Chimeric antigen receptor (CAR) T-cell therapy: Harnessing extracellular vesicles for enhanced efficacy

Pharmacological Research

CHIMERIC ANTIGEN RECEPTOR (CAR) T-CELL THERAPY: HARNESSING EXTRACELLULAR VESICLES FOR ENHANCED EFFICACY

--Manuscript Draft--

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Abstract:	A cutting-edge approach in cell-based immunotherapy for combating resistant cancer involves genetically engineered chimeric antigen receptor T (CAR-T) lymphocytes. In recent years, these therapies have demonstrated effectiveness, leading to their commercialization and clinical application against certain types of cancer. However, CAR-T therapy faces limitations, such as the immunosuppressive tumour microenvironment that can render CAR-T cells ineffective, and the adverse side effects of the therapy, including cytokine release syndrome. Extracellular vesicles (EVs) are a diverse group of membrane-bound particles released into the extracellular environment by virtually all cell types. They are essential for intercellular communication, transferring cargoes such as proteins, lipids, various types of RNAs, and DNA fragments to target cells, traversing biological barriers both locally and systemically. EVs play roles in numerous physiological processes, with those from both immune and non-immune cells capable of modulating the immune system through activation or suppression. Leveraging this capability of EVs to enhance CAR-T cell therapy could represent a significant advancement in overcoming its current limitations. This review examines the current landscape of CAR-T cell immunotherapy and explores the potential role of EVs in augmenting its therapeutic efficacy.
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	Lucia Languino lucia.languino@jefferson.edu expert

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Dipartimento
Scienze Mediche

Turin, 08/05/2024

To Pharmacological Research

Dear Editors,

Please find enclosed the revised version of the the Ms entitle, "**Chimeric Antigen Receptor (CAR) T-cell Therapy: harnessing Extracellular Vesicles for enhanced efficacy"** *by Beatrice Spokeviciute, Sharad Kholia, and myself* which we would like you to consider for publication in *Pharmacological Research* special collection "Engineered nanomedicines for potential pharmaceutical applications and novel therapeutic developments"

Based on the Reviewers' comments the manuscript has been revised. In particular, we thank the Reviewers for their criticisms since to address them the Ms has been substantially improved. The graphical abstract is also included.

The answers to Reviewers are attached.

We state that the material presented herein is original research, has not been previously published and has not been submitted for publication elsewhere while under consideration. There is no conflict of interest.

Yours Sincerely
Maria Felice Brizzi MD, PhD

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	Yes	***No***	***Not applicable***
Formatting - The submission will automatically be rejected if the first six questions are not marked "yes" (questions 1-6) or "not applicable" (limited to questions 3-6**)**			
1. Are all tables and figures numbered and appropriately titled with descriptive legends that permit stand-alone interpretation?	***X***		
2. Are all data shown on figures and tables mentioned in the text of the Results section?	***X***		
3. Are the whole un-cropped images of the original western blots from which figures have been derived shown as supplemental figures?			***X***
4. In case of human studies, has specific mention been made of the study compliance with the regulations of the country(ies) in which the study was carried out ?			***X***
5. In case of human studies, has the study been registered on an accessible international registry/database of clinical trials (e.g. EudraCT, ClinicalTrials.gov, ChiCTR, ANZCTR, JPRN and the like)			***X***
6. In case of studies on animals, is there a statement indicating compliance with regulations on the ethical treatment of animals including identification of the institutional committee that approved the experiments?			***X***
Introduction			
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8. Are the primary endpoints clearly described?	***X***		
Materials and Methods			
9. Are the sources of all materials clearly indicated?			***X***
10. Is(are) the chemical structure(s) of any new compound(s) presented as a figure in the manuscript or referenced in the literature?			***X***
11. Are the source(s) and passage number of cell lines indicated?			***X***
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15. Are quantified results (*e.g.* IC_{50} and/or EC_{50} values) of concentration- and dose-response experiments included in the report?			***X***
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17. Are all group sizes approximately the same?			***X***
18. Were the criteria used for excluding any data from analysis determined prospectively and clearly stated?			***X***
19. Was the investigator responsible for data analysis blinded to which samples/animals represent treatments and controls?			***X***
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21. Are the reported data displayed as the mean +/- an estimate of variability (SD, SEM) of three or more independent experimental replications?			***X***
22. Is the number of replications used to generate an individual data point in each of the independent experiments clearly indicated?			***X***
23. Were the statistical tests employed to analyse the primary endpoints predetermined as part of the experimental design?			***X***
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26. Were post-hoc tests used to assess the statistical significance among means?			***X***
27. Was the study exploratory rather than hypothesis-driven?			***X***
28. Were human tissues or fluids used in this study?			***X***
Results			
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30. Were MIQE guidelines followed in the quantitative analysis and presentation of PCR and RT-PCR findings?			***X***
31. Was a reference standard (positive or negative controls) included in the study to validate the experiment?			***X***
Discussion			
32. Are all the findings considered within the context of the hypothesis presented in the Introduction?			***X***
33. Are the primary conclusions and their implications clearly stated?			***X***
34. Are any secondary endpoints reported and are these sufficiently powered for appropriate statistical analysis?			***X***
35. Are the limitations of the current study or alternative interpretations of the findings clearly stated?			***X***
For Meta-analyses only			
36. Were the PRISMA reporting guidelines and checklist followed in the case of meta-analyses on randomised controlled trials?			
37. Were the MOOSE reporting guidelines followed in the case of meta-analyses on observational studies in epidemiology?			
38. Was the protocol submitted into the PROSPERO International prospective register of systematic reviews and a registration number obtained?			
Conflict of Interest/Financial Support			
39. Is the conflict of interest statement is included in the manuscript?	***X***		
40. Are all organisations providing funding for this work listed in the Acknowledgments?	X		

Feedback/suggestions on the checklist by the author

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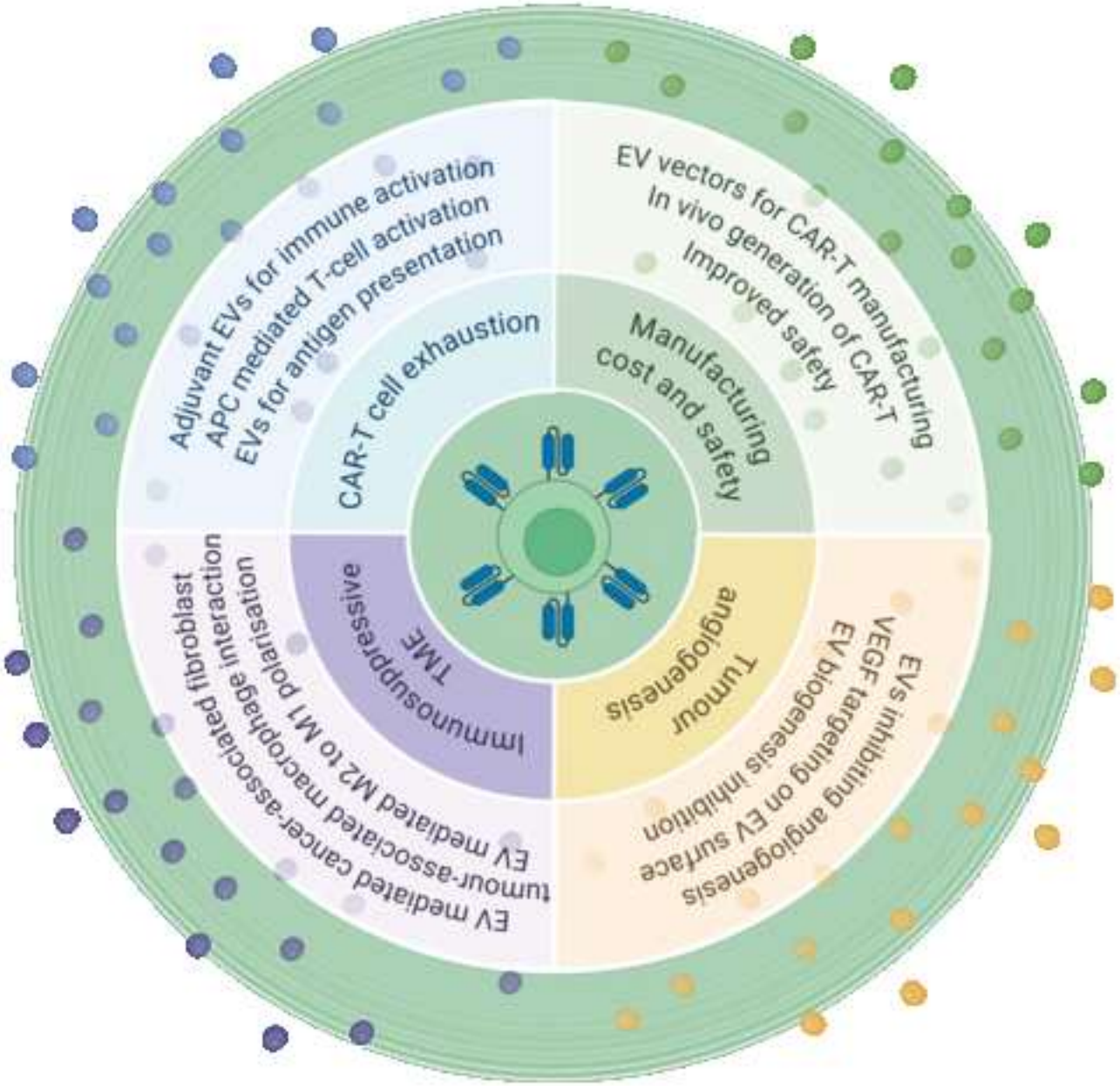

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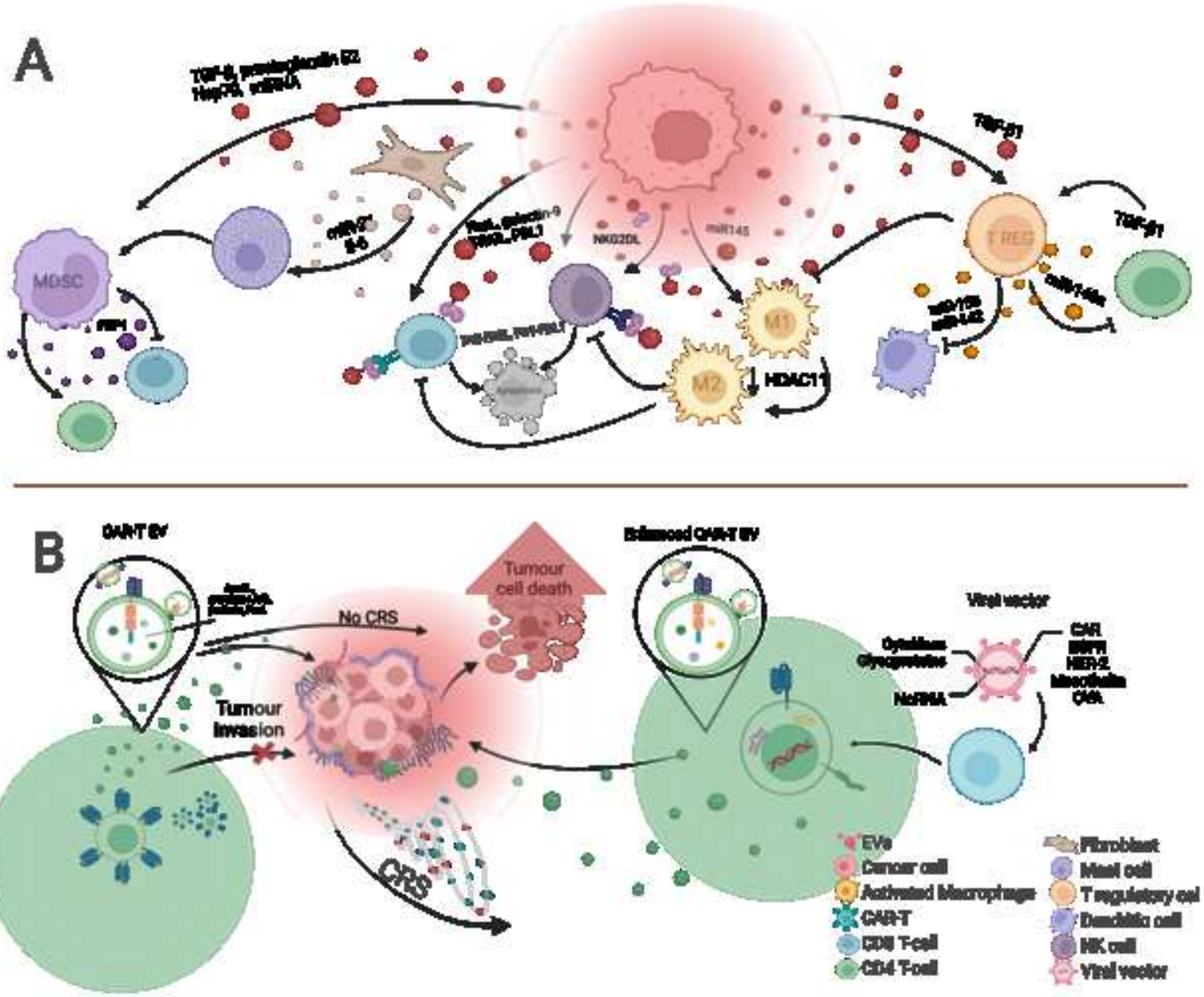

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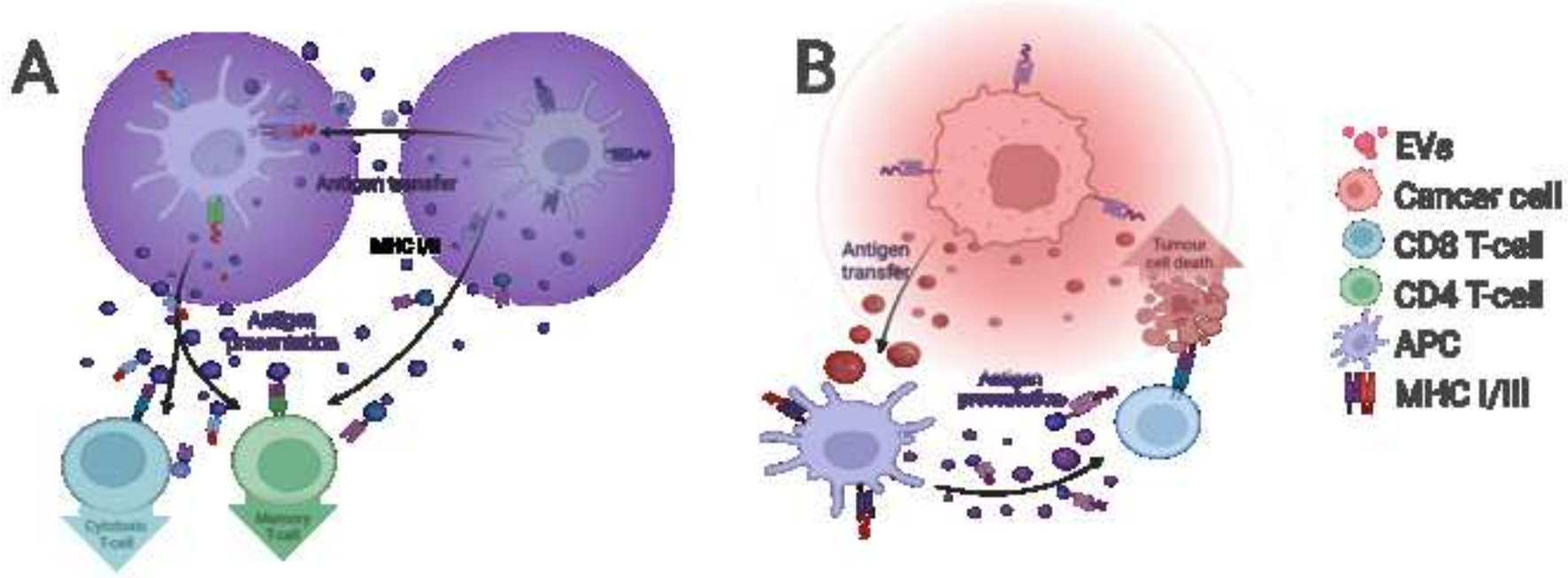

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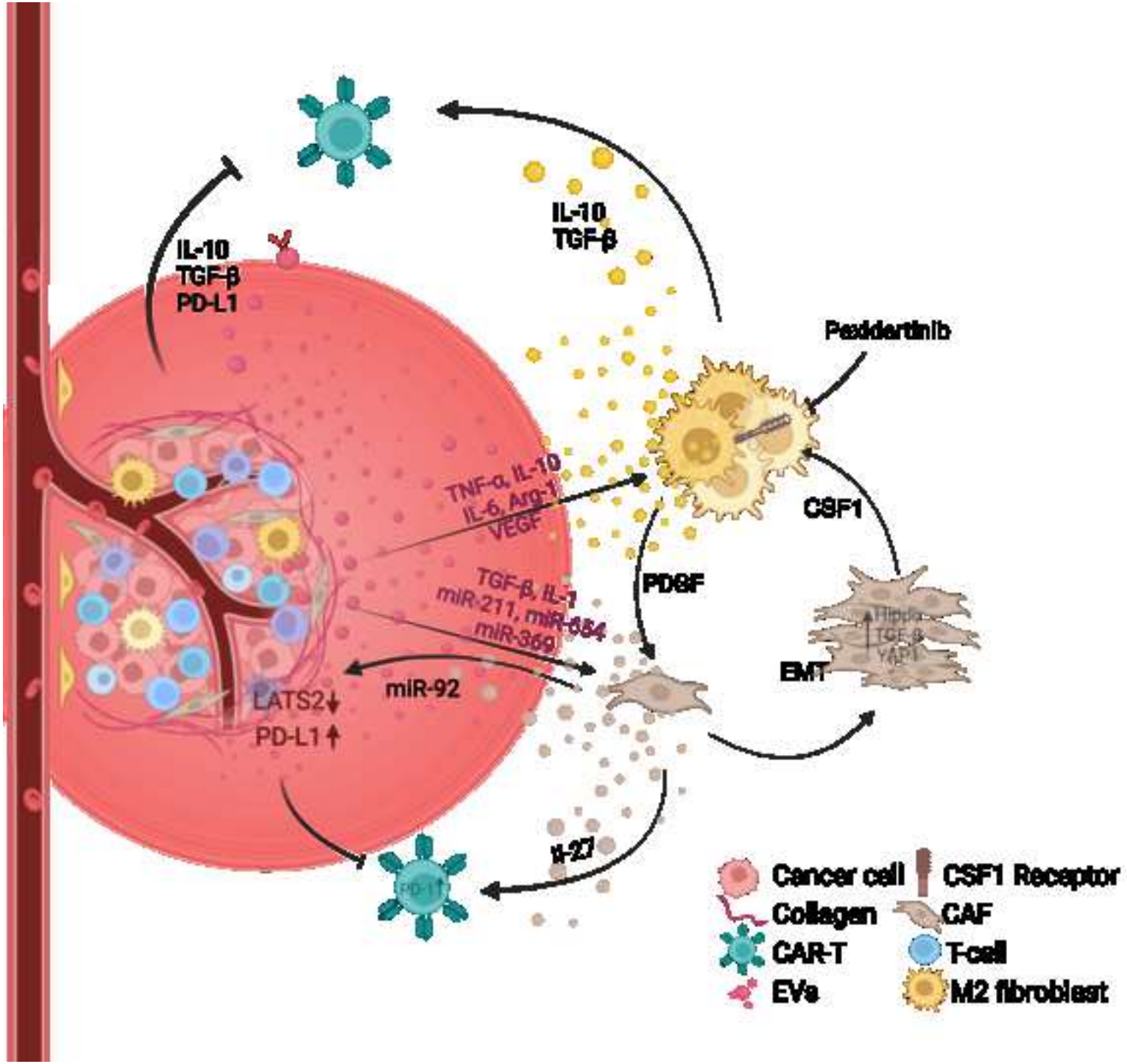

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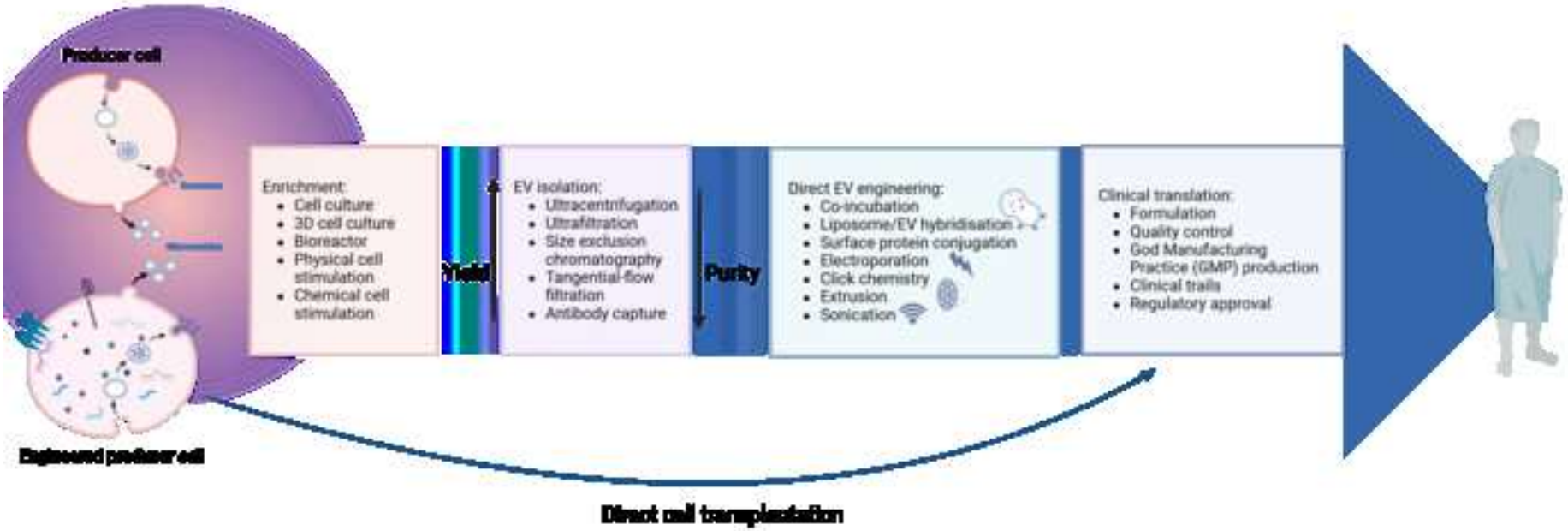

CHIMERIC ANTIGEN RECEPTOR (CAR) T-CELL THERAPY: HARNESSING EXTRACELLULAR VESICLES FOR ENHANCED EFFICACY

Beatrice Spokeviciute[1], Sharad Kholia[1], and Maria Felice Brizzi[1]*

Conflict of interest

The other authors have no conflicts of interest to declare.

CHIMERIC ANTIGEN RECEPTOR (CAR) T-CELL THERAPY: HARNESSING EXTRACELLULAR VESICLES FOR ENHANCED EFFICACY

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Running title: EVs as a potential tool to improve (CAR) T-Cell therapeutic efficacy

Key words: Extracellular vesicles, (CAR) T-CELL THERAPY, cancer, immune modulation, tumour microenvironment

Abstract

A cutting-edge approach in cell-based immunotherapy for combating resistant cancer involves genetically engineered chimeric antigen receptor T (CAR-T) lymphocytes. In recent years, these therapies have demonstrated effectiveness, leading to their commercialization and clinical application against certain types of cancer. However, CAR-T therapy faces limitations, such as the immunosuppressive tumour microenvironment (TME) that can render CAR-T cells ineffective, and the adverse side effects of the therapy, including cytokine release syndrome (CRS).

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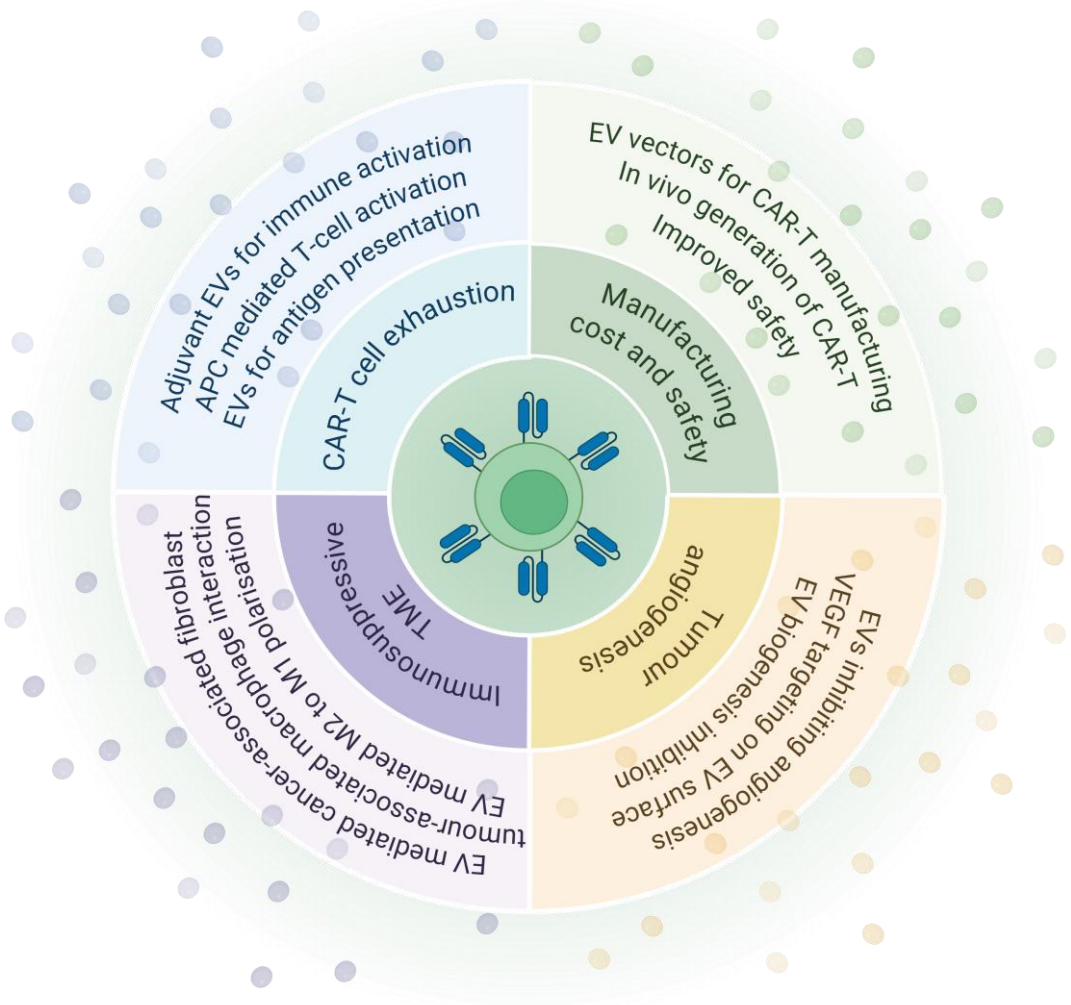

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Introduction

Cancer immunotherapy has revolutionised the fight against malignancies by leveraging the immune system to identify and destroy cancer cells. One of the most promising developments in this field is chimeric antigen receptor (CAR-T) cell therapy, which involves genetically engineering a patient's T-cells to express specific receptors that target and eliminate cancer cells. Despite its successes, CAR-T cell therapy faces significant challenges, including side effects, cost and accessibility, as well as the immunosuppressive tumour microenvironment (TME). Simultaneously, extracellular vesicles (EVs) have emerged as critical players in intercellular communication, carrying proteins, lipids, and nucleic acids that influence various cellular processes. EVs from both tumour cells and immune cells can modulate immune responses and the TME. Integrating the functionalities of EVs into CAR-T therapy offers a novel approach to enhance therapeutic efficacy and address current limitations. This review explores the interplay between CAR-Ts and EVs, aiming to highlight recent advancements and their potential to improve cancer treatment outcomes.

1. Background on immunotherapy and CAR-T cell therapy

Cancer immunotherapy has become a revolutionary approach in the fight against malignancies, harnessing the power of patients' own immune system to identify and destroy cancer cells. Having its origins dating back to the middle of the nineteenth century, when Wilhelm Busch first documented tumour mass reduction in patients upon intentional infection with Streptococcus pyogenes, and subsequent development of a superficial skin infection known as erysipelas [1]. This idea that the immune system could recognize and eliminate cancer cells gained more evidence with the extensive work of Dr. William Coley, who, in the late 1800s, observed tumour regression in patients following bacterial infections [2]. However, a lack of functional knowledge on immunity, autoimmunity, and adaptive immune response impeded the further development of immunotherapeutic treatments. Furthermore, the new discovery of radiotherapy at the beginning of the 20th century, and chemotherapy a few decades later put the development of cancer immunotherapy on the back burner [3]. Fortunately, discoveries in molecular biology and immunology, like the identification of interferons, T-cell receptors, and the development of monoclonal antibodies in the late 20th century, reignited interest in cancer immunotherapy, now with a more clear and targeted approach [2]. The 21st century has seen great breakthroughs in the field, with the discovery and approval of immune checkpoint inhibitors such as ipilimumab (CTLA-4 inhibitor) and embrolizumab (PD-1 inhibitor), which demonstrated remarkable efficacy across a set of malignancies. Immune checkpoint inhibitors aim to block inhibitory signals of cancer cells and enable the patients' own immune system to detect and eradicate them [4]. Further developments in immunology and genetic engineering allowed for a new type of immunotherapy to emerge called CAR-T cell therapy.

1.1 Brief history and development of CAR-T cell therapy

CAR-T is the patient's own T-cell, genetically engineered to express on its surface, highly specific chimeric antigen receptors that enable precise targeting and elimination of cancer cells. The essential components of CAR-T cells include an extracellular antigen recognition domain derived from an antibody's single-chain fragment variant (scFv), a transmembrane domain, and an intracellular signalling domain [5].

Dr. Yoshikazu Kurosawa and his team in Japan, were the first to construct chimeric receptors containing immunoglobulin derived variable, and T-cell receptor derived constant regions. Dr. Kurosawa was able to demonstrate that engineered T-cells were activated by increased Ca2+

signalling when exposed to bacteria expressing the target antigen versus bacteria not expressing it [6]. Not long after, in 1992, Dr. Zelig Eshhar and colleagues developed what is now known as first generation CAR-T cells, by creating a single-chain variable fragment (scFv) from a monoclonal antibody fused with a lymphocyte intracellular signalling domain sourced from CD3ζ [7]. First *in vivo* experiments demonstrated the effectiveness of these CAR-T cells in mouse models of human ovarian cancer [8], [9]. However, when this new therapy reached clinical trials, the results were less promising. CAR-T cells were generated utilising the scFv antibody-type receptor, which recognizes an epitope on carboxy-anhydrase-IX (CAIX), which is frequently overexpressed on clear cell renal cell carcinoma (RCC) [10]. These were administered to patients intravenously in two treatment cycles, totalling five treatments in nineteen days [11]. Unfortunately, all three patients involved in the study experienced significant liver toxicity and disease progression. Important observations were made nonetheless: CAR-T cells were detectable in the circulation for 32 to 53 days, depending on the detection method liver damage was due to on-target, off-tumour toxicity, which was proved by biopsy showing CAIX expression on the bile duct epithelial cells and T-cell infiltration in the surrounding area [11].

During this time intracellular T-cell signalling domain of CD3ζ as well as costimulatory domains CD28 or 4-1BB were added to the construct, which allowed the engineered T-cells to persist and expand after administration [12].

All these discoveries and improvements lead to first FDA approval of Tisagenlecleucel (Kymriah) in 2017 targeting CD19 positive B-cells for the treatment of relapsed or refractory B-cell acute lymphoblastic leukaemia [13]. Due to this success CAR-T cell therapy is approved as the standard of care for some forms of relapsed or refractory non-Hodgkin lymphoma, multiple myeloma, and adult and paediatric relapsed B-cell acute lymphoblastic leukaemia. However, ongoing clinical trials are being undertaken to investigate its potential in treating multiple myeloma, small cell neuroendocrine prostate cancer and small cell lung cancer, there is promising research suggesting the use of CAR-T as an antifibrotic therapy [14].

1.2 Side effects of CAR-T therapy

As successful and promising as CAR-T therapy seems to be, it does not come without its challenges. The most common and potentially severe side effect is cytokine release syndrome (CRS), caused by the increased release of interleukin-1 (IL-1) and interleukin-6 (IL-6), which in turn can result in nausea, fever, hypotension, encephalopathy and tachycardia and can progress to life-threatening complications [15].

Immune effector cell-associated neurotoxicity syndrome (ICANS) is another significant side effect, resulting in headache, confusion, seizure, encephalopathy, or even death. Both CRS and ICANS can be managed with the administration of the IL-6 receptor antagonist, tocilizumab, and the IL-1 receptor antagonist, anakinra [16]. Up to 95% of patients undergoing CAR-T treatment experienced some level of CRS, and up to 20% showed symptoms of neurotoxicity. The intensity of these side effects and steroid treatments to mediate them are also among the risk factors for severe bacterial and viral infections following CAR-T treatment [17]. Other side effects include low blood cell counts causing anaemia, bleeding, and tumour lysis syndrome, which results from the rapid destruction of cancer cells, leading to electrolyte imbalances and kidney damage. Moreover, since up to 27% of patients require admission to an intensive care unit to manage side effects, with mortality reaching 8%, it is important to work towards improving CAR-T therapy to both boost its efficacy and reduce significant adverse effects [18]. New approaches are needed to address this, and EVs might provide novel solutions.

1.3 Extracellular Vesicles (EVs)

EVs are a diverse group of membrane-bound particles that are secreted by all eukaryotic cells into the extracellular environment. They play crucial roles in intercellular communication, immune modulation, and various physiological processes [19].

Two main types of EVs - exosomes and ectosomes are classified based on their origin from different cellular compartments. Exosomes, which are small (approximately 50–150 nm in diameter), are formed when the endosomal membrane inwardly buds to create intraluminal vesicles and are released as EVs when the endosome merges with the plasma membrane. Ectosomes originate from outward protrusions of the plasma membrane, which are released into the extracellular space. Ectosomes vary in size, ranging from less than 100 nanometres to several micrometres in diameter, and include microvesicles (typically 0.2–1 μm in diameter) and large oncosomes (>1 μm) [20]. Additionally, apoptotic bodies, formed when cells fragment during cell death, are also considered EVs. Migrasomes are still under characterization, but they appear as a unique EV subtype resulting from retraction fibres during cell migration. In fact, their biogenesis mainly depends on the migration process, which involves cells, like immune and metastatic cells, displaying a mighty migration capability [22]. EVs can be further sub-categorised by their cellular origins, cargo and biological functions, like oncosomes, large oncosomes, melanosomes, immune cell-derived EVs, etc. [23], [24], [25]. Tumour-derived EVs, including oncosomes and large oncosomes, might be particularly relevant for diagnostic, prognostic, and therapeutic purposes since their cargo is uniquely enriched in oncomiRs such as miR-520g, β-catenin, heat-shock proteins, oncoproteins, and circulating tumour DNA [26], [27], [28]. It is therefore tempting to speculate that improving EV isolation, imaging, and characterisation can lead to even more complex classification and nomenclature of EVs [26], [27], [28]. Lipid nanoparticles (LNPs) and liposomes are often researched alongside EVs for therapeutic applications. Liposomes are artificially prepared spherical vesicles composed of cholesterol and phospholipid bilayers that can encapsulate aqueous solutions. LNPs are tiny particles made of lipids that can encapsulate therapeutic agents, such as RNA or small molecule drugs, and are used as drug delivery systems to protect the cargo and facilitate its entry into target cells [29].

The EV cargo is diverse and includes proteins, lipids, and various nucleic acids, such as mRNA, microRNA (miRNA), and other non-coding RNAs (ncRNAs). The composition of EV cargo reflects the cellular origin and the physiological state of the parental cell [30], [31]. Proteomics analysis of EVs has revealed the presence of various proteins involved in cell signalling, membrane trafficking, and immune regulation [31], [32]. Similarly, EVs contain a wide range of nucleic acids, including mRNA, miRNA, long non-coding RNA (lncRNA), and DNA fragments. These nucleic acids can be transferred to recipient cells, where they regulate gene expression and cellular functions [21]. EVs deliver cargo to recipient cells through several potential pathways that impact gene expression and nuclear access. These include endocytosis, fusion, or macropinocytosis, a type of endocytosis where a large volume of extracellular fluid and its contents are engulfed by the cell through the formation of large ruffles and cups of the plasma membrane [33], [34]. Their contents, including proteins, RNAs, and miRNAs, are then released into the cytoplasm, where they interact with intracellular signalling pathways or are directly transported to the nucleus. Nuclear localization signals on EV-associated proteins can facilitate the transport of EV cargoes into the nucleus through nuclear pore complexes [35]. Additionally, some EVs may merge with endosomal membranes, releasing their cargo directly into the cytoplasm, where they can influence gene expression by modulating transcription factors or interacting with DNA and RNA polymerases [21]. This nuclear delivery mechanism allows EVs to regulate gene expression by altering

transcriptional activity, epigenetic modifications, and RNA processing, significantly impacting cellular functions and contributing to tumour progression and metastasis [33].

EVs, therefore, can influence various cellular processes, including proliferation, differentiation, and apoptosis [36], [37], [38]. EVs derived from APCs, such as dendritic cells (DCs), can modulate the function of other immune cells by delivering antigenic peptides and immunomodulatory molecules [25]. EVs released from tumour cells can also modulate the anti-tumour immune response by promoting immune tolerance and inhibiting the cytotoxic action of T-cells and NK cells [39].

Understanding the biogenesis, cargo, and physiological functions of EVs may provide new insights into several pathologies, offering potential solutions. There is growing interest in EVs and their roles in intercellular communication and immune modulation. Integrating the functionality of EVs into CAR-T cell therapies could address and help modulate the limitations currently envisioned, and possibly enhance their therapeutic efficacy. This would be particularly relevant since CAR-T cell clinical application faces challenges such as CRS, neurotoxicity, the immunosuppressive TME, and the high manufacturing costs associated with this adoptive cell therapy. With this review, we aim to highlight the newest developments in EV research and their relevance in improving CAR-T therapy, hopefully leading to improved patient outcomes and providing new approaches for cancer treatments.

2. EVs as Vectors for CAR Expression: EVs for targeted delivery of CAR constructs to T-cells

Although CAR-T cell therapy has shown notable benefits such as fast reaction time and a curative potential ranging from 40% up to 70% of patients undergoing treatment, it does have its drawbacks [40]. Currently, all Food and Drug Administration (FDA) approved CAR-T treatments are manufactured using lentiviral or gamma-retroviral vector transduction [41]. One of the biggest factors limiting the accessibility of CAR-T therapy is its price, with a single CAR-T treatment costing from 400,000 to 500,000 USD in 2023 [42]. The cost of treatment is mainly incurred due to the need for specialised, accredited healthcare facilities, personnel, and equipment to produce large quantities of lentiviruses and quality control procedures [43].

The development of non-viral vectors could substantially reduce the manufacturing expenses of CAR-T by simplifying the scaling up of production schedules for gene therapy vectors. We have seen successful use of non-viral vectors in the form of LNPs utilised in the BioNTech/Pfizer COVID vaccine [44]. LNPs have been shown to be highly efficient at crossing cell membranes, and relatively easy and cheap to produce. Unfortunately, due to their synthetic composition, especially polyethylene glycol (PEG) containing lipids, some adverse effects of LNPs, like anaphylaxis, autoimmune disease, and compaction activation-related pseudo-allergy reactions have been observed [45]. EVs can act as a safer alternative to LNPs. EVs have been proposed as vectors or as vector coatings for increased internalisation and immune evasion [46]. EVs have a neutral advantage over LNPs as they are relatively inert, non-immunogenic, and biocompatible. Their ability to interact with immune cells makes them a great candidate for the development of new vector designs [47]. By using adeno-associated viruses (AAVs), it has been demonstrated that transfected HEK293T cells produce microvesicles containing viral vector capsids. These microvesicles had been shown to transfer genes *in vitro*, at a higher efficiency than AAVs isolated by a conventional method using the same genome copy number dose [48]. More efficient gene delivery *in vivo* was achieved using microvesicle-enveloped AAV when compared to free AAV capsids and successfully transduced genes in liver, retinal, and brain tissues [49], [50], [51].

Although AAV microvesicles do not address the issue of the cost and time associated with CAR-T manufacturin and their small gene packing capacity and transient expression are not optimal for CAR-T production, they do significantly improve the transfection efficiency and introduce the rationale for successful EV-mediated gene transfection *in vivo.*

The observation that EVs can be successfully applied to introduce various gene engineering proteins such as Cre recombinase (Cre) and CRISPR-associated protein 9 (Cas9), both *in vitro* and *in vivo*, further supports their importance in non-viral vector research [52], [53], [54], [55]. Specifically, Zhang et al. [56] utilised a two-in-one approach to create targeted CAR-T cells via CRISPR–Cas9 electroporation. It was demonstrated that inserting an anti-CD19 CAR, cassette into the programmed cell death protein 1 (PD1) gene locus enhances tumour cell eradication capabilities and a complete remission in five out of eight patients in the phase I clinical trial. Following this success, the clinical trial was expanded to include twenty-one patients, nine of whom stayed in full remission after a nineteen-months follow-up [57]. However, electroporation is not a sustainable option for gene transfection *in vivo,* and nanoparticles such as EVs can help address this issue.

Considerable research is being conducted on CAR-T cell therapy, with an increasing focus on nanomaterials to enhance efficacy and safety. Functionalised LNPs, modified with a CD3 antibody on their surface and loaded with a plasmid containing IL-6 short hairpin RNA and CD19-CAR, have been successfully used to generate CAR-T cells with IL-6 knockdown *in vivo* [58]. Mice treated with AntiCD3-LNP/CAR19+ shIL6 demonstrated sustained T-cell transfection and CAR-T production, leading to prolonged survival and significant tumour reduction. Furthermore, IL-6 inhibition contributes to CRS reduction, enhancing the safety of CAR-T therapy and facilitating its clinical application [58]. EVs, generated by engineering a lymphoblast cell line to express HLA-A2 and CD80 activated CD8+ T-cells and adding PD-L1, produced an immunoregulatory response in an *in vitro* model of type 1 diabetes [59]. This study, demonstrating EV-mediated immune modulation, has offered insights into designing EVs for tailored immunotherapy. Various methods are being used for EV engineering, either by acting directly on the EVs or their parental cells, to improve their loading, targeting, and circulation properties.

Direct EV engineering can include exogenous loading of nucleic acids, proteins, and smell molecules by co-incubation or by more physical methods like sonication, electroporation, or freeze-thaw cycling [60], [61], [62]. Liposomes, which are artificial vesicles used in drug delivery, differ from EVs in that they are synthetically composed of lipid bilayers able to encapsulate both hydrophilic and hydrophobic substances. EVs and liposomes have been demonstrated to form hybrid nanoparticles containing both liposome cargo and EV surface proteins and successfully deliver the CRISPR-Cas9 system as well as small interfering RNA (siRNA) to target cells [63], [64]. Hybrids of liposomes and EVs are produced either by lipid-film hydration followed by extrusion or by simple co-incubation to create a semisynthetic liposome/EV mix. The inclusion of EV membrane components results in functional differences. Indeed, hybrids are less toxic than liposomes, and gene-silencing effects are maintained. Additionally, intrinsic functionalities of EVs, like activating endothelial signalling pathways and stimulating endothelial cell migration, are preserved in hybrids. Therefore, hybrid nanoparticles can combine the benefits of both liposomes and EVs, offering an optimal non-viral vehicle for therapeutic cargo delivery.

LNPs and charged polymer nanoparticles used for cell transfection can develop a context dependent protein corona, including proteins like apolipoprotein E (ApoE), on their surface [65]. This feature is also shared by EVs and can subsequently alter the final density, diameter, stability, and interaction with target cells [66], [67].

Ivanova et al. [68] have demonstrated a series of techniques to engineer EVs for both their targeted delivery and protein cargo loading. EVs were isolated from 293F cells transfected with constructs designed for specific target monoclonal antibodies fused with CD81 tetraspanin on the EV surface. The antibody binding domains retained their IgG-binding capacity during the western blot procedure, reflecting high intrinsic stability against denaturation [68]. Protein loading of genetically engineered EVs involves a specific design where the tetraspanin CD63 is fused with a DnaB mini-intein cassette and Cre. The mini-intein cassette has a splicing domain modified to catalyse cleavage at its termini, releasing Cre from the CD63-Cre fusion protein, allowing it to be recruited into the EV lumen during biogenesis [68].

Chemical modifications like lipid modification, PEGylation, or click chemistry of EVs also allow for altering their surface properties, stability, and targeting [69].

However, modification of parental cells can produce ready to use EVs with desired cargo and surface modifications. This exact method was successfully used to produce virus-mimetic nanovesicles that can fuse with T-cells and deliver CAR constructs to them [70]. Additionally, plasmids can be used to transfect cells to secrete EVs containing target genes. Kanuma et al. [71] engineered Ovalbumin (OVA)-Ag fused with a CD63-expressing plasmid, enhancing the immunogenicity of DNA vaccines. Mice vaccinated with these engineered EVs exhibited effective Ag-specific T-cell responses.

Zhao et al. [70] furthered this approach by obtaining fusogenic EVs from RAW264.7 producer cells by initially transfecting them with a lentiviral vector containing sequences for T-cell viral fusogens p14 FAST protein (p14TF) and mutated hemagglutinin protein (MVTF). These viral proteins facilitate cell membrane fusion and cell entry by recognising CD3 on the surface of T-cells, followed by direct membrane incorporation on target cells. Cells successfully expressing fusogenic proteins were next transfected with a lentiviral vector of anti-CD19 CAR protein, resulting in cells expressing T -cell fusogens and anti-CD19 CAR protein. EVs were next isolated and infused intravenously in mice, where they were successful at reducing the growth of A20 B-cell lymphoma tumours. Furthermore, these EVs did not induce CRS in mice compared to classically manufactured anti-CD19 CAR-Ts, and repeat injections did not produce neutralising antibodies for the viral fusogenic proteins [70]. Predictably, this anti-CD19 expression on T-cells is transient, thereby preventing the development of CRS, while requiring repeat infusions. Nevertheless, this is a great example of how EVs can be used as non-viral vectors for CAR-T generation *in vivo* and provide the scientific rationale to develop an off-the-shelf therapy or a bridging approach before the next stage of treatment.

Despite significant advancements, producing EVs with adequate yield and minimal preparation remains difficult. To address these issues, Kojima et al. [72] developed the EXOsomal Transfer into Cells (EXOtic) devices. HEK-293T cells were transfected with a series of plasmids, resulting in EVs containing the RNA packaging device (CD63-L7Ae selectively binding to the C/D box RNA structure), targeting module (RVG-Lamp2b to target neuronal acetylcholine receptor subunit alpha-7 CHRNA7), cytosolic delivery helper (Cx43, an active mutant of gap junction protein Connexin 43, allowing for cellular intercommunication and transfer of materials), and catalase mRNA containing nluc-C/Dbox, allowing for its packaging by the CD63-L7Ae construct. This approach employs engineered EV-producing cells to overexpress a STEAP3-SDC4-NadB booster, resulting in an approximately 15-fold increase in EV yield. These producer cells were then transplanted in vivo, where they produced EVs containing catalase miRNA. These EVs were able to attenuate neuroinflammation in a mouse model of Parkinson disease. A similar approach could

be exploited to generate large quantities of highly customised EVs displaying cell specific targeting and tailored cargo to generate CAR-T cells in vivo.

However, the presence of viruses in EV preparations poses significant risks for clinical applications, necessitating thorough discussion and resolution. Viral vectors have several significant disadvantages, including high immunogenicity, limited insert size, the potential for insertional mutagenesis, and high production costs [73]. Moreover, controlling the precise insertion site of the gene is challenging. If the transgene integrates near a cancer-promoting gene and activates it, or if it deactivates a tumour-suppressing gene, the risk of inducing T-cell cancer increases [74]. As of December 31, 2023, 22 cases of T-cell cancer have been detected in patients treated with CAR-T cells [75]. Genetic sequencing in three cases has identified the CAR transgene within malignant clones, suggesting that CAR-T therapy might have contributed to the development of T-cell cancers. With over 27,000 doses of six approved CAR-T products administered in the United States, the incidence of T-cell cancers among recipients remains very low, even assuming that all reported cases are treatment-related. This demonstrates that, while the risk exists, it is relatively rare. In EV-based CAR-T transfection, similar risks could arise if the EVs inadvertently transfer oncogenic material or their cargo off-target integration. However, the mechanisms and rates might differ due to the distinct nature of EVs compared to viral vectors.

Additionally, using virally transfected cells for EV isolation in clinical applications presents several other risks. A major concern is the potential contamination of EV preparations with residual viral particles, which poses significant aforementioned safety risks [76]. Additionally, viral transfection can alter the biological properties of EVs, potentially affecting their therapeutic efficacy and safety profile [77]. Regulatory challenges also arise, since ensuring the complete removal of viral contaminants and proving the purity and safety of EVs can be difficult. Ensuring virus-free EV preparations is challenging due to the similarity in size and physical properties between viruses and EVs, making conventional purification methods less effective. Advanced purification techniques, such as high-resolution density gradients and immunoaffinity-based methods, must be developed and rigorously validated to minimise viral contamination. Regulatory guidelines and robust quality control measures should be established to ensure the safety and efficacy of EV-based therapies. Addressing these concerns is critical to advancing the clinical use of EVs and maximising their therapeutic potential while minimising associated risks. Nonetheless, the use of EVs as vectors remains a promising tool to replace conventional viral vectors due to their low immunogenicity, deep tissue penetration, natural ability to cross the blood brain barrier, cargo protection, and high engineering potential.

3. EVs as Mediators of Immune Response

EVs have been implicated in playing a role in various biological functions, including immunoregulation. All major immune cells, together with non-immune cells, release EVs enriched with numerous molecules that are involved in immunoregulation **(Fig. 1)** [78], [79].

For example, EVs released by APCs such as B-cells, macrophages, and DCs display the extracellular domain of major histocompatibility complex (MHC) class I and class II molecules on their surface, which can directly stimulate CD8 and CD4 T lymphocytes, respectively [80], [81], [82]. Furthermore, when administered at high concentrations or when the number of MHC complexes per EV is increased by direct co-loading with a peptide, these EVs can function effectively as antigen-presenting vesicles, significantly enhancing their T-cell stimulatory capabilities **(Fig. 2)** [83], [84]. Apart from direct stimulation, EVs can also influence T-cell activation

indirectly by transferring antigenic peptides to APCs, which then present these peptides to T-cells [85], [86]. For instance, intestinal epithelial cell-derived EVs rich in HLA-DR4 molecules loaded with human serum albumin peptide activate specific T-cell hybrids only in the presence of HLA-DR4 positive DCs. This suggests that EVs transfer the peptide from their HLA-DR4 molecules to the HLA-DR4 molecules on the DCs [85].

EVs can also transfer naive antigens to APCs. Tumour-derived EVs containing naive tumour antigens are efficiently taken up by DCs, which process and cross-present these antigens to tumour-specific cytotoxic T-cells (CTLs) [87], [88]. For instance, vaccinating mice with tumour-derived EVs has been shown to induce a strong CD8+ T-cell-mediated antitumour response against both the autologous tumour and other related tumours expressing the same tumour-rejection antigens [87]. Hence, this ability of EVs, either from tumour cells or immune cells, to efficiently deliver antigens to DCs for CTL cross-priming could potentially be applied to CAR-T cells to further enhance their anticancer abilities.

Apart from antigens, EVs can also transfer signals to immune cells that promote their activation. For instance, EVs derived from mast cells are highly enriched with heat shock proteins (Hsp60, and Hsp70) that promote the maturation of DCs in mice [89]. Whereas macrophages infected by various microbes release EVs enriched with microbial antigens as well as pathogen associated molecular patterns that elicit an inflammatory response by macrophages [90]. Other molecules carried by EVs include cytokines such as IL-1β, Fas-ligand (FasL), TNF-related apoptosis-inducing ligand (TRAIL), CD154, among others, that can activate cells of the immune system such as T, DC, and NK cells [91]. This intrinsic adjuvant effect of EVs could be exploited to further enhance the priming of CAR-T cells in the tumour environment.

EVs have also exhibited immunosuppressive properties. The classical example is tumour-derived EVs, which have been reported to suppress tumour-specific and non-specific immune responses. They are enriched with molecules such as FasL, galectin-9, TRAIL, PDL1 among others which tend to induce apoptosis in T-cells and NK cells through the Fas-FasL and PD1-PDL1 pathways [92], [93], [94], [95]. In addition, they can also suppress the natural killer group 2D (NKG2D)-dependent cytotoxicity of NK cells and CD8 T-cells, and influence the maturation of APCs such as DCs by inducing the production of IL-6, [96], [97]. Tumour derived EV cargo such as transforming growth factor-beta (TGF-β), prostaglandin E2, Hsp70, and miRNAs have been shown to drive monocyte differentiation into myeloid-derived suppressor cells (MDSCs), which in turn have the ability to suppress the anti-tumour immune response by supporting the propagation and function of regulatory T-cells (T-regs) [98]. Moreover, tumour derived EVs can also directly influence T-regs by enhancing their function and blocking the maturation of DCs and macrophages, mainly through a TGF-β1-dependent mechanism [97], [99].

As tumour-derived EVs are enriched with both tumour antigens and immunosuppressive mediators, it is not surprising that they have the ability to suppress tumour Ag-specific immune responses through modulation of both DC and macrophage function. For instance, in an OVA tumour antigen model, EVs derived from an OVA-expressing melanoma, enriched with OVA protein, efficiently suppressed an OVA-specific immune response [100]. In addition, in tumour-bearing mice, CD11b-positive blood derived EVs suppressed tumour Ag-specific responses through a MHC Class-II-dependent mechanism [101]. These observations suggest that tumour-derived EVs probably regulate the function of CD11b-positive APCs in the TME, which in turn release immunosuppressive MHC Class-II, CD11b-positive EVs into the circulation. The mechanism of action by which these endogenous EVs suppress tumour antigen-specific responses is yet to be clarified, but their role in tumour immune evasion is very crucial. Apart from

antigens and molecules, ncRNAs enriched within tumour-derived EVs can also contribute to their immunosuppressive properties. For instance, miR-145 enriched within tumour-derived EVs can influence the polarisation of macrophages towards an M2-type phenotype by downregulating the expression of histone deacetylase 11 [102].

Tumour-derived EVs also play a part in assisting tumour invasion and metastasis. This is mainly through the establishment of pre-metastatic niches by generating suitable microenvironments in distant metastatic sites [103], [104], [105]. For instance, melanoma-derived EVs administered in peripheral tissues preferentially target sentinel lymph nodes, preparing them to become remote niches for metastatic tumour growth [106]. Taken together, tumour-derived EVs can be said to be involved at multiple levels of tumour pathogenesis, from evading anti-tumour specific immune responses to aiding metastatic niche development [107], [108].

CAR-T-derived EVs have also been identified to play an anti-tumorigenic role similar to the cells they are derived from. They pose many potential advantages over the CAR-T cells they are derived from. For instance, CAR-T cells can trigger CRS following administration, which is recognised as one of the most prevalent and recurrent complications associated with CAR-T therapy. Conversely, the stable nature of CAR-T EVs, coupled with their inability to proliferate and limited lifespan, renders them a low-risk option for immunotherapy, with minimal collateral toxicity such as low incidence of CRS [109]. In line with this lower toxicity potential, CAR-T EVs also have a low immunogenicity and can easily cross tumour barriers, therefore enabling them to penetrate solid tumours where CAR-T cell therapy faces challenges due to restricted tumour infiltration [25].

T-cell-derived EVs are enriched with proapoptotic molecules such as Apo2L, granzymes A and B, perforin, and FasL, endowing them with cytotoxic capabilities and antigen specificity. When combined with CAR, as in the case of CAR-T-derived EVs, they become highly effective vectors for delivering pro-apoptotic signals to tumour cells [25].

Taking advantage of the fact that EVs carry the cargo of their parental cells, CAR-T cells could be engineered to release more EVs enriched with CAR [109]. In addition, the cells could also be engineered to express endogenous anti-tumourigenic molecules, which could be packaged in EVs together with the CAR to obtain more efficient CAR-T EVs. For instance, in a recent study, CAR-T cells were genetically modified to increase the expression of the endogenous non-coding RNA RN7SL1. This RNA activates signalling pathways through the pattern recognition receptors (PRRs), retinoic acid-inducible gene 1 (RIG-1), and melanoma differentiation-associated protein 5 (MDA5). The CAR-T EVs derived from these cells were notably enriched in RN7SL1, and upon uptake by innate immune cells within the TME, these EVs led to reduced MDSC development, decreased myeloid production of immunosuppressive TGFβ, and enhanced co-stimulation by DC subsets [110]. In another study, CAR-T EVs with EGFR and HER-2 specific CAR efficiently and specifically killed HER2+ and EGFR+ tumour cells in mouse xenograft models without impacting cells not expressing those molecules [109]. Whereas EVs derived from mesothelin-targeted CAR-T cells could target mesothelin-positive and triple negative breast cancer cells through the secretion of granzyme B and perforin [111]. These studies therefore prove that, in combination with CAR modification, T-cells could be engineered with other types of molecules (ncRNAs, cytokines, glycoproteins) to produce EVs that could act synergistically with the CAR-T cells as anticancer therapeutics. In addition, CAR-T EVs lack the expression of PD1, and their anti-tumour efficacy remains unaffected by recombinant PD-L1 treatment, therefore making them ideal as a potential complement to CAR-T cell therapy [109].

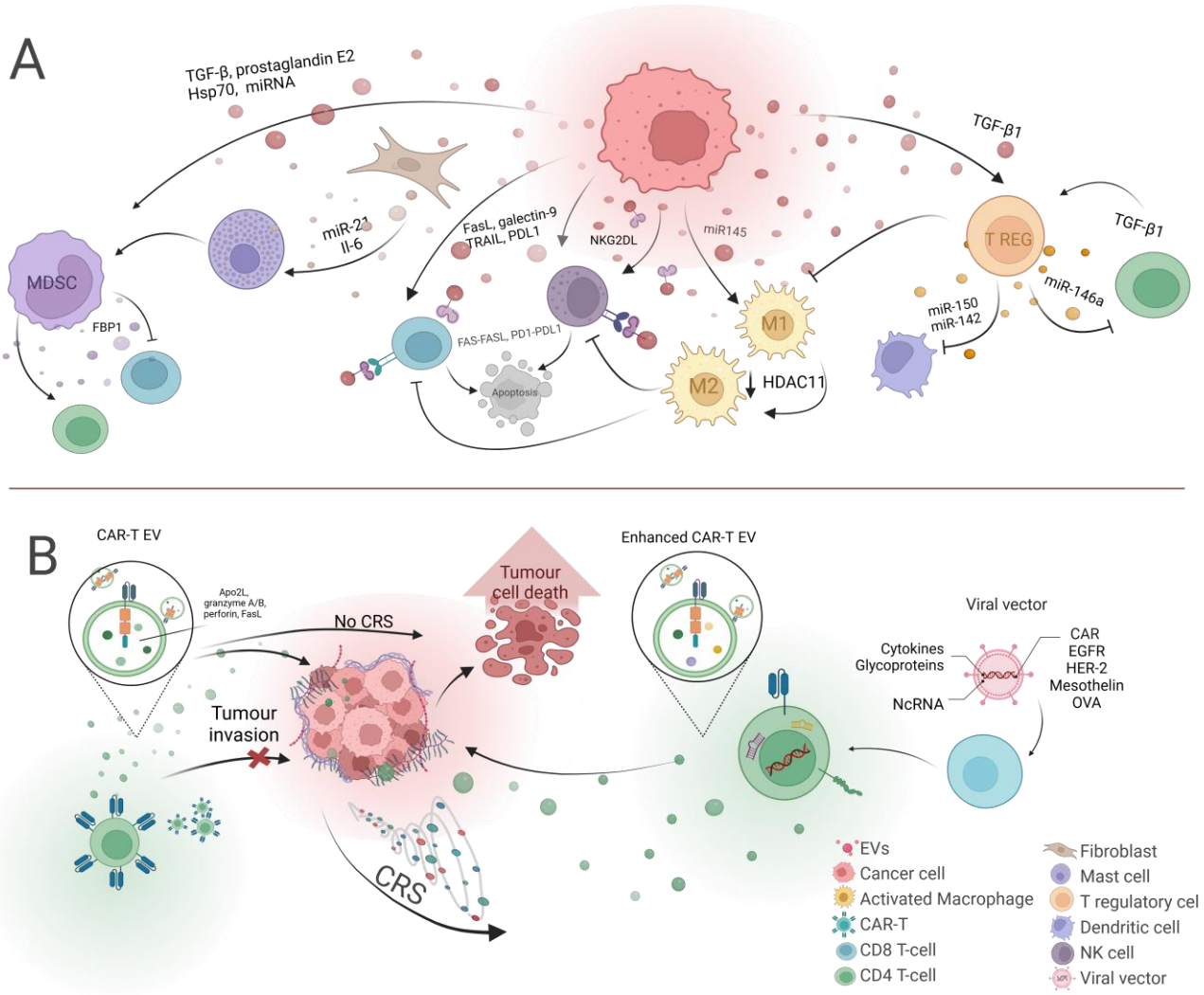

Fig. 1 EV mediated immune interactions. *Tumour EVs can suppress immune responses by delivering immunosuppressive proteins, growth factors, and miRNAs to target cells, including MDSCs and T-reg cells (**A**). CAR-T cell-derived EVs, enriched with cytotoxic molecules, show potential for reducing the risks associated with CAR-T therapy, such as CRS, while effectively infiltrating and killing tumour cells. CAR-T cells could also be further engineered to express other anti-cancerogenic molecules, including RNAs, cytokines and glycoproteins, which could further enhance their anti-tumour function (**B**). CAR-T cell-derived EVs, enriched with cytotoxic molecules, show potential for reducing the risks associated with CAR-T therapy, such as CRS, while effectively infiltrating and killing tumour cells. Created with BioRender.com*

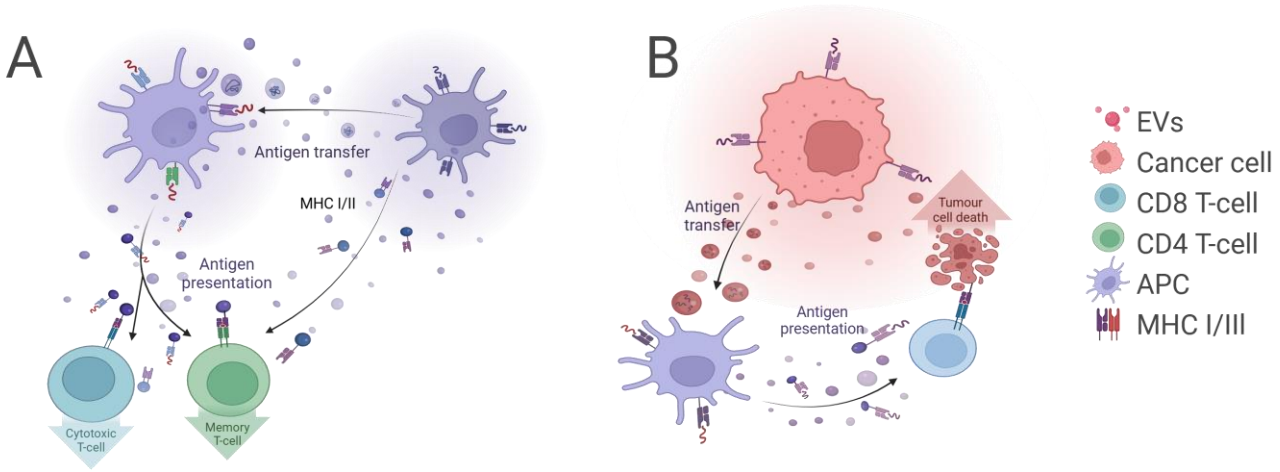

Fig. 2 EV-mediated immune activation. *EVs from antigen-presenting cells (APCs) like B-cells, macrophages, and DCs can directly stimulate T-cells by secreting EVs containing MHC class I and II molecules. These EVs can also transfer antigens to surrounding APCs, which then present them to T-cells, enhancing T-cell activation (**A**). Tumour-derived EVs can deliver naive antigens to DCs, which then present these antigens to cytotoxic T-cells, facilitating an anti-tumour response (**B**). Created with BioRender.com*

3.1 Overcoming tumour immunosuppressive factors in the TME through EV-mediated modulation

CAR-T therapy, originally groundbreaking in the treatment of haematologic malignancies, is increasingly being explored for its potential in targeting solid tumours. Unfortunately, the solid TME is highly immunosuppressive, creating both physical and chemical barriers that block CAR-T cells from effectively infiltrating and attacking the cancer cells [112]. Once cancer cells initiate uncontrolled proliferation, their secreted EVs start affecting and modifying surrounding cells, including immune cells [113]. T-cell exhaustion and the lack of tumour specific antigen targets also add complexity to the development of CAR-Ts for solid tumour treatment [114], [115]. The TME is characterised by a complex network of interactions between tumour cells, stromal cells, and immune cells, modulated by soluble factors and EV-mediated communication **(Fig. 3)** [116]. Cancer-associated fibroblasts (CAFs) and tumour-associated macrophages (TAMs) are the primary cell types found in the stroma of solid tumours and are known to promote tumour growth.

Tumour-promoting M2-like TAMs are induced by interleukin-10 (IL-10) and TGF-β, promoting the expression of IL-10, TGF-β, CCL, and surface proteins like CD204, CD206, and CD163. These macrophages, involved in immunosuppression, EMT, angiogenesis, and therapy resistance, differentiate into four subgroups (M2a, M2b, M2c and M2d) through different stimuli such as interleukins or glucocorticoids [117]. Each of these subgroups is specialised in its function in tissue remodelling, immunoregulation, phagocytosis, and tumour progression. EVs play an important role in tumour-induced macrophage polarisation. Tumour EVs can induce secretion of TNF-α, IL-10, IL-6 and increase mRNA expression of arginase-1 (Arg-1) and VEGF [118]. The M2d subgroup of macrophages is the main component of TME and is activated by Toll-like receptor (TLR) agonists and IL-6 signals [119]. M2d TAMs are highly responsible for tumour progression via their pro-angiogenic role inferred by their secretion of growth factors like vascular endothelial growth factor (VEGF) as well as IL-10 [120]. Additionally, M2 derived EVs induce CD8+ T-cell exhaustion via

miR-21-5p, targeting deubiquitinase YOD1, leading to yes-associated printerleukinotein 1 (YAP1)/β-catenin pathway activation [121].

Addressing these EV-mediated factors might increase the efficacy of CAR-T treatments for solid tumours. For instance, inhibition of VEGF signalling could restore normal vascularisation in renal carcinoma, colorectal cancers (CRC), stromal cancers, and breast cancers [122], [123]. Antibody-mediated inhibition of VEGF improved the efficacy of CAR-T cells in an *in vivo* model of glioblastoma (GBM) [124].

Inhibition of colony-stimulating factor 1 receptor (CSF1R) with pexidartinib (PLX3397), a small-molecule drug, has been able to revert TAMs into an antitumorigenic M1 phenotype and increase GBM survival *in vivo* [125]. Pexidartinib has also been shown to promote a shift in TAM polarisation and enhance CAR-T treatment by reducing the number of TAMs through cytokine/chemokine ligand-receptor inhibition [126], [127]. However, sotuletinib, another potent CSF1R inhibitor, has shown no improvement in CAR-T therapy in glioma-bearing mice [128]. Since CSF1R inhibitors can indiscriminately reduce the number of macrophages, there is a high possibility that specific subgroups of macrophages are essential for the CAR-T antitumor response. Liang et al. [129] have demonstrated that it is possible to specifically treat M2 cells with pexidartinib by utilising functionalized LNPs with the targeting ligand tuftsin and legumain protease-cleavable PEG chains. Featuring an "on/off" coating of legumain-sensitive PEG, this nano-system was designed to reduce LNP uptake due to the PEG shielding effect, while specifically enhancing recognition of M2-like TAMs overexpressing legumain. Once PEG is cleaved in response to legumain on M2 TAMs, the targeting ligand tuftsin is exposed and activated to stimulate TAM phagocytosis [129]. Plant-derived EVs have also been shown to alter M2 polarisation both *in vitro* and *in vivo*. A ginseng-derived nanoparticle promoted M2 to M1 polarisation via TLR4 and MyD88 signalling, increased reactive oxygen species production, and suppressed melanoma growth in mice by increasing M1 macrophages in tumour tissues [130]. These findings highlight the potential of M2-targeted therapies in modulating macrophage polarisation in the TME to enhance the efficacy of CAR-T cell treatment in solid tumours. However, in triple-negative breast cancer patients, these interactions seem more complex. Tumour-derived EVs, containing CSF1, influence macrophages with unique immune checkpoint expression and high T-cell chemoattractant secretion. These macrophages exhibit a blend of M1 and M2 features, challenging the traditional M1 vs. M2 dichotomy, and this simplified view does not apply to the complex context of the TME [131].

Nevertheless, CSF1 remains an interesting druggable target as it has been shown to play a role in a stable macrophage-fibroblast cell circuit. Fibroblasts have been found to produce CSF1 to support the growth and survival of macrophages [132]. Macrophages are more sensitive to growth factors in the environment, and fibroblasts are more sensitive to surrounding space and cell density [133]. Density-dependent gene expression in fibroblasts, regulated by the Hippo and TGF-β pathways, activate YAP1 to increase CSF1 expression, which elevates macrophage numbers through mechanical force and environmental sensing mechanisms [133]. While their exact interactions are not yet fully understood, CAFs and TAMs significantly influence disease progression, resistance to therapy, and clinical outcomes, working together synergistically.

Tumour-derived EVs can induce CAFs via the endothelial to mesenchymal transition (EndMT) pathway. *In vitro* 3D microfluidic models demonstrated that melanoma-derived EVs promoted EndMT and increased CAF differentiation from human umbilical vein endothelial cells (HUVECs) [134]. Uterine leiomyosarcoma (ULMS)-derived EVs contain upregulated miR-654-3p and miR-369-3p, which are highly expressed in the sera and tissues of ULMS patients and ULMS cell lines.

ULMS derived EVs were able to upregulate CAF markers, particularly actin alpha 2 (ACTA2), in fibroblasts after EV treatment, suggesting that these miRNAs carried by EVs contribute to the generation of CAFs [135]. In turn, CAFs further contribute to tumour progression, invasiveness, metastases, and therapy resistance through their secreted EVs in the TME. (Table 1)

Table 1. CAF EV cargo supporting tumour progression

Contents	Target	Involved process	Reference
miR-92	Breast cancer cells	LATS2/YAP1/PD-L1 axis induced T-cell depletion	[136]
Polycomb complex protein BMI-1(BMI1), integrin beta1(ITGB1)	Oral squamous cell carcinoma	Increased invasiveness and metastasis	[137]
lncRNA H19	Breast cancer cells	miR-497/ DNMT1 induced cell growth, metastasis, and chemoresistance	[138]
miR-876-3p	Oral squamous cell carcinoma	Enhanced cisplatin resistance *in vitro* via IGFBP3 downregulation	[139]
miR-18a-5p	Cervical cancer cells	Transmembrane protein 170B mediated proliferation, migration, apoptosis inhibition	[140]
miR-500a-3p	Prostate cancer cells	HSF1 increase via F-box and WD repeat domain containing 7 (FBXW7)	[141]
miRNA-92a	Breast cancer cells	EMT by G3BP2/TWIST modulation	[142]
miR-20a-5p	Colorectal cancer cells	EMT transition via PTEN downregulation	[143]
lncRNA NNT-AS1	Pancreatic ductal adenocarcinoma cells	miR-889-3p/HIF-1α regulated cell progression and anaerobic glycolysis	[144]

miRNAs, small non-coding RNAs, also play a role in tumour progression by regulating the expression of tumour-associated mRNAs. Melanoma cell-secreted miRNAs have been implicated in transforming normal fibroblasts (NF) into CAFs by altering CAF-related gene expression. Similarly, melanosomes, melanin-containing EVs, can induce NF to CAF transition through miR-211, which targets the tumour suppressor insulin-like growth factor 2 receptor (IGF2R), leading to increased collagen production and enhanced tumour cell motility. Although blocking miR-211

could reduce melanoma invasion, more research is needed to determine if melanosome miRNAs are absorbed by other cells in the melanoma microenvironment.

Additionally, EVs secreted by CAFs play essential roles in tumour progression. CAF-induced MDSCs directly inhibit T-cell function *in vitro*. However, inhibition of MDSC-EV biogenesis was able to restore normal T-cell proliferation [145]. Recent studies showed that CAF-released EVs, enriched with CD9 and CD63, significantly inhibit melanoma cell proliferation. Patients with CD9-positive CAF-derived EVs had better five-year disease-free survival, suggesting that CD9 in CAF-EVs could be a favourable prognostic marker for melanoma [146]. Developing targeted drugs based on these EVs could benefit clinical melanoma treatment.

Many tumour-derived EVs carry miRNAs identified to modulate macrophage fate. CRC cell-derived EVs contain aberrantly overexpressed miR-934, which was demonstrated to induce M2 macrophage polarisation by activating the PI3K/AKT signalling, via downregulation of PTEN [147]. The PI3K/AKT/mTOR pathway, which promotes M2 polarisation, has been shown to be activated by miR-106b, miR-25-3p, miR-130b-3p, and miR-425-5p encapsulated in HCT116 (human colon cancer) cell line-derived EVs [148], [149]. Exosomal ephrin type-A receptor 2 (EphA2) can also activate the PI3K/AKT/mTOR pathway in macrophages, promoting the progression of renal cell carcinoma [150].

TAMs, CAFs, and tumour cells interact in a dynamic and reciprocal manner that drives tumour progression. CAFs secrete cytokines and chemokines that recruit and polarise TAMs to an M2-like phenotype, supporting tumour growth and metastases. TAMs release growth factors and proteases that remodel the extracellular matrix and promote angiogenesis, facilitating tumour cell invasion. EVs from tumour cells and CAFs carry signalling molecules that further modulate the behaviour of TAMs and other stromal cells, enhancing their tumour-promoting functions and creating a supportive niche for tumour progression and metastasis. Addressing these complex interactions can help bring CAR-T treatment for solid tumours closer to patients. Utilising EVs as mediators of these interactions may also help to fine tune novel therapies and identify crucial targets.

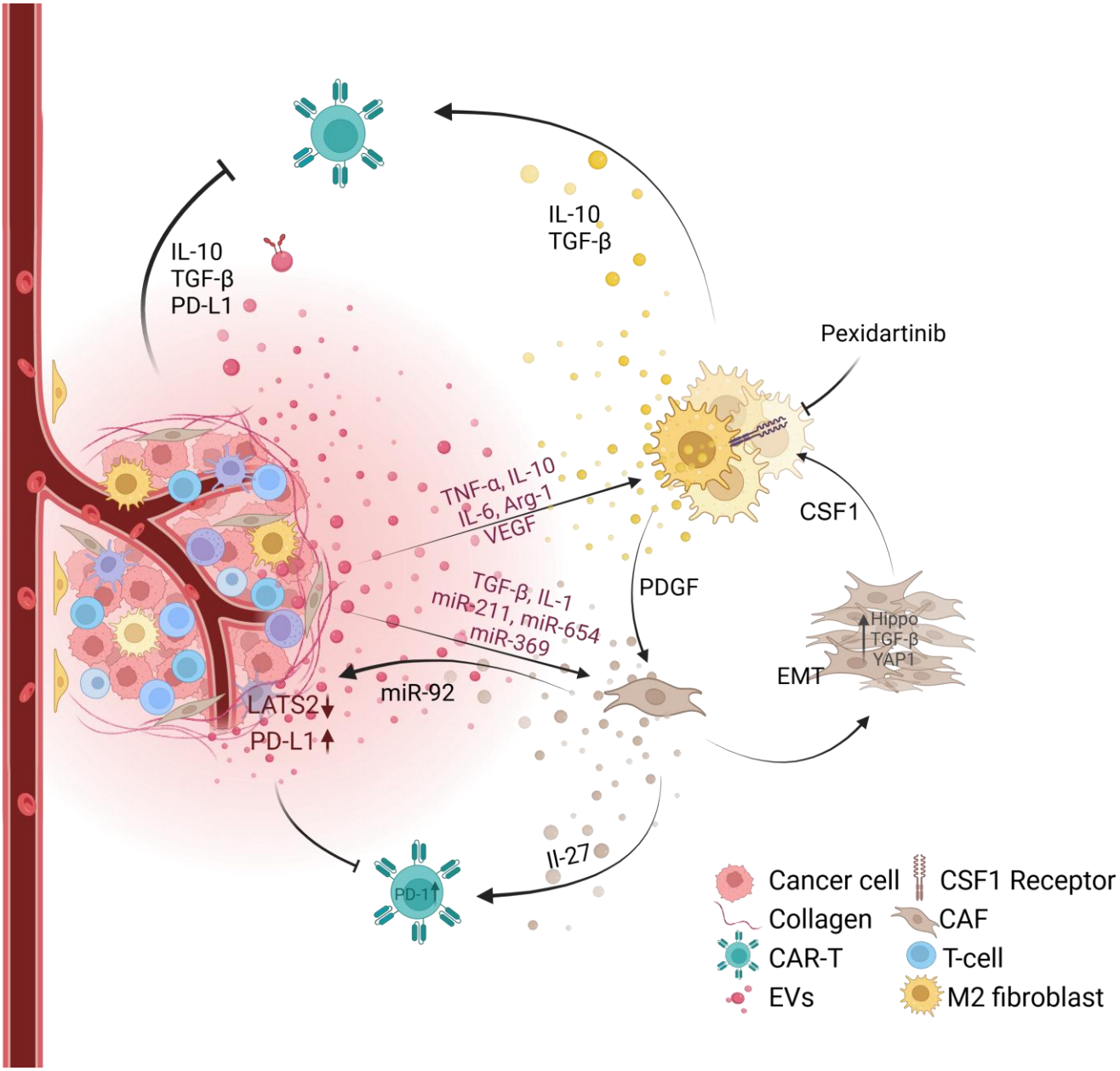

Fig. 3 Circular EV mediated interactions in TME. *The immunosuppressive TME consists of tumour cells, CAFs, and TAMs. These cells all release EVs enriched with cargo that suppress immune cells, including CAR-T cells, and also promote T-Reg cells, EMT, and EndMT. Therefore, creating a lethal cycle that promotes the growth and metastasis of the tumour. Created with BioRender.com*

3.2 Overcoming tumour angiogenesis in the TME through EV-mediated modulation

Hundreds of clinical trials are being carried out with CAR-T targeting solid tumours. The most common neoplasms being targeted include the liver, brain, pancreas, stomach, lungs, colorectal, breast, and ovarian [151]. However, the efficacy of CAR-T therapy is often hindered by the critical factors in solid tumour initiation and aberrant angiogenesis, which are frequently driven by EVs [140].

Various cancer types secrete EVs that promote angiogenesis, a crucial factor in solid tumour initiation and development. For instance, colorectal tumour perivascular cell-derived EVs (TPC-EVs) promote angiogenesis via the growth arrest-specific 6 (Gas6)/AXL receptor tyrosine kinase (Axl) pathway [152]. Whereas EVs from oesophageal squamous cell carcinoma cells enriched with cation-dependent mannose-6-phosphate receptors (M6PR) mediated the pro-angiogenic effects

both *in vitro* and *in vivo* [153]. Gastric cancer EVs carrying Angiopoietin-2 (ANG2) were also shown to activate PI3K/Akt signalling, inducing endothelial cell proliferation, migration, invasion, and tube formation *in vitro* and *in vivo* [140]. However, these pro-angiogenic mechanisms can also be targeted. Huang et al. [152] have shown that Gas6 or Axl pathway inhibition suppressed TPC-EV-induced angiogenesis both *in vitro* and *ex vivo*. Tumour-associated endothelial cells (TECs) have also been identified to release EVs, promoting angiogenesis in triple-negative breast cancer. Primarily through an increase in granulocyte colony-stimulating factor (G-CSF), due to upregulation of the mammalian target of rapamycin (mTOR) pathway, EVs blunt the anti-tumour immune response [108]. Additionally, it was found that blocking interleukin-3 receptor α (IL-3Rα) on TECs alters the cargo of TEC-derived EVs. This shift transforms their function from pro-tumorigenic to tumour-suppressing by enhancing cancer cell apoptosis and reducing cell viability and migration [154].

Bone marrow mesenchymal stromal cells (MSCs) treated with pro-inflammatory cytokines, secrete EVs enriched in TIMP metallopeptidase inhibitor 1 (TIMP-1), CD39, and CD73. Administration of these EVs inhibited angiogenesis by affecting both extracellular matrix remodelling and endothelial cell migration, inhibiting tumour progression *in vivo* [155]. Nasopharyngeal carcinoma (NPC) progression was also suppressed by the addition of limb-bud and heart positive NPC EVs *in vivo*. NPC EVs have also inhibited cellular proliferation, migration, and tube formation of HUVECs by downregulating vascular endothelial growth factor A (VEGFA) signalling [156].

Many cancer therapeutics are approved for targeting tumour angiogenesis, such as VEGF inhibitors like bevacizumab, sorafenib, sunitinib, and pazopanib. Unfortunately, their clinical outcomes are often limited, and patients frequently relapse due to acquired resistance [157]. CAF-derived EVs have also been shown to contain VEGF on their surface and activate vascular endothelial growth factor receptor 2 (VEGFR2) signalling even in the presence of VEGF inhibitors. Heparinase treatment of EVs was able to cleave VEGF from their surface and restore bevacizumab sensitivity in oral squamous cell carcinoma cells [158]. Developing drugs that specifically bind to VEGF/VEGFR on tumour-derived EVs or targeting the connection between VEGF and EVs could enhance the therapeutic effect of anti-angiogenic therapy.

Anti-VEGF-A antibodies have been shown to improve CAR-T cell persistence, infiltration, and distribution throughout the TME, delaying tumour growth, improving survival *in vivo*, and providing rationale to administer anti-angiogenic therapies alongside CAR-T [124], [159]. This preclinical data supports the combination of vascular normalising agents with CAR-T therapies, to potentially enhance the efficacy of CAR-T therapies in solid tumours.

EVs carrying ncRNAs also play a significant role in tumour angiogenesis. Glioblastoma stem cell (GSC) EVs have been shown to carry miR-148a and miR-9-5p, which are able to mediate the EV-associated angiogenesis. MiR-9 expression levels in GSC-EVs co-incubated with HUVEC, directly correlated with the resulting tubule formation count and length. In addition, silencing of miR-148a has been found to reduce the aberrant tumour vasculature in mouse models of GBM [160]. Nasopharyngeal carcinoma EVs suppress testis specific 10 (TSGA10) expression via miRNA-23a, and gastric cancer EVs containing the circular RNA circSHKBP1 regulate angiogenesis by sponging miR-582-3p, which results in human antigen R (HUR) upregulation and VEGF stabilisation [161], [162]. Circular RNA DCAF8 (hsa_circ_0014879) was also found to promote proliferation, migration, invasion, and EMT in hepatocellular carcinoma by interacting with miR-217 and promoting nucleosome assembly protein 1 like 1 (NAP1L1) expression [163]. Other EV-derived ncRNAs, like lncRNA MALAT1 in osteosarcoma and lncRNA-H19 in mice, also play crucial roles in promoting angiogenesis [164]. EVs secreted by ovarian cancer cells show

downregulation of miR-92b-3p, leading to enhanced angiogenesis and migration of endothelial cells via SOX4. To ameliorate this effect, Wang et al. [165] engineered the human ovarian cancer cell line (SKOV3) to overexpress miR-92b-3p and coated it with Arg-Gly-Asp peptide to specifically target HUVECs. These engineered EVs managed to suppress tumour-related angiogenesis and tumour growth in ovarian cancer. There are legitimate concerns about the potential risks of using tumour cells as a source of EVs for clinical applications. Despite engineering efforts, uncertain cargoes in tumour cell derived EVs could contribute to tumour progression. Therefore, it is crucial to ensure that the EV content is well characterised before clinical use. Unfortunately, often, the main hurdle to using EVs for cargo delivery is the consistent and efficient loading of the active agent.

EVs have been identified as crucial mediators in the TME, influencing cancer progression and response to therapy [166], [167], [168]. One way to address these effects is by controlling EV trafficking and protein metabolism involved in EV biogenesis. Compounds like calpeptin have been studied for inhibition of EV formation and release through their activity on cortactin, a cytoskeleton protein. Manumycin A inhibits EV biogenesis by blocking RAS activation, resulting in blocked ESCRT dependent EV biogenesis [169]. Fasudil (Y27632) affects EV release by blocking Rho-associated protein kinases, which act on myosin light chain, and LIM kinases, blocking these proteins and inhibiting EV release [169]. Additionally, inhibiting lipid metabolism pathways also has an effect on EV production. Pantethine has been shown to reduce EV formation in calcium stimulated, doxorubicin-resistant breast cancer cells (MCF7) by 24% [170]. Imipramine inhibits the acid sphingomyelinase activity, blocking the hydrolysis of sphingomyelin to ceramide, a process involved in membrane fluidity and EV generation. Imipramine significantly reduces EV secretion, showing a 77% reduction in total EV release *in vitro* [171]. However, recently, it has been demonstrated that imipramine can increase the systemic effects of extracellular UV radiation-damaged DNA loading and distribution of EVs [172]. It is therefore important to understand how these EV biogenesis-affecting drugs can influence both healthy cells and non-tumourigenic damaged cells. For example, extensive use of Y27632 has been observed to increase expression of insulin-like growth factor-binding protein 5 (IGFBP-5), which potentially transforms primary human dermal fibroblasts into CAF-like cells [173]. Imipramine acting on various receptors in the body can result in a series of side effects, like suppression of immune cells and associated infections, dizziness, tiredness, nausea, vomiting, and low blood pressure [174]. It is worth noting that since clinically used drugs have some side effects, treatment decisions should be based on a careful risk assessment, particularly when targeting biologically relevant mechanisms like EV biogenesis.

4. Conclusion

In this review, we summarised recent developments in CAR-T cell immunotherapy and discussed the many roles of EVs in cancer progression as well as their potential to improve CAR-T therapeutic efficacy. In the context of CAR-T therapy, EVs play a complex role, acting as mediators of tumour progression, responsible for increased terminal differentiation and functional exhaustion of CAR-T cells, and acting as potential rescuers in the hostile TME. While CAR-T therapy has revolutionised the treatment of haematologic malignancies, its efficacy in solid tumours is hindered by the immunosuppressive TME. Targeting EV-mediated pathways, such as those involving VEGF, immune-modulatory, or pro-angiogenic miRNAs, can potentially enhance CAR-T cell infiltration and effectiveness. Combining CAR-T therapy with strategies to inhibit or modulate EVs offers a promising approach to overcoming the barriers posed by the TME, such as

immunosuppression and abnormal angiogenesis, consequently enabling efficacious therapeutic use of CAR-T for all types of tumours.

Optimising EV production and cargo loading processes is essential to moving towards EV clinical application to enhance the efficiency of CAR-T therapy **(Fig. 4)**. Recent systematic review and meta-analyses of EV-based clinical trials have shown that EV-based therapy is safe, showing a low incidence of serious adverse events at 0.7% and adverse events at 4.4%. With no significant differences in serious adverse events between autologous and allogeneic administration or between engineered and non-engineered EVs [175]. Overall, this review highlights the relevance of continuing EV research to improve their clinical applications, as they appear to be well tolerated. Leveraging these observations and recent advancements in EV engineering, it should be expected that CAR-T-based therapies can be made more efficient by exploiting EV-mediated delivery of specific modulatory RNAs or proteins enhancing CAR-T cell persistence, targeting, and effectiveness. Such strategies, combined with robust EV isolation and characterization techniques, hold promise for translating EV-based therapeutics into clinically viable treatments.

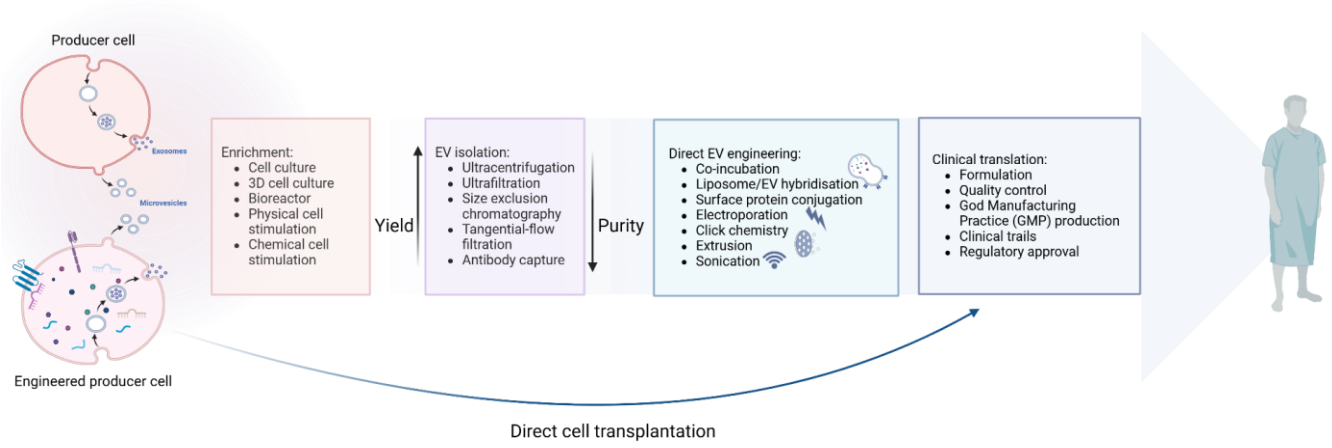

Fig. 4 EV manufacturing process for clinical applications. *EVs are enriched from producer cells by physical or chemical stimulation as well as different cell culture conditions. Isolation and purification processes include techniques such as ultracentrifugation and tangential flow filtration to ensure high EV purity and batch-to-batch consistency. Direct EV engineering is exploited to enhance cargo loading as well as surface functionalization to improve therapeutic efficacy and targeting specificity. For clinical translation, EVs must be formulated into a stable and effective product. Quality control measures are to be implemented to ensure consistency and safety, complying with GMP standards. The EVs can then undergo clinical trials to evaluate their efficacy and safety in humans. Successful trials lead to regulatory approval, enabling the EV-based therapy to be used in clinical settings. The entire process, from EV enrichment to regulatory approval, is crucial for the development of reliable and effective EV-based therapeutics.*

Acknowledgments and Disclosures

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