotoxic action to cancer cells not expressing the CAR-targeted antigen. In addressing tumor heterogeneity, this feature is particularly significant because antigen-negative malignant clones can evade immune surveillance. Upon activation, TRUCKs secrete IL-12 directly at the tumor site, promoting a localized inflammatory response that facilitates innate immune cell recruitment and enhances overall antitumor activity, even against antigen-escape variants (Chmielewski *et al.*, 2014). The fifth generation of CAR-T cells incorporates an additional intracellular module designed to enhance cytokine signaling. This typically includes a cytokine receptor β -chain fragment with binding motifs for transcription factors such as STAT3 or STAT5. Upon antigen engagement, this advanced receptor design initiates three distinct signaling pathways: CAR-T cell activation, the promotion of memory T-cell formation, and the stimulation of endogenous immune responses (Pagotto *et al.*, 2023). These cells are generally produced from the patient's own immune cells, a process known as autologous manufacturing. Recent advances have led to the development of universal CAR-T cells (UCAR-T) to overcome the limitations that come with autologous CAR-T therapies, such as manufacturing delays, variability and cost. These cells retain the same mechanism of action but are designed for allogeneic use, offering broader applicability and easier production. UCAR-T cells are engineered to minimize the risk of graft-versus-host disease (GvHD) by using genome editing techniques to disrupt HLA class I loci particularly by knocking out the HLA-A gene of the MHC complex in donor-derived T cells. These modifications allow for the creation of "off-the-shelf" CAR-T products that can be used across multiple patients, representing a major step toward scalable, readily available cell therapies (Pagotto *et al.*, 2023) (Fig.2).

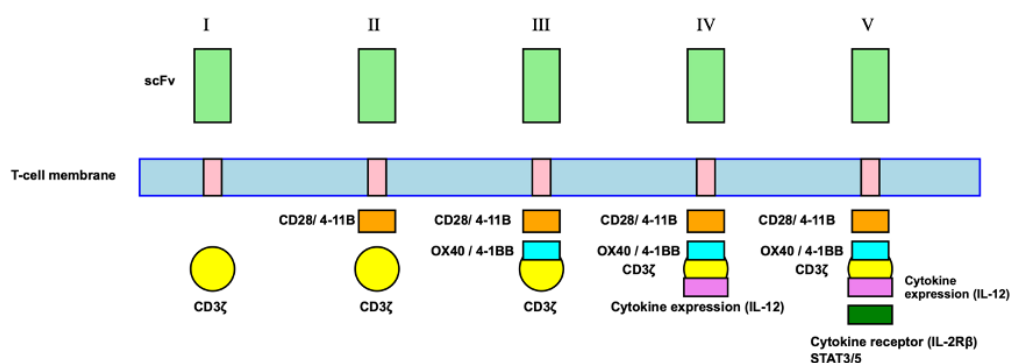


Figure 2 Schematic overview of CAR structural evolution. First-generation CARs contain only the CD3 ζ activation domain. Second- and third-generation CARs incorporate one or two co-stimulatory domains (e.g., CD28, 4-1BB, OX40). Fourth-generation CARs (TRUCKs) are engineered to induce cytokine secretion (e.g.,

IL-12) upon antigen engagement. Fifth-generation CARs include cytokine receptor signaling modules enabling activation of JAK–STAT pathways (STAT3/5).

1.3 Genetic Engineering of T Cells

The engineering process begins with the collection of T cells through leukapheresis, a technically complex procedure requiring the integration of advanced cellular and molecular biology techniques. This stage is performed under stringent quality control measures to ensure both the safety and therapeutic efficacy of the final cell product destined for patient infusion. Central to this process is the use of highly specific gene transfer methods, primarily involving viral vectors, most commonly retroviral and lentiviral vectors. Although regulatory agencies consider these vectors intermediate reagents, their use is strongly recommended under current Good Manufacturing Practices (GMP) guidelines (Delgado *et al.*, 2022). In the context of CAR-T cell production, viral vectors are used to stably integrate the CAR transgene into the genome of patient-derived T cells. This is achieved through reverse transcription of the vector RNA into DNA, followed by genomic integration, resulting in long-term expression of the CAR. Among the various viral systems, lentiviral vectors have demonstrated a superior safety profile in clinical trials due to their more favourable integration characteristics compared to retroviral vectors, leading to their broader clinical adoption (Montini *et al.*, 2009).

Viral vector production takes 10–14 days and involves “packaging” cells expanded in flasks and later transferred to culture chambers, where polyethyleneimine is added as a transfection reagent, alongside viral envelope and gag-pol components. Fixed amounts of plasmids encoding the CAR are maintained, and after transfection, the material is collected, clarified, ultrafiltered, and cryopreserved at –80 °C until use (Castella *et al.*, 2019).

1.4 CAR-T Manufacturing Protocol

Since CAR-T cells used for therapeutic purposes are classified as advanced therapy medicinal products, their collection and manufacturing are regulated by laws and standards set by authorities such as the EMA (European Medicines Agency), as well as national and regional agencies. Consequently, CAR-T cells must be produced in highly specialized laboratories following Good Manufacturing Practices (GMP) to ensure standardized, controlled, reproducible, and verifiable cellular products (Delgado *et al.*, 2022). The final product that satisfies quality requirements is obtained through leukapheresis a procedure that separates hematopoietic cells from peripheral blood. This process is crucial since a higher yield of T cells during this stage is linked to better therapeutic outcomes. Studies have identified patient- and disease-related factors associated with reduced T-cell

collection efficiency, defined as the lymphocyte count per product volume divided by the average lymphocyte count per processed peripheral blood volume. The main limiting factors include advanced age and disease-related conditions that can lower collection efficiency below 40%, high platelet counts that cause platelet aggregation in the centrifuge, and the timing of collection relative to disease phase and prior treatments. Clinical observations indicate that previous antineoplastic therapies can impair subsequent leukapheresis attempts, affecting both T-cell recovery and quality (Qayed *et al.*, 2022). Leukapheresis from peripheral blood cannot separate components with differing density gradients, thus yielding a mixed cell population. Monocytes, considered contaminants, can mature into macrophages and phagocytose essential components such as antibodies used in CAR-T production. Their numbers can also increase after G-CSF treatment, commonly administered following chemotherapy or radiotherapy (Qayed *et al.*, 2022). Once purified, the leukapheresis product can either be cryopreserved or used fresh. Fresh use shortens production time but requires initiation of the next step within 24–48 hours since cell viability declines rapidly; missing production slots can severely affect patient outcomes. Conversely, cryopreservation provides flexibility, allowing leukapheresis when the patient's health is optimal, ensuring healthier T cells. It also facilitates logistical management of shipping delays and extended processing times due to contaminant removal (Tyagarajan *et al.*, 2019). The next phase is genetic engineering and recently, non-viral gene transfer and genome-editing approaches have been proposed, though they remain in preclinical and early clinical phases and are not yet available for clinical application (Prommersberger *et al.*, 2021). For instance, the Sleeping Beauty transposon system combined with mRNA transfection still raises clinical safety concerns due to potential insertional mutagenesis and the need for repeated infusions (Hackett *et al.*, 2013). Following genetic modification, the CAR gene is introduced into T cells, which are then activated to stimulate proliferation. This activation can occur through antigen-presenting support cells or antibody-coated beads targeting CD3 and CD28 (Maude *et al.*, 2014). Interleukin-2 (IL-2) is commonly used to promote T-cell proliferation and effector function, though its systemic toxicity limits its use in CAR-T immunotherapy (Rokade *et al.*, 2024). Once activated, cells are transferred to controlled culture environments for expansion, ensuring both safety and quality. The main systems used include the WAVE bioreactor, a scalable platform for cellular expansion (Hollyman *et al.*, 2009); the G-REX system, which allows large-scale expansion with minimal manual intervention (Ludwig *et al.*, 2020); and the fully automated CliniMACS Prodigy®, which integrates enrichment, activation, transduction, and expansion in a single device (Palani *et al.*, 2023) (Fig.3).



Figure 3 GMP cell therapy manufacturing: CliniMACS Prodigy® Platform (Miltenyi Biotech)

Expansion continues until enough CAR-T cells is achieved. Throughout this process, quality control monitors cell viability (live, functional cells), size, concentration, polarity, and purity, ensuring a high proportion of uncontaminated CAR-T cells (Reddy *et al.*, 2020).

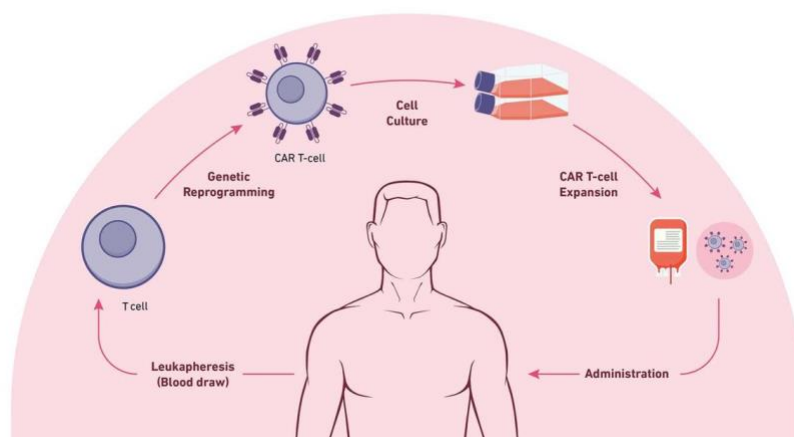


Figure 4 CAR-T manufacturing protocol steps (Biopharma)

Once the final cellular product is obtained, the infusion process begins. The patient is first prepared through a lymphodepleting regimen, typically involving chemotherapeutic agents such as cyclophosphamide or fludarabine. This preconditioning step aims to reduce the number of existing lymphocytes, thereby creating a favorable environment for the infused CAR-T cells to function effectively and proliferate in the patient. As with all previous stages, the infusion represents a critical and delicate phase; it is therefore performed in specialized medical centers equipped with intensive care units to manage potential severe adverse reactions (Gurretiez *et al.*, 2020). CAR-T cells are administered intravenously in a hospital setting as a single infusion, with the total number of infused cells according to the clinical protocol usually ranging from 1×10^8 to 1×10^9 cells (Roddie *et al.*, 2019) (Fig.4) Post-infusion monitoring is equally important, as it assesses the patient's response to therapy and enables prompt intervention in case of adverse effects, primarily cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS) (Sheth *et al.*, 2021). In

addition to immediate monitoring, long-term follow-up is essential to evaluate the durability of response, prevent or manage late-onset infections, and detect possible relapses (Schuster *et al.*, 2021).

2. CAR-T Cells in Cancer Therapy

2.1 *Application of CAR-T Therapy in Hematologic Malignancies*

The therapeutic goal of CAR-T cell is to identify and eliminate target cells with high precision and specificity. The introduction of this therapy has demonstrated remarkable remission rates, particularly in patients with hematologic malignancies that are resistant to conventional treatments, representing a breakthrough in modern medicine (Patel *et al.*, 2025). Over time, therapeutic strategies for hematologic cancers have evolved from chemotherapy, radiotherapy, and hematopoietic stem cell transplantation toward immunotherapy, involving monoclonal antibodies and, most notably, CAR-T cells. This shift has led to a significant improvement in treatment success rates and patient outcomes (Zhang *et al.*, 2022). At present, the main therapeutic applications of CAR-T cells, focus on malignant hematologic diseases such as various forms of leukemia, lymphomas, and multiple myeloma. CAR-T therapy has achieved notable success in acute lymphoblastic leukemia (ALL), a highly aggressive malignancy characterized by the proliferation of immature lymphoid cells. In this context, the U.S. Food and Drug Administration (FDA) has approved CD19-directed CAR-T therapy, which has achieved remission rates of nearly 80% within the first three months after infusion (FDA 2018). However, treatment resistance may occur due to CAR-T cell exhaustion or antigen loss, prompting the development of alternative markers and more specific CAR-T constructs (Ruella *et al.*, 2023; Rasche *et al.*, 2022). In chronic lymphocytic leukemia (CLL), the most common leukemia in adults, CAR-T cells targeting CD5/CD19-positive B lymphocytes have shown more modest results, with complete response rates around 25% and median survival of approximately 18 months (Porter DL *et al.*, 2015) (Gauthier J *et al.*, 2015). Nonetheless, ongoing studies highlight the role of CAR-T cells as a consolidation therapy for patients with partial or unsatisfactory remission following standard treatments (Scarfò *et al.*, 2016; Frey *et al.*, 2020). In acute myeloid leukemia (AML), CAR-T therapy is particularly challenging due to the lack of tumor-specific surface antigens and the risk of damaging healthy hematopoietic cells that share similar markers. To address these safety concerns, current research is investigating combined multi-target CAR-T approaches aimed at improving therapeutic efficacy while maintaining tolerability (Khwaja *et al.*, 2016; Haslauer *et al.*, 2021). In multiple myeloma (MM), a malignancy of plasma cells, traditional therapies such as chemotherapy, stem cell transplantation, and immunomodulatory drugs have proven largely palliative. Early CD19-targeted CAR-T treatments were ineffective due to low antigen expression on myeloma cells; however, the discovery of the B-cell maturation antigen (BCMA) as a new therapeutic target has produced

encouraging results, with response rates exceeding 80% in clinical trials (Rodríguez-Lobato *et al.*, 2020; Kumar *et al.*, 2008). Lastly, in lymphomas, which include Hodgkin and non-Hodgkin subtypes characterized by abnormal lymphocyte proliferation, CAR-T therapy targeting CD19 has significantly improved outcomes for patients with diffuse large B-cell lymphoma (DLBCL) resistant to standard chemoimmunotherapy (Wudhikarn *et al.*, 2023). Clinical trials have reported complete response rates of approximately 60%, with sustained partial responses in about 25% of patients for up to two years. Research is now expanding to other antigens, such as CD30, which has shown promising preliminary results, achieving partial responses in several patients (Cappell *et al.*, 2023; Meng *et al.*, 2025).

Overall, CAR-T cell therapy represents a significant innovation in the management of hematologic malignancies, offering personalized, targeted, and durable treatment options for patients who previously had limited or no therapeutic alternatives.

2.2 *Application of CAR-T Therapy in Solid Tumors*

Given the remarkable potential of CAR-T cells in combating hematologic malignancies, there is growing interest in expanding their therapeutic applications to solid tumors. However, the clinical success of CAR-T therapy in this field remains limited, representing an ongoing biotechnological and clinical challenge. To date, three main target antigens have been identified on the surface of malignant cells across various solid cancers: (1) mesothelin, a membrane protein expressed in tumors such as pleural mesothelioma and several carcinomas (notably pancreatic and ovarian) (Beatty *et al.*, 2014); (2) HER2, highly expressed on malignant cells in breast and gastric cancers and in some brain tumors, including glioblastoma (Ahmed *et al.*, 2017); and (3) GD2, found in certain melanomas and neuroblastomas, showing encouraging responses as a CAR-T target in preclinical and early-phase clinical trials (Locatelli *et al.*, 2025) (Mount *et al.*, 2018).

The first step toward developing effective and safe CAR-T cell therapies for solid tumors is the optimization of target antigen selection, which must satisfy two essential criteria: the antigen should be expressed on 100% of tumor cells to ensure comprehensive recognition and elimination, and it must be absent from healthy tissues to avoid off-target toxicity (Newick *et al.*, 2017). Nevertheless, achieving such specificity is still challenging, and concerns persist regarding the potentially lethal “on-target/off-tumor” effect, wherein CAR-T cells mistakenly recognize and attack healthy cells expressing the target antigen (Morgan *et al.*, 2010). Another critical barrier is tumor heterogeneity, referring to the variability of antigen expression among cancer cells, which limits CAR-T efficacy (Restifo *et al.*, 2012). Numerous strategies are being explored to overcome this challenge: (1) developing multi-target CAR-T constructs that recognize multiple tumor antigens simultaneously to

minimize resistance and maximize tumor eradication; (2) enhancing CAR-T cytotoxic mechanisms beyond traditional perforin/granzyme and Fas pathways; and (3) implementing immunohistochemical screening of patient tumors to identify optimal target antigens before treatment (Newick *et al.*, 2017). The tumor microenvironment (TME) poses an additional set of challenges to CAR-T infiltration and persistence. After the infusion, CAR-T cells must successfully migrate into and penetrate the tumor mass, a process hindered by several physiological barriers. The first of these is high interstitial pressure, largely due to the abundance of cancer-associated fibroblasts (CAFs), which form a dense stromal network. Preclinical studies suggest that engineering CAR-T cells to secrete fibrolytic enzymes can enhance tumor penetration and therapeutic efficacy (Caruana *et al.*, 2015). Secondly, the hypoxic and metabolically hostile environment of solid tumors impairs CAR-T function. Hypoxia is believed to suppress T-cell activation, proliferation, and effector function, although evidence remains partly controversial (Petrova *et al.*, 2018). Moreover, the TME often features low glucose levels, high lactate concentrations, and acidic pH, which further inhibit T-cell activity and cytokine production (Wang *et al.*, 2024). To counteract these suppressive conditions, researchers are developing multi-functional CAR-T designs capable of resisting immunosuppression mediated by regulatory T cells (Tregs) and of locally releasing pro-inflammatory cytokines within the tumor microenvironment to restore immune activity (Gwadera *et al.*, 2025). Altogether, these novel strategies represent the frontier of innovation in CAR-T therapy, aiming to translate its success from hematologic malignancies to the far more complex landscape of solid tumors.

2.3 *CAR-T Cell Therapy for Pancreatic Cancer*

CART-cell therapy exhibits remarkable efficacy in hematologic malignancies such as B-cell acute lymphoblastic leukemia and diffuse large B-cell lymphoma. However, translating this success to solid tumors, particularly pancreatic ductal adenocarcinoma (PDAC), remains a challenge. PDAC is characterized by a highly desmoplastic and immunosuppressive tumor microenvironment (TME) that limits T-cell infiltration and function. The tumor stroma is composed of dense extracellular matrix components, cancer-associated fibroblasts (CAFs), and stromal cells that form a physical barrier, preventing CAR-T cell infiltration into the tumor mass (Feig *et al.*, 2013; Stromnes *et al.*, 2017). Moreover, the TME contains abundant immunosuppressive cell populations, such as regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), and tumor-associated macrophages (TAMs), which inhibit T-cell activation and proliferation through the secretion of cytokines like transforming growth factor-beta (TGF- β) and interleukin-10 (IL-10) (Park *et al.*, 2022; Yu *et al.*, 2024). A second major limitation lies in antigen heterogeneity and lack of tumor-specific targets. Unlike hematologic cancers, where lineage-restricted markers such as CD19 can be safely targeted,

pancreatic cancer expresses tumor-associated antigens (e.g., mesothelin, CEA, MUC1, claudin-18.2) that are also found at low levels in normal tissues, raising the risk of on-target/off-tumor toxicity (Beatty & O'Hara, 2016; Farhangnia *et al.*, 2022). Additionally, PDAC exhibits dynamic antigen expression and clonal heterogeneity, enabling tumor cells to escape CAR-T recognition through antigen loss or downregulation, leading to disease relapse (Stromnes *et al.*, 2017). Another major challenge is CAR-T cell persistence and exhaustion. Within the nutrient-deprived and hypoxic pancreatic TME, CAR-T cells experience metabolic stress that limits their survival and cytotoxic function (Sukumar *et al.*, 2013). Chronic antigen stimulation, combined with inhibitory signaling through immune checkpoints such as PD-1/PD-L1 and CTLA-4, leads to functional exhaustion and diminished effector capacity (Klampatsa *et al.*, 2022). Manufacturing difficulties also contribute to limited success, as generating CAR-T cells capable of surviving and expanding in the hostile solid tumor milieu remains technically demanding (Azeez *et al.*, 2025). The most extensively studied targets in PDAC are mesothelin (MSLN), carcinoembryonic antigen (CEA), mucin-1 (MUC1), prostate stem cell antigen (PSCA), and claudin-18.2 (CLDN18.2) (Zhang *et al.*, 2023). Early-phase clinical trials using MSLN-directed CAR-T cells have demonstrated partial responses and disease stabilization in subsets of patients, but overall efficacy remains limited (Beatty *et al.*, 2018). To overcome these barriers, researchers are developing CAR-T cells engineered to secrete cytokines such as IL-12 or IL-18 to remodel the TME, as well as dual-targeting and switchable CAR constructs that reduce antigen escape (Peng *et al.*, 2024). Furthermore, localized delivery methods such as intraperitoneal or intratumoral infusion are being explored in mouse model to increase efficacy while mitigating systemic toxicity (Restle D *et al.*, 2025). Although durable clinical responses remain rare, continuous improvements in CAR design, antigen selection, and TME modulation suggest that CAR-T therapy could eventually play a meaningful role in the management of pancreatic cancer.

3. Extracellular Vesicles (EVs) and CAR-T Cell–Derived EVs

3.1 Structure and Biogenesis of Extracellular Vesicles (EVs)

Extracellular vesicles (EVs) are small, membrane-bound particles released by all cell types that carry biomolecules reflecting the features and functional state of their parental cells. EVs constitute a heterogeneous population, According to the MISEV2023 guidelines of the International Society for Extracellular Vesicles (ISEV), extracellular vesicles (EVs) can be classified based on their size into three main categories: small EVs (sEVs), typically with diameters less than 200 nm; medium EVs, with intermediate diameters; and large EVs, with diameters greater than 200 nm, which include entities such as apoptotic bodies. This size-based classification facilitates experimental analysis and enhances the comparability of results across laboratories, while acknowledging the limitations imposed by the various isolation and characterization methods employed in practice. Despite their different origins, all EVs share a lipid bilayer enriched in cholesterol, sphingomyelin, and phosphatidylserine, which protects their cargo from extracellular degradation (De Lellis *et al.*, 2021; Yáñez-Mó *et al.*, 2015) (Fig.5).

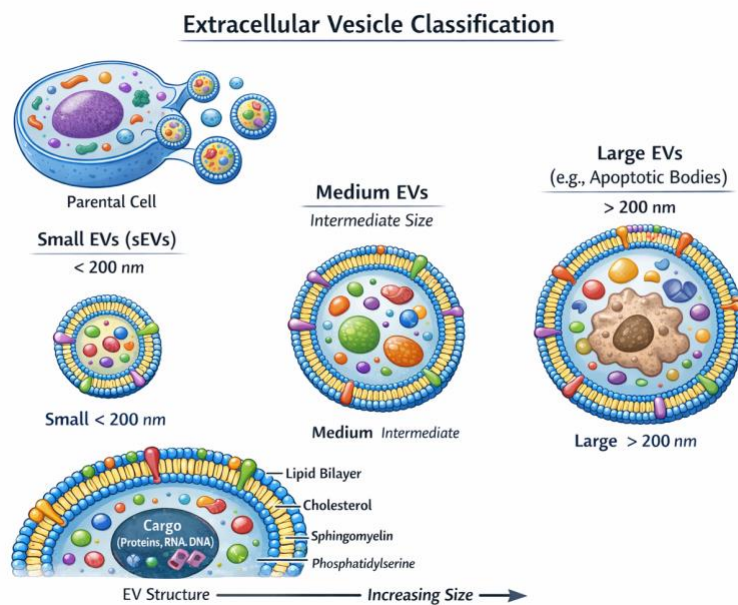


Figure 5 Classification of extracellular vesicles (EVs) by size. Small EVs (sEVs) have diameters <200 nm, while medium and large EVs include microvesicles and apoptotic bodies (>200 nm). All EVs share a lipid membrane rich in cholesterol, sphingomyelin, and phosphatidylserine that protects their biomolecular content. Latest EV classification based on the MISEV2023 guidelines

EVs are released under both physiological and pathophysiological conditions, and their characteristics are shaped by the originating cell type, the surrounding microenvironment, and external stimuli (Liu *et al.*, 2023). Consequently, body fluids contain a heterogeneous mixture of EVs derived from multiple parental cells, reflecting the diversity of cellular sources and physiological

states (Brocco *et al.*, 2021). By analyzing EV phenotypes, it is possible to identify their cellular origin, as EVs have been detected in body fluids and culture media and are considered dynamic indicators of tissue status, making them promising biomarkers for liquid biopsy applications (Yáñez-Mó *et al.*, 2015; Kalluri *et al.*, 2020). Beyond this diagnostic potential, EVs function as active mediators of intercellular communication, modulating target cell behavior through their molecular cargo. They interact with recipient cells via surface ligands and receptors, enabling binding, internalization, and activation of intracellular signaling pathways (Raposo & Stoorvogel, 2013; Van Niel *et al.*, 2017). EVs can transfer active receptors such as EGFRvIII and CCR5, influencing the phenotype and function of recipient cells (Al-Nedawi *et al.*, 2008; Ratajczak *et al.*, 2021). EV uptake occurs via multiple mechanisms, including phagocytosis, membrane fusion, micropinocytosis, macropinocytosis, lipid raft-mediated endocytosis, clathrin/caveolin-mediated endocytosis, and tunneling nanotubes (Liu *et al.*, 2023). After internalization, EV cargo can escape the endosomal pathway, avoiding lysosomal degradation, and deliver nucleic acids, proteins, and lipids to the cytoplasm of target cells, where they exert functional effects (Ribovski *et al.*, 2023). In pathological contexts such as pancreatic ductal adenocarcinoma, tumor-derived exosomes actively shape the tumor microenvironment, promoting angiogenesis, immune evasion, metastasis, and chemoresistance, with oncogenic cargo reprogramming stromal and immune cells to favor tumor progression (Costa-Silva *et al.*, 2015; Melo *et al.*, 2015; De Lellis *et al.*, 2021). Overall, EVs serve as versatile messengers that transfer biologically active molecules across cells and tissues, providing both diagnostic information and functional modulation of recipient cells, although the precise intracellular pathways of cargo release remain incompletely understood (Gurung *et al.*, 2021).

3.2 CAR-T Cell-Derived Extracellular Vesicles

The development of CAR-T cells-derived extracellular vesicles (CAR-T-EVs) is a promising and innovative extension of CAR technology, which combines the precision and safety of CAR-T cells with the flexibility and safety of cell-free therapy. These nanosized, membrane-enclosed vesicles are naturally secreted by CAR-T cells and share many molecular and functional features with their parental cells, including the presence of the CAR molecule on their surface (Fu *et al.*, 2019). The biogenesis of CAR-T-EVs follows the canonical pathways described for other EVs. (Pagotto *et al.*, 2023). During CAR-T cell activation and immune synapse formation, vesicle secretion is enhanced by the activity of Rab GTPases and SNARE proteins, which coordinate vesicular transport and membrane fusion (Tirziu *et al.*, 2026). The released CAR-T-EVs are enriched in cytolytic components such as granzyme B, perforin, and proinflammatory cytokines like IFN- γ and TNF- α , conferring them direct tumoricidal activity against antigen-expressing cancer cells (Lanuti *et al.*, 2025). Functionally,

CAR-T-EVs retain the antigen specificity of CAR-T cells while avoiding several safety concerns associated with live-cell therapies. Because they lack proliferative capacity and do not trigger massive cytokine secretion, CAR-T-EVs minimize the risk of cytokine release syndrome (CRS) and neurotoxicity, two major adverse effects of CAR-T cell therapy (Yang *et al.*, 2021; Fu *et al.*, 2019). Moreover, due to their small size and stability, CAR-T-EVs demonstrate improved tumor penetration, enabling them to infiltrate solid tumors more efficiently an area where conventional CAR-T therapies often struggle due to the physical and immunosuppressive barriers of the tumor microenvironment (Pagotto *et al.*, 2023). Through the transfer of immune-regulatory molecules, these vesicles can change the tumor milieu, enhancing antitumor immunity or sensitizing cancer cells to other therapies (Pagotto *et al.*, 2023). Beyond their intrinsic cytotoxic potential, CAR-T-EVs are being explored as biocompatible delivery systems for therapeutic molecules. Their natural targeting capabilities enable them to transport drugs, RNAs, or immunomodulatory agents directly to tumor cells, which reduces off-target toxicity (Huang *et al.*, 2025). Their low immunogenicity, stability in circulation, and ease of biomanufacturing make them a practical and scalable alternative to live CAR-T cell infusion (Pagotto *et al.*, 2023). Preclinical studies have provided compelling evidence of their efficacy. For instance, anti-CD19 CAR-T-EVs have shown strong cytotoxic effects against B-cell leukemia via perforin- and granzyme-mediated apoptosis, while EGFRvIII-directed CAR-T-EVs have demonstrated significant tumor suppression in glioblastoma models without inducing severe systemic inflammation (Lanuti *et al.*, 2025; Kale *et al.*, 2025). Notably, within the context of pancreatic cancer, a preclinical study reported that extracellular vesicles derived from CLDN18.2-targeted CAR-T cells carry functional CAR proteins and cytotoxic molecules, exhibit marked antigen-specific activity against pancreatic adenocarcinoma cells, and significantly reduce tumor growth while prolonging survival in murine models, without triggering systemic IL-6 release. Collectively, these findings highlight the particularly promising therapeutic potential of CAR-T-derived EVs in pancreatic malignancies (Qing *et al.*, 2025). In conclusion, CAR-T cell–derived extracellular vesicles constitute a next-generation immunotherapeutic approach, merging the precision of CAR-mediated targeting with the controllability of cell-free systems. The ability for these vesicles to modulate the tumor microenvironment, their biogenesis and molecular versatility support their potential as an innovative treatment approach. Ongoing studies aim to refine production, engineering, and delivery strategies, to facilitate the translation of cell-free CAR-based therapies into clinical applications (Ma *et al.*, 2025).

4. Rationale and objectives of the study

Pancreatic cancer is one of the most aggressive forms of cancer characterized by a distinctive desmoplastic stroma and by the presence of a highly immunosuppressive tumor microenvironment (Masugi *et al.*, 2022). Despite advances in treatment, there are still limited therapeutic options that may significantly improve disease progression. Chimeric antigen receptor (CAR-T) T cell therapy has revolutionized cancer immunotherapy, emerging as one of the most effective therapeutic technologies for hematological malignancies (Khan *et al.*, 2024). In this context, CAR-T therapies directed against antigens such as CD19 or BCMA have demonstrated high complete remission rates and significant clinical benefits in acute leukemias and refractory/recurrent lymphomas, leading to the approval of several products by international regulatory agencies and consolidating the role of CAR-T in the treatment of hematological malignancies (Ma *et al.*, 2025) (Zugasti *et al.*, 2025). Conversely, the application of CAR-Ts in solid tumors is challenging, since there is a lack suitable, homogeneous and specific tumor antigens. The solid tumor environment is characterized by a dense extracellular matrix, irregular vascularization. Moreover, the immunosuppressive microenvironment limits the infiltration and persistence of infused cells, while antigenic heterogeneity promotes immune evasion of tumor cells. (Xu *et al.*, 2025) Within this framework, there is a need to develop therapeutic approaches capable of reducing the adverse effects associated with cellular therapies in hematological malignancies and to improve treatment of many solid tumors, including Pancreatic Cancer (PC). An important goal of this project is to produce and test *in vitro* next-generation CAR-T cells directed towards specific PC antigens and their derived EVs. The selection of a target antigen, highly expressed in pancreatic tumors and poorly expressed in normal tissues, would allow safe administration and enhanced antitumor efficacy of CAR-T cells and their derived EVs against solid tumors. The choice of the target antigen B7-H3 (CD276) is based on established evidence demonstrating its high expression in PC and its limited expression pattern in healthy tissues (Miller *et al.*, 2024). Previous studies have indeed confirmed an overexpression of B7-H3 on both PC cells and components of the tumor microenvironment, making it a particularly attractive target for targeted immunotherapy strategies. (Inamura K *et al.*, 2018). In addition, recent studies showed that B7-H3 is a promising target for CAR-T cell therapy of solid tumors, including PC (Du *et al.*, 2019) (Li *et al.*, 2023).

Overall, the present work aims:

- To generate and characterize B7-H3 CAR-T cells;
- To generate and characterize B7-H3 CAR-T derived EVs;
- To evaluate the cytotoxic activity of B7-H3 CAR-T derived EVs *in vitro* against PC cell lines characterized by different levels of B7-H3 antigen expression;

5. Materials and Methods

5.1 *CAR-T Cell Generation*

Peripheral blood mononuclear cells (PBMCs) were obtained from leukapheresis products collected from healthy donors at Santo Spirito Hospital (Pescara, Italy) after written informed consent. T cells were activated using anti-CD3 and anti-CD28 antibodies in the presence of RPMI-1640 culture medium supplemented with heat-inactivated fetal bovine serum, L-glutamine, and antibiotics. Activation was conducted for 24–48 hours at 37°C in a humidified atmosphere with 5% CO₂. The CAR construct used encodes an antigen recognition domain derived from a single-chain variable fragment (scFv), followed by a hinge region, a transmembrane domain, and intracytoplasmic signaling domains containing CD3 ζ and costimulatory molecules. The CAR gene was cloned into a viral vector suitable for T cell transduction. Following transduction, CAR-T cells were expanded OpTmizer™ CTS™ T-Cell Expansion SFM (GIBCO) supplemented with 5% Human AB Serum (Sigma Aldrich), 1% Penicillin/Streptomycin (Pen/Strep, Dutscher, Bernolsheim, France) and 10 ng/mL IL-15 and IL-7, both from Miltenyi. Culture media was replenished regularly to sustain T-cell expansion. Expanded CAR-T cells were subsequently used for downstream phenotypic and functional analyses. Multi-parametric flow cytometry analysis was used for the study of CAR-T cell surface marker. Briefly, residual CAR-T cells were labeled in Stain Buffer (BSA 0.2%, BD Biosciences) with dye-conjugated antibodies in the dark for 15 min at room temperature. The antibodies used to analyze the T cell compartment and T cell exhaustion were: CD45, CD3 BV421, CAR PE, CD4 FITC, CD4 BV421, CD8 APCH7, PD-1 PE-Cy7, LAG-3 AlexaFluor 647, TIM-3 FITC, CD62L BV421, CD45RA PE-Cy7. After the staining the cells were washed in PBS, stained with 7AAD (BD Bioscience) to identify dead cells and analyzed using BD FACSCanto II Flow Cytometer (BD Biosciences).

5.2 *EVs Concentration and Isolation*

EVs concentration By Tangential flow filtration

Cell culture-conditioned media were first centrifuged at 1,000 $\times$ g for 10 min to remove residual cells. The supernatants were then concentrated using the Minimate™ EVO Tangential Flow Filtration (TFF) System (Pall Corporation, Port Washington, NY, USA) with a Minimate™ TFF Capsule containing a 300 kDa Omega™ membrane (Pall Laboratory, Port Washington, NY, USA). This filtration step removes solvents and small contaminants while gently retaining EVs of all sizes, which are much larger than 300 kDa, prior to isolation by sorting. The peristaltic pump speed was adjusted to maintain a feed pressure of 5 psi, a retentate pressure of 0 psi, and a filtrate pressure of 0 psi,

resulting in a transmembrane pressure ($TMP = ((P_{\text{feed}} - P_{\text{retentate}})/2) - P_{\text{filtrate}}$) of 2.5 psi. The retentate was collected and concentrated to a final volume of 3.5 mL. After concentration, the solution was recirculated through the system for 10 min to recover any EVs adhering to the membrane. An equal volume of PBS was then added to restore the initial sample volume, remove residual impurities, and improve EV recovery, followed by reconcentration to 3.5 mL. This cycle of recirculation and concentration was repeated three times in total.

EVs isolation by Immunomagnetic separation

A new protocol based on the CD3 positive immunomagnetic selection of EVs was optimized. Briefly, B7-H3.CAR-T cell culture-conditioned media was collected and centrifuged to remove residual B7-H3 CAR-T cells (340xg, 10 minutes). The EV containing medium was incubated with CD3 MicroBeads (Miltenyi Biotec, 25 $\mu\text{L}/\text{mL}$ of medium) for 1h at RT. Next, a LS Column (Miltenyi Biotec), placed in the magnetic field of a MACS Separator (Miltenyi Biotec), was rinsed for 3 times with 1 mL of CliniMacs PBS/EDTA Buffer (Miltenyi Biotec) supplemented with 0.5% HSA. Labeled cell culture-conditioned media were then loaded into the column and, after 4 washes using 1.5 mL of buffer each, the column was removed from the magnetic field and the magnetically retained CD3+ EVs were eluted using 5 mL of buffer into the column. EVs were finally analyzed by flow cytometry.

5.3 EVs Characterization

Nanoparticle tracking Analysis

Size and concentration of isolated EV samples were subsequently measured by nanoparticle tracking analysis (NTA) on a ZetaView instrument (Particle Metrix, Meerbusch, Germany) equipped with a 488 nm, 40 mW blue laser and a long-pass emission filter for blue laser excitation, as already reported (Piro et al., 2024). The autofocus was calibrated by the system to ensure clear particle visualization. Post-acquisition parameters were optimized and consistently applied across all measurements. For each sample, a minimum of 11 fields were recorded, with 20-second videos captured using the ZetaView software. For every measurement, a comprehensive report was generated, including all analyzed parameters such as the mean and median EV sizes.

Atomic Force Microscopy

To investigate the size distribution and morphology of the isolated EVs, Atomic Force Microscopy (AFM) analysis was performed using a MultiMode 8 AFM microscope with a Nanoscope V controller (Bruker, Billerica, MA, USA). The samples were prepared by depositing a drop of diluted suspensions of isolated vesicles on a SiO_2 wafer, followed by drying in the oven at 37 °C for 2 h and then at room

temperature overnight. These preparations were scanned by the silicon RTESPA-150 probe (rectangular geometry; cantilever resonance frequency, 150 kHz; and nominal spring constant, 5 N/m) in TappingMode™ in air. Images of 512 × 512 pixels were collected with different scan sizes of 1.5 μm² and were elaborated using NanoScope Analysis 1.8 software (Bruker, Billerica, MA, USA).

EVs Markers

Western Blot

Cells were collected and lysed in RIPA buffer supplemented with protease inhibitor cocktail. Protein concentrations were determined by the BCA Protein Assay (Thermo Scientific, Rockford, IL, USA) and 30 μg were subjected to electrophoresis followed by immunoblotting. The membranes were blocked in 5% nonfat dry milk for one hour at room temperature and incubated with the appropriate primary antibodies. Mouse monoclonal CD81 and Cytochrome C antibodies were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Mouse monoclonal CD63 and rabbit monoclonal Flotillin-1 antibodies were purchased from Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA) and Cell Signaling Technology, Inc. (Beverly, MA, USA), respectively. Then the membranes were incubated with either anti-rabbit or anti-mouse HRP-conjugated secondary antibodies. The blots were revealed by chemiluminescence using the SuperSignal West Pico Chemiluminescence Substrate (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions. β-actin was used as loading control.

Flow Cytometry

Extracellular vesicles (EVs), previously isolated as described above, were subsequently analyzed by flow cytometry. For intravesicular staining of EVs, samples were further fixed and permeabilized using 200 μL of Cytofix/Cytoperm (Becton Dickinson Biosciences) for 20 minutes in the dark at RT and stained with flotillin-1, perforin, or granzyme B antibodies for 30 minutes. Stained EV samples were properly diluted with PBS 1X and acquired by flow cytometry using a FASCVerse (BD Biosciences) instrument. To enhance flow cytometry sensitivity, the threshold was set on the APC channel, the flow rate was adjusted to avoid swarming effects. For each sample, 1x10⁶ events were acquired. All antibodies and reagents were titrated under the assay conditions. FMO controls were used.

Proteomic and bioinformatic analysis

At least 2x10⁶ isolated EVs were used for proteomic analysis as previously reported (citazione). Isolated EVs were lysed and further centrifuged at 500g for 5-10 minutes in Amicon Ultra-2 Centrifugal Filter Unit 100 KDa (Merck) to recover peptides after digestion of EV protein cargo for proteomic purposes. The filter-aided proteome preparation (FASP) digestion was carried out

overnight at 37°C with trypsin (Promega, Madison, WI). The proteins from each sample were analyzed in triplicate using LiquidChromatography-Tandem Mass Spectrometry (LC- MS/MS) with UltiMate™ 3000 UPLC chromatographic system (Thermo Fisher Scientific, Milan, Italy) coupled to Orbitrap Fusion™ Tribrid™ (Thermo Fisher Scientific, Milan, Italy) mass spectrometer. The subsequent bioinformatic processing was performed with Proteome Discoverer software (Thermo Fisher Scientific, Milan, Italy) using the Homo Sapiens research database complete with contaminating and reverse sequences. The GO (Gene Ontology) functional enrichment analysis was then carried out using the Ingenuity Pathway Analysis software (Qiagen, Hilden, Germany) and STRING software to evaluate protein-protein interactions (PPI). The False Discovery Rate (FDR) indicates the significance in terms of functional enrichment, shown as a corrected P-value using Benjamini-Hochberg post-hoc test.

5.4 Cell Culture

B7-H3 CAR-T cells were cultured in OpTmizer™ CTS™ T-Cell Expansion SFM (GIBCO) supplemented with 5% Human AB Serum (Sigma Aldrich), 1% Penicillin/Streptomycin (Pen/Strep, Dutscher, Bernolsheim, France) and 10 ng/mL IL-15 and IL-7, both from Miltenyi. Two different B7-H3 expressing human cell lines (L3.6pl and panc-1) were used for further in vitro experiments. L3.6pl purchased from ATCC, were cultured in RPMI-1640 with L-glutamine (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Dutscher) and 1% Pen/Strep. Panc-1 were purchased from ATCC and maintained in DMEM high glucose with L-glutamine supplemented with 20% FBS and 1% Pen/Strep. Cells were cultured in an incubator at 37 °C and 5% CO₂.

5.5 Quantification of EV protein content

To relate the EV numbers to the EV protein content, samples were quantified using the BCA Protein Assay (Thermo Scientific, Rockford, IL, USA).

5.6 Killing Assays with CAR-T Cells and CAR-T-Derived EVs

Killing analyses were assessed by flow cytometry. Briefly, target cells (L3.6pl or Panc-1 cell lines) were labeled with 0.5 μM CFSE (Thermo Fisher Scientific), according to the related manufacturer's instructions. Cells were further resuspended in RPMI-1640 or DMEM high glucose supplemented with 20% of FBS and seeded in 12-well round-bottom plates. L3.6pl or Panc-1 cell lines were co-cultured with B7-H3 CAR-T cells, testing different CAR-T cells:target ratios (2:1; 5:1; 10:1). After

24 hours of culture, cell samples were stained using a Live/Dead™ Fixable Far Red Dead Cell kit (Thermo Fisher Scientific). Samples were then acquired using a FACS Aria™ III instrument (BD Biosciences). CFSE positive target cells were analyzed for their viability and the percentage of CAR-T cell induced killing was calculated using the following formula: Killing (%) = $[1 - \text{viable target cells (sample)} / \text{viable target cells (control)}] \times 100\%$.

B7-H3 EVs or EVs obtained from non-transduced PBMCs were concentrated with TFF and then isolated by CD3+ Immunomagnetic Beads column resuspended in 100 µL of exosome-depleted RPMI 1640 complete medium. To remove FBS derived EVs, FBS was ultracentrifuged and further added to a commercial RPMI. L3.6pl and Panc-1 were seeded in a 12-well flat-bottom plate to a concentration of 7.5×10^4 cells in 2 mL of culture media and treated with 150µg of EV proteins, in standard cell culture conditions (37°C, 5% CO₂). After 24h and 48h of incubation, cells were collected and stained with 10 µL of 7-AAD (BD Biosciences) for 10 minutes, in the dark. All samples were diluted with 200 µL of PBS and acquired using a FACSVerse flow cytometer (BD Biosciences). At least 10,000 events/sample were acquired. The percentage of induced killing was calculated based on the 7-AAD+ gate, using the following formula:

Killing (%) = $[1 - \text{viable target cells (sample)} / \text{viable target cells (control)}] \times 100\%$.

5.7 B7-H3 CAR-T derived EVs in vitro MTT cytotoxicity assay

Cell viability was analyzed using MTT assay. Briefly, L3.6pl and Panc-1 cells were seeded in 96-well plates (10×10^3 cells/well) in RPMI-1640 and DMEM High glucose with 20% FBS, respectively. Cells were treated for 48 hours at various concentrations (25-50-100-150µg) of B7-H3 EVs at 37°C. Then medium was removed, 0.5 mg/mL MTT solution was added to each well, followed by incubation at 37 °C for 3 hours. The MTT solution was removed and replaced with dimethyl sulfoxide (DMSO, Euroclone) in order to dissolve formazan crystals. Absorbance of each well was quantified at 570 and 690 nm, using a Synergy H1 microplate reader (BioTek Instruments Inc., Winooski, VT, USA).

5.8 Flow Cytometry B7-H3 EV analysis

Cell culture supernatant samples were stained to detect EVs, as previously described. Briefly, 100 µl of cell supernatant was stained using a lipophilic cationic dye (LCD), to identify the whole compartment of EVs, fluorescein isothiocyanate (FITC)-conjugated phalloidin to exclude damaged EVs and anti CAR-T B7H3. After 45 minutes of staining at room temperature (RT) in the dark, the samples were properly diluted to avoid swarming effects and analyzed by flow cytometry (Symphony

A5, BD Biosciences, La Jolla, CA, USA). The flow cytometry trigger threshold was set on the APC channel (LCD emission channel) to enhance flow cytometry sensitivity. Mean fluorescence intensity (MFI) ratios, calculated as the ratio between the MFI of the stained sample and the related unstained control, were used to assess single marker expression level. Standardized beads, combined with the application of the Cytometer Setup and Tracking (CS&T) module, enabled daily alignment of instrument performance. We performed instrument calibration to support reproducibility of FC analysis. The PE Fluorescent channel was calibrated using Spherotech Ultra Rainbow (UR) quantitative particles (URCP-38-2K), following the manufacturer's instructions. Mean MFI values for calibrated PE channel were calculated. To analyse the interaction between EVs derived from B7-H3 CAR-T cells or non-transduced (NT) with target cells, EVs were labelled for imaging flow cytometry. Specifically, purified B7-H3 EVs and NT EVs were stained with pHrodo™ by incubating the samples for 30 minutes at RT in the dark. Labelled both EVs were subsequently used to treat L3.6pl cells as previously described. After 48 hours of co-culture, L3.6pl cells were acquired using the FACS Discover S8 imaging flow cytometer (BD Biosciences, San Jose, CA, USA), and a minimum of 20,000 events were collected per sample. Data analysis was carried out using FlowJo v11 (BD Biosciences, Ashland, OR, USA, 2025).

5.9 3D Spheroids Culture and Treatments

Spheroids were generated from L3.6pl pancreatic cancer cells using a Matrigel-based three-dimensional (3D) culture system in 96-well flat plates. Cells (8×10^2 cells/well) were suspended in a solution composed of 1/3 Matrigel (phenol red-free, growth factor-reduced, 10 mg/mL; BD Biosciences, Erembodegem, Belgium) and 2/3 spheroid plating medium. The spheroid plating medium was based on RPMI-1640 supplemented with 3% fetal bovine serum (FBS; Gibco), GlutaMAX (Invitrogen), penicillin/streptomycin (Gibco), 10 mM HEPES (Invitrogen), $1 \times \text{N2}$ and $1 \times \text{B27}$ supplements (Thermo Fisher Scientific), N-acetyl-L-cysteine (1.25 mM; Sigma-Aldrich), and nicotinamide (1 mM; Sigma-Aldrich). During initial spheroid formation, the ROCK inhibitor Y-27632 (10 μM ; Merck-Millipore, Temecula, CA, USA) was included in the culture medium and subsequently omitted once spheroids were established. Plates were incubated at 37 °C for 30 minutes to allow matrix polymerization, after which 100 μL of spheroid plating medium were added to each well. On day 5 after seeding, once L3.6pl-derived spheroids had formed, they were either left untreated (control) or treated with extracellular vesicles (EVs) derived from not-transduced lymphocytes (NT) or CAR-B7-H3 lymphocyte. EV treatments were performed every 48 hours, with half of the culture medium removed and replaced with fresh medium containing EVs. Spheroid area

was measured daily using image analysis software, and data were expressed as changes in spheroid area over time relative to baseline and reported graphically.

The cytotoxic activity of not-transduced lymphocytes (NT) or CAR-B7-H3 lymphocytes against L3.6pl-derived spheroids was evaluated using a lactate dehydrogenase (LDH) release assay (Cytotoxicity Detection Kit LDH; Roche, Cat. No. 11 644 793 001), as previously described (Di Donato et al., 2025 Cell Death and Disease). L3.6pl-derived spheroids were cultured for 10 days and subsequently co-cultured with lymphocytes at different effector-to-target (E:T) ratios (5:1, 10:1, and 20:1) for 7 hours. At the end of the co-culture period, plates were centrifuged at $250 \times g$ for 10 minutes, and cell-free supernatants were transferred to optically clear 96-well flat-bottom plates. Samples were incubated with freshly prepared reaction mixture for 30 minutes, after which LDH activity was measured at 490 nm using a multi-well plate reader (EnSpire, PerkinElmer).

Absorbance values were acquired in technical triplicates for each condition, and background absorbance was subtracted. Target cell lysis was calculated using the following

formula: % Cytotoxicity =
$$\frac{(LDH_{\text{co-culture}} - LDH_{\text{lymphocytes}}) - LDH_{\text{spheroids}}}{LDH_{\text{Triton}} - LDH_{\text{spheroids}}} \times 100$$

6. Results

6.1 Generation and characterization of B7H3 CAR-T cells

As outlined in the Rationale, B7-H3 antigen was selected as the target antigen for the generation of CAR-T (Fig.6_Panel A). Human T lymphocytes were isolated from peripheral blood mononuclear cells by CD3⁺ immunomagnetic selection. Subsequently, the selected CD3⁺ cells were activated using anti-CD3 antibodies and transduced with a specific CAR construct for the B7-H3 antigen (kindly provided by Professor Ignazio Caruana (Universitätsklinikum Würzburg) (Patent IT202200025593A1)).

Phenotypic characterization of anti-B7-H3 CAR-T cells was performed using CD3, CD4, and CD8 surface markers to identify the major T subpopulations in the generated product. The CD3 marker was employed to identify total T lymphocytes, while CD4 and CD8 markers were used to discriminate helper T cells and cytotoxic T cells, respectively (Fig.6_Panel B). CAR⁺ T-cell population (CD3⁺CAR⁺) consistently displayed a higher proportion of CD4⁺ cells compared with CD8⁺ cells across all analyzed samples, indicating a predominance of helper T-cell phenotype (CD3⁺CD4⁺) over the cytotoxic phenotype (CD3⁺CD8⁺) (Fig. 6, Panel B). This pattern is consistent across different experimental samples, indicating a stable phenotypic composition of the CAR-T product.

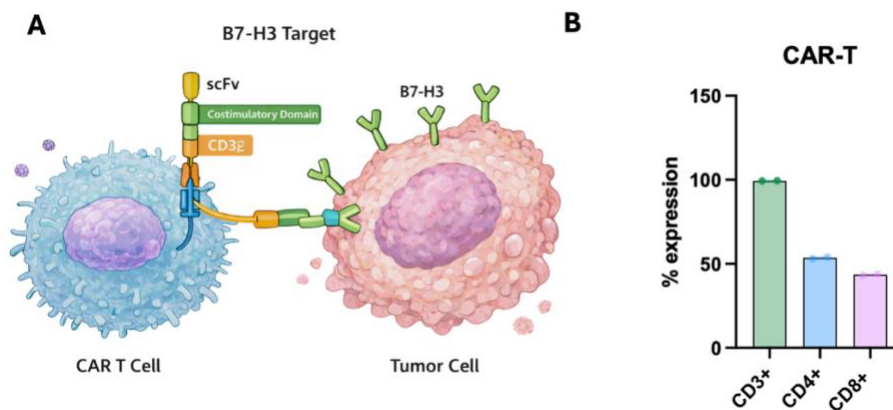


Figure 6 A) Schematic representation of B7-H3 CAR-T cells with chimeric antigen receptor. B) T-cell Subset Distribution in CAR-T Products. Phenotypic analysis of anti-B7-H3 CAR-Ts highlighted a predominance of the CD4⁺ population over CD8⁺ cells within CAR-positive cells.

We further characterized the phenotypic composition of freshly isolated CAR-T cells assessing the distribution of differentiation subsets (naïve, TCM, TEM, TEMRA) and the expression of activation markers (PD-1, TIM-3, and LAG-3) (Fig. 7).

CAR-T samples exhibited a lower frequency of naïve and T_EMRA cells compared with T_CM and T_EM subsets, suggesting a predominance of memory-like populations that may support sustained proliferative potential and effector function, while limiting highly differentiated or less adaptable

cells. This analysis revealed distinct differences in the expression of activation markers, including PD-1, TIM-3, and LAG-3. Interestingly, TIM-3 was the most highly expressed in CAR⁺ cells, potentially reflecting a state of early T-cell activation. Incidentally, these observations are preliminary and derived from a single donor, and further analyses are needed to confirm these trends across multiple samples.

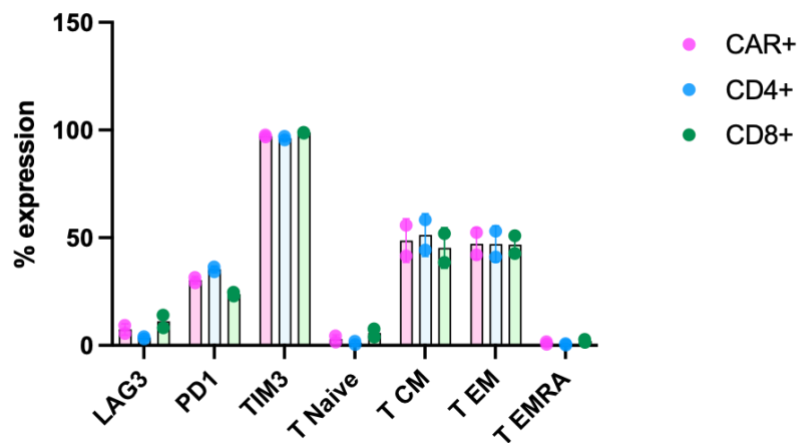


Figure 7 Phenotypic profile of CAR⁺ population. Phenotypic characterization of CAR-positive population (CAR⁺) through specific markers of activation (PD1, TIM3, LAG3) and differentiation markers (naïve, TCM, TEM, TEMRA).

In order to test *in vitro* the antitumor efficacy of B7-H3 CAR-Ts in pancreatic cancer, we evaluated the expression of the B7-H3 antigen in different pancreatic cancer cell lines candidate for these assays. We selected two PC cell lines with different expression of the antigen: Panc-1 (MFI ratio 23.4), and L3.6pl (MFI ratio 28.2). L3.6pl cell line showed slightly higher B7-H3 expression, as compared to Panc-1 cell line (Fig.8).

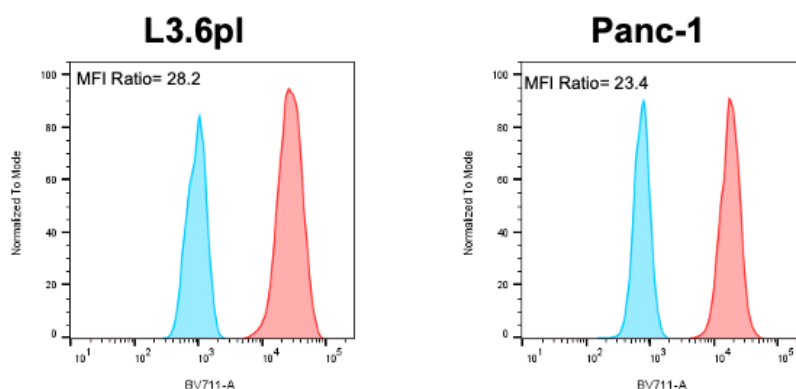


Figure 8 B7-H3 expression on pancreatic cancer cell lines. Surface expression of B7-H3 was analyzed on PC cell lines L3.6pl (MFI ratio: 28.2) and Panc-1 (MFI ratio: 23.4). Mean fluorescence intensity (MFI) ratios were calculated by dividing the MFI of the B7-H3–stained sample (red peak) by that of the corresponding fluorescence minus one (FMO) control (blue peak). Data are representative of three independent experiments.

After confirming B7-H3 expression on PC cell lines, a co-culture assay was performed to assess the cytotoxic activity of B7-H3 CAR-T cells, or untreated Not-Transduced (NT) lymphocytes (effector cells) against L3.6pl and Panc-1 (CFSE-labeled target cells). Co-cultures were carried out at different effector:target ratios (2:1, 5:1 and 10:1) and the cytotoxic effect of B7-H3 CAR-T or NT-T, in terms of percentage of dead target cells, was evaluated after 12 hours of incubation (Fig.9). Overall, for both PC cell lines, B7-H3 CAR-T cells showed significantly higher percentage of dead target cells, as compared to untreated NT-lymphocytes or medium-only controls.

In L3.6pl cells, which exhibit the highest B7-H3 expression, high levels of cytotoxic activity, in terms of percentage of dead target cells, were already observed at low effector-to-target ratios (2:1). In contrast, in Panc-1 cells, cytotoxic activity was observed at higher effector-to-target ratios (5:1 and 10:1).

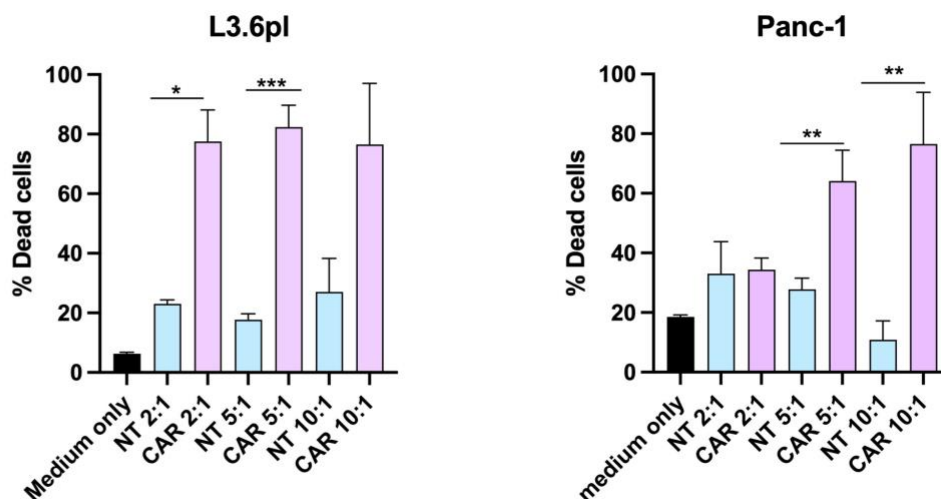


Figure 9 Cytotoxic activity of B7-H3 CAR-T cells against pancreatic cancer cell lines. A co-culture assay was performed to evaluate cytotoxic activity, expressed as the percentage of dead target cells, of B7-H3 CAR-T cells (CAR) or untreated not-transduced (NT) lymphocytes (effector cells) against L3.6pl and Panc-1 cells (CFSE-labeled target cells) at different effector-to-target ratios (2:1, 5:1, and 10:1).

6.2 Generation and characterization of B7-H3 CAR-T derived EVs

EVs were isolated from the culture supernatant of B7-H3 CAR-T cells, concentrated by tangential flow filtration and subsequently purified by immunomagnetic CD3⁺ selection. EV production was monitored over time in both CAR-T and untreated Not-transduced (NT) lymphocytes cultures by FACSVerse analysis (Fig.10). Both cell populations exhibited a progressive increase in EV production over 72 hours of culture. EV levels in CAR-T cell cultures were consistently higher than those observed in NT cells, indicating a greater vesicle output over time (Fig.10)

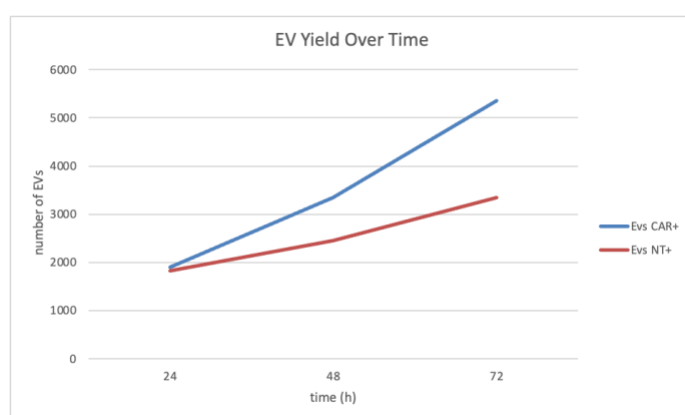


Figure 10 Extracellular vesicle (EV) production by CAR-T and Not-transduced T cells. EV production was monitored over time in cultures of B7-H3 CAR-T (EVs CAR+) and NT lymphocytes (EVs NT+) by FACSVerse analysis. CAR-T cells consistently released higher numbers of EVs compared with NT cells over the analyzed time points.

The diameter and size distribution of EVs were measured by Nanoparticle Tracking Analysis (NTA). According to NTA, the EVs derived from B7-H3 CAR-T showed a median diameter of 112.6 nm, with a peculiar size distribution (Fig.11).

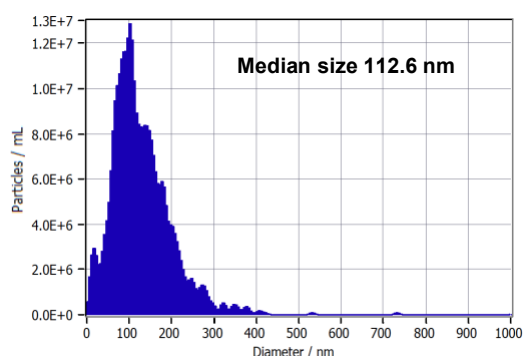


Figure 11 Size distribution and concentration of extracellular vesicles released by B7-H3 CAR-T cells. EVs secreted by B7-H3 CAR-T cells were analyzed by nanoparticle tracking analysis (NTA) to determine vesicle size (Diameter/nm) and concentration (particles/mL). Data represent measurements performed in triplicate.

EVs from B7-H3 CAR-T were evaluated by atomic force microscopy (AFM) to assess their shape and structural integrity (Fig.12). According to AFM, most of the EVs showed globular shape and were in isolated form (Fig.12).

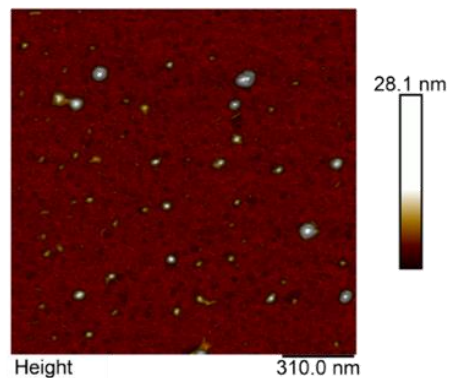


Figure 12 Characterization of B7-H3 CAR EVs by Atomic force microscopy. Atomic force microscopy (AFM) images showing the size, shape, and structural integrity of EVs. Scale bar 310nm. Data represent measurements performed in triplicate.

EVs were further characterized by western blot analysis and flow cytometry for two positive protein markers of EVs and one EV negative protein marker, according to current MISEV2023 guidelines. In particular, EVs expressed CD63 and Flotillin-1, but not cytochrome C that was exclusively detectable in cell lysates, as expected (Fig. 13_Panel A). In fact, cytochrome C is predominantly located within cell mitochondria, and it serves as a negative marker for EVs and, according to MISEV2023 guidelines, its detection in EV preparations would imply potential contamination with cellular remnants like debris or apoptotic bodies. Flotillin-1 and CD63 expression on EVs was confirmed and corroborated by Flow cytometry (Fig.13_Panel B).

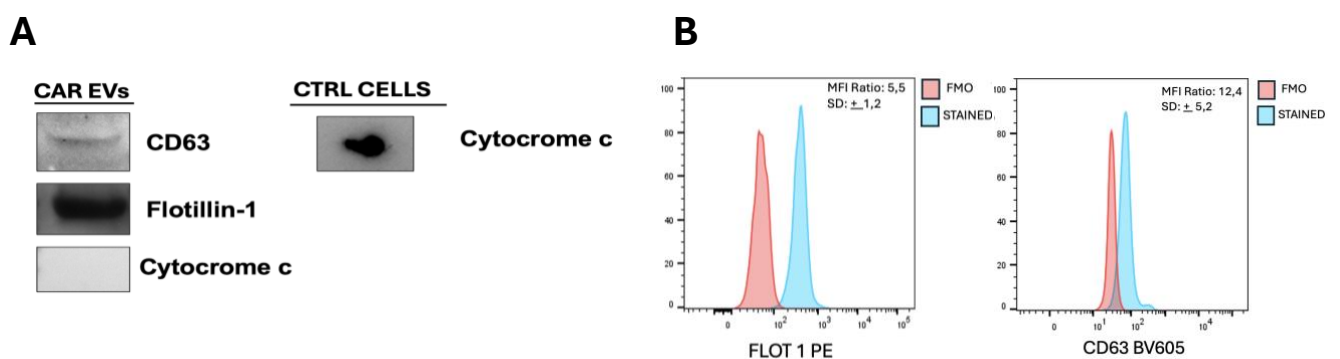


Figure 13 Characterization of EV protein markers. (A) Western blot analysis showing the presence of EV-positive markers CD63 and Flotillin-1, and the absence of the EV-negative marker cytochrome C, indicating that the EV preparation is free from cellular organelle contamination and apoptotic blebs. Parent CAR-T Cells were used as controls for cytochrome C expression. (B) Flow cytometry analysis confirming the expression of CD63 and Flotillin-1 in B7-H3CAR EVs. Mean fluorescence intensity (MFI) ratios were calculated by dividing the MFI of the B7-H3 EVs–stained sample (red peak) by that of the corresponding fluorescence minus one (FMO) control (blue peak).

Proteomic analysis was performed to additionally characterize B7-H3 CAR EVs. Proteomic profiling of EVs derived from B7-H3 CAR-T cells, identified 82 proteins that were interconnected within a

single functional network ($p=1 \times 10^{-16}$) as determined by STRING analysis (Fig.14_Panel A). According to *STRING Analysis*, 59 of the 82 identified proteins were involved in “Extracellular exosome” (FDR 1.64×10^{-35}) while 39 proteins were associated with the “*Immune System*” (FDR 6.96×10^{-12}), collectively confirming both the extracellular vesicle origin of proteins and their immune cell derivation (Fig. 14, Panels B–C).

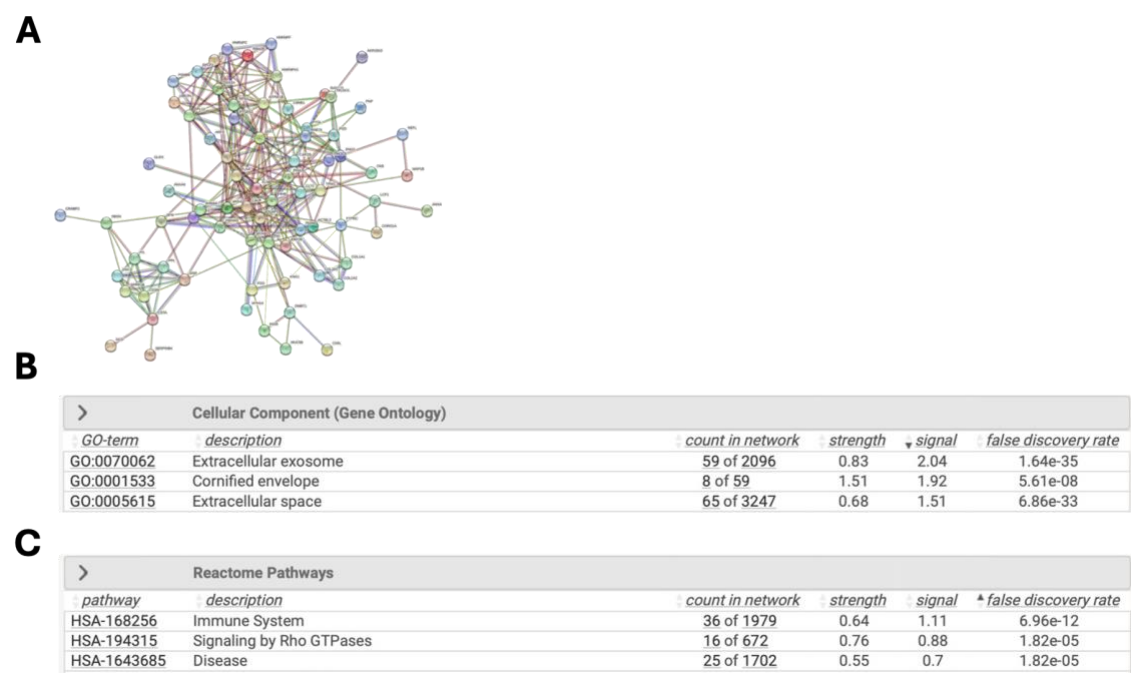


Figure 14 Proteomic analysis of EVs isolated from B7-H3 CAR-T Cells. (A) *STRING* network of 82 proteins identified in B7-H3 CAR EVs, showing a single interconnected functional network (PPI enrichment $p = 1.0 \times 10^{-16}$). (B) *STRING* analysis indicating that 59 of the 82 proteins are associated with “Extracellular Exosome” (FDR = 1.64×10^{-35}), consistent with the EV origin of the protein dataset. (C) *STRING* analysis indicating that 36 of the 82 proteins are associated with “Immune System” (FDR = 6.96×10^{-12})

6.3 In-Vitro cytotoxic activity of B7-H3 CAR-T derived EVs

We assessed the antitumor effect of EVs derived from B7-H3 CAR-T, or from Not-Transduced (NT) lymphocytes on PC cell viability using MTT, growth curves and 7AAD-killing assay.

A dose response MTT analysis was conducted by treating target PC cell lines L3.6pl and Panc-1 with growing doses of EVs (Fig. 15). The highest tested EV doses, expressed as micrograms of EV proteins, were 100 µg for L3.6pl cells and 150 µg for Panc-1 cells. After 48 hours of treatment, B7-H3 CAR EVs significantly affected cell viability of both tested PC cell lines in a dose-dependent manner in comparison to EVs derived from NT lymphocytes (Fig.15).

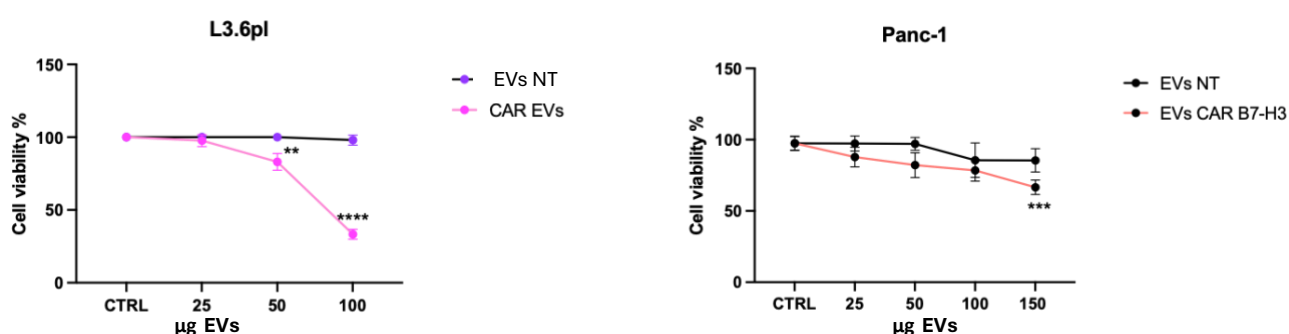


Figure 15 Effect of B7-H3 CAR EVs on the viability of PC cell lines. Cell viability of L3.6pl and Panc-1 cell lines was assessed by MTT assay after 48 hours of incubation with EVs CAR B7-H3 at the indicated concentrations, expressed as micrograms of EV proteins. Data are presented as mean $\pm$ SD of 3–4 replicates. Statistical significance was determined by comparison with cell viability after treatment with EVs derived from NT lymphocytes (EVs NT): * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

Growth curves of L3.6pl and Panc-1 cell lines were analyzed to evaluate the effects of treatment with 150 µg of EVs derived from B7-H3 CAR-T or from Not-Transduced (NT) lymphocytes at different incubation times (0, 24 and 48 h). As shown in Figure 16, treatment with EVs NT did not induce significant changes in L3.6pl cell line proliferation, as compared with the untreated control (CTRL). In contrast, treatment with B7-H3 CAR EVs resulted in a significant reduction in cell number at 48 h ($p < 0.01$), indicating an inhibitory effect on cell growth that became evident with prolonged exposure. A similar trend was observed in the Panc-1 cell line, where B7-H3 CAR EVs treatment resulted in a significant decrease in cell growth at 48 hours, as compared to control ($p < 0.05$), while NT EVs exerted only a limited and non-significant effect.

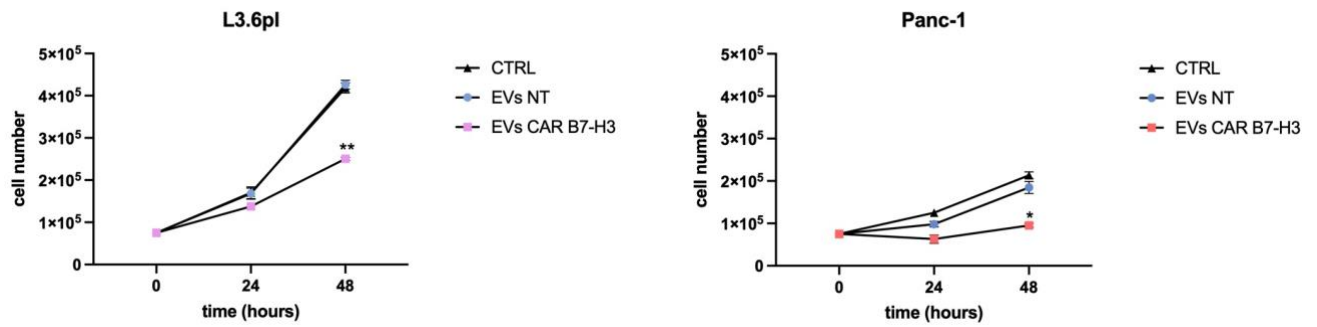


Figure 16 Effect of B7-H3 CAR EVs on growth of PC cells. Growth curves of L3.6pl and Panc-1 cell lines were evaluated following treatment with 150 µg of EVs at 0, 24, and 48 hours. EVs derived from B7-H3 CAR-T cells (EVs CAR B7-H3) significantly reduced cell proliferation at 48 hours compared with the untreated control (CTRL). NT EVs exerted only a non-significant effect. Statistical significance is indicated as ** $p < 0.05$ and * $p < 0.01$.

Subsequently, the cytotoxic effect of EVs derived from B7-H3 CAR-T or NT lymphocytes was evaluated on the same target cells L3.6pl and Panc-1. The target cells were treated with 150 micrograms of EVs, and dead cells were identified by flow cytometry after labeling with 7-AAD viability dye. For both cell lines, treatment with B7-H3 CAR-T –derived EVs resulted in a significant reduction in viable cell numbers at 48 hours (Fig.17).

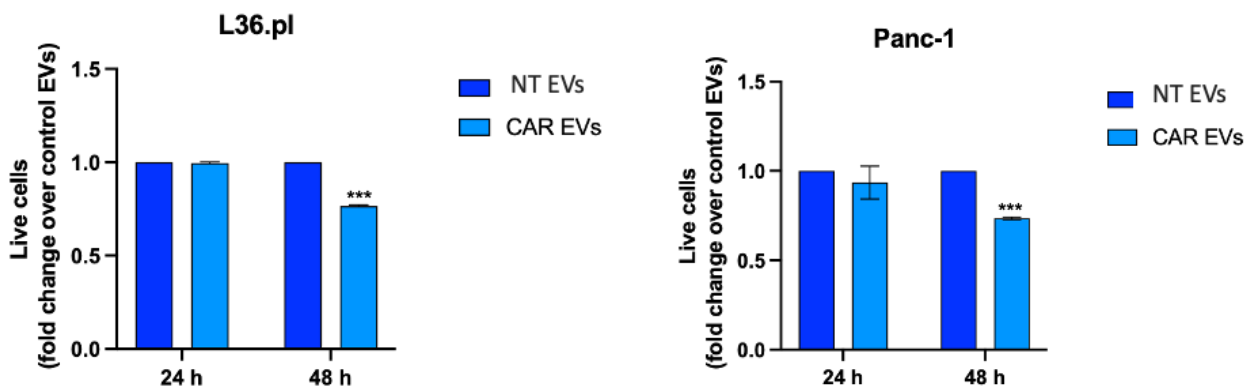


Figure 17 Effect on PC cell lines killing of B7-H3 CAR EVs. L3.6pl and Panc-1 cells were treated with 150 µg of EVs derived from B7-H3 CAR-T (EVs CAR B7-H3) or from NT lymphocytes (EVs NT) for 24 and 48 hours. Cytotoxic activity was evaluated by flow cytometry using the 7-AAD viability dye and calculating the number of living cells. The graphs represent the fold change of the number of live cells in the treated samples with EVs CAR B7-H3 over control EVs. Statistical significance is indicated as *** $p < 0.001$

Overall, results from all three assays indicate that treatment with B7-H3 EVs for 48 hours significantly increased tumor cell death in both pancreatic cancer cell lines. Notably, the effect was particularly evident in the L3.6pl cell line, especially in terms of cell viability and proliferation, as shown by viability assays and growth curve analyses.

6.4 Mechanisms involved in B7-H3 EVs cytotoxic activity

To investigate the cytotoxic mechanisms of B7-H3 CAR EVs, we analyzed whether they carried granzyme B and perforin, two effector molecules that play a key role in cytotoxicity of parental CAR-T cells (Fig. 18). These cytotoxic proteins are essential for the induction of tumor cell apoptosis, and their detection within EVs provides insight into the functional and therapeutic potential of the vesicles. As shown in Figure 18, all analyzed EVs carried the characteristic cytotoxic molecules perforin and granzyme B. Therefore, it is conceivable that mechanisms involved in the cytotoxic activity of B7-H3 CAR EVs rely at least in part on perforin and granzyme B.

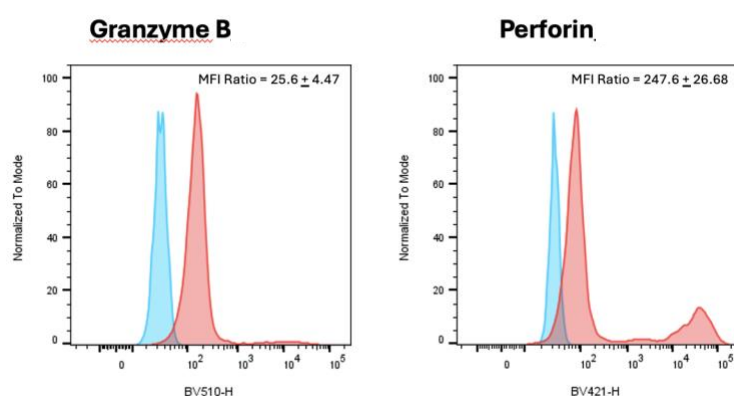


Figure 18 Flow cytometry analysis of Granzyme B and Perforin expression in B7-H3 CAR EVs. Representative histograms show B7-H3 CAR EVs stained for Granzyme B or Perforin. Mean fluorescence intensity (MFI) ratios were calculated by dividing the MFI of the positively stained sample (red peak) by that of the corresponding FMO control (blue peak). Data represent measurements performed in triplicate.

CAR expression is one of the key players in the mechanisms of CAR-T cell targeted cytotoxicity. Therefore, we also assessed CAR expression on extracellular vesicles (EVs) released by B7-H3 CAR-T cells (Fig. 19). Our analysis demonstrated that EVs derived from B7-H3 display the CAR receptor on their surface, indicating that T lymphocytes after transduction secrete EVs carrying the specific CAR. Furthermore, CAR-T cell-derived EVs show high surface expression of the CAR receptor, exceeding that observed in parental cells per surface unit (Fig.19).

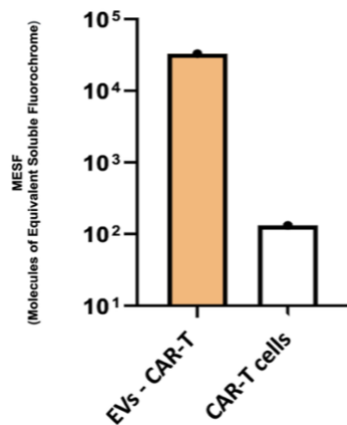


Figure 19. CAR Expression on B7-H3 EVs vs CAR-T Cells. CAR-T cell-derived EVs show high surface expression of the CAR receptor, exceeding that observed in parental cells per surface unit.

To verify whether CAR expression favored the interaction and the internalization of **B7-H3 CAR EVs** with target PC cells, a critical step for the exertion of cytotoxic activity, we assessed the ability of EVs to enter L3.6pl target cells. In fact, efficient CAR-mediated uptake is expected to enhance the intracellular delivery of cytotoxic effector molecules, thereby increasing tumor cell susceptibility to CAR⁺ EV-mediated killing. As shown in Figure 20, EVs derived from B7-H3 CAR-T cells exhibited significantly higher internalization in L3.6pl cells compared with EVs from Not-transduced (NT) cells. Imaging flow cytometry revealed that pHrodo-labeled B7-H3 CAR EVs were efficiently internalized by target cells, supporting the role of the CAR in mediating specific and targeted EV uptake. Overall, the increased internalization of B7-H3 CAR EVs is consistent with an enhanced targeting and cytotoxicity, as compared to EVs derived from Not-Transduced (NT) lymphocytes.

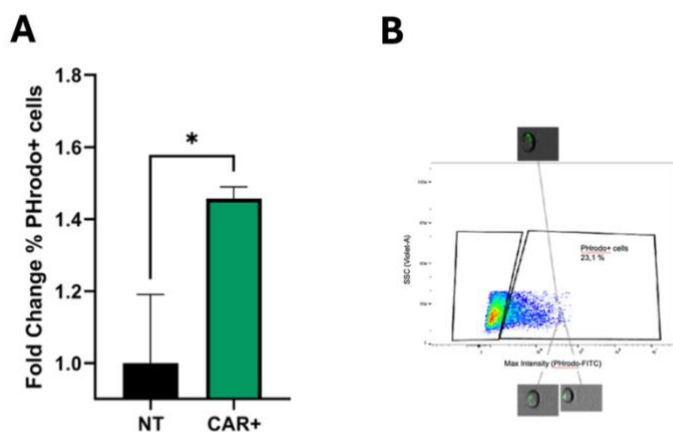


Figure 20 Internalization of CAR-B7-H3 EVs into L36PL cells. A) Histograms represent the fold change (over NT) of percentage of pHrodo-labeled cells after treatment with EVs derived from non-transduced lymphocytes (NT) or B7-H3 CAR EVs (CAR+). Compared with NT EVs, B7-H3 CAR EVs exhibited increased internalization, consistent with an enhanced cytotoxic potential. Statistical significance is indicated as * $p < 0.05$. B) Imaging flow cytometry analysis showing efficient internalization of pHrodo-labeled B7-H3 CAR EVs by L3.6pl target cells.

6.5 Cytotoxic Effect of B7-H3 CAR-T-derived EVs on 3D Pancreatic Cancer L3.6pl Spheroids

To assess the cytotoxic activity of B7-H3 CAR-T cells (CAR) against L3.6pl cells, a lactate dehydrogenase (LDH) release assay was performed in 3D tumor spheroids. The LDH release assay proved to be a reliable quantitative method to assess CAR T cytotoxic activity on PC spheroids. Treatments were carried out at increasing effector:target ratios (5:1, 10:1 and 20:1) and the cytotoxic effect of B7-H3 CAR-T or Not-Transduced lymphocytes (NT), in terms of percentage of lysed PC cells, was evaluated at 14 days of incubation (Fig.21). The percentage of cytotoxic activity was calculated using a standardized formula that normalizes LDH release to both the maximum release control (Triton X-100) and spontaneous release, allowing a direct comparison across the different experimental conditions.

The results showed an increase of cytotoxic activity as a function of the effector-to-target (E:T) ratio, with a more marked effect observed after treatment with B7-H3 CAR T compared with NT lymphocytes. In particular, cytotoxic effects after treatment with B7-H3 CAR T at 10:1 or 20:1 ratios, were associated with a significant increase in the percentage of lysed PC cells (Fig.21).

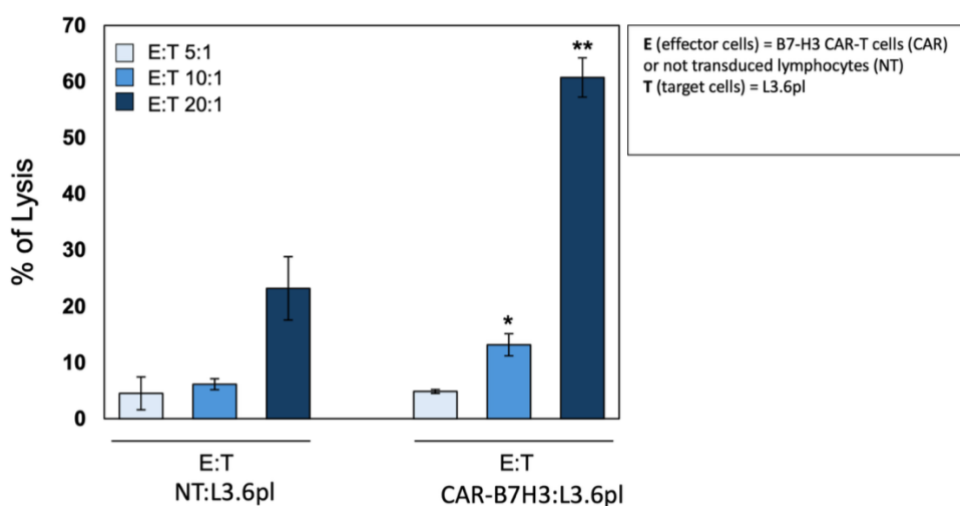


Figure 21 Cytotoxic activity of B7-H3 CAR-T on PC spheroids by LDH release assay. B7-H3 CAR-T or not transduced lymphocytes (NT) were cocultured with L3.6pl spheroids at different effector-to-target ratios (5:1, 10:1, and 20:1). Cytotoxicity Percentage of lysed PC cells was calculated by normalizing LDH release compared to maximum release control (Triton X-100 treated spheroids) and spontaneous release (lymphocyte-free spheroids). The data represent the mean $\pm$ SD of three technical replicates for each condition.

6.6 Cytotoxic Effect of B7-H3 CAR-T-derived EVs on 3D L3.6pl Spheroids

In line with the cytotoxic activity of B7-H3 CAR EVs observed in 2D models of L3.6pl PC cells, we evaluated whether EV-induced morphological changes and cell death in the 3D models.

Treatment with B7-H3 CAR EVs resulted in a progressive reduction in the size of L3.6pl spheroid models, which was significantly more marked as compared to spheroids treated with NT-derived EVs at 14 days (Fig. 22).

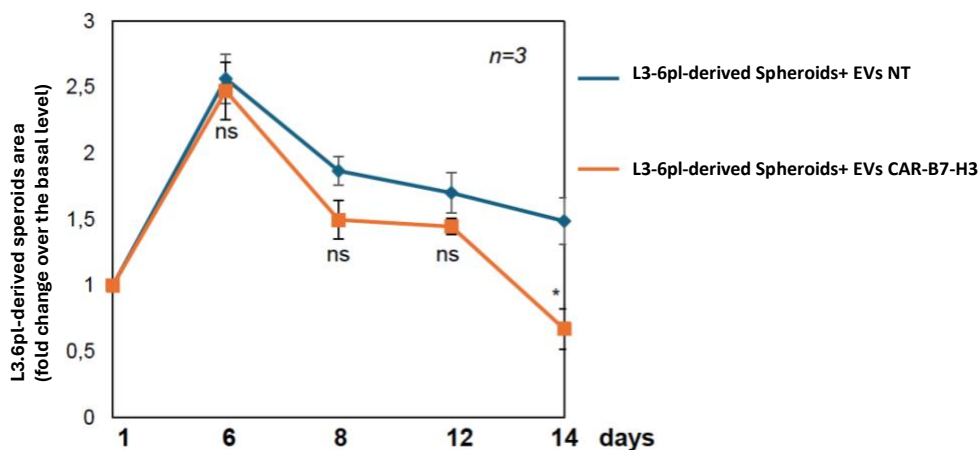


Figure 22 Quantitative analysis of L3.6pl spheroids treated with B7-H3 EVs.

(B) Quantitative analysis of spheroid size over time. Graphs show the variation in tumor size over time (fold change vs. basal level). B7-H3 CAR EV treatment results in a progressive and significant reduction in spheroid size, which was significantly more marked as compared to spheroids treated with NT-derived EVs at 14 days, highlighting cytotoxic effects in the 3D L3.6pl model.

7. Discussion and conclusions

Pancreatic cancer (PC) remains one of the most lethal malignancies, characterized by a desmoplastic stroma, immunosuppression, and remarkable resistance to conventional therapies. The dense extracellular matrix, abnormal vasculature, and abundance of immunosuppressive cell populations severely limit drug penetration and immune cell infiltration, contributing to the limited efficacy of currently available therapies. (Feig *et al.*, 2013; Stromnes *et al.*, 2017; Masugi *et al.*, 2022). Despite significant advances in cancer immunotherapy, pancreatic cancer continues to represent a major clinical challenge with an urgent need for innovative and more effective treatment strategies. Chimeric antigen receptor (CAR) T-cell therapy has revolutionized treatment of hematologic malignancies, achieving high and durable response rates in diseases such as acute lymphoblastic leukemia, lymphomas, and multiple myeloma, leading to the approval of several CAR-T products worldwide (June *et al.*, 2018; Zugasti *et al.*, 2025). However, the translation of CAR-T therapy to solid tumors has been largely unsuccessful to date. Major obstacles include the lack of truly tumor-specific antigens, antigen heterogeneity, limited trafficking and infiltration of CAR-T cells into tumor, and functional suppression within the immunosuppressive microenvironment (Newick *et al.*, 2017; Restifo *et al.*, 2012). In pancreatic cancer, these obstacles are compounded by the desmoplastic stroma and a chronically inflamed microenvironment, driving CAR-T cell exhaustion and severely constraining therapeutic effectiveness. (Stromnes *et al.*, 2017; Klampatsa *et al.*, 2022). In recent years, extracellular vesicles (EVs) have emerged as key mediators of intercellular communication and as promising tools for therapeutic applications. EVs are nanosized, membrane-bound vesicles capable of transferring biologically active molecules, including proteins and nucleic acids, to recipient cells, thereby modulating their phenotype and function (Raposo & Stoorvogel, 2013; Yáñez-Mó *et al.*, 2015). Building on this concept, EVs derived from CAR-T cells have been proposed as a novel cell-free immunotherapeutic platform. CAR-T-derived EVs retain the antigen specificity and cytotoxic machinery of their parental cells while lacking proliferative capacity, thereby potentially reducing the risk of severe toxicities such as cytokine release syndrome and neurotoxicity (Lanuti *et al.*, 2025; Fu *et al.*, 2019). Preclinical studies have demonstrated that CAR-T-derived EVs exert antigen-specific cytotoxicity and display improved tumor penetration in solid tumor models, including pancreatic cancer, highlighting their translational potential (Sani *et al.*, 2024; Qing *et al.*, 2025).

The present study explored the therapeutic potential of extracellular vesicles derived from B7-H3 CAR-T cells in pancreatic cancer *in vitro*. B7-H3 was selected as a target antigen for CAR-T cell engineering, based on its high expression in pancreatic cancer and its limited distribution in healthy

tissues, supporting its suitability as a tumor-associated antigen (Inamura *et al.*, 2018; Du *et al.*, 2019; Li *et al.*, 2023).

As a first step we generated B7-H3 CAR-T cells from human peripheral blood T lymphocytes producing a stable and phenotypically defined CAR-T product. Phenotypic analysis revealed a consistent predominance of CD4⁺ T cells within the CAR-positive population, suggesting a helper T-cell-enriched product. Notably, CD4⁺ CAR-T cells have been reported to contribute to sustained antitumor responses *in vivo* through cytokine production (Bove *et al.*, 2023). Additionally, the observed distribution of naïve and memory subsets suggests that CAR-T cells retain a differentiation profile compatible with effector function, while maintaining features associated with proliferative capacity and longevity. Collectively, these findings indicate that B7-H3 CAR-T cells display a stable phenotype, suitable for functional and translational studies. Notably, B7-H3 CAR-T cells consistently released higher levels of extracellular vesicles over time, as compared with non-transduced lymphocytes, suggesting that CAR expression, together with the enhanced activation state of engineered T cells, may promote vesicle biogenesis and secretion. Antitumor activity of B7-H3 CAR-T cells was subsequently evaluated *in vitro* using pancreatic cancer cell lines expressing different levels of B7-H3. Co-culture assays demonstrated that B7-H3 CAR-T cells induced tumor cell killing that was significantly higher than not-transduced (NT) lymphocytes. Notably, in L3.6pl cells, which express the highest levels of B7-H3, cytotoxic activity was observed even at low effector-to-target ratios and was higher than NT-lymphocytes across all tested effector-to-target ratios. Conversely, in Panc-1 cells, which express lower levels of B7-H3, killing increased proportionally to effector-to-target ratios and was higher than NT-lymphocytes only at the highest effector-to-target ratios tested. The lower effect of NT lymphocytes vs. CAR-T and the higher cytotoxicity at low effector-to-target ratios in cells expressing higher B7-H3 indicate that the specific interaction between CAR and its target favors cytotoxicity.

Following the characterization of B7-H3 CAR-T cells, we characterized EVs produced by parent CAR-T cells. These EVs displayed a size distribution consistent with small extracellular vesicles (112.64 nm) as confirmed by NTA and exhibited a globular shape according to AFM analyses. The expression of canonical EV markers (CD63 and Flotillin-1) and the absence of cytochrome C further confirmed the purity of preparations in accordance with MISEV2023 guidelines. Proteomic profiling of EVs derived from B7-H3 CAR-T cells, identified 82 proteins interconnected within a single functional network ($p=1 \times 10^{-16}$), as determined by STRING analysis. Moreover, proteins in “*Extracellular exosome*” and “*Immune System*” were highly enriched (FDR 1.64×10^{-35} and 6.96×10^{-12} , respectively) in EV preparations, collectively confirming both their extracellular vesicle origin and immune cell derivation.

The main aim of this study was to assess the therapeutic potential of B7-H3 CAR-EVs *in vitro*; thus, we evaluated whether these vesicles exerted a cytotoxic effect on target PC cells. Treatment of pancreatic cancer cell lines with CAR EVs in 2D models resulted in a dose-dependent PC cytotoxicity and affected target cell growth, whereas EVs derived from Not-transduced lymphocytes exerted minimal or no effects. This observation is in line with the therapeutic potential of B7-H3 CAR-EVs on PC targets expressing this antigen.

We investigated mechanisms involved in the cytotoxicity exerted by CAR. As first mechanistic evidence, we show that EVs derived from B7-H3 CAR-T cells display the CAR receptor on their surface, indicating that T lymphocytes after transduction secrete EVs carrying the specific CAR. It is conceivable that the presence of the CAR molecule on the surface of EVs may enable antigen-specific interactions, enhancing EVs targeting to B7-H3-expressing cells. In line with this prediction, imaging flow cytometry revealed that B7-H3 CAR EVs were internalized more efficiently by L3.6pl target cells than EVs derived from Not-transduced lymphocytes. The enhanced uptake of pHrodo-labeled CAR EVs supports a CAR-dependent mechanism of EV internalization, likely mediated by antigen recognition. This higher internalization of CAR- vs. NT-derived EVs is consistent with their higher cytotoxic activity and with the role of CAR in conferring specificity in a cell-free EV-based context, similarly to parental CAR-T cells. In addition to these mechanisms regarding targeting and uptake of EVs, another mechanism involved in cytotoxicity is linked to the EVs cargo. In this regard, this study shows that B7.H3 CAR EVs carry granzyme B and perforin that are well known to play a crucial role in cytotoxic activity of parental CAR-T cells.

Finally, we evaluated the antitumor activity of CAR-T cells and CAR-T-derived EVs in three-dimensional PC spheroid models. These models reproduce interactions between cells and matrix providing a better environment, as compared to 2D models, to assess efficacy or resistance to therapeutic EVs. B7-H3 CAR-T cells demonstrated increased cytotoxicity in spheroids in an effector-dependent manner, as compared to NT-lymphocytes, confirming their ability to infiltrate and kill tumor cells in a complex 3D architecture. Notably, in analogy to parental cells, B7-H3 CAR EVs induced marked structural disruption and cell death in L3.6pl spheroids, with a significant decrease in spheroid area over time. These results highlight the capacity of CAR-derived EVs to exert direct cytotoxic effects in a three-dimensional tumor model, independent of the presence of effector cells.

Overall, these findings indicate that, similarly to parental cells, B7-H3 CAR-T-derived EVs retain CAR, granzyme B and perforin expression, they are efficiently internalized by target PC cells and exert consistent cytotoxic effects across both two-dimensional and three-dimensional PC models, supporting their potential as a versatile cell-free immunotherapeutic platform for pancreatic cancer.

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