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Immunotherapy resistance and advancing adaptive cell therapeutics

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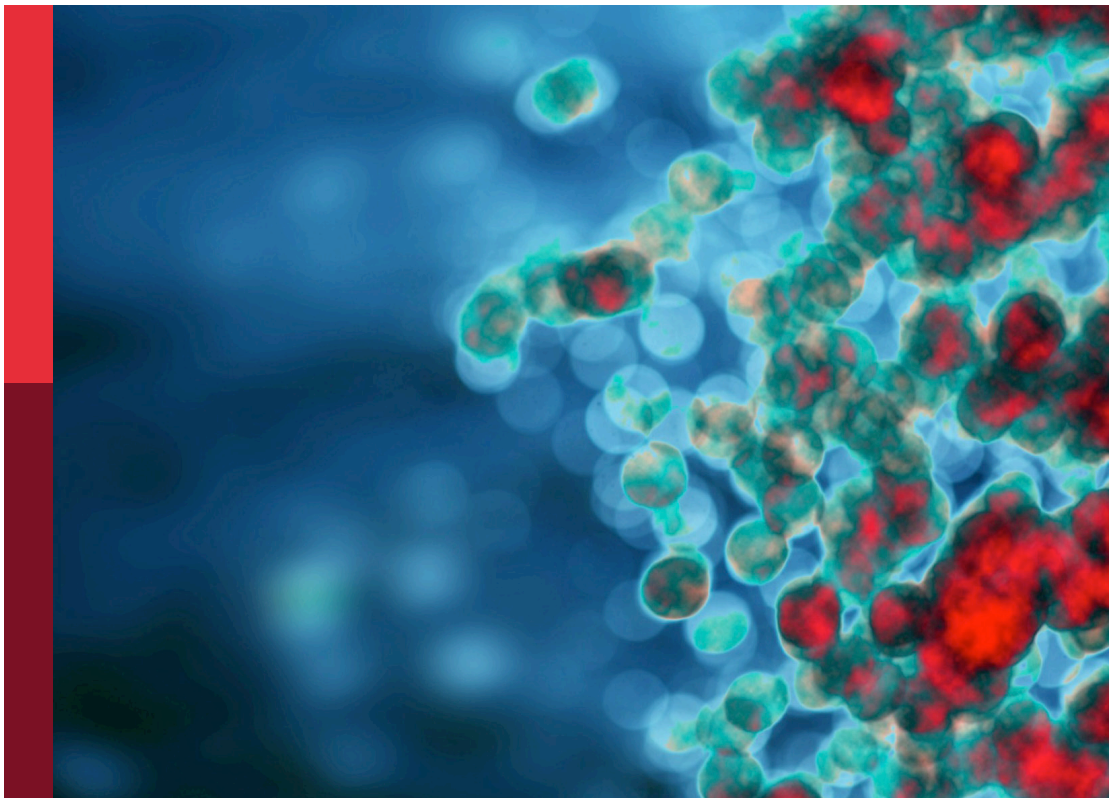

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Immunotherapy resistance and advancing adaptive cell therapeutics

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Editorial: Immunotherapy resistance and advancing adaptive cell therapeutics

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Editorial on the Research Topic
Immunotherapy resistance and advancing adaptive cell therapeutics

Advances in the understanding of immune biology and the development of a newer generation of immunotherapies have ushered in a new stage in combating many conditions, including cancer, autoimmune diseases, and infectious diseases. The principal focus of this type of therapy is to modulate the host immune response using antibodies, vaccines, cytokines, and cells. However, it is important to note that the immune responses are dynamic and constantly developing. The response to immunotherapy varies among patients and includes their pathophysiological environment, metabolism, and genetic factors. Additionally, Advanced Cell Therapeutics (ACT) is becoming more prevalent in the treatment of all kinds of diseases; still, a substantial number of patients are experiencing resistance to many of these treatments, necessitating further advancements. Therefore, researchers are pursuing different modifications to ongoing therapeutic options to overcome immunotherapy resistance and emphasize the promising frontier of adaptive cell therapeutics in overcoming these challenges.

Chimeric antigen receptor cell therapies

CAR-T cells have been used with considerable success to treat hematologic cancers. However, manufacturing autologous CAR-T cells is challenging because of the quantity and quality of the patient's T cells, which can compromise the clinically applicable dose of CAR-T cells, increase the risk of relapse during production, and cause manufacturing difficulties. One approach to overcoming these challenges is an "off-the-shelf" production derived from allogeneic T cells from peripheral blood (PB), embryonic or iPSC-derived cells, and umbilical cord blood (UCB). Rassek et al. compared the autologous T cells with these two allogeneic sources in production time, cost, quality of cells, availability, quality control, applicability, T cell exhaustion, and graft-versus-host disease risk. There is preclinical and clinical evidence from phase I trials for UCB-derived CAR cells. One of the advantages of using UCB-derived CAR cells is the abundance of cells in cord blood banks, which makes it possible to obtain young, naive natural killer (NK) cells, T cells, and other types of cells, including mesenchymal stem cells (MSCs). Although cord blood needs *in vitro* purification and expansion, it effectively has a lower level of checkpoint inhibitors, such as PD1, LAG3, and TIM3 expression, than allogeneic PB-derived CAR cells.

Regardless of the cell source, T cells and NK cells are the main effector cells carrying CARs for cancer therapy. A main concern with CAR-T or CAR-NK cells is their ability to migrate and persist in the tumor. All immune cells migrate via a chemokine gradient into the tumor microenvironment. In the case of myeloma, the bone marrow is the main site, and the chemokine CXCL12 may attract NK and T cells, thereby increasing the likelihood of therapy success. Moles et al. developed a BCMA CD28 zeta CAR-NK cell with bicistronic CXCR4 or $CXCR4^{R334X}$ surface receptor expression. Both receptors increase the *in vitro* migration and cytotoxicity of CAR-NK cells against $RAJI^{BC\ eMA}$, and trogocytosis. CXCR4 expression also identifies a lower amount of antigen that is necessary for BCMA-CAR activation, evidencing a potential recognition of $BCMA^{low}$ cells.

Another interesting strategy to increase CAR potential is changing the scFv (single chain Fragment variant) to a Variable Heavy domain of a Heavy chain (VHH) molecule. Hanssens et al. tested a library of VHH-CARs derived from camelid-found heavy-chain-only antibodies (HCAbs) as an antigen-binding moiety against the CS1 antigen for multiple myeloma. Several VHH-CAR T cells could be activated *in vitro*, exhibited cytotoxicity, and were able to migrate to the tumor *in vivo*. Nonetheless, *in vitro* predictions failed to indicate the best VHH behavior.

For solid tumors, ACT is challenging due to low cell migration, an inhibitory microenvironment, and especially antigen expression heterogeneity within the tumor. CAR-T cells, NK cells, dendritic cell-based vaccines, and tumor-infiltrating lymphocytes (TILs) are the main strategies presented for the treatment of solid tumors. Several studies were reviewed by Ao et al. on biliary tract malignancies, intrahepatic cholangiocarcinoma, extrahepatic cholangiocarcinoma, and gallbladder cancer. These tumors are aggressive, with a poor prognosis and an overall survival rate of only a few months despite chemotherapy. These tumors have a low incidence in Western countries, but a 40-fold higher incidence in Asian countries. A combination of ACT may be tested in the future as an alternative to the current chemotherapy to improve the prognosis.

In ACT, especially when expanding and re-injecting TILs or CAR-T cells, the functional quality of these cells could become critical. For example, the meta-analysis by Wan et al. indicated that high PD-1 expression on CD8+ cells based on 20 studies involving 3,086 patients, was linked to poorer overall survival (Yan et al.). However, if the injected T cells have already acquired an exhausted phenotype such as PD-1 upregulation in the tumor microenvironment, their anti-tumor efficacy may be compromised. Furthermore, the study suggested that the use of checkpoint inhibitors such as Pembrolizumab in combination with chemotherapy could lead to cytokine release syndrome or hemophagocytic lymphohistiocytosis (Qin et al.). Therefore, strategies that either select for non-exhausted adaptive T cells or genetically knockout T cells for checkpoints are needed to enhance ACT outcomes.

Adaptive cell and CAR cell-based therapies are currently in clinical use for cancer, especially hematological cancers, but face challenges in terms of availability and cost, which limit their use for more patients. On the other hand, adaptive cell therapies for autoimmune diseases are in Phase 1 or 2 clinical trials. Fu et al. found that autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, and multiple sclerosis can be managed by pharmacotherapy or ACT. In the latter, regulatory T cells (Tregs), CAR-Treg cells, chimeric auto-antibody receptor T cells, regulatory NK cells, and tolerogenic dendritic cells can be used as ACT. Although none of these strategies are approved for autoimmune diseases, a combination of drugs and ACT seems promising for the future.Various approaches are being investigated to enhance ACT and immunotherapeutics, including using machine learning to integrate multi-omics data for precise prognostic modeling. Yan et al. identified an immunogenic cell death-related signature (ICDRS) using single-cell and bulk RNA sequencing data, offering valuable insights into tumor immune evasion in bladder cancer. This approach could make it possible to identify patient-specific features, enabling more personalized ACT or its combination with immunotherapeutic strategies.

In conclusion, ACT and advanced immunotherapeutics hold immense potential for treating cancer and autoimmune diseases by developing and engineering immune cells in different ways alongside CAR receptors. However, current challenges such as resistance, cell exhaustion, and manufacturing hurdles persist; ongoing innovations and personalized approaches could pave the way for more effective and accessible therapies.

Author contributions

LB: Writing – original draft, Writing – review & editing. AY: Writing – original draft, Writing – review & editing.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Scrutiny of chimeric antigen receptor activation by the extracellular domain: experience with single domain antibodies targeting multiple myeloma cells highlights the need for case-by-case optimization

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Introduction: Multiple myeloma (MM) remains incurable, despite the advent of chimeric antigen receptor (CAR)-T cell therapy. This unfulfilled potential can be attributed to two untackled issues: the lack of suitable CAR targets and formats. In relation to the former, the target should be highly expressed and reluctant to shedding; two characteristics that are attributed to the CS1-antigen. Furthermore, conventional CARs rely on scFvs for antigen recognition, yet this withholds disadvantages, mainly caused by the intrinsic instability of this format. VHHs have been proposed as valid scFv alternatives. We therefore intended to develop VHH-based CAR-T cells, targeting CS1, and to identify VHHs that induce optimal CAR-T cell activation together with the VHH parameters required to achieve this.

Methods: CS1-specific VHHs were generated, identified and fully characterized, *in vitro* and *in vivo*. Next, they were incorporated into second-generation CARs that only differ in their antigen-binding moiety. Reporter T-cell lines were lentivirally transduced with the different VHH-CARs and CAR-T cell activation kinetics were evaluated side-by-side. Affinity, cell-binding capacity, epitope location, *in vivo* behavior, binding distance, and orientation of the CAR-T:MM cell interaction pair were investigated as predictive parameters for CAR-T cell activation.

Results: Our data show that the VHHs affinity for its target antigen is relatively predictive for its *in vivo* tumor-tracing capacity, as tumor uptake generally decreased with decreasing affinity in an *in vivo* model of MM. This does not hold true for their CAR-T cell activation potential, as some intermediate affinity-

binding VHHs proved surprisingly potent, while some higher affinity VHHs failed to induce equal levels of T-cell activation. This could not be attributed to cell-binding capacity, *in vivo* VHH behavior, epitope location, cell-to-cell distance or binding orientation. Hence, none of the investigated parameters proved to have significant predictive value for the extent of CAR-T cell activation.

Conclusions: We gained insight into the predictive parameters of VHHs in the CAR-context using a VHH library against CS1, a highly relevant MM antigen. As none of the studied VHH parameters had predictive value, defining VHHs for optimal CAR-T cell activation remains bound to serendipity. These findings highlight the importance of screening multiple candidates.

KEYWORDS

CAR-T cells, VHH, multiple myeloma, adoptive cell transfer, hematology

1 Introduction

Multiple Myeloma (MM) is a cancer of mature, antibody-producing B cells (plasma cells) that grow uncontrollably in the bone marrow. They thereby disturb the bone formation process and hematopoietic equilibrium, leading to characteristic bone lesions, hypercalcemia and general anemia (1). They also produce excessive amounts of dysfunctional immunoglobulin molecules (M- or paraprotein), causing renal problems (2). Worldwide, MM accounts for 14% of all hematological cancers, making it the third most observed one. It is considered a treatable but incurable malignancy, with a 5-year overall survival rate of 54% (3).

Standard induction therapy for newly diagnosed MM patients usually comprises a combination of immunomodulatory drugs (e.g., lenalidomide) and corticosteroids (e.g., dexamethasone), most often combined with proteasome inhibitors (e.g., bortezomib) (1, 4). This regimen is supplemented with anti-CD38 monoclonal antibody (mAb)-therapy (daratumumab) in some cases. For all eligible patients, induction therapy is followed by autologous stem cell transplantation (SCT). Subsequent maintenance therapy may be bortezomib- or lenalidomide-based (4). Although these regimens are often initially successful, relapse with an increased tolerance to previous treatment regimens is a commonly observed phenomenon. Upon relapse, combination treatment options are diverse and adjusted to the patient specifically (2).

It is established that the MM tumor microenvironment is highly immunosuppressive, among others due to disturbed cytokine production by malignant, stromal and immune cell populations and an outbalanced programmed death-1 (PD-1)/PD-1 ligand (PD-L1) immune checkpoint axis (5). This leads to a malfunctioning innate and adaptive immune environment, involving both myeloid and lymphoid actors. Increased understanding about these aberrances, together with observed graft-versus-myeloma effects in early allogenic SCT trials, highlight the potential added value of immune therapy for MM (6). Forms of adoptive cell transfer currently being evaluated for the treatment of MM include T-cell receptor (TCR)-modified T-cells, (allogenic) chimeric antigen receptor (CAR)-T cells, (CAR)-natural killer (NK) cells and tumor-infiltrating lymphocytes. Furthermore, immune checkpoint inhibitors, bi-specific T-cell engagers and cancer vaccination strategies are under (clinical) evaluation (6). Of these, adoptive cell transfer, and more particularly CAR-T cell therapy holds great promise due to the unseen curative outcomes observed in other hematological malignancies, namely high-grade lymphomas and leukemias (7).

CAR-T cells are patients' own T-cells that are genetically modified to express a transmembrane CAR (8). This receptor can recognize a tumor antigen, expressed on the surface of tumor cells, with its extracellular domain. Classically, a monoclonal antibody (mAb)-derived single-chain variable fragment (scFv) is incorporated to achieve tumor antigen recognition (9, 10). Upon antigen encounter, the intracellular T-cell co-stimulatory (most often CD28- and/or 4-1BB-derived) and CD3ζ T-cell activation domains are responsible for engaging a cytotoxic T-cell response toward the malignant cell. After *ex vivo* modification and expansion of these patient-derived T-cells, they are administered back to the patient, where they thus act as a living drug (11).

Historically, most of the evolution of CAR design has been focused on the optimization of the intracellular portion of the receptor, in order to achieve maximal T-cell activation (10). The extracellular antigen-binding moiety has received less attention, as many mAbs against relevant tumor antigens are (clinically) available. However, the use of the artificial mAb-derived scFv format has been linked to some limitations in CAR-T cell efficacy (9, 12). Firstly, scFvs lack natural stability and therefore need to be artificially linked. This instability has been associated with aggregation, antigen-independent (tonic) signaling and subsequent premature T-cell exhaustion (13, 14). Secondly, their non-human nature limits CAR-T cell persistence *in vivo*, as anti-CAR immune responses have been observed in patients upon

relapse (15). Thirdly, because these molecules are usually derived from clinically validated mAbs, there is often no step of (structural) optimization of the scFv domain included in the development of these CARs, as a scFv selection procedure is not required (9, 11). In recent years however, the importance of optimizing this extracellular protein domain has become increasingly recognized, and alternatives to the classical scFv format are rapidly emerging, as reviewed elsewhere (9). One example of such alternative CAR design incorporates a Variable Heavy domain of Heavy chain (VHH) molecule, derived from camelid-found heavy-chain-only antibodies (HCAbs) as an antigen-binding moiety. HCAbs compare to mAbs by lacking light chains and constant heavy-1 (C_H1) domains (16). The antigen-binding part therefore consists of only one protein domain, the VHH. Evolutionarily, this has ensured that the VHH domain behaves as a monomer and is therefore intrinsically more stable compared to a scFv (16). It also allows for more straightforward VHH screening and selection, as the availability of (immune) libraries is more evident (16). Furthermore, VHH immunogenicity is expected to be lower in comparison to scFvs (17), as there is a high sequence resemblance with human VH sequences of family III. At this point, no reports of anti-VHH CAR immune responses with a neutralizing effect on CAR-T cell therapy have been reported from clinical trials (18), and VHH humanization protocols are available (9, 19, 20).

In the last decades, CD19-directed CAR-T cell therapy has astonished the medical landscape with its curative outcomes in certain forms of leukemia and lymphoma (21). This raises hope for its potential in other hematological malignancies, including MM (11). However, as CD19 is usually not expressed by plasma cells, clinical success of these well-established CAR-T cell products in MM has been limited (11). Higher success rates have been achieved with B-cell maturation antigen (BCMA)-targeted CAR-T cells, with currently two FDA-approved CAR-T cell products (i.e., idecabtagene vicleucel and ciltacabtagene autoleucel) as a result (7). Although the observed overall response rates are high, a progression-free survival period of more than 1 year is observed in under 50% of patients treated (22, 23). The most commonly reported causes for this are antigen shedding and CAR immunogenicity, which can lead to anti-CAR immune responses with a therapy-neutralizing effect (11, 18, 22). Hence, other target proteins are under investigation. Of these, SLAMF7/CS1, GPRC5D, CD138 and CD38 are showing different clinical success rates, while data for newer tumor antigens like CD70, NKG2DL and κ-light chain are to be expected (11). Particularly of interest as an alternative cancer antigen for MM CAR-T cell therapy is CD2 subset 1 (CS1, CD319 or signaling lymphocytic activation molecule family member 7 [SLAMF7]). Indeed, CS1 is highly expressed by > 95% of both healthy as well as malignant plasma cells (11, 24), and expression is retained after multiple lines of therapy (25). Expression on healthy tissue is lower, limited to hematopoietic cell lineages (NK, T, B, and dendritic cells; monocytes and macrophages) and absent on hematopoietic stem cells (11, 26).

These observations have provided the rationale for this study, which consists of developing a new form of CAR-T cell therapy for MM, targeting CS1. To this end, we aimed to develop an optimized CAR through variation in the antigen-binding part of the receptor, as well as determine which specific parameters of this extracellular part are crucial to achieve potent CAR-T cell activation. The influence of differences in affinity, cell-binding capacity, epitope location, cell-to-cell distance and binding orientation were examined as possible determinant factors.

2 Materials and methods

2.1 Cell culture

Bacterial cell lines used include *E. coli* TG1 (Sigma Aldrich), *E. coli* WK6 (American Type Culture Collection, ATCC), *E. coli* NEB5-α (New England BioLabs) and *E. coli* XL1-Blue (Agilent) and were all cultured in lysogeny broth. Mammalian cell lines used include cancer cell lines OPM2 ($CS1^{pos}$, ATCC), JJN3 ($CS1^{neg}$, ATCC) and murine $CS1^{pos}$ 5T33vt, described before (27) –, which were all cultured in Roswell Park Memorial Institute 1640 culture medium (Gibco), supplemented with 10% (v/v) fetal bovine serum, 2 mM L-Glutamine, 1% (v/v) penicillin-Streptomycin, 1% (v/v) non-essential amino acids and 1 mM sodium pyruvate, all from Thermo Scientific. The lentiviral vector (LV) production cell line HEK293T was obtained from ATCC and cultured in Dulbecco's Modified Eagle Medium culture medium (Gibco), equally supplemented. Human reporter T-cell line Jurkat-67 (2D3) contains the gene for eGFP under an NFAT-driven promoter, as described before (28), and was cultivated in equally supplemented Iscove's Modified Dulbecco's Medium (Gibco).

2.2 Identification of CS1-specific VHHs

2.2.1 Immunization and VHH library construction

Recombinant CS1 proteins (CS1-(his)C) were produced and purified by U Protein Express (Utrecht, The Netherlands). Briefly, HEK293-E 253 cells were transiently transfected with DNA encoding the extracellular portion of either human or murine CS1, C-terminally fused to a hexahistidine tag. Purification from the supernatant was ensured by subsequent Immobilized Metal Affinity Chromatography (IMAC) and Size Exclusion Chromatography (SEC). A llama was immunized by weekly injections of a mixture of 100 μg of recombinant human and murine CS1 proteins, combined with Gerbu adjuvant, over a period of 6 weeks. Total mRNA extraction from 10^7 peripheral blood lymphocytes, isolated from 100 ml blood sample, yielded 40 μg of mRNA which was used to generate the immune VHH phagemid library via procedures described elsewhere (29).

2.2.2 Biopanning

For VHH selection, the VHH library was cloned into a pMECS phagemid vector, as previously described (29). Phages expressing the VHHs on their surface were produced after transformation of *E. coli* TG1 cells and infection with M13 VCS helper phages. Four rounds of biopanning were performed on the biotinylated variant of human CS1 (CS1-(his)C-PEG4-biotin), custom-produced by U Protein Express. As it was intended to select human CS1-specific VHHs, we will further refer to human CS1 as CS1. In each round,

these phages were incubated with CS1-(his)C-PEG4-biotin (100 nM in rounds 1 and 2; 10 nM in rounds 3 and 4) and phage selection was performed via magnetic streptavidin beads (New England Biolabs). Phage elution was obtained with 50 mM dithiothreitol. Harvested phages were infected into *E. coli* TG1 cells for VHH production (single colonies) and generation of the VHH sub-library for further rounds of panning.

2.2.3 VHH screening via ELISA

A randomized selection of single *E. coli* TG1 colonies carrying a VHH-pMECS plasmid was made and bacterial colonies were produced at 2 ml culture scale in lysogeny broth medium. Periplasmic production of hexahistidine- and hemagglutinin (HA)-tagged VHHs was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Crude periplasmic extracts were obtained via freeze-thawing and CS1 binding was evaluated in an enzyme-linked immunosorbent assay (ELISA) on 100 ng recombinant CS1, coated on Nunc MaxiSorp plates (Invitrogen). This by subsequent staining with murine anti-HA-tag mAb (Sigma Aldrich) and alkaline phosphatase-coupled goat anti-mouse mAb (Sigma Aldrich), as described (29, 30). A tripling of the 405 nm absorption signal compared to background was used as a threshold to identify candidates as positive. Positive VHH clones were sequenced.

2.3 Selection and production of CS1-specific VHHs

2.3.1 Flow cytometry

VHH binding to cell-expressed CS1 protein was evaluated in flow cytometry on $CS1^{pos}$ MM cells (OPM2). Periplasmic extract was used, containing hexahistidine- and HA-tagged VHHs at unknown concentration, of which cell binding was detected by subsequent staining with mouse-anti-HA IgG1 mAb and phycoerythrin (PE)-labeled anti-mouse IgG1 mAb (BD Biosciences). Cell fluorescence was measured on a FACSCanto Flow Cytometer (BD Biosciences). Data analysis was performed using the FlowJo 10.9.0 software (BD Biosciences).

2.3.2 Off-rate screening

The off-rate dissociation constant of binding (k_d) is concentration-independent and can therefore be determined using surface plasmon resonance (SPR) technology on periplasmic extract. To this end, biotinylated recombinant CS1 protein (U Protein Express) was coated on a streptavidin chip in a Biacore T200 instrument. Periplasmic extract in hepes-buffered saline (HBS) was run over the chip and dissociation was monitored for 600 s in HBS. The chip was regenerated using 0.1 M glycine HCl (pH 2.0) between different measurements. Evaluation was performed using the Biacore T200 2.0 evaluation Software (GE Healthcare).

2.3.3 VHH production and purification

VHHs were cloned in the pHEN6c production vector (31) via described procedures (29). These pHen6c vectors were transformed into chemo-competent *E. coli* WK6 cells and periplasmic VHH expression was induced using 1 mM IPTG. Periplasmic extracts were obtained via osmotic shock and from there hexahistidine-tagged VHHs were purified by subsequent IMAC and SEC – as described (29). A non-targeting control VHH (R3B23)- described elsewhere (32)-, was produced in parallel, following identical procedures.

2.4 Molecular and *in vivo* characterization of purified CS1-specific VHHs

2.4.1 Flow cytometry

To detect VHH binding to cell-expressed antigen, $CS1^{pos}$ OPM2 cells were incubated with the VHHs at 200 nM for 1 h at 4°C. Detection was performed by staining for the C-terminal hexahistidine tag using a primary mouse anti-His IgG1 mAb (Biolegend), followed by a secondary allophycocyanin (APC)-labeled anti-mouse IgG1 mAb (Biolegend). A positive control for antigen-expression by the target cells (APC-mouse anti-human CS1 IgG2bκ mAb; Biolegend) and its isotype control (APC-mouse IgG2bκ isotype control; Biolegend) were included. All staining steps were performed according to manufacturer's instructions. Cell-fluorescence was evaluated using the BD FACSCelesta Cell Analyzer (BD Biosciences) and data analysis was done with the FlowJo 10.9.0 software (BD Biosciences).

2.4.2 Affinity determination via SPR

Kinetic parameters for VHH binding to CS1 were determined via SPR on a Biacore T200 instrument (GE Healthcare). To that end, 5 µg/ml recombinant CS1 protein in 10 mM sodium acetate, pH 4.0 (VWR International) was coupled on a 100 mM N-hydroxysuccinimide (GE Healthcare) and 400 nM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hypochloride (GE Healthcare)-activated CM5 sensor chip. Blocking of residual binding sites was achieved with 1 M ethanolamine-HCl (GE Healthcare). A 1/2 VHH dilution series of the purified VHHs, ranging from 100 nM to 3.125 nM (with duplicate at 50 nM), in HBS (pH 7.4) was run over the CS1-coated chip at 25°C. Binding association was allowed for 180 s and dissociation for 300 s. CM5 chip regeneration was ensured by 0.1 M glycine HCl (Sigma Aldrich) at pH 2.0. The association (k_a), dissociation (k_d) and equilibrium dissociation constants (K_D) were calculated with the Biacore T200 2.0 evaluation Software (GE Healthcare) using the '1:1 binding with drift and RI2' fitting model.

2.4.3 ^{99m}Tc radioactive labeling, *in vivo* microSPECT/CT imaging and *ex vivo* biodistribution

After sedation via isoflurane inhalation (Vetflurane, Virbac; 5% induction and 2.5% maintenance), $10x10^6$ $CS1^{pos}$ OPM2 cells in 100 µl phosphate buffered saline (PBS) were inoculated subcutaneously in the right flank of female, 6-week-old CB17/lcr-*Prkdc*^{SCID}/lcrlcoCrl mice (Charles River). At 18-19 days post inoculation, tumors were palpable (100 - max. 500 mm^3), allowing

biodistribution studies. To this end, hexahistidine-tagged VHHs were site-specifically radiolabeled with ^{99m}Tc, as previously described (33). Next, 100 µl of the ^{99m}Tc -labeled VHHs was injected intravenously in isoflurane-sedated animals. At 50 min post injection (p.i.), mice were sedated with 75 mg/kg ketamine (Ecuphar) + 1 mg/kg medetomidine (Virbac) via intraperitoneal injection and subjected to a 2 min microCT and a 20-minute pinhole-SPECT scan at 1 h p.i. of the radiotracer. Image analysis was performed with the AMIDE 1.0.4. and OsiriX MD 11.0 software. Sedated animals were sacrificed via neck dislocation after imaging. Organ collection for *ex vivo* biodistribution analysis was performed at 90 min p.i. and radioactive uptake in selected organs was measured using a γ-counting instrument (2480 WIZARD2 Automatic Gamma Counter, Perkin Elmer). Data were normalized to organ weight and corrected for radioactive decay. All animal experiments were approved by the ethical committee for use of laboratory animals of the Vrije Universiteit Brussel (Brussels, Belgium) (license number 19-281-2).

2.5 Generation of VHH-CAR encoding lentiviral particles (LVs)

2.5.1 Cloning

To generate a CAR-encoding pHR'-derived lentiviral transfer plasmid, a dsDNA oligonucleotide molecule containing a second-generation CAR sequence with adequate pHR' vector overhangs of 20 nucleotides was designed and ordered to-demand from Integrated DNA Technologies (IDT). This gBlock was assembled into a BamHI/SpeI-linearized (Thermo Scientific) pHR'-derived lentiviral transfer plasmid (described before by Breckpot et. al. (34)), via the Gibson Assembly Method (IDT). This ensured integration of the CAR sequence in the triple helix and 3' ΔU3 long terminal repeat-containing pHR' backbone, upstream of an Internal Ribosomal Entry Site (IRES) and a truncated Nerve Growth factor Receptor (tNGFR) reporter gene (as described (34)) and downstream of a cytomegalovirus (CMV) promoter. The lay-out of the CAR backbone is depicted in Figure 1.

VHH cloning into the lentiviral CAR backbone was performed via PstI/BstEII restriction from the pHEN6c production vectors, followed by T4 DNA ligation (Thermo Scientific), according to manufacturer's instructions. Resulting transfer plasmids were transformed into commercially available competent NEB5-α *E. coli* (New England Biolabs) and subsequently into XL1-Blue *E. coli* (Thermo Scientific) for larger scale plasmid production. Transformation was executed using the TransformAid Bacterial Transformation Kit (Thermo Scientific). Large scale plasmid purification was performed using the NucleoBond Xtra Maxi kit (Macherey-Nagel). Quality control included plasmid yield and purity assessment via OD_{260}/OD_{280} measurements on a Implen NanoPhotometer (Westburg), gel electrophoresis to verify plasmid integrity and correct restriction digestion (1.5% agarose gel, Mupid One Electrophoresis apparatus, Advance Co. Ltd.), and sequence verification (NightXpress Mix2Seq Kit, Eurofins Genomics).

2.5.2 HEK293T cell transfection

Transfer plasmids were co-transfected with the envelope-encoding plasmid pMD.G and the packaging plasmid pCMVΔR8.9 at a 3:1:2-ratio into HEK293T cells, as described before (34, 35). Both the pMD.G and pCMVΔR8.9 plasmid were a kind gift of D. Trono (University of Geneva Medical School, Geneva, Switzerland). Culture supernatant containing lentiviral particles (LVs) was harvested at 48 h and 72 h after transfection.

2.5.3 LV concentration

Subsequent 0.22 µm filtration and ultracentrifugation for 90 min at 64074 x g (Beckman SW28 rotor; Optima LE-80K ultracentrifuge; Beckman Coulter) of the culture supernatant was performed to pellet and concentrate the LVs, which were resuspended in PBS supplemented with 10 µg/ml protamine sulphate (LeoPharma). LV titers were determined by titration of a serial dilution on HEK293T cells, as described (36). Evaluation of transduction was done at 72 h post transduction via flow cytometry using the BD FACSCelesta Cell Analyzer, after staining for the tNGFR reporter protein with APC-coupled anti-NGFR mAb; Biolegend). Data analysis was done with the FlowJo 10.9.0 software (BD Biosciences) and titers were calculated using following formula:

$$\frac{\#cells\ at\ time\ of\ transduction\ x\ fraction\ of\ infected\ cells\ x\ dilution\ factor}{transduction\ volume\ in\ ml}$$

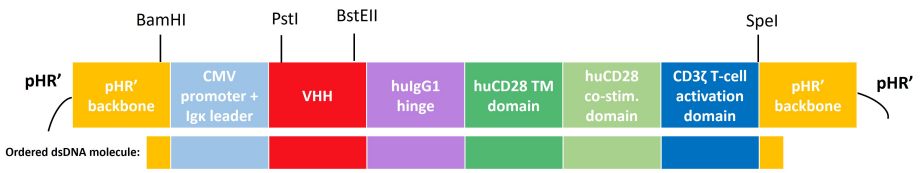

FIGURE 1
Schematic overview of the CAR construct and its subdomains the pHR'-derived lentiviral transfer plasmid. A second-generation human CAR was used, incorporating an Igκ leader sequence, a cytomegalovirus (CMV) promoter, a CS1-specific or non-targeting control VHH, a hinge region derived from human IgG4, a transmembrane and intracellular co-stimulatory domain derived from human CD28 and a human CD3ζ-derived T-cell activation domain. Relevant (unique) restriction sites are indicated above. Below the construct, the part that was ordered as a dsDNA molecule (gBlock) from Integrated DNA Technologies (IDT) is highlighted. CMV, cytomegalovirus; TM, transmembrane; hu, human; co-stim., co-stimulatory.

2.6 2D3 reporter T-cell transduction and selection of stable 2D3-CAR cell lines

Transduction was obtained by incubating 10^5 2D3 reporter T-cells with the LVs at a multiplicity of infection (MOI) of 10 in culture medium enriched with 10 µg/ml protamine sulphate (28). Transduction efficiency was evaluated in flow cytometry via incubation with biotinylated CS1 antigen (U Protein Express), followed by PE-coupled streptavidin (eBioscience). Cell fluorescence was measured on a BD FACSCelesta instrument and data analysis was done with the FlowJo 10.9.0 software (BD Biosciences). Cell lines with a stable transduction rate of > 95% were obtained by fluorescence activated cell sorting (FACS), after staining with biotinylated CS1 antigen and PE-coupled streptavidin. FACS was performed on a BD FACSAria™ III Cell Sorter. 2D3 cells stably expressing a VHH-CAR on the surface are further on denoted as 2D3-[VHH number].

2.7 Evaluation of T-cell activation potential by different VHH-CARs

2.7.1 CAR-T cell activation assay

2D3-CAR cells were co-cultured with OPM2 ($CS1^{pos}$) or JJN3 ($CS1^{neg}$) target MM cells (referred to as the stimulated and unstimulated condition, respectively) at an E:T ratio of 10:1 in supplemented IMDM culture medium at 37°C, 5% CO_2. Green fluorescence was followed-up in real-time in the IncuCyte ZOOM apparatus over a period of 40 h. Subsequently, cells were stained with biotinylated CS1 protein (U Protein Express) for CS1-specific 2D3-CARs or biotinylated anti-VHH mAb (GenScript) for 2D3-R3B23, followed by PE-coupled streptavidin for further analysis in flow cytometry. Antigen expression by the target cell lines was confirmed in flow cytometry after staining with APC-labeled anti-CS1 IgG2bκ mAb (Biolegend) and APC-labeled IgG2bκ isotype control mAb (Biolegend). Flow cytometry measurements were performed on a BD FACSCelesta apparatus (BD biosciences) and data analysis was done with the FlowJo 10.9.0 software (BD Biosciences).

2.7.2 Co-culture competition assays

MM cells were pre-incubated with a 1 µM saturating concentration of different soluble VHHs (1 h, 37°C, 5% CO_2), after which 2D3-CAR cells were added at a 1:1 ratio. Evaluation of CAR T-cell activation was performed via flow cytometry as described above at 40 h post co-incubation.

2.8 Estimation of binding orientation by *in silico* modeling

In silico 3D simulations of VHH-CAR:CS1 interactions were made via the online AlphaFold2 (v1.5.2) software (37). Evaluation of obtained structure predictions was performed in PyMol (v.4.6.0). To estimate the binding distance, distances were determined between 1) the C-terminal serine residue S(128) of the VHH and the most membrane-distal point of CS1 D(49); and 2) the C-terminal serine residue S(128) of the VHH and CS1 membrane anchor point A(219). Using the Pythagorean theorem, the distance between the CAR anchor point of the VHH S(128) and the membrane anchor point of CS1, as projected onto the axis of the extracellular part of CS1, was calculated. Bindings angles were calculated between the most membrane-distal residue of CS1 D(49); the membrane anchor point of CS1 A(219) and the terminal serine in the VHH S(128). A visual representation of the determined distances and angles is provided in Figure 6C.

2.9 Statistical analysis

Statistical analysis of *ex vivo* biodistribution experiments was performed by one-way ANOVA (multiple t-tests) in which each CS1-specific VHH was compared to the non-targeting control VHH R3B23, described before (32). For the analysis of the CAR activation assay, the difference between the stimulated and unstimulated condition was used as a measure of (specific) T-cell activation. Via one-way ANOVA (multiple t-tests), each antigen-specific VHH-CAR was compared to the non-targeting control VHH-CAR R3B23. Additional information about the number of replicates for each assay is provided in the adequate figure legends. All analyses were performed using the GraphPad Prism 9.1.0 software. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$; not significant (n.s.), $P>0.05$.

3 Results

3.1 Identification of anti-CS1 VHHs and evaluation of affinity characteristics

For CS1-specific VHH identification, a VHH immune phage display library was constructed after llama immunization with recombinant human and murine CS1 proteins. This library was displayed on the tip of bacteriophages and then subjected to four rounds of biopanning in solution on biotinylated CS1 protein.

After panning, a randomized selection of 285 VHH clones from different rounds of selection was produced in crude form, which was screened in ELISA for binding to immobilized recombinant CS1 protein. From these, 254 were considered positive for binding. Sequencing revealed these to be 81 unique VHH molecules. Flow cytometry analysis showed that all identified subjects bind $CS1^{pos}$ OPM2 cells, and a subsequent off-rate screening confirmed these observations. From these 81 identified clones, 19 VHHs, belonging to eight different VHH families - according to the standard ImMunoGeneTics (IMGT) numbering system (38) -, were selected for production, purification, and in-depth characterization (Figure 2). The parameters used for this selection were the observed off-rate (k_d), sequence differences, the presence of stop codons in the sequence, as well as sequence prevalence.

Further characterization of the produced and purified VHHs involved screening in flow cytometry and affinity determination

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          <-----------FR1----------> <---CDR1---> <------FR2------> <--CDR2--> <-----------------FR3-----------------> <----CDR3-----> <---FR4--->
          1        10        20        30        40        50        60        70        80        90        100       110          120
          |********|*********|****** ***|******** *|*********|***** ****|***** ****|*********|*********|*********|**** *****|*11****** **|********

VHH-2     DVQLVESGG-GLVQPGDSLRLSCTAS GRTF----ENNF MGWFRQPPGKEREFVAA VGWN--TGRG YYTDSVK-GRFTISRDSAKNTVYLQMNSLKPEDTAVYSC NAPLQSLDRRLPGPY WGQGTQVTVSS
VHH-6     DVQLVESGG-GLVQAGGSLRLSCAAS GRTF----EDYF MAWFRQIPGKEREFVAA VGWN--TGRG YYTDSVK-GRFTISRDSAKNTLYLQMDSLKPEDTAVYYC NAPLQSLDRRLPGPY WGQGTQVTVSS
----------------------------------------------------------------------------------------------------------------------------------------------
VHH-17    DVQLVESGG-GLVQPGDSLRLSCTAS GFTF----SNYP MSWVRQAPGKGPEWISH INTG--GGST YYADSVK-GRFTISRDNAKNTLYLQMNSLKPEDTALYYC ARGDPFK--YSGSYL RGQGTQVTVSS
----------------------------------------------------------------------------------------------------------------------------------------------
VHH-22    DVQLVESGG-GLVQPGGSLRLSCAAS GFTF----SSYD MSWVRQAPGKGPEWVSH ITTG--GGST HYDDSVK-GRFTISRDNAKNTLYLQMNSLKPEDTALYYC ARGDPF----DSSRD WGQGTQVTVSS
VHH-24    DVQLVESGG-GMVQPGGSLRLSCAAS GFTF----SSYD MSWVRQAPGKGPEWVSH ITTG--GGST HYDDSVK-GRFTISRDNAKNTLYLQMNSLKPEDTALYYC ARGDPF----DSSRD WGQGTQVTVSS
VHH-28    DVQLVESGG-GLVQPGGSLRLSCAAS GFTL----DDYA MSWVRQAPGKGPEWVSY INTG--GGST YYDDSVK-GRFTISRDNAKNTLYLQMNSLKAEDTALYYC ARGDPF----QRPKY WGQGTQVTVSS
VHH-29    DVQLVESGG-GLVQPGGSLRLSCAAS GFTF----SSYA MSWVRQAPGKGPEWVSH INTG--GGST YYDDSVK-GRFTISRDNAKNTLYLQMNSLKPEDTALYYC ARGDPF----DEYRY WGQGTQVTVSS
VHH-49    DVQLVESGG-GMVQPGGSLRLSCAAS GFTF----SSYD MSWVRQAPGKGPEWVSY INTG--GGST YYDDSVK-GRFTISRDNAKNTLYLQMNSLKAEDTALYYC ARGDPF----QRPKY WGQGTQVTVSS
VHH-51    DVQLVESGG-GMVQPGGSLRLSCAAS GFTF----SSYD MSWVRLAPGKGPEWVSY INTG--GGST YYDDSVK-GRFTISRDNAKNTLYLQMNSLKAEDTALYYC ARGDPF----QRPKY WGQGTQVTVSS
VHH-53    DVQLVESGG-GMVQPGGSLRLSCAAS GFTF----SSYD MSWVRQAPGKGPEWVSY INTG--GGST YYDDSVK-GRFTISRDNAKNTLYLQMNSLKAEDTALYYC ARGDPF----QRPKY WGQGTQVTVSS
VHH-56    DVQLVESGG-GLVQPGGSLRLSCAAS GFTF----SSYG MSWVRQAPGKGPEWVSH INTG--GGST YYADSVK-GRFTISRDNAKNTLYLQMNSLKPEDTALYYC ARGDPW----GHDPS WGQGTQVTVSS
VHH-57    DVQLVESGG-GMVQPGGSLRLSCAAS GFTF----SSYG MSWVRQAPGKGPEWVSH INTG--GGST YYADSVK-GRFTISRDNAKNTLYLQMNSLKPEDTALYYC ARGDPW----GHDPS WGQGTQVTVSS
----------------------------------------------------------------------------------------------------------------------------------------------
VHH-61    DVQLVESGG-GMVQPGGSLRLSCAAS GFTF----SSYG MSWVRQAPGKGPEWVSY INVG--GGST YYDDSVK-GRFTISRDNAKNTLYLQMNSLKPEDTALYYC ARGDPYS--DHPQRT WGQGTQVTVSS
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VHH-63    DVQLVESGG-GLVQPGGSLRLSCTVS GLTF----SDYD MSWVRQAPGKGPEWVSF INTG--GGST YYDDSVK-GRFTISRDNAKNTLYLQMDSLKPEDTALYYC ARGDPY----SDPKY WGQGTQVTVSS
VHH-65    DVQLVESGG-GMVQSGGSLRLSCAAS GFTF----SSYD MSWVRQAPGKGPEWVSY INTG--GGST YYDDSVK-GRFTISRDNAKNTLYLQMNSLKAEDTALYYC ARGDPF----QRPKY WGQGTQVTVSS
VHH-71    DVQLVESGG-GMVQPGGSLRLSCAAS GFTF----SSYD MSWVRQAPGKGPEWVSF INTG--GGST YYDDSVK-GRFTISRDNAKNTLYLQMNSLKPEDTALYYC ARGDPY----SDPKY WGQGTQVTVSS
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VHH-73    DVQLVESGG-GMVQSGGSLRLSCAAS GFTF----SSYD MSWVRQAPGKGPEWVST INTG--GSST YYDDSVK-GRFTISRDNAKNTLYLQMNSLKPEDTALYYC ARGGP-----YSRDY WGQGTQVTVSS
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VHH-78    QVQLQESGG-GLVQPGGSLRLSCAAS GSIF----SILA MGWYRQAPGKQRELVAS VTNG---GST NYADSVK-GRFTISRDNAKNTVYLQMNSLKPEDTAVYYC NVRPY-----SDYDS WGHGTQVTVSS
----------------------------------------------------------------------------------------------------------------------------------------------
VHH-79    QVQLQESGG-GLVQPGGSLRLSCAAS GSIF----SILA MAWYRQAPGKQRELVAS ITNG---GST NYADSVK-GRFTISRDNDKNTVYLLMNSLKPEDTAVYYC NPRPMP---WGTYDY WGQGTQVTVSS
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FIGURE 2
Overview of the protein sequences of the 19 unique CS1-specific VHHs identified. Residue numbering is displayed according to the standard ImMunoGeneTics (IMGT) numbering system (38). Different VHH families are separated by dashed lines. FR, framework region; CDR, complementarity-determining region.

via SPR. Out of the 19 candidates, 17 VHHs were confirmed to bind cell-expressed CS1 in flow cytometry to different extents (Figure 3; Table 1). These findings were confirmed in SPR experiments (Figure 3B), where VHH-2 to VHH-73 showed binding to human CS1 with an affinity range of K_D = 0.33 nM (VHH-61) to K_D = 49.70 µM (VHH-2), as summarized in Table 1. The two non-binding compounds VHH-78 and VHH-79 were identified as murine CS1 (muCS1) binding VHHs in follow-up SPR experiments on plate-coated muCS1 (Supplementary Figure 1) and flow cytometry experiments on muCS1^{pos} 5T33vt cells (Supplementary Figure 2). None of the identified VHHs were cross-reactive for human and murine CS1.

Taken these results, as well as sequence differences and VHH production yields into consideration, a further selection of 10 VHHs was done, i.e., VHHs 2, 6, 17, 29, 53, 57, 61, 63, 71 and 73 (highlighted in bold in Table 1).

3.2 In line with their affinity for CS1, different VHHs target CS1^{pos} MM tumors to a different extent *in vivo*

We next investigated whether the *in vitro* affinity parameters of anti-CS1 VHHs also translated into an *in vivo* tumor-targeting capacity. To this end, the VHHs were site-specifically radiolabeled with ^{99m}Tc on their C-terminal hexahistidine tag. Radioactive compounds were next injected intravenously into subcutaneous MM-bearing (CS1^{pos} OPM2 cells) mice. A 20 min microSPECT/CT scan was performed at 1 h p.i. to visualize the *in vivo* biodistribution in living animals (Figure 4A). At 90 min p.i., animals were sacrificed to quantify radioactive uptake in isolated organs. Tumor uptake ranged from 0.48 ± 0.07% injected activity (IA)/g for VHH-2 to 7.50 ± 1.35% IA/g for VHH-53, with uptake of the non-targeting control VHH-R3B23 being 0.27 ± 0.12% IA/g (Figure 4B).

A statistically relevant elevated tumor uptake (p-value < 0.0001) compared to the non-targeting control VHH-R3B23 was observed for VHH-6 (6.29 ± 1.81% IA/g), VHH-29 (4.75 ± 0.29% IA/g), VHH-53 (7.50 ± 1.35% IA/g), VHH-61 (6.57 ± 0.79% IA/g) and VHH-71 (4.02 ± 0.71% IA/g). Intermediate affinity VHH-6, VHH-29 and VHH-71 (5 nM > K_D > 1 nM) equally show mediocre tumor accumulation, whereas high affinity VHH-53 and VHH-61 (K_D < 1 nM) are the most potent tumor-targeting compounds.

These experiments further revealed that compounds displaying a weaker affinity for the target protein (K_D > 5 nM, as measured in SPR, Table 1), are the ones showing the lowest tumor uptake *in vivo*. This accounts for VHH-2, with a tumor uptake of 0.48 ± 0.07% IA/g (p-value = 0.9995), VHH-17 (1.05 ± 0.50% IA/g, p-value = 0.8001), VHH-57 (0.85 ± 0.01% IA/g, p-value = 0.9526), VHH-63 (1.03 ± 0.45% IA/g, p-value = 0.8173) and VHH-73 (0.52 ± 0.08% IA/g, p-value = 0.9994). These correlations highlight the importance of a strong affinity of the VHHs toward their target antigen to achieve adequate tumor targeting *in vivo*. Equally was the tumor-to-muscle-uptake elevated for VHH-6, VHH-29, VHH-53, VHH-61 and VHH-71, being the intermediate-to-high affinity compounds. Tumor-to-blood-uptake ratios were elevated for VHH-53, VHH-61 and VHH-71 and the tumor-to-bone ratios for VHH-6 and VHH-53 (Table 2). As expected due to the renal clearance route of VHH-sized compounds, accumulation in the kidneys was high.

3.3 The CAR-incorporated VHH has an affinity-independent influence on T-cell activation kinetics

It was next investigated which of these VHHs ensured good T-cell activation when incorporated in a CAR (Figure 1). To that end, 2D3 reporter T-cells were used. These are Jurkat-76 cells that

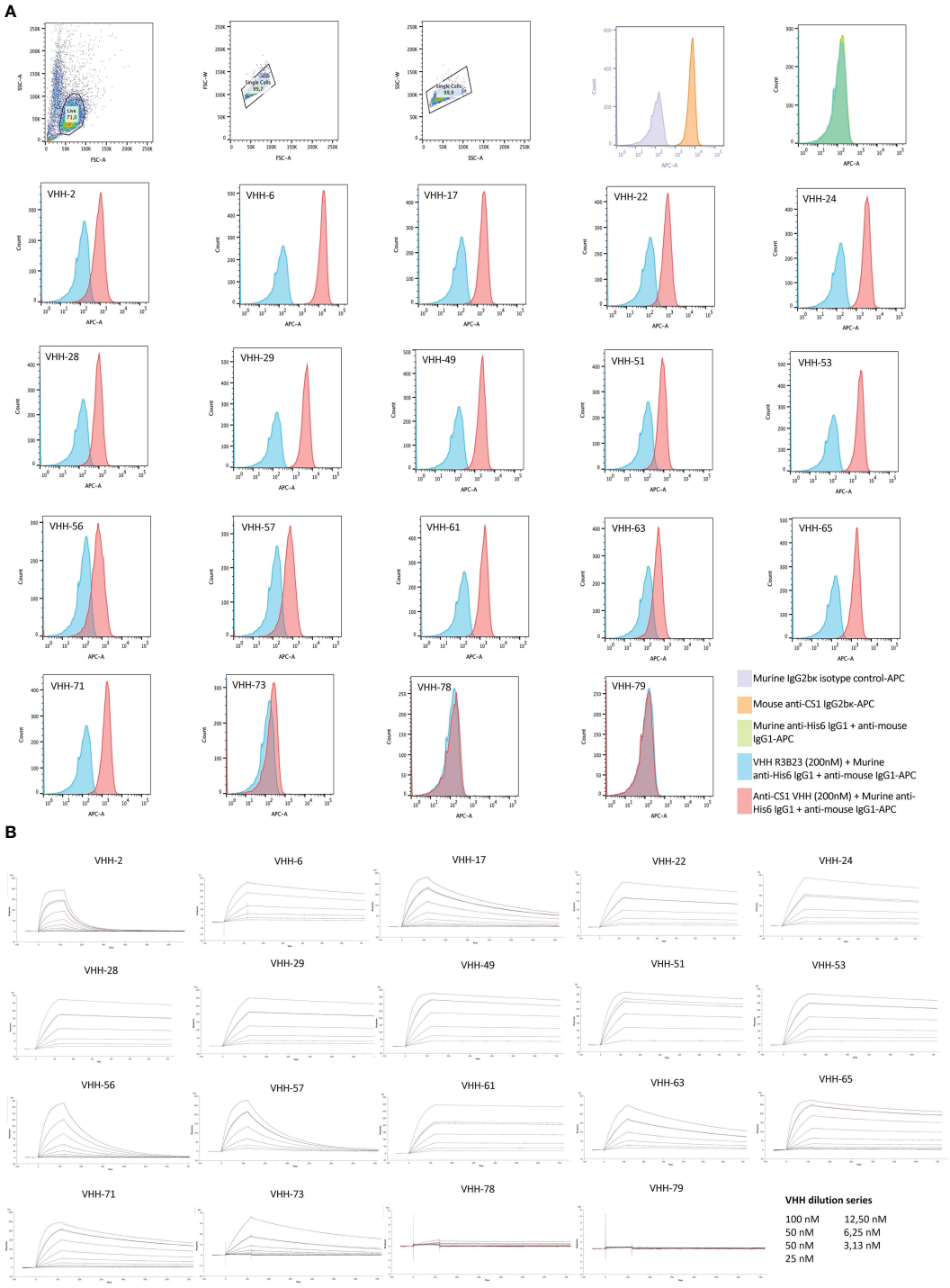

FIGURE 3
Binding characteristics of the selected CS1-specific VHHs. **(A)**: Flow cytometry results to confirm VHH binding to cell-expressed CS1 protein on $CS1^{POS}$ OPM2 MM cells. Cell binding of the CS1-specific VHHs at 200 nM is shown by the red histograms, relative to binding of the non-targeting control VHH R3B23 at 200 nM (blue histograms). CS1 expression by the target OPM2 cells was confirmed with an APC-labeled anti-CS1 mAb (orange histogram), relative to its APC-labeled isotype control mAb (purple histogram). The situation in which no VHH was added to the cells is displayed by the green histogram (n=1); **(B)**: Sensograms showing VHH association and dissociation from a CS1-coated CM5 sensor chip in SPR at different VHH concentrations, in a 1/2 dilution series ranging from 100 nM to 3.13 nM with a duplicate measurement at 50 nM. From this, kinetic binding parameters k_a, k_d and K_D were calculated, using the '1:1 binding with drift and RI2' fitting model in the Biacore T200 2.0 evaluation Software (GE Healthcare), (n=1).

express enhanced green fluorescent protein (eGFP) in response to CD3ζ signaling, as they express eGFP under control of a nuclear factor of activated T-cells (NFAT)-driven promotor. 2D3 cells were stably transduced with the different VHH-CARs (Figure 5A). As flow cytometry staining was performed by addition of biotinylated antigen followed by PE-labeled streptavidin, the observed shift in mean fluorescence intensity (MFI) is affinity-dependent. Hence, the relatively lower shift in fluorescence

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TABLE 1 Summary of the affinity parameters of the produced CS1-specific VHHs.

VHH	ΔMFI [VHH] = 200 nM	K_D (nM)	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})
2	**6.09**	**49.72**	**6.41 x 10^5**	**3.18 x 10^{-2}**
6	**101.30**	**3.80**	**2.10 x 10^5**	**7.90 x 10^{-4}**
17	**13.03**	**9.01**	**3.66 x 10^5**	**2.99 x 10^{-1}**
22	7.29	4.00	1.20 x 10^5	4.80 x 10^{-4}
24	20.61	4.70	1.00 x 10^5	4.70 x 10^{-4}
28	6.58	1.20	2.10 x 10^5	2.50 x 10^{-4}
29	**29.45**	**1.60**	**1.50 x 10^5**	**2.40 x 10^{-4}**
51	14.29	0.60	4.90 x 10^5	2.90 x 10^{-4}
56	5.33	0.94	3.00 x 10^5	2.80 x 10^{-4}
53	**22.09**	**0.81**	**3.70 x 10^5**	**3.00 x 10^{-4}**
56	4.72	36.07	2.79 x 10^5	1.00 x 10^{-2}
57	**4.80**	**24.70**	**3.50 x 10^5**	**8.50 x 10^{-3}**
61	**10.34**	**0.33**	**2.50 x 10^5**	**8.10 x 10^{-5}**
63	**3.37**	**9.80**	**1.90 x 10^5**	**1.90 x 10^{-3}**
65	11.43	2.32	4.23 x 10^5	9.79 x 10^{-4}
71	**11.24**	**4.29**	**3.72 x 10^5**	**1.60 x 10^{-3}**
73	**1.35**	**35.76**	**7.44 x 10^4**	**2.54 x 10^{-3}**
78	2.07	*N.A.*	*N.A.*	*N.A.*
79	0.77	*N.A.*	*N.A.*	*N.A.*

Shift in Mean Fluorescence Intensity (ΔMFI) is determined as the difference in MFI measured in flow cytometry between the CS1-specific VHH at a concentration of 200 nM and the nontargeting control VHH R3B23 at an equal concentration on CS1pos OPM2 cells (n=1). Kinetic binding parameters of VHHs toward plate-coated antigen (k_a, k_d, and K_D) are determined via SPR on recombinant CS1 protein (n=1). VHHs that were selected for further evaluation are indicated in bold. N.A. = not applicable; SPR, surface plasmon resonance.

observed for VHH-2, in line with its inferior affinity for CS1 (Table 1). This method of staining simultaneously confirms the functional expression of the CAR on the surface of the transduced cells, and the appropriate protein folding of the incorporated VHH. The observed transduction rates were confirmed to be stable over time.

Next, the different 2D3-CAR cell lines were co-cultured with either huCS1^{pos} (OPM2) or huCS1^{neg} (JJN3, Figure 5A) MM target cells for a period of 40 h (referred to as the stimulated and unstimulated condition, respectively) and expression of eGFP was monitored in real-time using IncuCyte Zoom Live cell analyses (Figure 5B). It appeared that VHH-2 and VHH-6 were the most potent CAR T-cell activators, while VHH-61, VHH-63 and VHH-29 showed the least antigen-specific eGFP upregulation, *i.e.*, CAR-T cell activation, even though especially VHH-29 and VHH-61 belong to the highest affinity binders toward CS1 (Table 1). This implies that there is no direct link between affinity and CAR-T cell activation potency. As expected, neither untransduced (wild type, WT), nor VHH-CAR-R3B23-transduced 2D3 cells displayed upregulation of eGFP when co-cultured with either of the target cell lines. Also, no upregulation of eGFP was observed in any of the CS1-specific 2D3-CAR cell lines upon co-cultivation with CS1^{neg} JJN3 cells (Supplementary Figure 3).

These results were afterwards confirmed in flow cytometry, where the difference in eGFP expression, measured in terms of MFI in the CAR^{pos} cell fraction, was calculated between the stimulated and the unstimulated condition (ΔMFI). Compared to the non-targeting control cell line 2D3-R3B23 (ΔMFI = 3.33 ± 26.10), only the untransduced 2D3 cell line (ΔMFI = 17.33 ± 50.90) and 2D3-73 (ΔMFI = 114.67 ± 237.58) failed to lead to significant T-cell activation (Figure 5C). All other VHHs proved capable of ensuring CAR-T cell activation (p-values < 0.0001, except for VHH-17), be it to different extent. VHH-6 (ΔMFI = 7064.33 ± 312.62) and VHH-2 (ΔMFI = 6425.67 ± 362.95) showed the most potent activation, confirming IncuCyte observations. These are followed by VHH-71 (ΔMFI = 4752.00 ± 451.60), VHH-29 (ΔMFI = 3575.33 ± 275.65), VHH-53 (ΔMFI = 3326.33 ± 238.55), VHH-57 (ΔMFI = 2731.67 ± 246.73) and VHH-63 (ΔMFI = 2653.00 ± 384.05) that enabled mediocre CAR-T cell activation. VHH-61 (ΔMFI = 1606.00 ± 211.17) and VHH-17 (ΔMFI = 777.00 ± 235.11, p-value = 0.0096) were the least capable of initiating antigen-specific CAR-T cell activation. No direct link between the capability of initiating CAR-T cell activation and any of the specific VHH parameters described above (affinity, cell-binding capacity or *in vivo* tumor-tracing capability) was observed.

3.4 Competition studies suggest a role for the VHH-bound epitope in CAR-T activation, which cannot be confirmed by *in silico* VHH-antigen interaction modeling

Since no direct link was observed between the capacity of 2D3 cell activation by the different VHH-CARs and the affinity, the cell-binding capacity or the *in vivo* behavior of the VHHs, the influence of the VHH-bound epitope was next investigated. To this end, competition co-culture assays were set up in which an excess of a particular soluble VHH was added to the 2D3-VHH-CAR:target cell co-culture. In case the addition of soluble VHH causes a reduction or blockage of another VHH-based CAR-T cell's activation (i.e., competition measured by a lowering of eGFP signal), this reflects their binding capacity for the same epitope.

It was observed that two groups of VHHs could be distinguished, since competition was observed specifically between VHH-2 and VHH-6 in group 1 and between VHHs 17, 29, 53, 57, 61, 63, 71 and 73 in group 2 (Figure 5D). In line, the VHHs belonging to group 1 ensured the highest CAR-T cell

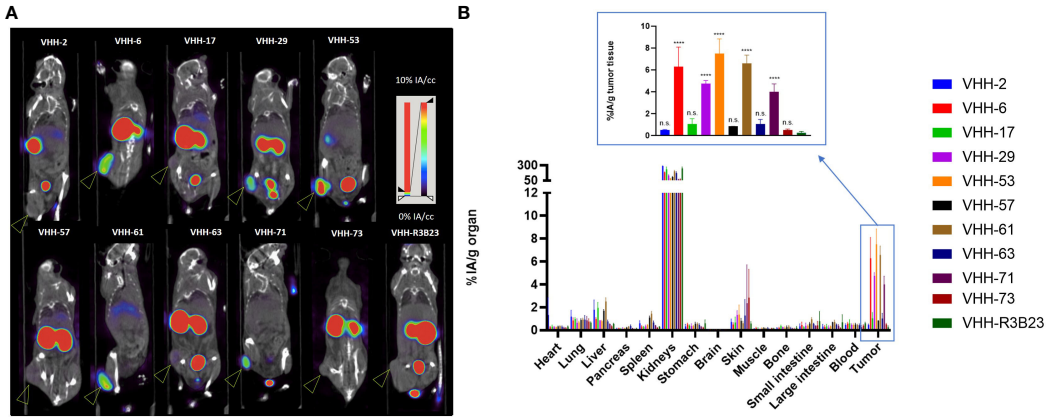

FIGURE 4
Radiolabeled anti-CS1 VHHs target MM tumors in mice to different extents. **(A)**: Representative microSPECT/CT images of tumor-bearing mice at 1 h p.i. with ^{99m}Tc-radiolabeld anti-CS1 VHHs (n = 3). Subcutaneous OPM2 tumors are indicated with an arrow. **(B)**: *Ex vivo* biodistribution data, obtained at 90 min p.i. with ^{99m}Tc-radiolabeld CS1-specific VHHs. Graphs show the mean ± standard deviation (SD) radioactive uptake, normalized to organ or tissue weight and corrected for radioactive decay (n = 3). P-values are determined using one-way ANOVA (multiple t-tests) in which each CS1-specific VHH was compared to the non-targeting control VHH-R3B23. *P< 0.05; **P< 0.01; ***P< 0.001; ****P< 0.0001; not significant (n.s.), P > 0.05.

activation in the CAR activation assay (Figures 5B, C), while the VHHs in group 2 consistently evoked lower activation. These similarities between the VHHs within each group suggest a potential link between the VHH-bound epitope and the capability of CAR-T cell activation.

To verify the hypothesis of the importance of the targeted epitope for CAR-T cell activation, we performed *in silico* modeling of the different VHH-CS1 interactions. For this purpose, the online artificial intelligence-driven self-learning algorithm AlphaFold2 was used, which is able to propose structural protein interactions with a high probability (37, 39), based on sequence information (Figure 6). According to this modeling, CS1 consists of an unstructured intracellular domain, a transmembrane alpha helix and two extracellular domains, of which the membrane-distal unit is responsible for the CS1 autointeraction (Figures 6A, B). The different hexahistidine-tagged VHHs were modeled together with CS1, from which structural information about the binding interaction was retrieved (Figure 6D).

It was assessed whether VHH binding occurred on the membrane-proximal or membrane-distal extracellular domain of CS1 and whether this binding interfered with the CS1 auto-association domain. Furthermore, the distance between the membrane-anchor point of CS1 and the C-terminus of the VHH was calculated, as well as the angle of binding between the VHH-axis and the membrane anchor of CS1 (Figure 6C). These may be relevant activation parameters, as they have a direct influence on the intercellular distance between the CAR-T and the target cell. In line with the kinetic segregation model for classical T-cell activation, it has

TABLE 2 Tumor-to-blood, -bone and -muscle radioactive uptake ratios of anti-CS1 VHHs.

VHH	Tumor/blood		Tumor/bone		Tumor/muscle	
2	1.08 ± 0.02	ns	2.72 ± 0.68	ns	3.32 ± 0.30	ns
6	11.07 ± 3.94	ns	34.83 ± 18.65	*	35.86 ± 19.01	*
17	1.79 ± 0.13	ns	2.67 ± 0.77	ns	8.75 ± 4.71	ns
29	10.17 ± 1.85	ns	17.31 ± 0.29	ns	73.46 ± 32.23	****
53	17.01 ± 6.30	**	45.17 ± 33.39	**	45.63 ± 15.43	**
57	1.68 ± 0.30	ns	3.13 ± 1.02	ns	4.52 ± 1.12	ns
61	14.23 ± 3.07	*	21.30 ± 8.79	ns	39.26 ± 8.85	*
63	2.26 ± 0.36	ns	6.07 ± 3.08	ns	5.49 ± 0.30	ns
71	18.21 ± 13.25	**	25.68 ± 8.52	ns	34.10 ± 7.79	*
73	1.30 ± 0.27	ns	5.97 ± 2.99	ns	3.01 ± 0.01	ns
R3B23	0.44 ± 0.21		1.52 ± 0.94		1.70 ± 0.78	

These values are calculated using the *ex vivo* biodistribution data. P-values are determined using one-way ANOVA (multiple t-tests) in which each CS1-specific VHH was compared to the nontargeting control VHH-R3B23 (n=3). *P< 0.05; **P< 0.01; ***P< 0.001; ****P< 0.0001; not significant (n.s.), P > 0.05.

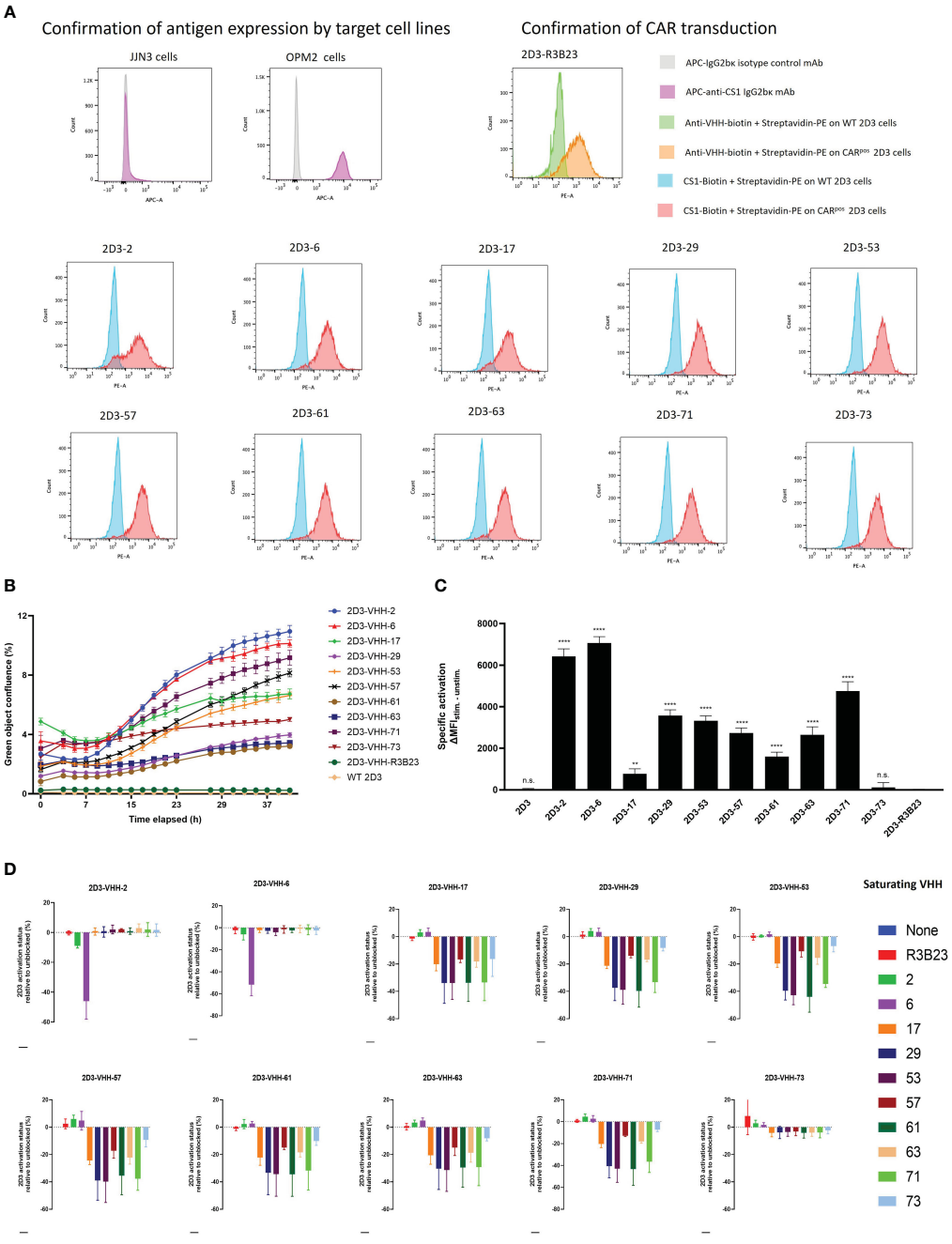

FIGURE 5
CS1-specific VHH-CAR-T cell activation assessment. **(A)**: Confirmation of antigen expression by the target cell lines (JJN3 and OPM2) and CAR-expression by the 2D3 cell lines transduced with the different CS1-specific VHH-CARs and the non-targeting negative control VHH-R3B23-CAR via flow cytometry (n=1). **(B)**: Real-time follow-up of green fluorescence in the IncuCyte Zoom Live-Cell Analysis System of co-cultures of $CS1^{pos}$ OPM2 MM cells with the different CS1-specific 2D3-VHH cell lines, the negative control 2D3-VHH-R3B23 and the wild type (WT) 2D3 cell line at a (1:10) cell ratio. Data are quantified as mean green object confluence (%) ± SD (n=3). **(C)**: CAR-T-cell activation levels as defined by the difference in MFI (ΔMFI) between the stimulated (40 h (1:10) co-cultured with $CS1^{pos}$ OPM2 cells) and the unstimulated (40 h (1:10) co-cultured with $CS1^{neg}$ JJN3 cells) condition, in the CAR^{pos} cell fraction, for each CS1-specific 2D3-VHH-CAR cell line (n=3). Statistical analysis was performed by one-way ANOVA (multiple t-tests), where each CS1-specific 2D3-CAR cell line was compared to the non-targeting control 2D3-CAR R3B23 cell line. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; not significant (n.s.), P>0.05. **(D)**: CAR-T cell activation status (defined by the percentage of eGFPpos cells within the CARpos cell fraction) of 40 h (1:1) 2D3-CAR:OPM2 co-cultured cell lines after pre-incubation of the OPM2 cells with a 1 µM excess of soluble VHH for 1 h at 37°C. Data are quantified in terms of percentage activated cells (i.e., eGFPpos cells within the CARpos cell fraction) ± SD after normalizing to the condition in which no soluble VHH is added to the co-culture (n=3). WT, wild type (untransduced cells); MFI, mean fluorescence intensity.

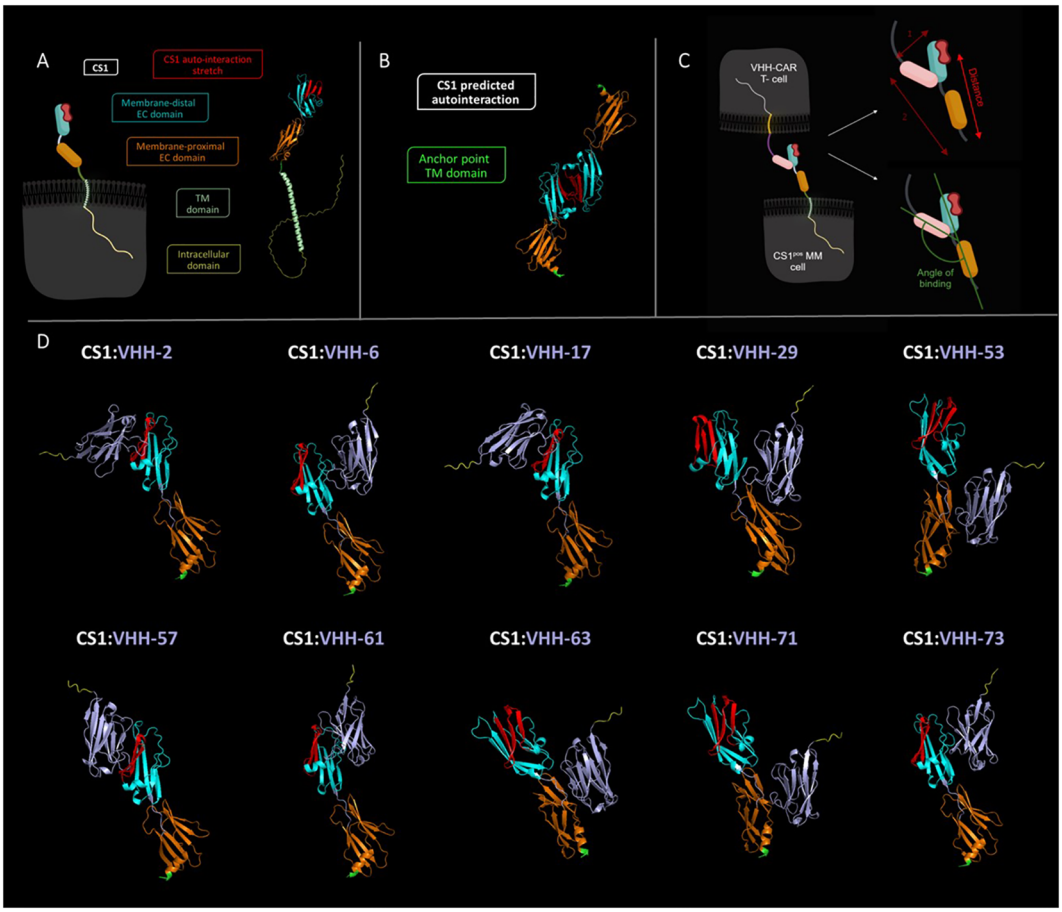

FIGURE 6
In silico predicted structural interaction of the different VHH-CS1 interaction pairs. **(A)**: Left: schematic representation of the cell-expressed CS1 molecule. Right: structural modeling of the CS1 protein. The intracellular part of CS1 is colored yellow, the transmembrane helix is colored in light green, the membrane proximal extracellular domain is shown in orange and the membrane-distal extracellular domain is marked in cyan. The latter contains the predicted protein stretch where CS1 auto-interacts, indicated in red. **(B)**: Structural modeling of the CS1:CS1 auto-interaction. The point where CS1 is membrane-anchored is indicated in green. The membrane-proximal and membrane-distal extracellular domains are displayed in orange and cyan blue, respectively. The auto-interaction protein stretch is colored red. **(C)**: Left: schematic representation of the $CS1^{POS}$ MM cell:VHH-CAR-T cell interaction. Right: Schematic representation of de manner of intercellular distance calculation (top) and of binding angle determination (bottom). The VHH molecule is indicated in pink. **(D)**: Structural modeling of the different VHH-CS1 interaction pairs. VHHs are colored purple with a yellow C-terminal hexahistidine tag. The CS1 membrane-anchoring residue is indicated in green. Modeling was done using the online AlphaFold2 Artificial Intelligence Deep Learning software and images were processed in PyMOL 4.6.0. TM, transmembrane; EC, extracellular. Schematic images shown in panels A and C were created in Biorender.

been hypothesized that this is relevant for adequate activation of a CAR-T cell.

Table 3 summarizes the observations made for the different VHHs. These are listed in order of decreasing 2D3 activation potential (based on the activation values calculated in Figure 5C). Four out of the ten VHHs are predicted to bind the membrane-proximal extracellular domain of CS1, while six bind the auto-interaction domain. From these, three compounds also seem to bind a protein stretch overlapping with the CS1 auto-interaction epitope. Binding distances range from 20.31 Å (VHH-71) to 90.16 Å (VHH-57) and the binding angle varies between 76.3° (VHH-17) and 132.2° (VHH-71). No clear link between either of these parameters and the potential of CAR-T cell activation was observed, nor with the data from the competition assay. While the AlphaFold2 algorithm has already proven its predictive power (37, 39), it should be noted here that this information remains prediction-based.

4 Discussion

In this study, a side-by-side comparison was made of different VHHs, their characteristics and their ability to lead to T-cell activation when incorporated into a CAR. This was evaluated in the context of MM, a highly relevant cancer type for the optimization of CAR-T cell therapy. We opted to target CS1, often mentioned as a promising next-generation MM antigen, and generated a panel of CS1-specific VHHs, which were fully characterized as soluble compounds and as components of VHH-based CARs.

Although the affinity proved to be predictive for the *in vivo* MM tumor-tracing ability of the VHHs to a certain extent, no link was discovered between the affinity and the ability of CAR-T cell activation. Remarkably even, the relatively weak binding VHH-2 proved to be among the most capable CAR-T cell activating VHHs,

TABLE 3 Summary of the observations made by *in silico* modeling of the different CS1-VHH interaction pairs.

VHH	CS1 binding domain	Overlap with CS1 auto-inter-action domain	Binding distance (Å)	Angle of binding (°)	VHH	CS1 binding domain	Overlap with CS1 auto-inter-action domain	Binding distance (Å)	Angle of binding (°)
6	Membrane distal	No	82.58	99.0	57	Membrane distal	Yes	90.16	124.0
2	Membrane distal	Yes	68.79	80.1	63	Membrane proximal	No	26.29	129.5
71	Membrane proximal	No	20.31	132.2	61	Membrane distal	No	89.37	120.2
29	Membrane proximal	No	60.25	109.3	17	Membrane distal	Yes	66.38	76.3
53	Membrane proximal	No	41.63	126.6	73	Membrane distal	No	78.18	95.2

For each VHH, it was estimated whether the membrane proximal or –distal extracellular protein domain of CS1 is bound and whether the epitope of VHH binding coincides with the CS1 autointeraction protein stretch. Binding distances were determined between the membrane-anchor point of CS1 and the C-terminus of the VHH, as projected onto the extracellular CS1-axis. The angle of binding was calculated between the axis of each VHH and the extracellular CS1-axis, as represented in Figure 6C. Results are displayed in order of decreasing CAR-T cell activation potential (as defined by DMFI in the 2D3 activation assay, determined in Figure 5C).

while – in line with its affinity for CS1 -, MM cell binding and *in vivo* MM tumor tracing capacity are inferior. A possible link between the targeted epitope of the VHH and CAR-T activation was suggested by competition CAR-T cell activation studies with excess soluble VHH added to the co-culture, but this relationship could not be confirmed by *in silico* structure modeling of the VHH-antigen interactions. The latter was used to estimate whether the intercellular distance and/or the orientation of CAR-target binding influence the T-cell activation potential. Here too, no cause- and-effect relationship was discovered. Of note is that these conclusions were drawn based on the read-out of an eGFP-based reporter T-cell line.

In particular, we have worked with 2D3 cells to assess the influence of the antigen-recognizing domain on T-cell activation. This is an immortalized cell line that is genetically engineered to specifically map (differences in) T-cell activation kinetics (40, 41). In order to make a critical comparison of different binding domains while excluding all other possible factors- biological or other -, a universal screening platform was required. 2D3 cells served that purpose, as they allow the variation of one CAR component solely. Yet, they come with the limitation that no direct estimate can be made of the *in vivo* therapeutic effect and/or possible cytotoxicity of the designed CARs; which are highly important parameters when a clinical translation of the CAR-T cells is envisaged. To further develop the VHH-CARs for therapeutic purposes, *in vivo* evaluation of the compounds is primarily required. However, to estimate the therapeutic potential of the designed CARs in a highly immune-complex disease such as MM, this must be investigated in clinically relevant animal models. These should be fully immune competent and representative of human disease in terms of tumor cell localization, behavior, disease progression and immune system involvement. Such models exist for murine MM disease (42, 43), but are currently not available for the human variant.

Indeed, by making use of a reporter cell line, our data can only state that it is of utmost importance to evaluate a (sub)library of different antigen-binding compounds side-by-side, while already in the structure of a CAR. Therefore, this should be regarded as a proof-of-concept comparison study, aimed at conveying a fundamental message about the importance of multiple candidate-screening when designing CAR-T cells.

In summary, our data simultaneously demonstrate the importance of carefully selecting the extracellular part of a CAR and the serendipity to which this design is subject. Multiple classical VHH parameters were investigated, including affinity, cell binding potential, *in vivo* behavior, epitope location, intercellular T-cell to target cell distance and orientation of the CAR-antigen interaction. Even though important differences were observed in the extent to which different VHHs can activate a CAR-T cell, none of the VHH parameters investigated had a predictive value for this. Therefore, the classical workflow of VHH selection does not match the needs for optimal VHH-CAR design.

Within the medical context, VHHs are mainly used as smaller, soluble, antigen-specific alternatives for the classically large mAbs (44). Therefore, it is important that the compound shows highly discriminative binding to target tissue versus non-target tissue. When certain conditions are met, such as sufficient (*in vivo*) specificity and (thermo)stability, this is generally obtained by compounds that display a high affinity (ideally < 10 nM) for the (cell-expressed) target protein (44, 45). The general flow of the VHH selection protocol, as described here, is considered standard, both by our research group (27, 30, 35, 46, 47), as by related research groups (48, 49), and other groups in the world (50), as well as by industry (45). It is optimized for finding highly stable, soluble pharmaceuticals with strong affinities for target proteins of all kinds, and has resulted in moieties binding different types of proteins, both extracellular (27, 35, 47) and intracellular (51). In fact, this selection procedure is optimized for identifying and selecting *in vivo* tracers. However, the workflow also contains a few bias points for selecting such soluble compounds. Firstly, the initial phase of the selection is performed on crude periplasmic extract, which automatically filters

for the highly produced and aqua-stable VHHs in ELISA and flow cytometry. Secondly, an off-rate screening creates bias toward high-affinity compounds with a slow off-rate. Thirdly, thermostability assays and production yield measurements, as well as sequence-dependent parameters are often considered when selecting VHHs. These parameters are less relevant when applications such as VHH-based CAR-T cells are intended.

Instead, it is believed that in order to obtain maximal CAR-T cell activation, the formation of a functional immunological synapse between the T-cell and the target cell is important. The kinetic segregation model for T-cell activation states that the balance of presence of kinases (mainly Lck) versus dephosphorylases (mainly CD45) in the T-cell:antigen-presenting cell interaction zone (immunological synapse) is determinant for TCR downstream signaling and thus for T-cell activation. This balance is mainly influenced by spatial restrictions that appear upon T-cell:target cell interactions.

CD45 enzymes are abundantly present on the membrane of resting T-cells, resulting in sufficient dephosphorylation of the TCR immunoreceptor tyrosine-based activation motifs (ITAMs) to maintain a basal, inactive T-cell status. However, these molecules are relatively bulky and are excluded from the immunological synapse upon TCR-pMHC (peptide major histocompatibility complex) and CD58-CD2 cell adhesion interactions. Consequently, the ITAM phosphorylation equilibrium is outbalanced, and T-cells become activated as a result of downstream intracellular phosphorylation events (52, 53).

This model highlights the importance of the formation of a spatially correct immunological synapse, which is equally relevant in CAR-T cells, as similar signal transduction pathways are determinant for T-cell activation (53). The immunological synapse has been described to have an ideal intermembrane distance of around 15 nm in the center (where TCR-pMHC interactions are mainly taking place) to 100 nm at the edges, where granules and cytokines are exchanged and receptor-ligand pairs are the main interaction molecules (54). As the VHH molecule is anchored to the CAR via a (large) flexible linker, it is difficult to make a concrete estimate of the effective intercellular distance. However, *in silico* modeling of the various VHH-CS1 interactions shows that there are relevant differences between different VHH-CAR:CS1 pairs at this level. These observed differences did not translate directly to differences in CAR-T cell activation potential, and therefore it was assumed that neither location of binding, nor VHH binding orientation are predictive parameters for CAR-T cell activation.

These conclusions are based on sequence-driven computational estimates for the protein structures and interactions. It should be noted here that these are artificial intelligence-based structure interaction predictions, which are not supported by experimental data. Although the AlphaFold2 algorithm has already proven its predictive power for more evolutionary conserved protein interactions (37, 39), it is noteworthy that the program is considered slightly less powerful and therefore less trustworthy for the prediction of antigen-antibody binding. This is because less evolutionary background (on which the modeling is based) is available for these types of interactions (55). As these data do not coincide with the experimental data retrieved from the competition activation assay, one might argue on the accuracy of these modeled interactions. To precisely know the orientation of interaction, experimental data on the interaction structure would be necessary, which could for example be obtained via X-ray crystallography. This is however a technically challenging method that is not recommended to screen complete VHH libraries. All in all, our data demonstrate that the VHH:antigen interaction, as currently computationally modeled, has no predictive value for the VHH-CAR-T cell activation potential. Whether this is due to a lack of accuracy of the prediction or due to a missing link between binding orientation and activation potential, remains an open question.

Within the immunological synapse, the affinity of a TCR for its target pMHC molecule is relatively low, i.e., 1 µM to 100 µM. As such, cells with low target pMHC expression do not trigger T-cell activation and T-cell overstimulation (which may lead to premature exhaustion) is avoided (56). CARs harboring a mAb-derived scFv usually have a (sub)nanomolar affinity for their target (57). This while various studies have shown that an unnecessary increase in affinity of a CAR is more likely to lead to higher toxicity and a shorter T-cell life span (56–58).

Chmielewski and colleagues have identified a ceiling of $K_D = 10^{-8}$ M, below which the affinity no longer contributes to enhanced T-cell function (58). It has also been reported that lower affinity CARs are more capable of correctly distinguishing cancerous tumor antigen-overexpressing cells from healthy cells with normal expression levels of a tumor antigen (59). However, this is a balance of sensitivity and selectivity in which (the degree of) antigen expression is also important. Indeed, different studies in different contexts show different results. For example, an optimal CAR affinity in the micromolar range was found for target antigens ICAM-1, CD38 and HER2 (56, 60, 61). Meanwhile, CARs with a nanomolar affinity appeared optimal for tumor antigens EGFR, EGFRvIII and CD123 (62–64). This of course is also related to the avidity of the CAR-antigen interaction, which is subject to not only the affinity, but also to the expression levels of both the CAR on the T-cell surface and the antigen on the cancer cell. Clinical data suggest that the importance of generating an interaction with adequate avidity may prevail over attaining a desired affinity (65).

In any case, these studies highlight the importance of case-by-case fine-tuning of the affinity of a CAR for its target antigen, and the advantage of using the weakest possible affinity binder that is still sufficiently sensitive. Moreover, the balance between selectivity and sensitivity will be even more important in solid tumors, where antigen expression often also occurs on vital healthy tissue. In a hematological context, antigen expression is more likely to be limited to hematopoietic cell lineages, rendering selectivity less critical. All in all, these studies demonstrate that the antigen-binding portion of a CAR has a major impact on its functionality, and that case-by-case selection is needed. When classical scFvs are incorporated, this is less obvious, as they are usually adopted from existing and clinically validated mAbs. Furthermore, the lack of (immune) libraries makes it less straightforward to evaluate multiple scFvs side-by-side.

VHHs have this advantage over scFvs, as they are monomeric by nature and selection usually starts from relatively small immune libraries. Furthermore, VHH-based CARs have already proven their functionality in the clinic, in particular in the context of MM. In the 2022 FDA-approved ciltacabtagene autoleucel (cilta-cel) CAR, antigen-recognition is ensured by two (bi-epitopic) BCMA-

specific VHHs (66). This is the second clinically approved CAR-T cell product for the treatment of MM. Idecaptagene vicleucel (ide-cel), also directed against BCMA, was previously approved. In the latter, antigen recognition is provided by a classic scFv format.

Despite the high initial success rates of these therapies, MM relapse remains an important problem, mainly caused by BCMA shedding. These findings demonstrate the power of CAR-T cell therapy for MM, but also its unfulfilled potential due to suboptimal choice of antigen. Therefore, other target antigens are under investigation. Of these, CS1 is of particular interest due to its retained expression after several lines of therapy and its expression pattern that is limited to hematological cell (sub)types. Several CS1-directed CAR-T cell therapies are under investigation, both in a preclinical and clinical stage (11, 67).

Our study combines both optimization points of current CAR-T cell research for MM. In particular, we use a CAR directed against the promising target CS1, and achieve this using a VHH molecule selected out of a library as being optimal in the tested CAR design. Furthermore, we have developed several CS1-specific VHHs that may be complementary to each other. Indeed, antigen expression throughout therapy is an important parameter for its success rate. The combination of therapy and diagnostic imaging is therefore an interesting concept that is increasingly gaining attention in the field of personalized medicine. Since VHHs are ideally suited as radiodiagnostic tools, it would be possible to monitor the status of CS1 expression before and during CS1-targeted (CAR-T cell) therapy. To this end, however, it is important that competition for target binding between the diagnostic and the therapeutic tool is avoided. Thus, compounds that bind a different epitope are needed. Our data lend themselves ideally to such a scenario, as the compounds that perform well *in vivo* do not necessarily correspond to those that provide good CAR-T cell activation, and two different compounds can be optimized according to their intended use.

Finally, it is important to note that although we demonstrate the importance of the incorporated VHH for T-cell activation, this is not the only point of optimization of CAR design and several other factors that may affect CAR-T cell behavior are under investigation (68). These include the other CAR subunits, such as the hinge and transmembrane region (69), as well as the incorporated co-stimulatory molecule(s) (70). The best-known CARs to date are second generation, implying that a single co-stimulation domain is incorporated, mostly 4-1BB or CD28-derived (9, 68). For example, third-generation CARs harbor a combination of two co-stimulatory domains and are currently under clinical evaluation (71). Fourth-generation CARs seek their added value through the local secretion of immune stimulatory cytokines, mainly IL-12 (72).

Another potential influencing parameter that is increasingly gaining attention is the composition and phenotype of the harvested and infused T-cells. As such, the ideal composition of $CD4^+/CD8^+$ T-cell fractions and the added value of eliminating regulatory T-cells are important fields of research (73, 74). Additionally, data show that younger, more stem cell-like phenotypes of T-cells may exhibit superior *in vivo* efficacy, owing to reduced or delayed exhaustion (75, 76). Furthermore, there are many variables in *ex vivo* modification protocols, genetic CAR constructs and manner of cell modification that might influence T-cell behavior (68). In particular, the benefit of reducing CAR-T cell manufacturing time is also gaining interest, as this may not only save patients with rapidly progressing disease and reduce overall costs, but could also potentially be of therapeutic added value, as reduced culturing time might result in a younger (i.e., more stem cell-like) T-cell phenotype (68, 77, 78).

All in all, there are many different variables that determine the behavior and efficacy of CAR-T cells. Our data show that although it has historically received little attention, the antigen-binding component certainly is one of them and that adequate screening is needed.

5 Conclusion

Taken together, our results demonstrate that the antigen-binding part of a CAR has a major influence on its functionality, as well as on the activation kinetics of modified CAR-T cells. This emphasizes the importance of selecting, or in the case of scFvs, optimizing this protein domain. We scrutinized several parameters of VHHs trying to link them – without success-, to a superior CAR-T cell response. Consequently, CAR-T cell research remains subject to serendipity and case-by-case selection of the optimal antigen-binding moiety is still needed. Hence, the importance of screening multiple candidate binding domains directly in a CAR configuration cannot be underestimated. In this regard, VHHs have an important advantage over mAb-derived scFvs, due to the easy availability of immune libraries.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Materials. Further inquiries can be directed to the corresponding author.

Ethics statement

Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used. The animal study was approved by ethical committee for use of laboratory animals, Vrije Universiteit Brussel. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

HH: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Software, Visualization, Writing – original draft. FM: Data curation, Formal analysis, Investigation, Writing – review & editing. YDV: Conceptualization, Data curation, Formal analysis, Methodology, Writing – review & editing. QL: Conceptualization, Data curation, Methodology, Writing – review & editing. JP: Methodology, Writing – review & editing. PD: Data curation, Investigation, Methodology, Writing – review & editing. TDG: Data curation, Methodology, Software, Writing – review &

editing. CG: Investigation, Writing – review & editing. KDV: Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Writing – review & editing. KB: Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Writing – review & editing. ND: Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1389018/full#supplementary-material

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Hemophagocytic lymphohistiocytosis/cytokine release syndrome secondary to neoadjuvant pembrolizumab for triple-negative breast cancer: a case study

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As indications for immune checkpoint inhibitors for breast cancer continue to expand, rare toxicities will emerge that require careful consideration and multidisciplinary management. We report the case of a 40-year-old female receiving neoadjuvant pembrolizumab and chemotherapy for locally advanced triple-negative breast cancer who developed cytokine release syndrome (CRS)/hemophagocytic lymphohistiocytosis (HLH). CRS/HLH secondary to pembrolizumab are scarcely documented in the literature and, to our knowledge, have never been reported in the context of neoadjuvant treatment for breast cancer.

KEYWORDS

breast cancer, immunotherapy, toxicity, cytokine release syndrome, hemophagocityc lymphohistiocytosis

Introduction

Triple-negative breast cancer (TNBC) is an aggressive malignancy associated with poor prognosis and high risk of early relapse (1). TNBCs are heavily infiltrated by immune cells (2), and a high tumor-infiltrating lymphocyte count has been associated with improved survival (3–5), providing a biological rationale for the use of immune checkpoint blockade in TNBC. Pembrolizumab is a monoclonal antibody that blocks programmed death (PD)-1, resulting in improved anti-tumour activity by tumor-infiltrating T lymphocytes. The practice changing phase III Keynote-522 trial investigated the addition of pembrolizumab

to neoadjuvant chemotherapy for patients with early stage TNBC and found improved pathologic complete response rates (pCR 64.8% vs. 51.2%) and event-free survival (84.5% vs. 76.8% at 36 months) when compared to chemotherapy alone (6–8). Pembrolizumab in combination with chemotherapy has also become standard of care for patients with metastatic TNBC and combined positive score >10 based on the Keynote-355 data (9).

As the number of patients treated with immune checkpoint inhibitors (ICIs) in the curative and palliative setting for TNBCs grows, rare toxicities will emerge that require careful consideration and multidisciplinary management. Cytokine release syndrome (CRS) and hemophagocytic lymphohistiocytosis (HLH) secondary to pembrolizumab are scarcely documented in the literature and, to our knowledge, have never been reported in the context of neoadjuvant treatment for breast cancer. HLH is a hyperinflammatory syndrome that occurs due to an overactivated immune response (10). While the primary form of this disease occurs in children, secondary HLH can occur in the context of cancer, infection or autoinflammatory disorders (10). CRS is an excessive systemic immune response characterized by the release of cytokines such as interleukin-6 (IL-6), interferon gamma, tumor necrosis factor alpha, IL-2, and IL-10 by large numbers of activated lymphocytes (11). We present the case of a 40-year-old patient who developed HLH/CRS after exposure to pembrolizumab in the neoadjuvant setting for TNBC.

Case report

A 40-year patient initially presented to her family physician with left axillary tenderness. A mammogram demonstrated a solid mass with irregular margins in her left breast with multiple left axillary lymph nodes, and biopsy confirmed TNBC. She underwent further staging imaging with a bone scan, computed tomography (CT) neck, chest, abdomen and pelvis and magnetic resonance imaging (MRI) breast. Imaging demonstrated locally advanced disease with nodal involvement but no distant metastases, and she was staged as cT2N2. Multidisciplinary cancer conference consensus was to proceed with curative intent treatment, and she was initiated on the Keynote-522 regimen.

She completed four cycles of treatment with carboplatin, paclitaxel, and pembrolizumab. She had a single episode of non-neutropenic fever, a mild intermittent localized rash affecting the face treated with topical corticosteroid, and a rhinovirus infection resulting in a 1-week delay. After cycle 4, she developed a right eye chemosis and was seen urgently by ophthalmology. This was deemed to be unrelated to immunotherapy and was treated with lubricating eye drops. She then proceeded with the first cycle of doxorubicin, cyclophosphamide, and pembrolizumab.

Eight days later, our patient presented to her local emergency room with fevers. She was found to be neutropenic, admitted to hospital, and treated with antibiotics and acyclovir. She developed an acute kidney injury, with a creatinine that initially increased to 118 μmol/L and rapidly progressed (Table 1). This was thought to be secondary to hypovolemia, antibiotics, and antivirals. She received granulocyte-colony stimulating factor (G-CSF) with improvement in her neutrophil count, defervescence, and clinical improvement. However, after 6 days of antibiotics, she developed recurrent fevers over 40°C and became tachycardic. Her antibiotics were broadened, septic workup was repeated, and immune toxicity workup was suggested by medical oncology on call. She then had sudden deterioration ten days into her hospital admission resulting in a pulseless electrical activity arrest, associated with rapidly evolving multiorgan failure including hepatitis, renal failure, respiratory failure, and a rapidly progressive purpuric and tense bullous rash that started peripherally and spread centrally (Figure 1). After resuscitation, she was admitted to intensive care where she was intubated, sedated with propofol, and commenced on vasopressor support. A bedside echocardiogram was normal. After consulting with medical oncology, she was started on pulse dose methylprednisolone and, once stabilized, she was transferred to the intensive care unit for continuous renal replacement therapy (CRRT) and ongoing management.

At the time of her transfer, the differential diagnoses included Stevens-Johnson syndrome (SJS), drug rash with eosinophilia and systemic symptoms (DRESS), and immunotherapy-related toxicity. She was then found to have an elevated ferritin (>120,000) and triglycerides (11), raising the possibility of HLH. Her viral investigations were negative. Dermatology, hematology, medical oncology, infectious diseases, and rheumatology were consulted. Her H-Score demonstrated a 98%–99% probability of HLH, and she was started on the HLH-2004 protocol with etoposide. High dose methylprednisone 1g daily was continued. Bone marrow biopsy did not demonstrate clear cytologic evidence of active phagocytosis, though did demonstrate histiocytes containing residual cellular debris, leading to some disagreement amongst our pathologists regarding whether there was evidence of hemophagocystosis. Skin biopsy demonstrated thrombotic vasculopathy, and dermatology felt that her rash was not secondary to SJS or DRESS. Bullous pemphigoid was also ruled out [direct immunofluorescence (DIF) negative]. The HLH protocol was continued as her ferritin, C-reactive protein (CRP), liver enzymes, and renal function improved, and her respiratory status stabilized. Her methylprednisolone dosing was decreased to 100 mg/day, and then further weaned to 80 mg/day. After the third dose of etoposide, she developed profound pancytopenia, and the fourth dose was held. She was recommenced on G-CSF and was given both platelet and red cell transfusions. Throughout, she required extensive wound care related to the bullous rash, which ultimately resulted in desquamation covering approximately 50% of her body (Figure 1).

Unfortunately, 13 days after the initiation of the HLH-2004 protocol, our patient began to clinically worsen. She became febrile, required increased ventilator support, and her CRP began to rise rapidly (from a low of 28 mg/L to a peak of 242 mg/L over 4 days). Cultures were obtained and, while pending, she was treated with tocilizumab due to ongoing consideration of cytokine release syndrome. She received two doses (8 mg/kg), 24h apart. Eventually, one of four blood cultures was positive for *Candida lusitaniae*, and she was started on casopfungin, later narrowed to fluconazole. Her clinical status improved, she had no further fevers, and there was rapid improvement in her CRP which fell from a peak of 267 mg/L to 8 mg/L in the 3 days following tocilizumab.

TABLE 1 Laboratory parameters over time.

Lab value	*Reference value*	Admission	PEA arrest day	HLH-2004 D1	HLH-2004 D4	HLH 2004 D8	Pre- Toci dose 1	Pre- Toci dose 2	Post- Toci	Discharge
		Day 0	Day 10	Day 13	Day 16	Day 20	Day 27	Day 28	Day 30	Day 48
Ferritin	*4–205 μg/L*			128,619	20267	4573	3213	3627	3949	3237
LDH	*120–315 U/L*			5210	559	414		188		359
CRP	*0–1 mg/L*		317.1	312.6	98.4	28.0	242.6	267.6	28.3	0.4
AST	*10–35 U/L*			1186	64	84	13	8	15	33
ALT	*8–40 U/L*	31	29	615	124	131	50	37	40	127
Cr	*0–85 umol/L*	91	379	531	211	163	123	128	122	111
WBC	*4.0–11.0 x10 9/L*	1.18	6.33	17.5	23.0	5.8	0.5	0.7	6.2	16.0
Neut	*2.0–7.50 x 10 9/L*	0.11	2.91	14.13	22.36	4.78	0.02	0.05	3.00	13.87
Hb	*115–155 g/L*	86	79	64	78	78	87	84	87	85
Plt	*150–450 x10 9/L*	83	101	50	66	64	11	15	34	119

If the lab value of interest was not available for a specified timepoint but a value within 1–2 days was available, this is provided in place.
PEA, pulseless electrical activity; LDH, lactate dehydrogenase; CRP, C-reactive protein; AST, aspartate transaminase; ALT, alanaine transaminase; Cr, creatinine; WBC, white blood cells; Neut, neutrophils; Hb, hemaglobin; Plt, platelets; Toci, tocilizumab.

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She was eventually able to come off CRRT and was extubated 23 days after her initial intubation. She was transferred to the ward, where she continued to receive extensive wound care and was eventually discharged home eight weeks after her initial presentation to hospital. Follow-up CT chest, abdomen and pelvis, and MRI breast have demonstrated a complete radiographic response of the breast cancer and eventual resection showed a complete pathologic response. Complete timeline of the events is found in Figure 2.

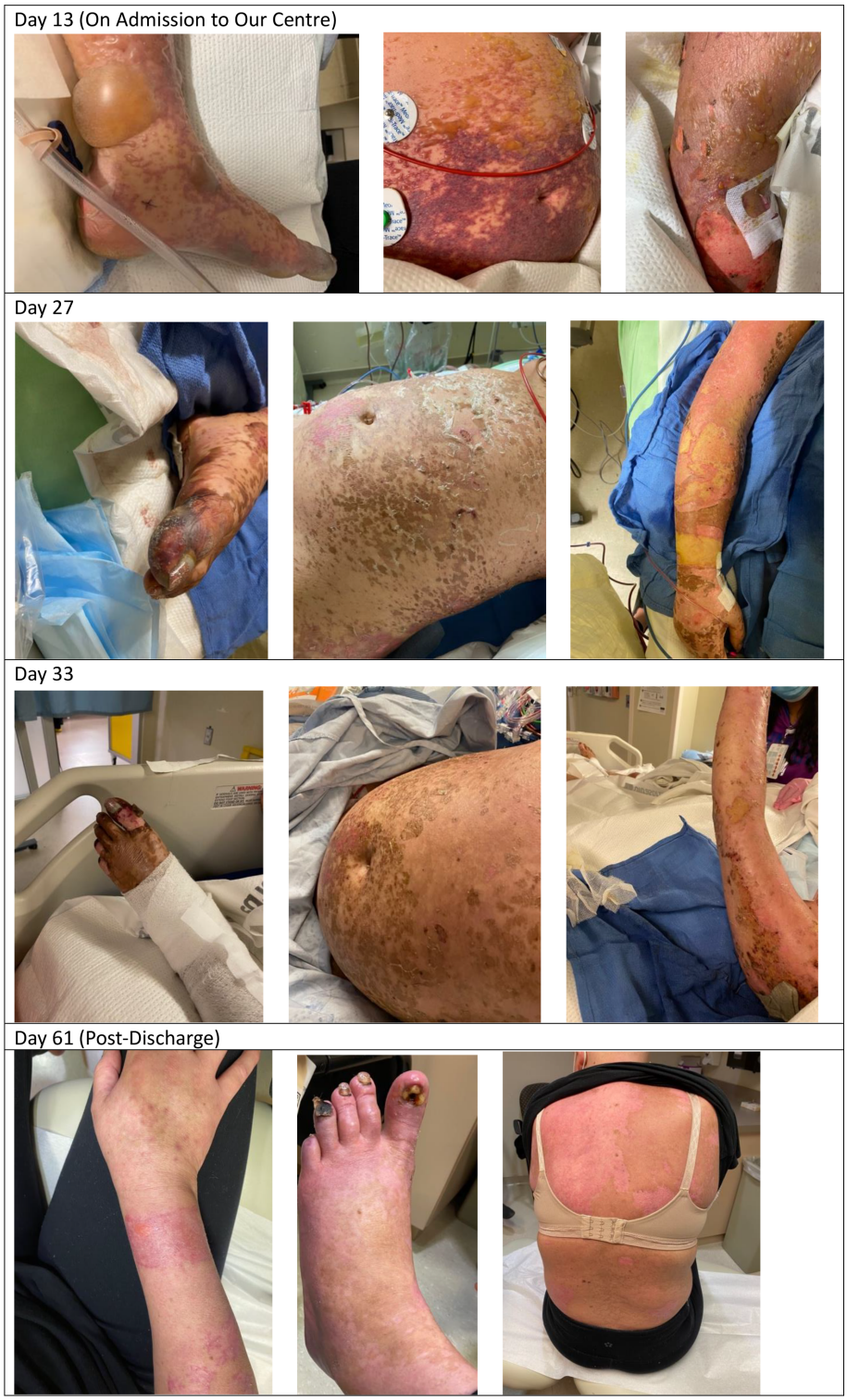

FIGURE 1
Dermatological toxicities over time.

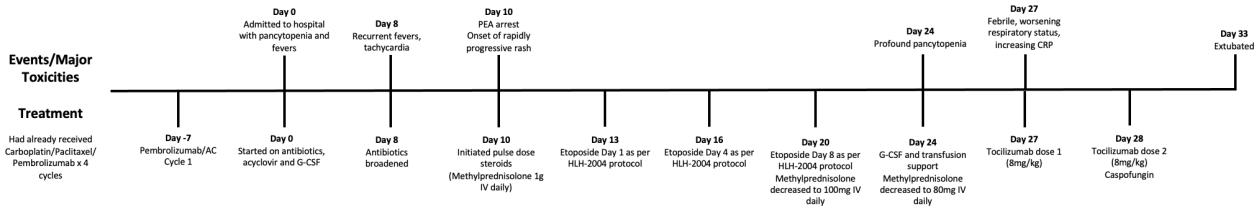

FIGURE 2
Timeline of events.

Discussion

CRS and HLH have overlapping clinical and biochemical features, which can confound the diagnosis. Immunotherapy has been implicated as a potential cause of both syndromes. Common clinical features include fever, malaise, hypotension, hypoxia, and end organ toxicity. Overlapping biochemical and laboratory findings include elevated creatinine, transaminitis, and elevated inflammatory markers including CRP. Moreover, a CRS-variant with HLH-like manifestations is recognized in patients receiving CAR T cells (12). CRS and HLH are serious complications from immunotherapy, with fatal outcomes occurring in approximately 10% of patients (13).

Although CRS and HLH are more associated with chimeric antigen receptor T-cell therapy and bispecific T-cell engagers, a growing number of published case reports describe CRS and HLH secondary to ICIs (14–32). In a 2020 analysis of the World Health Organization global database of drug-related adverse events, there were 43 reported cases of CRS associated with PD-1/PD-L1 therapy (33). A systematic review of hyperinflammatory syndromes such as CRS and HLH from ICI identified 49 articles and 189 patients and found that pembrolizumab was the most commonly implicated ICI (13). Most case reports have been in the context of metastatic disease, and none have been documented in patients receiving neoadjuvant treatment for breast cancer.

The diagnostic criteria for HLH requires five of eight of the following: fever, splenomegaly, cytopenias affecting greater than two of three lineages, hypertriglyceridemia and/or hypofibrinogenemia, hemophagocytosis in the bone marrow, spleen or lymph nodes in the absence of malignancy, low or no NK cell activity, ferritin greater than 500 μg/L and a soluble IL-2 (sCD25) greater than 2400 U/mL (34). Very high ferritin levels often lead clinicians to consider the diagnosis of HLH, and a ferritin > 10,000 μg/L reportedly has a sensitivity of 90% and a specificity of 96% for macrophage activation syndromes/HLH (35). However, ferritin may also be elevated in the context of sepsis and critical illness, although typically not to the same degree (36). Moreover, CRS may also cause hyperferritinaemia (19); the degree of ferritin elevation appears to correlate with the severity of CRS (37). The elevated triglycerides in this case also increased the H-score. Propofol, which was used for sedation in this patient, can cause elevated triglycerides (38) and could have further confounded the diagnosis. To try and clarify the diagnosis, a soluble CD25 level prior to steroid administration was ordered but not resulted due to limitations on how long samples are held at peripheral hospitals. The uncertainty regarding the presence of hemophagocytosis on the bone marrow further complicated the diagnosis. Although hemophagocytosis is not required for a diagnosis of HLH, it may have aided in distinguishing between HLH and CRS (10, 39).

Despite the clinical, biochemical, and diagnostic overlap of CRS and HLH, the two entities are, in theory, treated differently. HLH is classically treated using the HLH-2004 protocol, which combines IV etoposide and high-dose dexamethasone (40). In contrast, depending on the grade of CRS, tocilizumab and high-dose corticosteroids are the recommended treatments (41). However, in the context of CAR-T–associated HLH, the CAR-T-cell-therapy-associated TOXicity (CARTOX) Working Group have suggested patients be initially managed as per the CRS pathway with anti-IL-6 therapy and high-dose steroids. If there is no improvement after 48h, consideration should then be given to treating with etoposide as per the HLH-2004 protocol (42). Similar treatment sequencing may be beneficial in patients developing HLH/CRS overlap syndromes in the context of ICI.

In reviewing cases of HLH secondary to immunotherapy, we found variability in management. Some patients were treated with the HLH-2004 protocol (22, 27, 31, 43, 44), while others were treated with corticosteroids alone (23, 24, 26, 28, 32, 35, 45–47). Some centers utilized combinations of corticosteroids and other agents such as intravenous immunoglobulin (IVIG) (29, 48), mycophenolate mofetil (MMF) and cyclosporin (30), anakinra (29, 49), tocilizumab (31, 50), and infliximab (25). The management of CRS from immune checkpoint inhibition also varied among published case studies, from high-dose steroids alone (16, 17, 20, 21) to combination therapy with steroids, MMF, plasma exchange, and IVIG (18). Given the diagnostic uncertainty in our case, and given the biochemical and clinical improvement seen after the initiation of the HLH protocol, treatment was continued; however, high-dose steroids were also continued to manage possible CRS. With subsequent deterioration, IL-6–directed therapy was instituted with tocilizumab and, in parallel, antifungals were administered. It remains unclear whether this second improvement was predominantly due to tocilizumab or appropriate management of fungemia.

The cutaneous involvement in this case was unusual. Our patient's severe bullous rash began approximately 9 days after she initially presented with fevers. In the literature, there are seven case reports describing ICI-induced HLH with an associated rash. Four of the HLH case reports documented a maculopapular rash (22, 23, 43, 49). Sasaki et al. (46) reported pembrolizumab associated HLH with an erythema multiforme-like, full body rash. Choi et al (48) described a pruritic truncal rash that progressed to papules and erosions, and eventually peripheral and oropharyngeal bullae, but skin biopsies from this case were consistent with SJS. There are two

documented cases of CRS from ICI with an associated rash. Tsutsui et al. (20) described CRS secondary to ipilimumab and nivolumab with a progressive rash that started on the patient's neck and spread peripherally with mucosal involvement. A skin biopsy was consistent with toxic epidermal necrolysis. Amlani et al. (15) describe a patient who developed a purpuric eruption on his legs after ICI, which progressed to his entire body.

Our case was also unusual given the curative intent of treatment. Adjuvant or neoadjuvant ICI is currently approved for use in TNBC (7), melanoma (51), Bacillus Calmette-Guerin (BCG) unresponsive non-muscle invasive bladder cancer (52), non-small cell lung cancer (53), renal cell carcinoma (54), esophageal cancer (55), and is being investigated in many other disease sites. Recent positive data using neoadjuvant immunotherapy for patients with estrogen receptor–positive breast cancer, including Keynote-756 (56) and Checkmate-7FL (57), may further expand the number of eligible patients. ICIs in these patient populations will pose unique challenges, including possible exposure of younger, curative intent patients to potentially life-threatening and long-term side effects.

Conclusion

This is the first case report in the literature of HLH/CRS in a patient with breast cancer being treated with curative intent. As ICI indications expand further into the adjuvant and neoadjuvant population, careful consideration must be given to rare but potentially life-threatening complications in patients receiving curative therapy. Prompt recognition and early collaboration with specialists is vitally important to avoid fatal outcomes.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by Queen's Research and Ethics Board. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for publication of this case report was provided by the patient. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

LP: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. BM: Writing – review & editing, Methodology, Investigation. PH: Writing – review & editing, Methodology, Investigation. TH: Writing – review & editing, Methodology, Investigation. BW: Writing – review & editing, Supervision, Resources, Methodology, Investigation, Conceptualization.

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Conflict of interest

BW reports honoraria from AstraZeneca and Novartis, and grant funding from Bristol Myers Squibb (BMS) unrelated to this work.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CXCR4 has a dual role in improving the efficacy of BCMA-redirected CAR-NK cells in multiple myeloma

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Multiple myeloma (MM) is a plasma cell disease with a preferential bone marrow (BM) tropism. Enforced expression of tissue-specific chemokine receptors has been shown to successfully guide adoptively-transferred CAR NK cells towards the malignant milieu in solid cancers, but also to BM-resident AML and MM. For redirection towards BM-associated chemokine CXCL12, we armored BCMA CAR-NK-92 as well as primary NK cells with ectopic expression of either wildtype CXCR4 or a gain-of-function mutant CXCR4^{R334X}. Our data showed that BCMA CAR-NK-92 and -primary NK cells equipped with CXCR4 gained an improved ability to migrate towards CXCL12 *in vitro*. Beyond its classical role coordinating chemotaxis, CXCR4 has been shown to participate in T cell co-stimulation, which prompted us to examine the functionality of CXCR4-cotransduced BCMA-CAR NK cells. Ectopic CXCR4 expression enhanced the cytotoxic capacity of BCMA CAR-NK cells, as evidenced by the ability to eliminate BCMA-expressing target cell lines and primary MM cells *in vitro* and through accelerated cytolytic granule release. We show that CXCR4 co-modification prolonged BCMA CAR surface deposition, augmented ZAP-70 recruitment following CAR-engagement, and accelerated distal signal transduction kinetics. BCMA CAR sensitivity towards antigen was enhanced by virtue of an enhanced ZAP-70 recruitment to the immunological synapse, revealing an increased propensity of CARs to become triggered upon CXCR4 overexpression.

Unexpectedly, co-stimulation via CXCR4 occurred in the absence of CXCL12 ligand-stimulation. Collectively, our findings imply that co-modification of CAR-NK cells with tissue-relevant chemokine receptors affect adoptive NK cell therapy beyond improved trafficking and retention within tumor sites.

KEYWORDS

BCMA, chimeric antigen receptor, NK cells, multiple myeloma, chemokine receptor CXCR4, adoptive T cell therapy

Introduction

Chimeric antigen receptor (CAR)-T cell immunotherapy has emerged as an effective therapeutic option for relapsed and refractory multiple myeloma (MM) (1, 2). Results from the pivotal KarMMa (3) and CARTITUDE-1 (4, 5), studies have demonstrated the efficaciousness of BCMA CAR-T cell therapy to induce deep remissions for heavily pretreated MM patients. Nevertheless, the majority of recipients eventually relapse (3, 4, 6).

Mechanisms of acquired resistance to BCMA CAR-T cell therapy include immune escape due to target antigen loss. The exact mechanism for immune escape is more complex than previously reported for CD19 CAR-T cells since complete BCMA antigen loss is less frequent (7, 8). Other CAR-T cell- and microenvironmental-related factors for treatment failure include CAR-T cell exhaustion, limited migration to and persistence within the tumor niche, and the immunosuppressive BM microenvironment. Furthermore, the generation of suboptimal autologous CAR-T cells due to T cell dysfunction in pre-treated patients (9) has prompted the development of 'next generation' cellular products to improve patient outcomes (10).

CAR-modified natural killer (NK) cells are a powerful, allogeneic alternative to autologous CAR-T cells. While exhibiting comparable effector functions, off-the-shelf CAR-NK cells offer several advantages over CAR-T cells including an intrinsic ability to eliminate tumor cells in an HLA-unrestricted manner, a favorable safety profile with a negligible risk of graft-versus-host disease, or cytokine release syndrome, and more economical methods for CAR-NK production (11). Indeed, clinical studies demonstrated the efficacy and safety of CAR-NK cells derived from cord blood (12), induced-pluripotent stem cells (iPSCs) (13) and cell lines (14). Nevertheless, effective anti-tumor immunity is reliant upon the ability of adoptively transferred NK cells to breach the tumor niche (15). NK cells navigate towards malignant tissues *via* their chemokine receptors, which respond to cognate chemokines expressed in the tumor microenvironment (TME) (11). E*x vivo*-manipulation of primary NK cells has been shown to hinder penetration of the TME, in part, due to chemokine receptor downmodulation (16–18). For these reasons, engineering strategies to stably equip effector NK cells with tissue-relevant chemokine receptors have shown promise in pre-clinical studies for solid tumors (18–21) and hematological malignancies (16, 22, 23).

NK cell cytotoxic function is exerted without prior immune sensitization through secretion of perforin and granzyme-containing lytic granules. A prerequisite is the formation of the immunological synapse (IS) (24), and after target cell conjugation, granule content is released within minutes and can effectuate pro-apoptotic pathways in target cells in a serial manner (25). NK cells can also kill tumor cells by caspase-dependent death receptor engagement by their cognate ligands, e.g., TRAIL and FasL (26). These effector functions are regulated by several activating and inhibitory receptors which simultaneously recognize a multitude of ligands expressed on target cells. A dominance of NK cell activation receptor signals endows NK cells with innate abilities to mediate effector functions, i.e., NKG2D signaling is mediated through the adaptor molecules DAP10 and DAP12, where DAP12 recruits ZAP-70 and Syk to initiate NK cell activation (27–29). Receptor-proximal signaling molecules activate the signaling cascade, containing AKT, MAPKs, ERK1/2, JNK1/2, and p38. Alternatively, NK cells can couple *via* CD16/FcγRIIIA to target cells decorated with a therapeutic antibody (30).

The homeostatic chemokine receptor CXCR4 coordinates trafficking of NK cells towards the BM niche, where its ligand CXCL12 is constitutively expressed. Problematically for adoptive CAR-NK cell therapy of MM, CXCR4 expression is progressively lost during NK cell maturation, coinciding with an increase of the chemokine receptor CXCR3, which generally promotes NK cell mobilization from the BM into the periphery (31). NK cell homing to and retention within the BM has been shown to correlate with improved acute myeloid leukemia (AML) control (15, 32) However, the chemokine TME is sufficiently skewed during MM development, whereby CXCL12 downmodulation is paralleled by increased expression of CXCR3 cognate ligands, creating pathological conditions generally restrictive to NK cell infiltration (22). This provides the rationale for reestablishing the responsiveness of NK cells to BM-associated CXCL12 to improve MM disease control. Beyond their classical role coordinating chemotaxis, previous reports in T cells have shown that recruitment of chemokine receptors within the IS serves to enhance T cell activation (33–35). To this date, a role for chemokine receptors as co-receptors in CAR-NK cell activation has not been described.

Here we asked to what degree does CXCR4 impact BCMA CAR-NK cell anti-tumor efficacy beyond BM homing and

accumulation. We explored whether enforced CXCR4 expression in NK cells exerted a co-stimulatory role in the context of CAR-induced NK cell activation. Based on our findings we conclude that co-modification of BCMA CAR-NK cells with CXCR4 exceeds functionalities related to improved trafficking and retention within tumor sites.

Materials and methods

Generation of γ-retroviral vectors

The fully humanized anti-human BCMA CAR was generated and used as described (36). The human CXCR4 coding sequence was cloned downstream to the anti-human BCMA CAR sequence linked via a self-cleaving peptide P2A. The entire CAR and CXCR4 sequences were synthesized by GeneArt, and cloned into the retroviral vector MP71. For construction of a CXCR4-only expressing vector, a PCR reaction with primers to introduce a NotI and a EcoRI restriction site was run on the human CXCR4 coding sequence downstream of the aforementioned P2A site contained in a MP71 retroviral vector. The MP71 plasmid was digested with NotI and EcoRI, followed by ligation of the CXCR4 PCR sequence into the vector backbone.

The mutant form $CXCR4^{R334X}$ was generated by Gibson assembly. Briefly, the MP71 plasmid BCMA CAR-CXCR4 was digested with BsteII and EcoRI. An overhang extension PCR was used to create a CXCR4-fragment with a stop codon at amino acid position 334. For construction of all other CXCR4 mutant forms, CXCR4 was cut with BsteII and EcoRI and replaced with mutant CXCR4-fragment R134N ($CXCR4^{G\ protein\text{-}def.}$) sequences. All sequences were verified by restriction digest and Sanger sequencing.

Cytotoxicity assay

NK-92 and YTS NK cell lines cells stably transduced with various CAR constructs were seeded in 96-well U-bottom plates and mixed with a fixed number of $5x10^4$ $Raji^{BCMA}$ target cells. SP6-CAR NK-92 cells were used as a negative control, and tumor cells only were used for calculation of NK-92 cell-specific killing. Co-cultures were kept for 17 hours and stopped by inclusion of ice-cold FACS buffer. Cells were resuspended in blocking buffer supplemented with Fx True stain. To distinguish effector and target cells, anti-CD56-PE-Cy7 and anti-CD19-FITC antibodies in combination with 7-AAD for dead cell exclusion were used. Samples were acquired on a FACS Canto II flow cytometer (BD Biosciences), or a MACSQuant X analyzer (Miltenyi Biotec) and further analyzed with FlowJo v. 10.0.8 software.

Multiple myeloma cell line and primary multiple myeloma cells

Bone marrow-derived cells from three MM patients were obtained freshly and further purified using a Pancoll gradient before freezing. The study was conducted according to the Declaration of Helsinki and in accordance with local ethical guidelines; written informed consent of all patients was obtained. The diagnosis of MM was made by expert Charité-University Medicine Berlin hematologists and pathologists, and it integrates cell morphology, immunohistology, marker expression in flow cytometry, and pathophysiological behavior. Thawed target cells were used in co-cultures similar to MM.1S-GFP^+ cell line. NK-92 effector cells in co-culture were labeled with eFluor670 or with anti-CD56, and after 6 hours target MM cells were defined by $CD38^{high}$ and $eFluor670^-$. A 7-AAD staining was used for live-dead cell discrimination.

BCMA-bead stimulation and signal transduction

$1x10^6$ CAR-transduced NK-92 cells were pelleted by centrifugation at 400x g for 5 min. Cells were resuspended with BCMA protein-coupled magnetic beads (6 µg/mL; ACRO Biosystems) in PBS. A biotinylated BCMA fragment covers the N-terminal aa 1–54 that includes the cognate epitope for the scFv BCMA. BCMA-beads were added to NK-92 cells at 37°C for 5 min. Following the incubation period, the cells were placed on ice and lysed with ice-cold 2X RIPA buffer (300 mM NaCl, 100 mM Tris-HCl, 2% NP-40, 1% $C_{24}H_{39}NaO_4$, 0.2% SDS) containing protease and phosphatase inhibitors. The lysate was incubated on ice for 30 min and then centrifuged at 13,000 g for 20 min. Supernatant was processed for immunoblot analysis.

Endocytosis assay

To quantitate the kinetics of BCMA-CAR surface downregulation, CAR-transduced NK-92 cells were seeded in 1 ml of RPMI/0.5% BSA medium and kept at 37°C in a waterbath. 100 µl BCMA protein-coupled magnetic beads (Acro Biosystems) were washed, resuspended in medium and added to the cell suspension at 37°C. Cells were incubated for 10 min, centrifuged and resuspended in medium at 37°C. Aliquots were taken and added to an equal volume of ice-cold FACS buffer (PBS, 0.5% BSA, 0.05% NaN_3). Cell suspensions were immediately centrifuged at 4°C and resuspended in FACS buffer. For detection of CARs, a PE-coupled goat anti-human IgG antibody (Southern Biotech) was used. Cells were then centrifuged and resuspended in fixation buffer (BioLegend), followed by analysis on a FACS Canto II instrument.

Ethics approval

The recruitment of voluntary blood and bone marrow donors was conducted according to the declaration of Helsinki and in accordance with local ethical guidelines. The study was approved by the responsible ethic committee at Charité-University Medicine Berlin (EA2/142/20).

Results

CAR-CXCR4 co-expression improves migration of NK cells towards BM-associated CXCL12 *in vitro*

To gauge the migration of NK cells towards tissue-specific chemoattractants, we measured expression levels of chemokine receptors CXCR3, CXCR4 and CCR6 and adhesion molecule CD62L on the clinically relevant cell line NK-92. NK-92 cells were devoid of CCR6 and CD62L, possessed negligible levels of CXCR4 and ubiquitously expressed high levels of CXCR3 (Supplementary Figures S1A, B).

To assess if ectopically expressed CXCR4 coordinates chemotaxis of BCMA CAR-expressing NK cells towards CXCL12, we developed a BCMA CAR retroviral vector (CAR) (36) equipped with either a wildtype (WT), CXCR4 (CAR-CXCR4) or mutant CXCR4 (CAR-CXCR4^{R334X}) chemokine receptor sequence (Figure 1A). The CXCR4^{R334X} sequence is a C-terminally-truncated, gain-of-function mutant with increased signal transduction, reduced desensitization, and impaired receptor internalization (37–41). Retroviral transduction of NK-92 cells with either the CAR, CAR-CXCR4 or CAR-CXCR4^{R334X} construct induced comparable levels of BCMA CAR expression (Figures 1B, C). Substantially higher levels of CXCR4 were detected on NK-92 cells transduced with the bicistronic CAR-CXCR4 or CAR-CXCR4^{R334X} constructs as compared to CAR NK-92 cells (Figure 1D). Furthermore, CXCR4 expression was greater on CAR-CXCR4^{R334X} NK-92 cells than CAR-CXCR4 NK-92 cells (Figures 1D, E).

Next, we generated CAR and CAR-CXCR4 primary NK cells (pNK; CD3^{-} CD56^{+}) from peripheral blood (Figure 1F). These cells were transduced and expanded in the presence of IL-2 and IL-15 using (Supplementary Figure S1C) anti-CD2/CD355 MACS activator beads. On Day 9 (2-days post-transduction), gating on the CD56^{+}CD16^{high} population, we observed substantially higher levels of CXCR4 expression on CAR-CXCR4 and CAR-CXCR4 R334X transduced pNK cells (Figure 1F). CXCR4 expression also increased modestly in CAR pNK cells, indicating endogenous CXCR4 upregulation was not induced by viral infection (12, 23, 41). Co-modified CAR-CXCR4 pNK cells expressed CXCR4 at two- to threefold higher levels (gMFI) when compared to pNK cells transduced with the conventional CAR (Figures 1F, G).

We functionally characterized the activity of ectopically-expressed CXCR4 by quantitating phosphorylation of downstream signaling modules in response to a 5 min CXCL12 exposure. CXCL12 addition triggered phosphorylation of p44/42 MAPK (ERK1,2) (Figure 1H; Supplementary Figure S1D) in CAR-CXCR4 and CXCR4^{R334X} NK-92 cells to a larger extent than in CAR NK-92 cells. We further confirmed AKT phosphorylation in CAR-CXCR4 NK-92 cells (Supplementary Figures S1E, F) after 5 min CXCL12 co-incubations (42).

CAR-CXCR4 co-expression led to fourfold improved chemotaxis towards CXCL12 (25 ng/ml) compared to NK-92 cells expressing the conventional CAR (Figure 1I). Along these lines, both CAR-CXCR4 and CAR-CXCR4^{R334X} pNK cells featured a higher migratory ability towards CXCL12 than CAR pNK cells (Figure 1J). For context, serum concentrations in MM patients are reportedly in the range of 453 ± 124 pg/mL (43). Despite a modest upregulation of endogenous CXCR4 in pNK cells (Figure 1F), they retained an inefficient ability to migrate towards CXCL12 at the concentrations indicated. Collectively, incorporation of either a WT or mutant CXCR4 sequence into a BCMA-CAR-encoding retroviral vector leads to the expression of a fully-functioning protein capable of instigating enhanced migration towards CXCL12 *in vitro*.

Enforced CXCR4 expression enhances the cytolytic efficacy of CAR-NK cells *in vitro*

Previous reports in T cells have shown that recruitment of chemokine receptors within the IS enhances TCR-induced signaling and IS longevity (33, 35). Canonically, these co-stimulatory effects have been attributed to a chemokine stimulus acting on the cognate chemokine receptor. However, the conditions for non-conventional CAR-dependent signaling processes in NK cells are unknown. Hence, we first investigated whether enforced CXCR4 expression boosts the antigen-dependent cytolytic efficacy of BCMA CAR-NK cells. CAR, CAR-CXCR4, CAR-CXCR4^{R334X} and SP6-control (irrelevant CAR binder) NK-92 cells were co-cultured with an engineered BCMA-expressing Raji target cell line (Raji^{BCMA}) (Supplementary Figure S2). The proportion of viable Raji^{BCMA} cells remaining in culture after 17 hours was quantified by flow cytometry and showed that CAR-CXCR4 and CAR-CXCR4^{R334X} NK-92 cells possessed a 15–25% higher antigen-dependent killing capacity compared to CAR NK-92 cells at lower effector-to-target ratios (E:T: 0.125:1) (Figures 2A, B). To exclude effector cell type-specific peculiarities, we conducted analogous experiments with the NK cell line YTS (44) (Supplementary Figure S3A). Indeed, CXCR4-equipped CAR YTS cells exhibited a higher killing capacity against Raji^{BCMA} cells compared to conventional CAR YTS cells (Supplementary Figures S3B, C).

We next performed a CD107a (LAMP-1) secretory lysosome degranulation assay to scrutinize whether CXCR4 overexpression endows BCMA CAR-NK-92 cells with enhanced antigen-dependent cytolytic efficacy (45, 46). Following 30 min co-cultures with Raji^{BCMA} cells, we detected substantially higher levels (approximately 20%) of LAMP-1 expression on the surfaces of CAR-CXCR4 and CAR-CXCR4^{R334X} NK-92 cells (Figures 2C, D). This difference was diminished after 60 min, indicating that CXCR4 supports a rapid mobilization of lytic granules following CAR activation (Figure 2D).

Whereas previous reports have postulated a synergistic, mutually-reciprocal state of TCR- and chemokine-induced receptor activation (47, 48), others have alluded to a state whereby CXCL12-induced signals do not override those emanating from TCR activation (49, 50). Consistent with the latter, we found that CXCL12 supplementation did not enhance antigen-dependent degranulation among CAR-CXCR4 or CAR-CXCR4^{R334X} NK-92 cells (Figures 2E–G). This was, in part, because CAR NK cells lacking CXCR4 overexpression exhibited similar low-level gain-of-

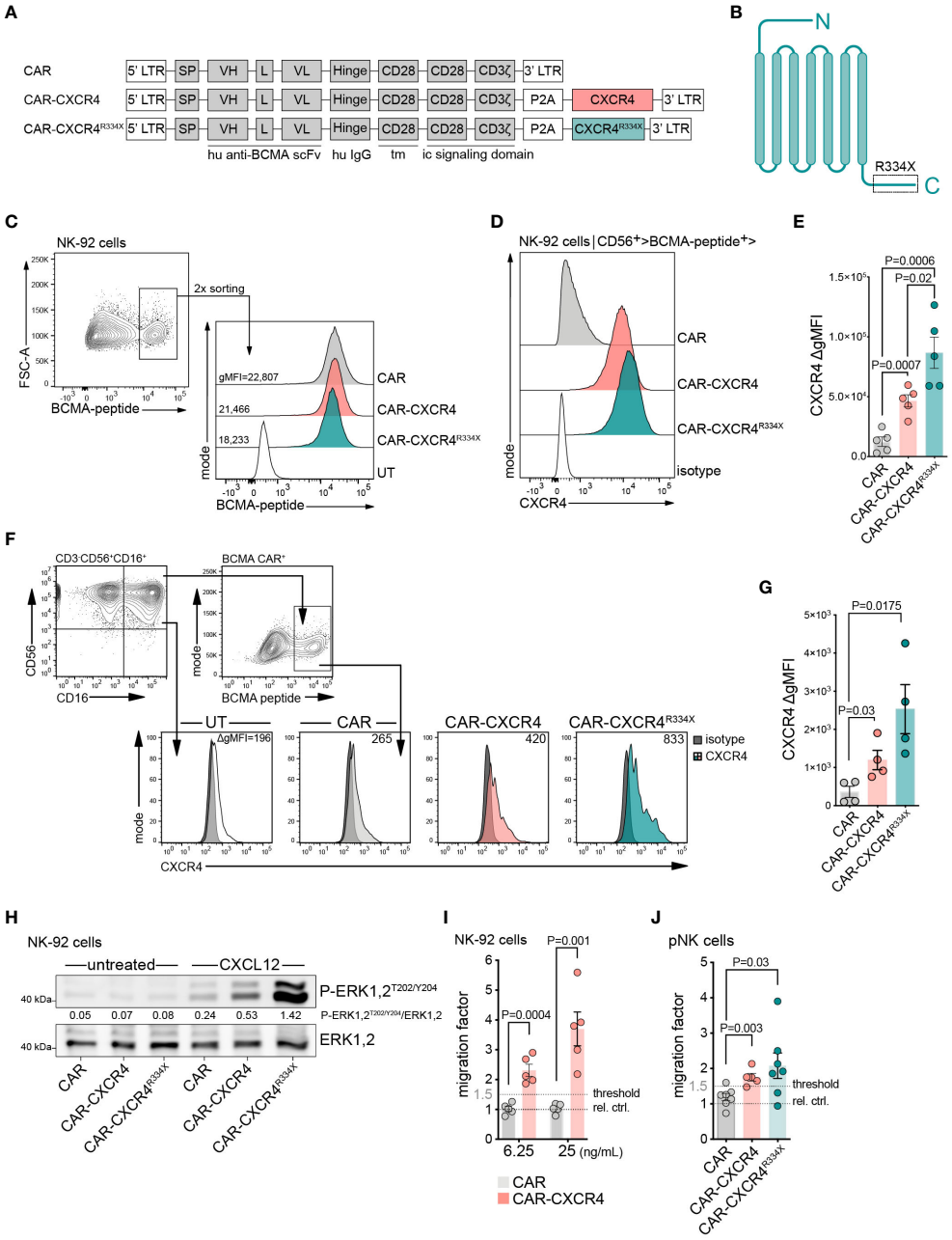

FIGURE 1
Enforced CXCR4 expression improves migration of BCMA-CAR NK cells towards chemotactic CXCL12 gradients. **(A)** Schematic of retroviral constructs encoding a BCMA-CAR (CAR), equipped with either a WT CXCR4 (CAR-CXCR4) or mutant CXCR4 (CAR-CXCR4^{R334X}) chemokine receptor sequence. LTR, long terminal repeat; tm, transmembrane region; ic, intracellular signaling domain; SP, signal peptide. **(B)** Schematic of heptahelical CXCR4^{R334X} mutant. **(C)** FACS plots depicting the generation of BCMA-CAR^{+} NK-92 cells. NK-92 cells underwent retroviral transduction with the aforementioned BCMA-CAR-encoding constructs. BCMA-CAR^{+} expressing NK-92 cells, as detected by cognate FITC-conjugated BCMA-peptide, were enriched twice by FACS to generate stable BCMA-CAR^{+} NK-92 cell lines. **(D)** Of these BCMA-CAR^{+} NK-92 cells, the expression of CXCR4 was assessed by flow cytometry, which was **(E)** quantified as ΔgMFI (values represent CXCR4 stained cells minus isotype control). Statistics calculated by an unpaired t-test, error bars ± SEM. **(F)** FACS histograms showing CXCR4 expression on pNK cells at day +9 of *ex vivo* expansion (2-days post-transduction; untransduced (UT); isotype, dark filled histogram; one representative example is shown. The gating strategy used to define CD56^{+}CD16^{+} pNK cells is shown on the left plot. NK cells were stimulated with IL-2 (500 U/ml) and IL-15 (20 ng/ml), in the presence of anti-CD2/CD335 activator beads. **(G)** Quantification as in **(E)**, ΔgMFI values are CXCR4 stained cells minus isotype control. An unpaired t-test was applied, error bars ± SEM. **(H)** Western blot assessing P-ERK1,2$^{T202/Y204}$ phosphorylation of untreated and CXCL12-stimulated (25 ng/ml, 5 min) CAR, CAR-CXCR4 and CAR-CXCR4^{R334X} NK-92 cells. Numbers between the gel lines give the ratios between P-ERK 1,2 and total ERK1,2. **(I)** Transwell migration assay assessing migratory capacity of CAR (grey bars) and CAR-CXCR4 (pink bars) NK-92 cells exposed to 6.25 ng/mL or 25 ng/mL CXCL12 for 4 hrs. Relative control (rel. ctrl.) represents unstimulated cells. Statistics calculated by an unpaired t-test, error bars ± SEM. **(J)** Quantification of migrated CAR (grey bar), CAR-CXCR4 (pink bar) and CAR-CXCR4^{R334X} (green bar) pNK cells exposed to 25 ng/mL CXCL12. Threshold set arbitrarily at 1.5-fold. Statistics calculated by an unpaired t-test, error bars ± SEM. P values are given. NK cells were used at day 12–15 after start of the culture. Data points in E, G, I, J represent independent biological replicates.

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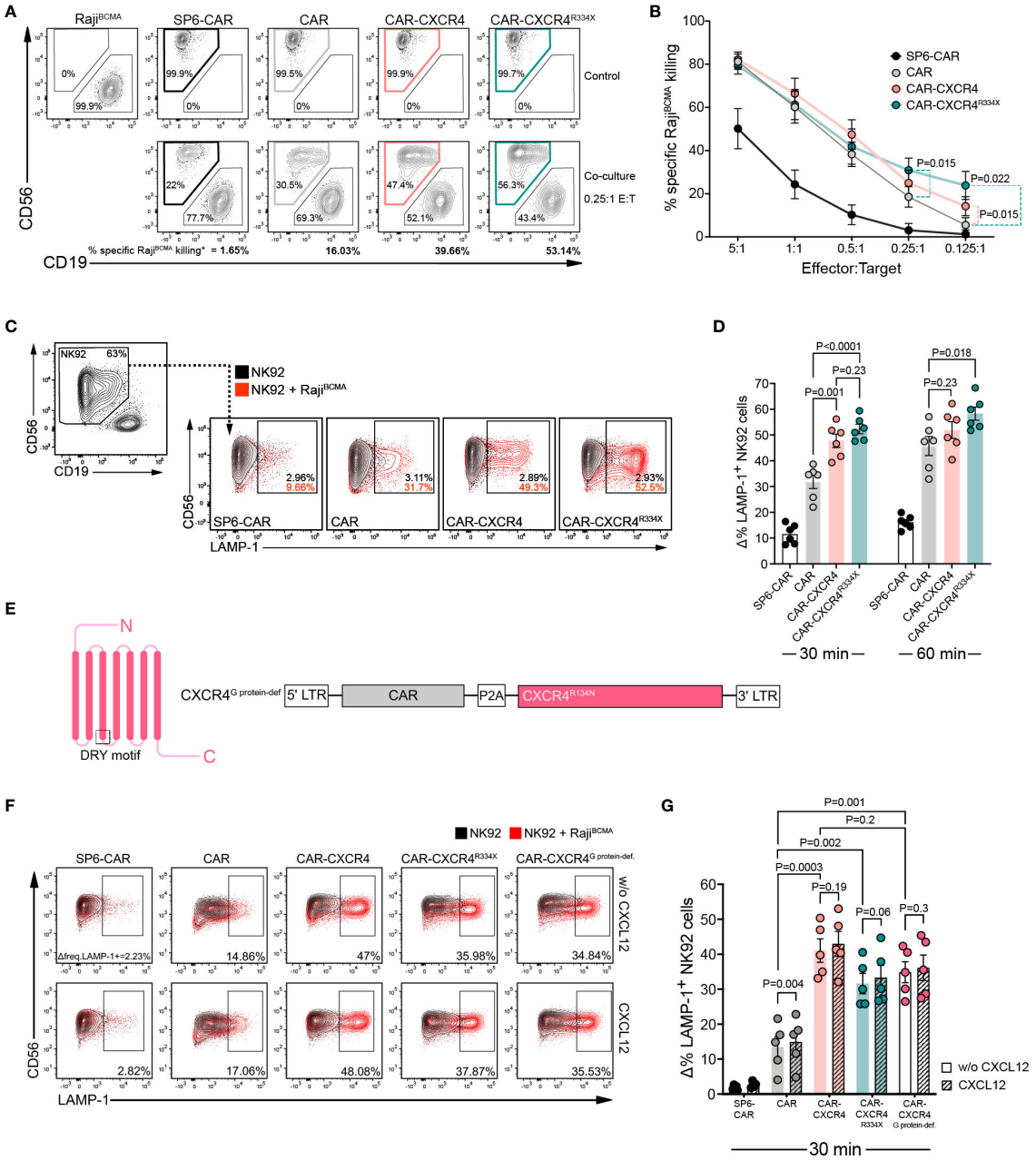

FIGURE 2
CXCR4 co-expression enhances cytolytic activity of BCMA-CAR NK cells in the absence of CXCL12 stimulation. **(A)** A flow cytometry-based cytotoxicity assay was performed by co-culturing CAR-transduced NK-92 cells and Raji^{BCMA} target cells. Representative FACS plot depicting a 17-hour co-culture between Raji^{BCMA} cells with either SP6-CAR as negative control, CAR, CAR-CXCR4 or CAR-CXCR4^{R334X} NK-92 cells at a 0.25:1 E:T ratio. Top panel displays Raji^{BCMA}- or transduced NK-92-only controls. Bottom panel shows the co-cultures between Raji^{BCMA} cells (CD19$^+$) and transduced-NK-92 cells (CD56$^+$). The percentage of specific-Raji^{BCMA} cell killing is calculated using the formula: Killing rate %= (1- (tumor cells in coculture/tumor cells only)) x 100. (dependent upon E:T ratio). Numbers in the gates are the percentages of effector (CD56$^+$) and target cells (CD19$^+$), respectively. **(B)** Quantification of specific-Raji^{BCMA} cell killing (%) for the aforementioned co-cultures at the indicated E:T ratios (n=13–18 independent biological replicates). Statistics calculated by a paired t-test. **(C)** Representative FACS plot of a degranulation assay detecting LAMP-1 expression on CD56$^+$ SP6-CAR as negative control, CAR, CAR-CXCR4 and CAR-CXCR4^{R334X} NK-92 cells following co-culture with Raji^{BCMA} cells for 30-min. Frequencies of LAMP-1$^+$ NK-92 cells were calculated by subtracting percentage LAMP-1$^+$ expression on transduced-NK-92 cells alone (grey plot) by percentage LAMP-1$^+$ expression on NK-92 cells co-cultured with Raji^{BCMA} cells (red plot). Numbers within gates are the frequencies in percent of LAMP-1$^+$ cells. **(D)** Quantification bar plots of **(C)** after 30- and 60-min stimulation. Statistics calculated by an unpaired t-test. **(E)** Schematic of G protein-deficient CXCR4^{R134N} mutant and retroviral construct. The position of the conserved DRY motif is indicated. **(F)** Representative FACS plot of a degranulation assay following a 30-minute co-culture between transduced NK-92 cells (including CAR-CXCR4^{R134N} mutant) and Raji^{BCMA} cells in the presence or absence of 25 ng/ml CXCL12. Numbers below gates are the frequencies in percent of LAMP-1$^+$ cells upon stimulation. **(G)** Quantification of aforementioned degranulation assays (n=5) with (CXCL12; hatched bars) or without (w/o CXCL12; solid bars) CXCL12. Statistics calculated using an unpaired t-test comparing different constructs and a paired t-test assessing each construct with or without CXCL12. Error bars ± SEM. P values are given; data points in **(D)**, **(G)** represent independent biological replicates.

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function (Figures 2F, G). We also generated a CXCR4 mutant with a defect in G protein coupling (R134N) in the conserved DRY motif (CAR-CXCR4$^{G\ protein\text{-}def.}$) (Figures 2E–G). This mutation does not affect downstream PI3K-activation, tyrosine phosphorylation of focal adhesion proteins and chemotaxis (51). When linked to the BCMA CAR in our bicistronic constructs, we confirmed that CAR-CXCR4, CAR-CXCR4^{R334X} and CAR-CXCR4$^{G\ protein\text{-}def.}$ NK-92 cells maintained a superior antigen-dependent degranulation capacity (Figures 2F, G). Our observations imply a hierarchy in which the enhanced killing capacity originates from CAR-driven activation of CXCR4 signaling nodes.

CXCR4-equipped CAR NK-92 cells are efficacious *in vitro* against MM cell lines and primary patient-derived MM cells

To rule out an artifact of enhanced BCMA expression in the transfected Raji^{BCMA} cell line, we compared the antitumor cytolytic activity of CAR and CAR-CXCR4 NK-92 in co-cultures with the MM cell line MM.1S and with four patient-derived primary MM specimen. In agreement with the improved cytolytic activity against Raji^{BCMA} cells, CXR4 co-expression endowed CAR NK-92 cells with an enhanced cytolytic capacity against both cell types exhibiting endogenous BCMA expression (Figures 3A–D). CAR-CXCR4 NK-92 cells gained a 25% higher antigen-dependent killing capacity for MM.1S cells compared to CAR NK-92 cells, visible at lower effector-to-target ratios (E:T: 0.125:1) (Figure 3B). The FACS-based killing assay was corroborated by a degranulation assay, using the MM.1S cell line as a stimulus (Supplementary Figures S4A, B). Similar to the MM.1S cell line, CXCR4 co-expression endowed CAR NK-92 cells with much stronger cytolytic capacity against primary MM cells (Supplementary Figure S4C) at low effector to target ratios (E:T: 0.25:1), which even exceeded the gain-of-function compared to Raji^{BCMA} and MM.1S cells (Figures 3C, D). Notably, the frequencies of MM cells within patient-derived primary bone marrow aspirates are much lower than homogenous target cell populations as for Raji^{BCMA} and MM.1S cell lines.

To exclude a CAR-independent response of chemokine receptor overexpression, we also generated NK-92 cells expressing CXCR4 only. We studied the innate killing capacity of CXCR4 NK-92 cells in a co-culture with MM.1S cells. Isolated CXCR4 overexpression was not sufficient to endow NK-92 cells with enhanced degranulation capacity. Their innate reactivity towards MM.1S tumor cells was similar to NK-92 cells only (mean LAMP-1 upregulation: 2.3%; CXCR4 NK-92: mean 3.4%), and substantially weaker than CAR NK-92 cells. This result indicated that the gain of cytotoxic capacity in NK-92 cells depends on a co-stimulatory function of the chemokine receptor in the process of BCMA CAR activation (Supplementary Figures S4D–F).

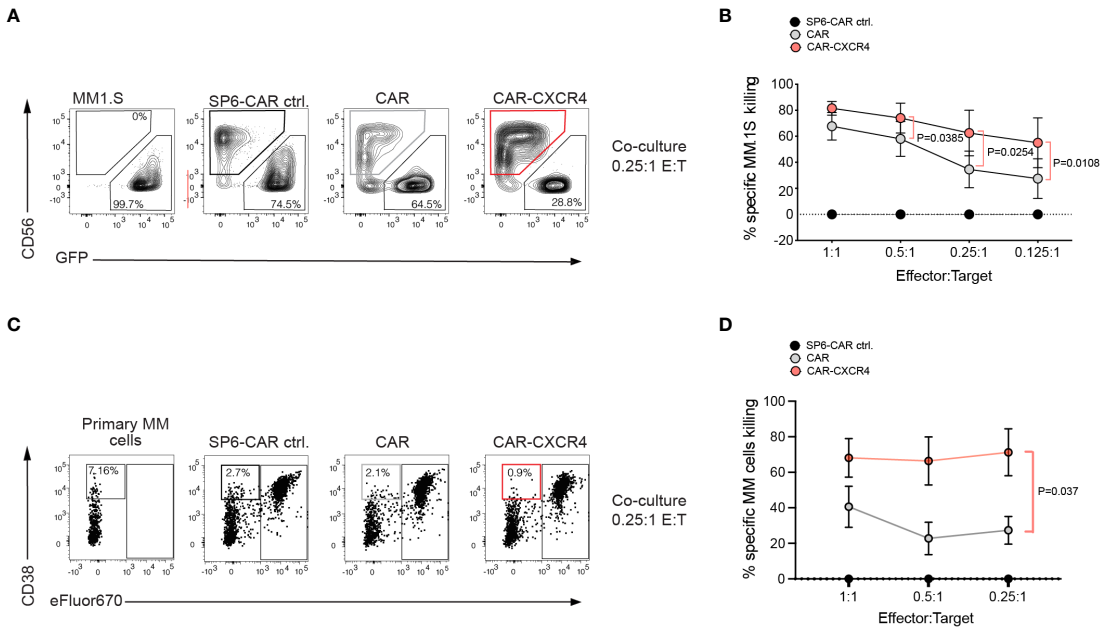

FIGURE 3
Multiple Myeloma cells with endogenous BCMA expression are more susceptible to BCMA CAR NK-92 cells equipped with the CXCR4 chemokine receptor. **(A)** A flow cytometry-based cytotoxicity assay was performed by co-culturing CAR-transduced NK-92 cells and MM.1S-luc.GFP target cells. Representative FACS plot depicting a 6-hour co-culture between MM.1S-luc.GFP cells with either SP6-CAR as negative control, CAR, or CAR-CXCR4 NK-92 cells at a 0.25:1 E:T ratio. Effector NK-92 cells were detected by anti-CD56 staining, and target cells were GFP labeled for discrimination. Numbers on the bottom gates are percentages of viable tumor target cells after co-culture. **(B)** The percentage of specific-MM.1S-luc.GFP cell killing is calculated as in Figure 2A). (dependent upon E:T ratio). Values displayed are mean ± SEM (n=4 independent experiments). Statistics calculated by a paired t-test. **(C)** A representative FACS plot of co-cultures between primary MM specimen and NK-92 effector cells. Effector cells were labeled with eFluor670^{+} for better distinction from MM target cells, defined by CD38^{+high} eFluor670^{-}. Because of the low frequency of primary MM target cells in the specimen, killing rates were calculated relative to the negative control, SP6 CAR. Statistics calculated by a paired t-test; n=4 independent donor samples tested. **(D)** Formula to quantify the killing rate is Killing rate %= (1- (tumor cells in coculture with test CAR/tumor cells in coculture with SP6 CAR control)) x100.

CAR-CXCR4 co-expression prolongs CAR surface expression and augments trogocytosis

To dissect the mechanisms underlying the increased cytolytic activity of CAR-CXCR4 NK-92 cells, we focused on CAR stabilization and the initiation of proximal or distal signaling. Because reorganization and accumulation of chemokine receptors within the IS of conventional T cells with antigen-presenting cells (APC) has been shown to stabilize the IS (33, 35), we explored whether ectopic CXCR4 overexpression affects BCMA-CAR internalization during antigen-engagement. CAR and CAR-CXCR4 NK-92 cells were exposed to cognate BCMA peptide-coated magnetic beads (BCMA-beads) and examined for the surface expression of the BCMA-CAR over-time. BCMA-beads offer the operational advantage of selectively binding to the BCMA-CAR cognate receptor. Although both CAR and CAR-CXCR4 NK-92 cells progressively lost BCMA-CAR surface expression after BCMA-bead exposure, CAR-CXCR4 NK-92 cells prolonged BCMA-CAR surface expression to a greater extent than CAR NK-92 cells (Figures 4A, B). BCMA-bead stimulation did not affect CXCR4 surface expression, but on the contrary, CXCL12 induced internalization of its cognate receptor (Figures 4C, D). These observations suggest that the integrity of the BCMA CAR-driven IS is maintained in the presence of abundant CXCR4. Exposing CAR- and CAR-CXCR4 NK-92 cells to BCMA-beads produced no striking difference in IFN-γ secretion (Supplementary Figure S5).

Recently, trogocytosis induced by CAR-activation on NK cells was associated with higher levels of degranulation towards target cells (52). To assess the degree of trogocytosis induced by CAR engagement, we quantified the ratio between $CD56^+/CD19^+$ double-positive ($TROG^+$) and $CD56^+/CD19^-$ ($TROG^-$) BCMA CAR-expressing NK-92 cells in response to $CD19^+$ $Raji^{BCMA}$ target cell exposure. CAR-CXCR4 (mean $TROG^+/TROG^-$=1.04 ± 0.59) and $CAR\text{-}CXCR4^{R334X}$ (1.17 ± 0.53) NK-92 cells triggered trogocytosis to a substantially higher degree than CAR NK92 cells (0.79 ± 0.47) (Figures 4E, F). We then asked whether trogocytosis modulated effector function of CAR-CXCR4 NK-92 cells by assessing the level of degranulation. Whereas minimal levels of LAMP-1 expression were detected on $CAR\text{-}CXCR4^{TROG-}$ NK92 cells (mean % ΔLAMP-1+ cells= 3.39 ± 2.14), a larger proportion of $CAR\text{-}CXCR4^{TROG+}$ NK92 cells had undergone degranulation (64.02 ± 8.74) (Figures 4G, H). Prolonged expression of the BCMA CAR at the cell surface and higher levels of trogocytosis following target cell challenge suggest that CAR-CXCR4 co-modification equips NK92 cells with a substantially enhanced effector function.

Engineered CXCR4 expression boosts BCMA-induced proximal signaling by enhanced ZAP-70 activation

CAR engagement results in the formation of a nonclassical, disorganized IS characterized by the rapid initiation of proximal signaling, and quicker recruitment of cytotoxic granules (53–55). It was recently determined that the sensitivity of a CAR-T cell towards antigen is approximately 1000-times reduced compared to that of conventional antiviral T cells, at least in part due to inefficient recruitment of ZAP-70 to the engaged CAR (56). Considering that chemokine receptor signal transduction relies on ZAP-70 (48, 57) recruitment and furthermore, promotes chemokine-enrichment in T cell synapses, we asked whether enforced CXCR4 during CAR engagement would augment ZAP-70 recruitment. We visualized the presence of phosphorylated ZAP-70 (P-ZAP-70) within the synapse of engaged CAR, CAR-CXCR4 and $CAR\text{-}CXCR4^{R334X}$ NK-92 cells following a short co-culture with $Raji^{BCMA}$ cells (Figures 5A, B). Thereafter, we quantified the expression (MFI) of P-ZAP-70 within IS. These data showed that P-ZAP-70 expression was significantly higher within the synapse of CAR-CXCR4 and $CAR\text{-}CXCR4^{R334X}$ NK-92 cells compared to CAR-NK-92 cells, which was most pronounced after 5 min (Figure 5B). After 20 min, synaptic P-ZAP-70 MFI values in CAR NK-92 cells were comparable to those obtained in CAR-CXCR4 NK-92 cells, further suggesting that CXCR4 supports the initial events triggered by CAR engagement. Of note, CXCR4 expression in $Raji^{BCMA}$ target cells interfered with the quantitation of synaptic CXCR4 recruitment in APC-conjugated CAR NK cells.

Immunoblot analysis of protein lysates derived from short-term (5 min) BCMA-bead stimulated NK-92 cells revealed a significant enhancement of pZAP-70 expression in CAR-CXCR4 and $CAR\text{-}CXCR4^{R334X}$ modified NK-92 cells (Figures 5C, D). We further inspected the signaling kinetics of antigen-engagement over-time by comparing induction of ERK1,2, which occurs downstream of ZAP-70 activation. After only one min exposure to BCMA-beads, ERK1,2 phosphorylation was significantly increased in CAR-CXCR4 NK-92 cells compared to CAR NK-92 cells. We conclude that BCMA-CAR activation is more potent when NK-92 cells are equipped with CXCR4 (Figures 5E, F).

Enforced CXCR4 expression lowers the antigen amount necessary for BCMA-CAR activation

CXCR4 expression could influence the formation of mature IS by enabling increased integrin inside-out (33) signaling. Using a conjugate formation assay we quantitated stable IS formation between CAR NK effector cells and $Raji^{BCMA}$ target cells. All NK-92 CAR constructs were devoid of integrin LFA-1 (open conformation) and adhesion molecule CD62L, but ubiquitously expressed CD11b and CD44 (Figure 6A). Conjugate formation after 30 min was indistinguishable between those cell types, addition of CXCL12 had no stimulatory impact in this process (Figures 6B, C). Potentially, conjugate formation could occur earlier than 30 min, which would be consistent with an earlier activation of the signaling cascade when CXCR4 is overexpressed. This was not further investigated herein.

Inadequate CAR reactivity towards low-density cognate antigen is a major source of treatment failure (58). Insufficient recruitment of ZAP-70 to the engaged CAR has emerged as an important cause of reduced CAR sensitivity (56). Because enforced CXCR4 expression enhances the presence of activated

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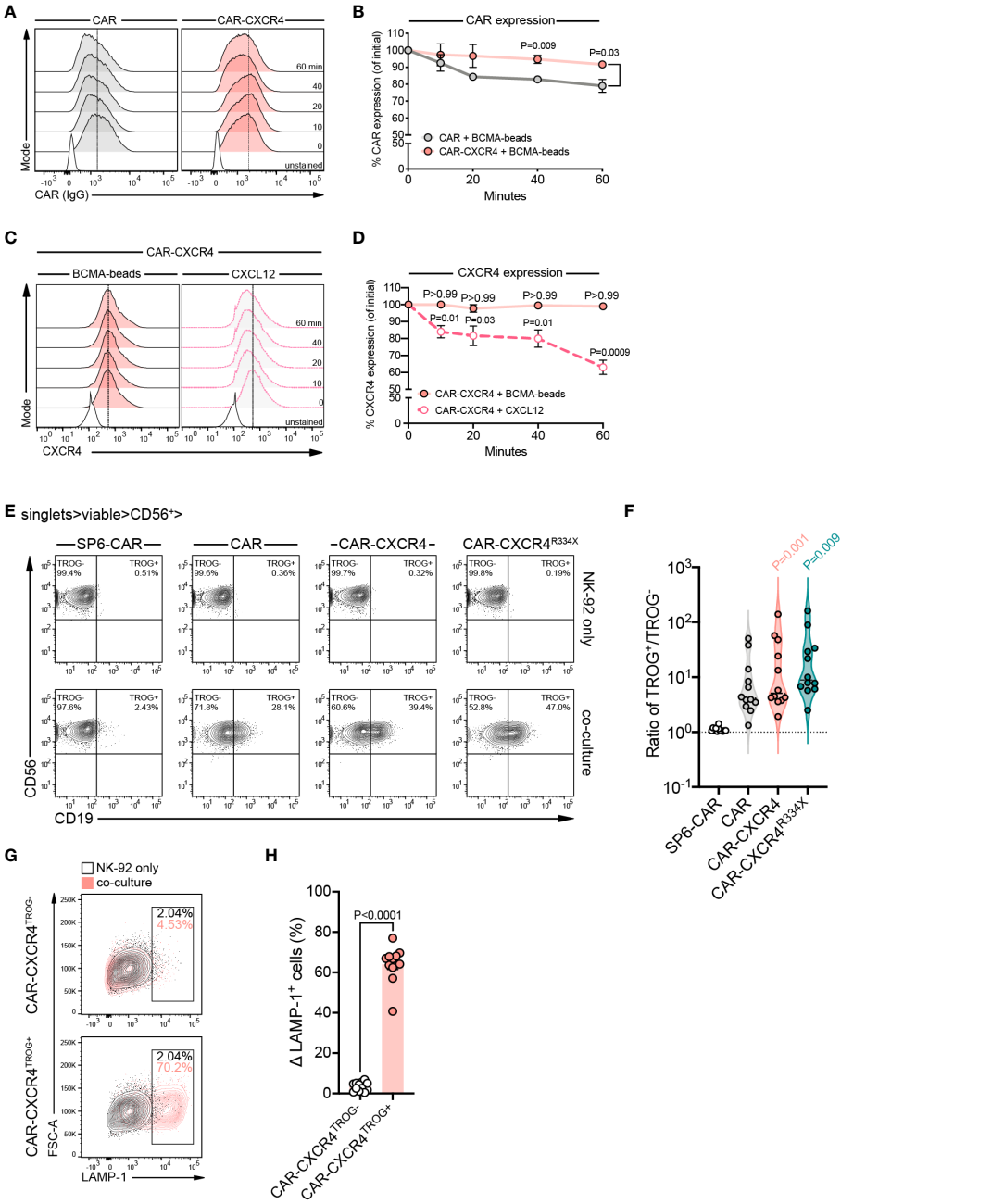

FIGURE 4
CXCR4 overexpression prolongs BCMA-CAR surface expression and is associated with enhanced levels of CAR-mediated trogocytosis. **(A)** Representative FACS histograms showing the kinetics of BCMA-CAR surface expression (as detected by IgG staining) on CAR (grey histograms) and CAR-CXCR4 (pink histograms) NK-92 cells following exposure to BCMA-beads for the indicated timepoints. **(B)** Quantification of BCMA-CAR surface expression. gMFI values were set arbitrarily at 100% at 0 min. The change in gMFI over time was quantified as a percentage of initial surface expression (n=3 independent biological replicates). Statistics calculated using an unpaired t-test (CAR versus CAR-CXCR4). **(C)** Representative FACS histograms of CXCR4 expression on CAR-CXCR4 NK-92 cells after exposure to BCMA-beads (pink histograms) or CXCL12 (grey histograms; pick-dotted line) for the indicated timepoints. **(D)** Quantification of CXCR4 surface expression, calculated as for **(B)** (BCMA-beads n=4; CXCL12 n=5 independent biological experiments). Statistics calculated using a paired t-test (BCMA-beads; timepoint versus '0 min') or Wilcoxon test (CXCL12; timepoint versus '0 min'), where appropriate according to sample size and distribution. **(E)** Representative FACS plots depicting the proportion (%) of $CD56^+$ $CD19^+$ SP6-CAR, CAR, CAR-CXCR4 and CAR-$CXCR4^{R334X}$ NK-92 cells (ordinarily $CD19^-$) following 30 min co-culture with $CD19^+$ $Raji^{BCMA}$ target cells, indicative of CAR activation-induced trogocytosis. Cell gating was on single cells (FSC-W/FSC-H). Top panel shows 'NK-92 only' control cells, while the bottom panel represents the co-culture. **(F)** Violin plots quantifying the ratio of SP6-CAR (white plot), CAR (grey), CAR-CXCR4 (pink) and CAR-$CXCR4^{R334X}$ (green) NK-92 cells that had undergone (TROG+) or had not yet experienced (TROG-) trogocytosis of $Raji^{BCMA}$ target cells (TROG+/TROG-). Individual datapoints for independent biological replicates are shown. Statistics calculated using a paired t-test. **(G)** Representative FACS plots detecting LAMP-1 expression on the aforementioned CAR-$CXCR4^{TROG-}$ ($CD56^+$ $CD19^-$; top panel) and CAR-$CXCR4^{TROG+}$ ($CD56^+$ $CD19^+$; bottom panel) NK-92 cells following a 30 min co-culture with $Raji^{BCMA}$ cells. Frequencies of $LAMP\text{-}1^+$ CAR-$CXCR4^{TROG-}$ or CAR-$CXCR4^{TROG+}$ cells were calculated by subtracting percentage $LAMP\text{-}1^+$ expression on CAR-CXCR4 NK-92 cells alone (black contour plot) by percentage $LAMP\text{-}1^+$ expression on either CAR-$CXCR4^{TROG-}$ or CAR-$CXCR4^{TROG+}$ NK-92 cells (pink contour plot). Numbers within gates are the frequencies in percent of $LAMP\text{-}1^+$ cells. **(H)** Quantification bar plots of **(G)**. Data points represent independent biological replicates. Statistics calculated by a paired t-test. P values are given; statistical significance $P<0.05$. Error bars ± SEM.

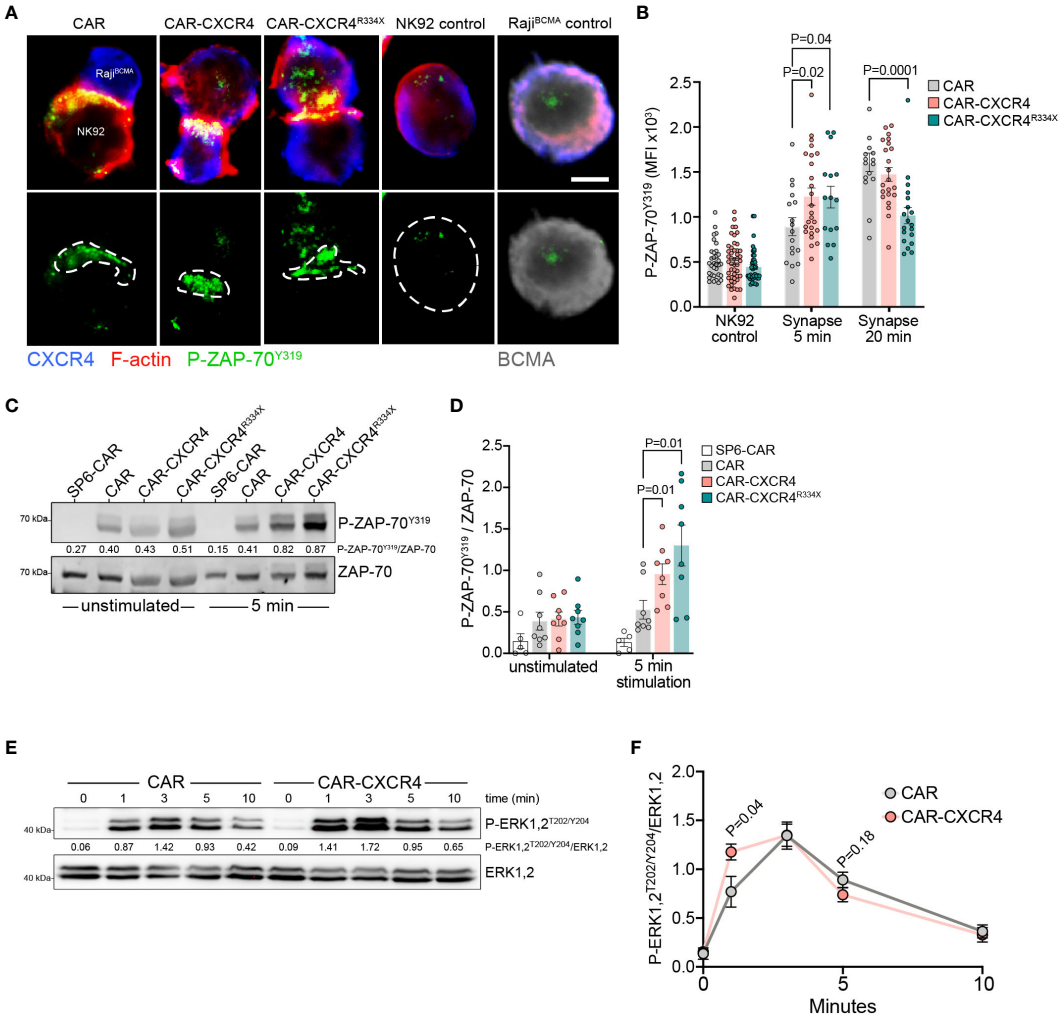

FIGURE 5
Enforced CXCR4 expression is associated with increased P-ZAP-70^{Y319} recruitment into the IS and boosted BCMA-induced proximal signaling. **(A)** Exemplary single plane micrographs depicting IS formation between Raji^{BCMA} cells and CAR, CXCR-CXCR4 or CAR-CXCR4^{R334X} NK-92 cells during 5 min co-incubations. The immunological synapse was identified by accumulation of actin cytoskeleton at the interface of NK-92 cells and Raji^{BCMA} target cells. Cells were probed for CXCR4 (blue), P-ZAP-70^{Y319} (green), F-actin (red; representative of IS interface) and BCMA (grey; target cell marker). The region of interest (ROI) represents the IS, where P-ZAP-70^{Y319} localizes to. Scale bar, 5 µm. Representative image, acquired on an LSM-980 airyscan microscope, equipped with a 63x Plan Apochromat NA 1.40 oil objective. Digital images were obtained with ZEN software (Zeiss), and further processed with Fiji. **(B)** Quantification of phosphorylated P-ZAP-70^{Y319} MFI within the ROI following 5- or 20-minute co-incubations. CAR=grey bar, CAR-CXCR4=pink bar and CAR-CXCR4^{R334X}=green bar. Statistics calculated using an unpaired t-test. Each data point represents one effector-target NK-92-Raji^{BCMA} cell conjugate. At least n=2 independent experiments were performed. **(C)** Representative immunoblot assessing P-ZAP-70^{Y319} phosphorylation of unstimulated and BCMA-bead-stimulated (5-minute) SP6-CAR control, CAR, CXCR-CXCR4 and CAR-CXCR4^{R334X} NK-92 cells. **(D)** Quantification (densitometry) of P-ZAP-70^{Y319} phosphorylation for the aforementioned Western blots (n=8 independent biological replicates), calculated by dividing P-ZAP-70^{Y319} signal intensity by total ZAP-70 signal intensity. SP6-CAR control=white bars, CAR=grey bars, CAR-CXCR4=pink bars, and CAR-CXCR4^{R334X}=green bars. Individual datapoints shown. Statistics calculated using a Mann-Whitney test. **(E)** Kinetics of ERK1,2 phosphorylation assessed by immunoblotting of BCMA-bead stimulated CAR-transduced NK-92 cells. In C) and E), numbers between the gel lines give the ratio between phosphorylated ZAP-70 and phosphorylated ERK 1,2 and their total protein forms ERK1,2 or AKT, respectively. **(F)** Quantification of P-ERK1,2$^{T202/Y204}$ occurrence. The ratio of total ERK1,2 and phosphorylated P-ERK1,2 is given (n=6 independent biological replicates). Statistics calculated using an unpaired t-test. Error bars ± SEM.

ZAP-70 within the IS (Figures 5A–D), we examined whether the presence of CXCR4 equips BCMA-CAR NK cells with an improved ability to recognize target cells with lower antigen amounts. We exposed CAR and CAR-CXCR4 NK-92 cells to lowering amounts of BCMA-beads and assessed the induction of downstream ERK1,2 signaling. CAR-CXCR4 NK-92 cells recognized and transmitted signals when antigen availability was lower compared to its conventional CAR counterpart (Figures 6D, E). More specifically, at a BCMA-bead concentration of 5 and 1.67 µL, CAR-CXCR4 NK-92 cells were still able to trigger ERK1,2 phosphorylation (Figure 6D). These data suggest that enhanced ZAP-70 recruitment at the IS, afforded by the presence of synthetic CXCR4, would enable CAR-CXCR4 NK-92 cells to eliminate lower-antigen-expressing tumor cells.

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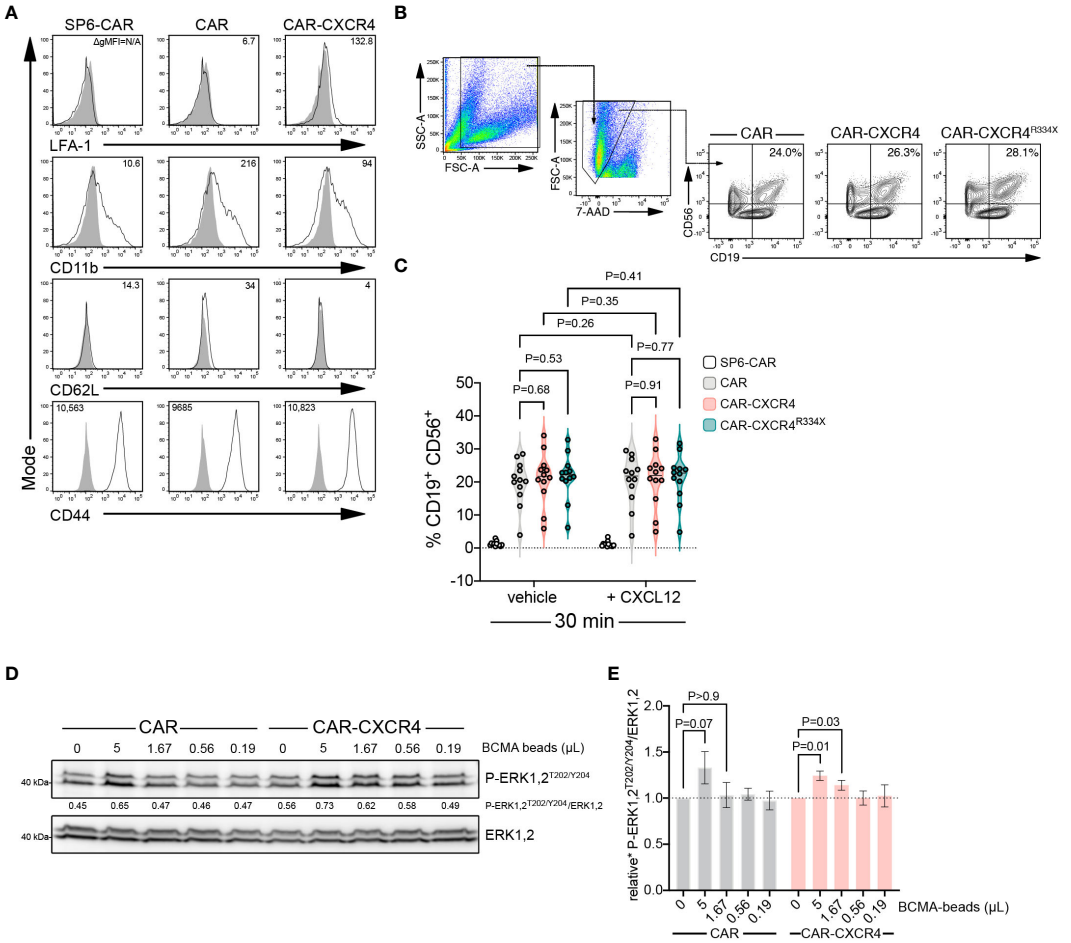

FIGURE 6
Enforced CXCR4 expression lowers the antigen threshold for BCMA-CAR activation and heightens BCMA-CAR sensitivity. **(A)** Flow cytometry analysis of adhesion molecule expression in BCMA-CAR and CAR-CXCR4 enhanced NK-92 cells. Numbers in the histograms indicate gMFI values, grey solid graphs depict isotype controls. **(B)** CAR-transduced NK-92 cells and Raji^{BCMA} target cells were co-cultured as in Figures 2C, F. Conjugate formation was performed either with or without CXCL12 supplementation (25 ng/ml). After 30 min, cells were stained for CD56 and CD19. Numbers in the gates indicate the percentage of cells forming conjugates. **(C)** Quantification of conjugate formation, as determined by cell doublets (FSC-H/FSC-W) and CD56$^+$/CD19$^+$ double positive cells. SP6-CAR ctrl=white plot, CAR=grey plot, CAR-CXCR4=pink plot, and CAR-CXCR4^{R334X}=green plot. Individual datapoints for biological replicates are shown. An unpaired and paired t-test was applied, where appropriate. **(D)** Representative immunoblot examining rapid P-ERK1,2$^{T202/Y204}$ phosphorylation of CAR and CXCR-CXCR4 NK-92 cells exposed to decreasing amounts of cognate BCMA antigen (BCMA-beads; 5, 1.67, 0.56, 0.19 and 0 µL) for 5 minutes. **(E)** Quantification (densitometry) of P-ERK1,2$^{T202/Y204}$ phosphorylation for the abovementioned western blots (n=7 independent biological replicates), calculated by P-ERK1,2$^{T202/Y204}$ signal intensity divided by ERK1,2 signal intensity. CAR=grey bars and CAR-CXCR4=pink bars. Statistics calculated using a Wilcoxon test.

Discussion

The findings reported herein demonstrate that engineered overexpression of CXCR4 bestows BCMA CAR-NK-92 cells with an increased ability to eliminate BCMA-expressing tumor cells *in vitro*. We show that CXCR4 possesses additional functions beyond its classical role of coordinating cell migration towards CXCL12 gradients. In fact, linked to the redistribution of CAR-T and CAR-NK cells into the tumor niche, reports have demonstrated that gains in anti-tumor efficacy are merely due to locally augmented effector cell populations. Indeed, enhancing homing capacity has been shown to enrich their accumulation within malignant tissues and, consequently, elicit tumor suppression of solid cancers (18–20), and hematological malignancies (16, 23, 32). However, pertaining to the co-stimulatory effects of chemokine receptors in TCR-mediated T cell activation (35, 47, 59), we also argue that outfitting BCMA CAR-expressing NK-92 cells with CXCR4 endows these cells with an enhanced ability to kill BCMA antigen-expressing tumor cells on a cellular level.

The degree to which chemokine receptors play a co-stimulatory role during T cell activation without their chemokine ligand (59) has garnered some debate. On the one hand, studies have shown that recruitment of CXCR4, CCR5 (35) and CCR7 (33) into the IS after TCR-engagement relies on chemokine ligand binding, as chemokine receptor enrichment within the T cell-APC contact site was reduced in the absence of chemokine ligands. CXCL12 has been suggested to prompt the physical association of CXCR4 with the TCR. The resultant recruitment of ZAP-70 *via* SH2-domain binding to TCR-associated phospho-ITAMs prolonged ERK kinase activity, intracellular Ca^{2+} signaling and cytokine secretion (60). Thus, it

would appear that chemokine receptor triggering on T cells is a precondition for chemokine receptor-mediated co-stimulation. On the other hand, Felce et al. demonstrated that CXCR4 organization within the IS was not influenced by CXCL12 supplementation, nor did CXCR4 form complexes with the TCR (49). Kremer et al. and Dinkel et al. collectively showed that TCR crosslinking transactivated CXCR4 in the absence of CXCL12 *via* GRK2-induced phosphorylation of the cytoplasmic $CXCR4^{S339}$ residue. The latter was in turn shown to highjack PREX1-Rac1 signaling to augment cytokine secretion (20, 61). Hence accumulative data suggest that CXCR4 activation and transactivation can occur irrespective of CXCL12 binding and downstream of TCR ligation.

Here, we provide multiple lines of evidence that demonstrate the enhanced anti-myeloma effects of CXCR4 co-modified BCMA CAR-NK cells in a chemokine-independent manner. Firstly, we co-cultured BCMA CAR-transduced NK-92 or YTS effector cells with target cells in the absence of exogenous chemokine, revealing that CXCR4-expressing CAR-NK cells feature elevated cytotoxic activity. Secondly, degranulation assays further supported the notion that CXCR4 acts as a co-receptor during CAR-engagement by triggering rapid activation and cytolytic efficacy in NK-92 cells, independent of CXCL12. Thus, CXCL12 does not override TCR-induced (49, 50), and, as evidenced in this study, CAR-induced signaling. To rule out the possibility that EBV-transformed $Raji^{BCMA}$ target cells provoke CXCL12 secretion (62) or contribute towards other NK stimulatory signals, we used BCMA-peptide-decorated microbeads. Antigen-independent stimuli involve integrin conjugation or Fc-receptor signaling that can confound BCMA CAR-dependent signaling. BCMA-beads stimulated CAR activation in a strictly antigen-dependent and chemokine-independent manner. Our data demonstrating the accelerated kinetics of ERK1,2 and ZAP-70 phosphorylation in CXCR4-expressing NK-92 cells are in line with enhanced BCMA CAR activation, which results in increased cytotoxic capacity *in vitro*.

How can we reconcile a CXCR4-dependent, but CXCL12-independent, co-stimulatory role of this receptor with previous reports on ligand-dependent CXCR4, CCR5 or CCR7 functionality in TCR-expressing T cells? To our knowledge, the mechanistic interplay between CARs and cooperating chemokine receptors have not been addressed in NK cells. Furthermore, fundamental differences exist between the formation of TCR- and CAR-induced synapses. Reports have demonstrated that CAR-antigen complexes form disorganized synapses in which the architecture deviates from a TCR-pMHC synapse by lack of defined central, peripheral and distal supramolecular activation complexes. Indeed, organized TCR-antigen synapses allow for a lower threshold for antigen recognition (53, 63). In fact, although CARs outperform TCRs with regard to antigen binding, proximal signaling in the CAR synapse is strongly attenuated, which results in reduced responsiveness to low antigen levels.

This effect appears to result from insufficient ZAP-70 recruitment to ligated CARs, followed by decreased signal activation. Importantly, low responsiveness facilitates immune escape, which has been reported for CD19 and CD22 CAR-T cell therapies (64–66). In our report, we revealed an enhanced accumulation of P-ZAP-70 within the synapse of CXCR4-expressing BCMA CAR-NK-92 cells, indicating that activation of ZAP-70 at the synapse is accelerated. Cycling and activation of ZAP-70 is integral to signal amplification and Ca^{2+} fluxing, resulting in the induction of downstream signaling cascades (67, 68). Together, CXCR4 can partially compensate for the drawbacks associated with CAR-induced synapses to foster detection of antigen at lower levels given earlier and more profound ZAP-70 activation.

CXCR4-equipped NK cells were able to activate the ERK1,2 signaling pathway at significantly lower microbead-bound antigen amounts than BCMA CAR NK cells. It seems plausible that BCMA-CAR sensitivity towards antigen can be fine-tuned by CXCR4 overexpression. Moreover, CAR-triggered signaling is more rapid, and consequently, cytotoxic granule release at the synapse followed by target cell killing is faster (53). Although the mechanistic link between the initial signal (the CAR-antigen encounter) and chemokine-independent CXCR4 co-stimulation remains to be further explored, it is conceivable that crosstalk induced by activated ZAP-70 may affect signal strength and onset.

TCR deubiquitination impairs receptor downregulation, which consequently promotes enhanced T cell responses (69, 70). As CARs emulate much of TCR-proximal signaling, it has been suggested that CAR downmodulation might attenuate tumor cell killing capacity (71, 72). Our results support the notion that counteracting CAR downmodulation via CXCR4-co-expression may improve overall CAR-T effector functions, a matter which has to our knowledge not been addressed in NK cells. We consider it likely that extended surface residency delays endocytosis and lysosomal degradation (73).

Collectively, we suggest that the mechanisms underlying this observation involves (i) CXCR4-mediated P-ZAP-70 recruitment into the synapse, (ii) the stabilization of the synapse and CAR surface deposition, and (iii) intensified antigen-dependent CAR activation by hijacked or colluding signaling nodes. We propose that the homeostatic chemokine receptor CXCR4 acts as a co-receptor during the formation of a CAR-mediated IS, which gains relevance in NK cells that have endogenously low CXCR4 expression.

Data availability statement

The datasets presented in this article are not readily available because data generation only refers to *in vitro* data from common cell lines. For primary MM samples, patient data are anonymized and not visible or available to the researchers, no further storage of data derived thereof. Requests to access the datasets should be directed to arehm@mdc-berlin.de.

Ethics statement

The studies involving humans were approved by Ethic Committee at Charite University Medicine Berlin, No. EA2/142/20; EA2/216/18. The studies were conducted in accordance with the

local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

MWM: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. HE: Investigation, Methodology, Resources, Writing – review & editing. LM: Formal analysis, Investigation, Methodology, Visualization, Writing – review & editing. MM: Investigation, Methodology, Writing – review & editing. AF: Investigation, Methodology, Writing – review & editing. MB: Methodology, Writing – review & editing. MS: Investigation, Methodology, Writing – review & editing. KG: Investigation, Methodology, Writing – review & editing. VG: Investigation, Methodology, Writing – review & editing. JR: Resources, Supervision, Writing – review & editing. KM: Supervision, Writing – review & editing. JG: Supervision, Writing – review & editing. JH: Methodology, Resources, Writing – review & editing. JK: Resources, Writing – review & editing. BV: Resources, Supervision, Writing – review & editing. UH: Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing. AR: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing.

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Conflict of interest

AR and UH filed a patent application for the BCMA CAR used in this manuscript PCT/WO2017211900A1. AR and UH receive research funds from Fate Therapeutics San Diego, CA. JR, KM, JG, and BV are employees of Fate Therapeutics.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1383136/full#supplementary-material

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Breaking barriers: advancing cellular therapies in autoimmune disease management

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Autoimmune diseases occur due to a dysregulation within the immune system, leading to an aberrant assault on the organism's own tissues. The pathogenesis of these conditions is multifactorial, encompassing intricate interplays among genetic predispositions, environmental determinants, and hormonal fluctuations. The spectrum of autoimmune diseases is broad, impacting a multitude of organ systems, with notable examples such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), multiple sclerosis (MS), psoriasis, and vitiligo. Despite substantial progress in therapeutic interventions over recent years, a definitive cure for autoimmune diseases has yet to be realized, with existing modalities largely providing palliative care. Cellular therapy is considered the fourth pillar in the management of oncological disorders subsequent to surgical resection, radiotherapy, and chemotherapy. Cellular therapies have shown potential in augmenting immune competence and eliminating of targeted neoplastic cells in a spectrum of cancers. As targeting specific molecules on the surface of autoreactive B and T cells, such as CD19, BCMA, CD20, and CTLA-4, cellular therapies are emerging as promising approaches for the treatment of autoimmune diseases. This review delineates the advancements in the application of cellular therapies applied recently for autoimmune diseases and proposes considerations for the advancement of novel therapeutic strategies.

KEYWORDS

autoimmune diseases, cellular therapy, B cells, CD19, Tregs

1 Introduction

The immune system, comprising immune organs, cells, and molecules, holds a pivotal position in the recognition and elimination of antigenic foreign entities, as well as in the coordination with other organic systems to maintain homeostasis and physiological equilibrium within the organism. However, once the immune system's inability to discern between foreign antigens and self-host cells results in the development of classical or pathological autoimmunity, culminating in tissue damage and inducing autoimmune diseases (1). Autoimmune diseases, ranking as the third most common category of illnesses after cancer and cardiovascular diseases, affect an estimated 5-8% of the global population, with a combined prevalence of 10.2%—impacting 13.1% of women and 7.4% of men (2). Characterized by the disruption of immune tolerance, the etiology of autoimmune diseases is intricate, encompassing genetic, environmental, and hormonal factors (3). To date, over 100 distinct autoimmune diseases have been identified, with prevalent examples including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and multiple sclerosis (MS) (4, 5). These conditions not only inflict substantial distress upon affected individuals but also pose a major global socio-economic challenge (6).

Reversing disrupted immune tolerance presents a significant challenge in the management of autoimmune diseases, with existing therapeutic strategies primarily aimed at disease control rather than cure (7). The advent of traditional synthetic disease-modifying antirheumatic drugs (DMARDs) and biologic DMARDs has led to substantial improvements in the prognosis of patients afflicted with autoimmune rheumatic diseases. Nevertheless, the majority of patients necessitate continuous drug therapy to ameliorate symptomatology. Nonspecific therapeutic approaches, predicated on the use of hormones and immunosuppressants, systematically suppress the immune system, thereby preventing autoreactive immune cells from assaulting host-body. This method, while effective, carries the risk of inducing severe adverse effects, including an increased susceptibility to infections (8). Biological agents, particularly monoclonal antibodies (mAbs) targeting TNFα and IL-6R, have demonstrated superior efficacy and reduced toxicity in comparison to conventional therapeutics. Despite these advancements, the necessity for repeated antibody infusions and the challenge of achieving a durable and effective restoration of immune homeostasis persist. Furthermore, the immunogenicity associated with the prolonged administration of antibodies poses an unresolved concern (9). Collectively, these therapeutic modalities often necessitate ongoing or intermittent drug administration, which is associated with the potential for cumulative long-term side effects. Most critically, they provide palliative relief and may slow disease progression, yet fail to reverse organ damage or physical disability (10).

With the continuous advancements in immunobiology and synthetic biology, along with the rapid progress of clinical-scale genetic engineering and gene editing technologies, various emerging cellular therapies have significantly advanced tumor therapy and other diseases (11). Cellular therapy has gradually become an important branch in the field of tumor treatment. It is mainly divided into two categories: native immune cell therapy and engineered immune cell therapy. Of native immune cell therapy, the patient's own immune cells are primarily utilized, which are activated or expanded ex vivo and then reinfused into the patient to enhance the body's immune response to tumor cells. Engineered immune cell therapy, on the other hand, involves genetically modifying immune cells to enable more precise recognition and destruction of tumor cells.

Targeted cellular therapies against autoreactive immune cells are considered a potential treatment for a series of autoimmune diseases. Chimeric Antigen Receptors (CARs), pioneered in the late 1980s, are synthetic transmembrane proteins with high specificity for target antigens (12). These receptors can redirect lymphocytes to recognize and exert their effects under specific conditions, offering non-major histocompatibility complex (MHC)-restricted recognition of cell surface components. Currently, various CAR T-cell therapies targeting different tumor antigens are either in clinical trials or have been approved for marketing. For instance, GD2 CAR T-cell therapy is specifically designed for neuroblastoma and other solid tumors that express the GD2 antigen (13). Mesothelin CAR T-cell therapy targets malignant pleural mesothelioma and other tumors expressing mesothelin (14). BCMA CAR T-cell therapy is primarily applied to patients with BCMA-positive multiple myeloma (15), and HER2 CAR T-cell therapy is directed against breast cancer and other tumors with HER2 overexpression (16, 17). Among these therapies, CD19 CAR T-cell therapy has garnered significant attention due to its remarkable efficacy in treating CD19+ B-cell hematological malignancies and has become one of the most successful cases in clinical application (18). Owing to its specificity and the induction of durable remission in autoimmunity, CAR cell therapy has been applied to the treatment of autoimmune diseases (19), with CAR-T cells, chimeric autoantibody receptor T cells (CAAR-T cells), and CAR-NK cell being used to deplete pathological immune cells, such as B cells, autoreactive B or T cells, and helper antigen-presenting cells (APCs), yielding favorable outcomes.

Furthermore, other cells are also under investigation for the treatment of autoimmune diseases, including regulatory T cells (Tregs), mesenchymal stem cells (MSCs) and hematopoietic stem cells (HSCs). This review discusses the advancements and challenges in cellular therapy for autoimmune diseases, providing insights for the development of novel therapeutic approaches targeting autoimmunity.

2 Autoimmune diseases

The epidemiology of autoimmune diseases exhibits variability in incidence and prevalence rates, which becomes intricate when accounting for differences in age, gender, ethnicity, and additional demographic characteristics. The pathogenesis of autoimmune diseases involves a multitude of factors: 1. Microbiological Factors: Infections by bacteria and viruses are implicated in triggering autoimmune responses. 2. Environmental Influences: Exposure to chemicals, prolonged ultraviolet radiation, and lifestyle factors such as smoking and alcohol consumption may

precipitate or intensify autoimmune reactions. 3. Genetic Predispositions: Certain human leukocyte antigen (HLA) alleles are significantly associated with the susceptibility to develop autoimmune diseases. 4. Hormonal effects: Fluctuations in hormone levels, gender and the use of certain medications, play a crucial role in disease onset, with women being disproportionately affected by specific autoimmune conditions (Figure 1). SLE is noted to have a prevalence ranging from 20 to 150 cases per 100,000 individuals, with a consistent increase in both incidence and prevalence across geographic regions (20). MS is typically diagnosed within the age range of 20 to 50 years (21). RA can affect individuals of any age, with peak incidence occurring between 50 and 59 years of age (22). SLE, MS, and RA are among the most prevalent and significant autoimmune diseases affecting patients, making them a central focus of research in the field of autoimmune disorders. This part will mainly provide a comprehensive review of these three diseases.

2.1 Systemic lupus erythematosus

SLE is a severe autoimmune disease characterized by a loss of immune tolerance, culminating in the immune system's attack on the body's healthy cells and tissues. This leads to immune complex-mediated inflammation across various organs, including the heart, kidneys, and skin (23). The mortality rate of patients with SLE is approximately 2–3 times higher than that of the general population, with geographical variations in prevalence and a female-to-male ratio of about 9:1 (24), affecting certain ethnic groups disproportionately, such as African Americans, American Indians, and Asians (25). The primary factors of mortality in SLE patients include renal disease, cardiovascular disease, and infections. Despite the approval of three novel treatments for SLE and lupus nephritis in recent years, the therapeutic management for the majority of patients has seen minimal evolution since the last century, with long-term outcomes characterized by high morbidity and mortality (26).

The complex immunopathogenesis of SLE encompasses a multitude of factors including genetics, environment, hormonal influences, epigenetics, and immune modulation, all of which can impact the immune system either individually or in concert (24). A key aspect of this complexity is the impaired clearance of apoptotic cells, which leads to the accumulation of cellular debris (Figure 2). This debris can activate normally dormant autoreactive lymphocytes, potentially evading self-tolerance under conditions of recurrent or chronic stimulation (27). Furthermore, neutrophils, particularly low-density granulocytes, may exacerbate the intricate interplay between innate and adaptive immune responses. Through increasing the production of pro-inflammatory cytokines and forming neutrophil extracellular traps that contain immunostimulatory proteins and self-antigens, such as double-stranded DNA, the inflammation is perpetuated (28). In addition to these cellular mechanisms, the IFN-1 signaling pathway, which mimics a sustained antiviral response, is associated with lupus susceptibility and may amplify the autoimmune reaction. This amplification can be manifested by the enhancement of autoreactive humoral activity. Genome-wide association studies (GWAS) have identified at least 132 lupus susceptibility loci (29). The functional significance of these variants

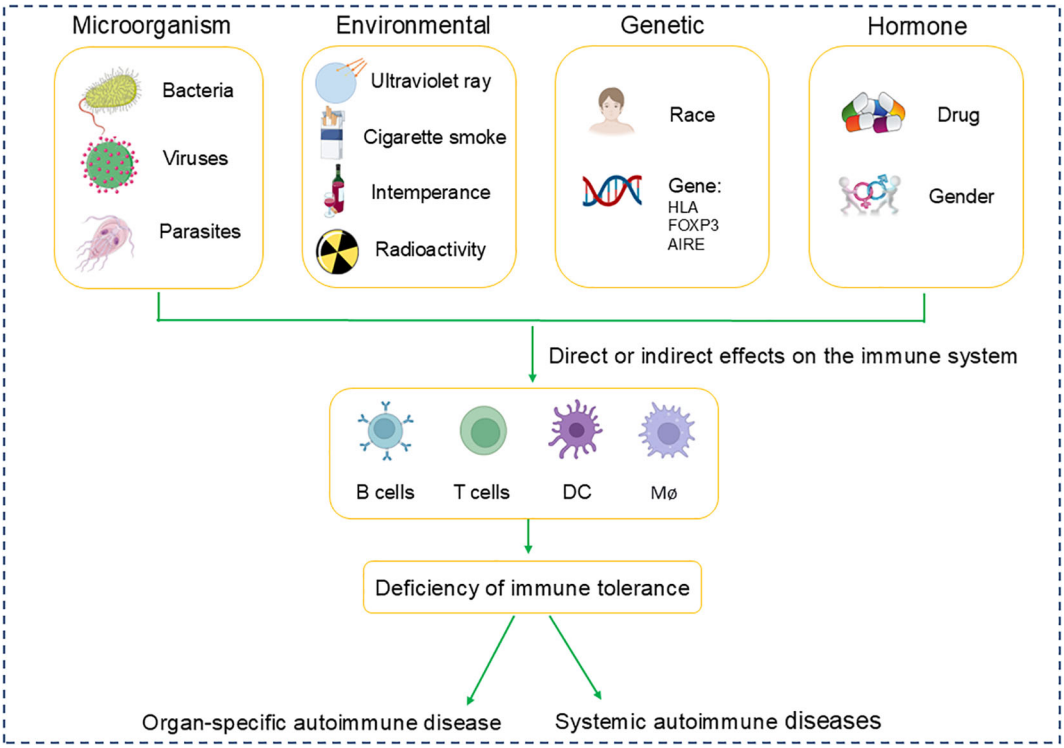

FIGURE 1
Pathogenic factors in autoimmune diseases.

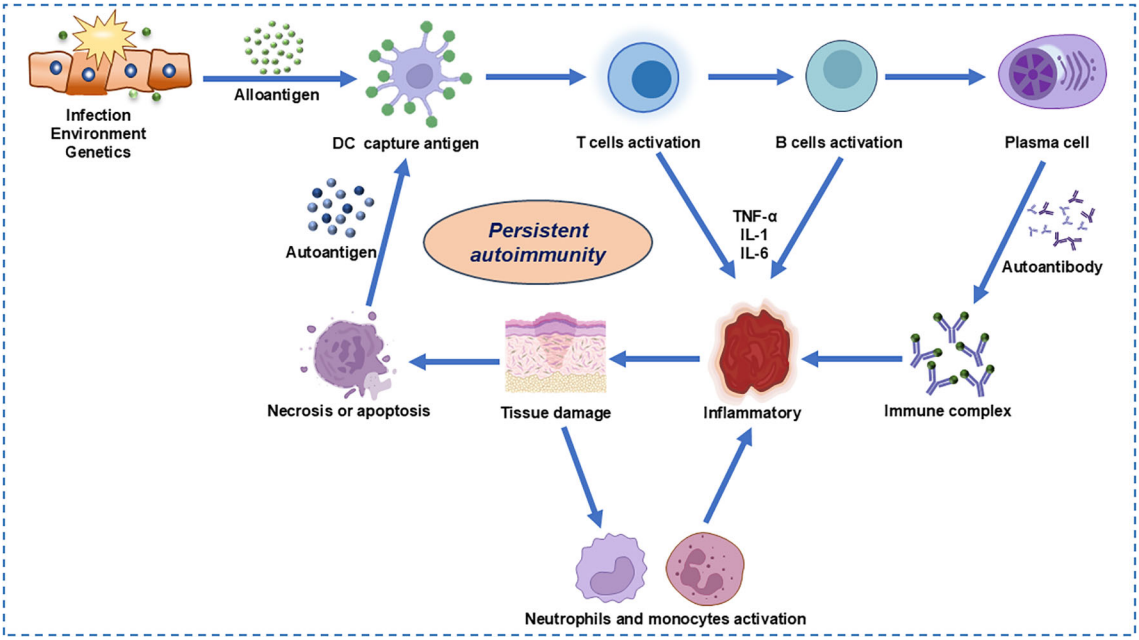

FIGURE 2
Cellular and molecular pathogenesis in autoimmune diseases.

and their potential role in lupus expression remain largely unknown. Additionally, sex hormones and environmental influences may contribute to immune system dysregulation in genetically predisposed individuals (5). Current therapeutic approaches include glucocorticoids, immunosuppressants, and belimumab, which often fail to achieve drug-free remission.

2.2 Multiple sclerosis

MS is the most prevalent chronic inflammatory disease of the central nervous system (CNS) and the most common nontraumatic disabling condition affecting young individuals, impacting over 2 million people worldwide. It affects females at a ratio of approximately 3:1 and remains incurable. The majority of patients are diagnosed between the ages of 20 and 40, although children and the elderly can also be diagnosed with MS (30). Typical presenting syndromes of MS include, but are not limited to, monocular vision loss due to optic neuritis, limb weakness or sensory loss due to transverse myelitis, diplopia resulting from brainstem dysfunction, or ataxia caused by cerebellar lesions (31). MS can manifest as an inflammatory disorder characterized by episodes of neurological symptoms followed by partial or complete remission (relapsing-remitting MS, RRMS, in 85% of cases), or as a progressive disease (primary progressive MS, PPMS). Over time, RRMS may evolve into a progressive phase of the disease known as secondary progressive MS (SPMS) (32).

It remains unclear whether MS is attributable to a single cause or a combination of factors, as few specific triggers have been identified. Epstein-Barr virus (EBV), ultraviolet B (UVB) radiation, cigarette smoking, and vitamin D, in conjunction with an individual's genetic background, all play significant roles in the development of MS. The prevalence of MS increases with latitude, a gradient closely related to UVB exposure, which stimulates the production of vitamin D in the skin. Low levels of vitamin D, reduced dietary intake of vitamin D, decreased outdoor activity, and increased susceptibility to MS associated with genetic polymorphisms linked to low vitamin D levels all correlate with the pathogenesis of MS (33). Furthermore, genetic factors influence susceptibility to MS, as approximately one-eighth of patients have a family history of the disease (34). GWAS conducted on samples collected from thousands of MS patients and matched controls have identified over 200 genetic variants that elevate the risk of developing MS, with the most significant association being linked to the *HLA-DRB1*1501* haplotype (32).

2.3 Rheumatoid arthritis

RA is an organ-specific autoimmune disease primarily characterized by symmetric peripheral polyarthritis. It may also affect other organs, including the lungs, heart, blood vessels, skin, and eyes (35). The patients typically present with symmetric polyarthritis of the hands and/or feet, which can progress to joint destruction if left untreated (36). In individuals diagnosed with RA, there is often an overlap with other diseases or comorbidities, including cardiovascular disease, chronic lung disease, and periodontitis (37). RA affects approximately 0.5% to 1% of the global population, with a higher prevalence in regions farther from the equator, and the prevalence in women is two to three times greater than that in men. RA is classified as an autoimmune disease due to the presence of autoantibodies, such as rheumatoid factor (RF) and anticitrullinated protein antibodies (ACPAs), in most cases (38).

The etiology of RA remains unknown; however, certain risk factors are associated with an increased likelihood of developing the condition. These factors include the family history of RA or other autoimmune diseases, smoking, poor dental health, and viral infections. Several infectious agents are recognized as potential pathogens or promoters of RA, including EBV, retroviruses, bacterial superantigens, and mycoplasmas, as well as specific microorganisms such as *Porphyromonas gingivalis* and *Prevotella intestinalis*. The pathogenesis of RA is complex, with genetic susceptibility playing a crucial role. First-degree relatives of individuals with RA take a risk of developing the disease that is 2 to 5 times greater than that of the general population (39). GWAS conducted on a large scale have identified more than 100 loci associated with RA (40), including TNIP2, WISP1 and TNFRSF11A (41), and these alleles increase the risk of the disease and dramatically affect immune pathways. The most relevant alleles include the "common epitope" (SE) on the *HLA-DRB1* locus and protein tyrosine phosphatase (PTPN22) (42).

GWAS have unveiled shared genetic variations across multiple diseases, providing crucial clues for understanding the genetic foundations of autoimmune diseases conditions. By GWAS analysis, researchers have identified some common genetic markers for SLE, MS, and RA. Variant in the HLA region is particularly prominent in these diseases; as a key component of the human leukocyte antigen system, the polymorphisms of HLA genes are closely associated with susceptibility to autoimmune diseases (41). Specifically, the presence of *HLA-DRB1*04:01* and *HLA-DQB1*03:01* alleles significantly increases the risk of RA in individuals and promotes the development of an autoimmune response by affecting autoantigen presentation and T-cell responses. Additionally, specific variations in the PTPN22 gene, which is involved in the regulation of T-cell signaling, have been shown to be associated with an increased risk of various autoimmune diseases, including SLE and RA (29, 43). STAT4 plays a key role in Th1, Th17, and Tregs, and the single-nucleotide polymorphisms (SNP) rs7574865 for STAT4 is associated with increased risk of SLE and RA (44). The TNFSF13B gene, which is involved in the survival and differentiation of B cells, has variations that are associated with an increased risk of SLE (45). The IRF5 gene plays a role in the immune response, and several SNPs (rs4728142, rs2004640, rs1744583) and small insertions/deletions in the IRF5 gene or regulatory regions have been validated to independently cause SLE, MS and RA by altering gene expression, splicing, and RNA stability (46) These findings indicate that although SLE, MS, and RA have different clinical manifestations, there is a genetic overlap, which may reflect common mechanisms in their immune regulatory pathways. The outcomes of GWAS are of significant importance for elucidating the complex genetic structure of autoimmune diseases, advancing the development of precision medicine, and developing new therapeutic approaches.

Autoimmune diseases exhibit a pronounced sexual dimorphism, with a higher incidence rate among females than males for numerous conditions. This gender bias may stem from a convergence of factors, including sex chromosome disparities, hormonal influences, the activity of the Xist ribonucleoprotein complex, gender-based immune cell variations, environmental exposures, and genetic predispositions. The presence of two X chromosomes in females could contribute to distinct immunomodulatory gene expression patterns, as the X chromosome is enriched with such genes, and variations in X chromosome number may influence susceptibility to autoimmune diseases (47). Men with Klinefelter syndrome (XXY) have an extra X chromosome, which causes them to experience significant impairments in fetal germ cell (FGCs) development, such as stagnation of FGCs at an early age, abnormal dosage of X-linked genes, aberrant interactions of Sertoli cells with FGCs, and inhibition of the TGF-β signaling pathway to improve FGCs differentiation (48). Disturbances in these molecular mechanisms not only affect their reproductive health, but are also associated with an increased risk of autoimmune diseases. Patients with KS may have abnormalities in their immune system, including abnormalities in the number and function of immune cells and a chronic inflammatory state, which together may put them at higher risk for autoimmune diseases (49). Recent studies have further revealed how abnormalities in gene dosage on the X chromosome can affect the risk of autoimmune diseases, particularly in patients with KS. The extra X chromosome leads to increased expression of X-linked genes such as *Tlr7*, a single-stranded RNA sensor associated with the pathogenesis of autoimmune diseases. Research has found that in KS patients, the X chromosome does not undergo inactivation in FGCs, resulting in an excess of X-linked gene dosage, which may directly impact the function of immune cells and the regulation of autoimmune responses. Additionally, a 3.2MB region of the X chromosome has been translocated to the Y chromosome, and this previously unreported X-Y translocation may influence the risk of autoimmune diseases in KS patients (50). These findings underscore the importance of X chromosome dosage control for maintaining normal immune function and provide a new perspective on how gender affects the pathogenesis of autoimmune diseases, highlighting the significance of gender differences in autoimmune disease research. Additionally, fluctuations in sex hormones like estrogen and progesterone may perturb immune cell development and activity, disrupting immune tolerance. The Xist RNP complex, responsible for X chromosome silencing in females, also forms molecular complexes implicated in autoimmune pathogenesis (51). Furthermore, environmental factors such as infections and ultraviolet radiation may impact the immune system in a gender-specific manner, and certain genetic variants are more prevalent in women, potentially increasing their risk for autoimmune diseases. The interplay among these factors likely drives the higher incidence of autoimmune diseases in women, highlighting the complexity of sex-based differences in disease susceptibility.

3 Emerging cell-based therapies for autoimmune diseases

The underlying causative factors of autoimmune diseases remain largely unknown; however, two main reasons contribute to the failure of immune tolerance (1) the presence of autoantibodies and (2) the

presence of disease-associated autoreactive lymphocytes (52). The complexity and heterogeneity of the mechanisms underlying immune dysregulation in autoimmune diseases present numerous challenges to develop novel therapeutic strategies that offer specific, long-lasting efficacy with minimal side effects (53). The treatment of autoimmune diseases currently relies predominantly on pharmacotherapy, which includes hormones, immunosuppressants, immunomodulators, and anti-inflammatory drugs (Figure 3). The mechanisms of these medications in treating autoimmune diseases are diverse, encompassing the suppression of inflammatory responses, modulation of immune system functions, and reduction of the number or activity of autoreactive cells. For instance, glucocorticoids broadly inhibit immune responses, while immunosuppressants such as cyclophosphamide and methotrexate reduce the number of immune cells by inhibiting DNA synthesis and cellular proliferation (54). Biologic DMARDs, such as infliximab and anifrolumab, are monoclonal antibodies targeting specific inflammatory mediators or immune cells, allowing for a more precise regulation of immune responses (55). JAK inhibitors reduce the signaling of inflammatory cytokines by suppressing the activity of JAK kinases (56). Natural compounds like curcumin and resveratrol also show therapeutic potential due to their broad targets, favorable safety profiles, and potential immunomodulatory effects (57). Although existing treatment modalities can control the disease to a certain extent, the complexity of autoimmune diseases often necessitates personalized treatment and the combined use of multiple drugs to achieve optimal efficacy. Moreover, pharmacological treatments often come with limitations and side effects, such as the severe side effects associated with long-term dependence on glucocorticoids and immunosuppressants, as well as the high costs and treatment limitations of biologic agents (58). Despite the introduction of improved therapies for autoimmune conditions in recent decades, further advancements are necessary. Ongoing research of cellular therapy strategies holds the potential to transform the traditional treatment paradigm for these diseases, offering the possibility of achieving sustained, targeted immune modulation while preserving essential protective immune functions.

Cell-based therapies (Figure 3) offer significant advantages over antibodies and cytokines in restoring immune tolerance due to the persistence and efficacy of these therapeutic cells *in vivo* (59). Such treatment approaches include the expansion of IL-10-expressing TR1 cells (60) and the engineering of Treg cells to express specific TCRs or CARs (61, 62), which modulate immune responses and contribute to the suppression of excessive autoimmune reactions. Moreover, MSCs provide a novel strategy for the treatment of autoimmune diseases by secreting anti-inflammatory factors and promoting tissue repair. HSCs, as multipotent stem cells capable of differentiating into various immune cells, offer a new perspective on immune modulation. CAR-T cells have shown promise in the treatment of autoimmune diseases by eliminating pathologically activated immune cells or re-establishing immune tolerance in affected organs (11). CAAR-T cells and CAR-NK cells utilize their specificity and enhanced targeting capabilities to provide innovative avenues for the treatment of autoimmune diseases. The innovative application of these cellular therapy strategies not only broadens our understanding of the treatment of autoimmune diseases but also provides a diverse array of options for future clinical therapies.

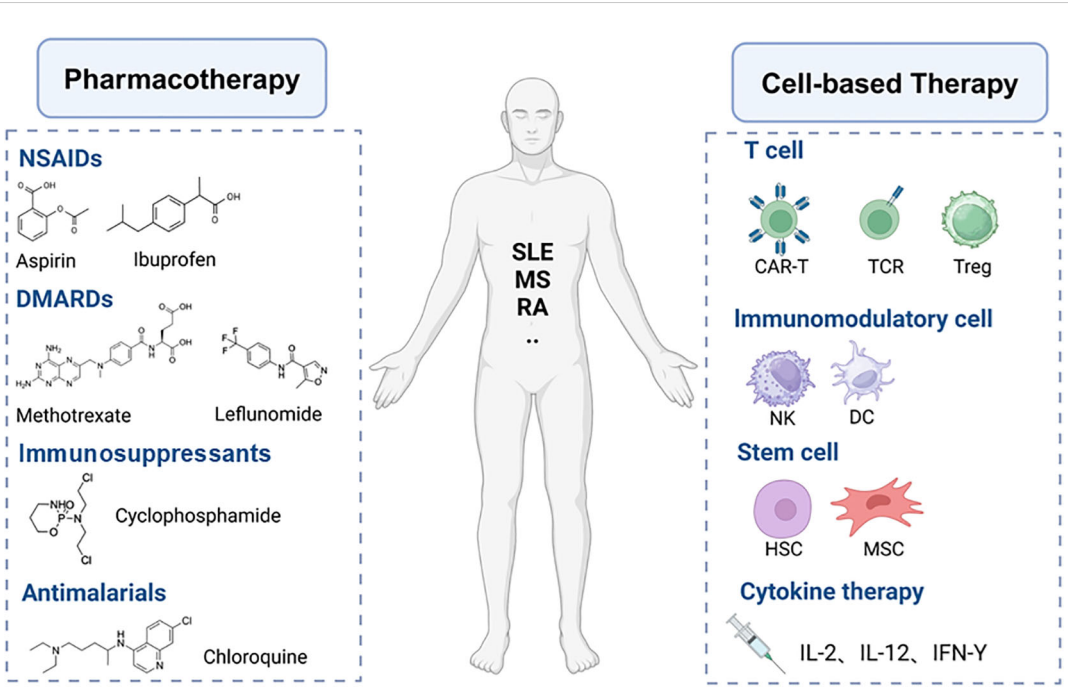

FIGURE 3
Pharmacotherapy and cell-based therapy for autoimmune diseases.

3.1 Treg cells

Tregs constitute 5-7% of CD4+ T cells and develop both directly in the thymus (thymus-derived Tregs, tTregs) and in peripheral tissues (peripherally-derived Tregs, pTregs). They possess the ability to suppress immune responses and promote tissue repair, ensuring that the immune system maintains a proper balance between its responses to foreign antigens and self-antigens (63). Treg cells are located in inflamed tissues and localized secondary lymphoid organs, where they exert their immunosuppressive functions. The number and functionality of Treg cells are altered in autoimmune diseases, resulting in defects in immune tolerance, abnormalities in immunoregulation, increased inflammation, and the proliferation of autoimmune cells. Treg cells confer immune tolerance through various mechanisms, including the secretion of anti-inflammatory soluble mediators such as interleukin-10 (IL-10), transforming growth factor-beta (TGFβ), and interleukin-35 (IL-35). They also achieve this by depleting interleukin-2 (IL-2) and expressing cytotoxic T lymphocyte antigen 4 (CTLA-4), CD39, CD73, and other inhibitory cell-surface receptors (64). Furthermore, Tregs regulate the direct or indirect targeting of T cells by APCs. For instance, the binding of CTLA-4 to CD80/CD86 on APCs induces the enzyme indoleamine-2,3-dioxygenase (IDO) (65). Additionally, Treg binding to APCs can result in the removal of cell surface molecules (a process akin to phagocytosis), thereby altering co-stimulation and antigen presentation (66).

Several therapeutic strategies based on the function of Treg cells have been proposed to restore tolerance in affected tissues. Transcription factors in Treg cells play a pivotal role in maintaining immune tolerance, preventing autoimmunity, modulating immune responses, promoting immune homeostasis, and supporting transplantation tolerance. Forkhead box P3 (Foxp3) plays a crucial role in the differentiation, development, and functional stabilization of Tregs. Fluctuations in Foxp3 protein levels can alter the stability of Treg function. Such variations are associated with a spectrum of significant immune-related disorders in humans. These include infectious diseases, autoimmune diseases, allergic diseases, tumorigenesis and metastasis, as well as transplant rejection. The Foxp3 gene has thirteen mutations, including five within the forkhead domain, one causing a frameshift due to a nucleotide deletion, and another disrupting the leucine zipper domain through base deletion. Such mutations may destabilize the mRNA, resulting in reduced levels of normal Foxp3 protein in T cells. This can lead to immune-mediated disorders such as diabetes, lymphadenopathy, and cytokine storms *in vivo* (67). The therapeutic potential of Foxp3+ Tregs has been demonstrated in various preclinical models such as graft-versus-host disease (GVHD) (68), type 1 diabetes mellitus (T1D) (69), systemic lupus erythematosus (70), inflammatory bowel disease (71) and multiple sclerosis (72). Currently, over 50 clinical trials are either underway or have been completed to assess the safety and efficacy of Treg cell therapy for the treatment of conditions such as renal or liver transplantation, atypical forms of pemphigus, systemic lupus erythematosus, inflammatory bowel disease, autoimmune hepatitis, allergies, and asthma (73).

Treg cells play a crucial role in suppressing immune responses and maintaining immune homeostasis. The infusion of polyclonal Treg cells has demonstrated a favorable safety profile. The efficacy of this approach has been established in patients with type 1 diabetes mellitus and those with GvHD, with additional clinical trials currently underway for patients with Crohn's disease and aspergillosis. IL-2 is a vital factor in promoting Treg cell expansion by regulating the STAT5 signaling pathway and enhancing Foxp3 expression, which is essential for Treg cell function and proliferation (74). Consequently, IL-2 analogs, such as IL-2/antibody complexes and IL-2 mutant proteins, represent promising therapeutic strategies for treating autoimmune diseases. In August 2013, a phase I/IIa study (NCT02084238) led by Peking University People's Hospital showed that the administration of low-dose IL-2 has been shown to stimulate Treg cell expansion of patients with various autoimmune conditions involving (75). Amgen's AMG-592, an IL-2 mutein with increased regulatory T cell selectivity, is in phase I/II trials in systemic lupus erythematosus and graft-versus-host disease, which also showed enhanced Treg cell expansion after low-dose IL-2 infusion in patients with SLE and type 1 diabetes (76) However, the limitations of IL-2 therapy include its short half-life, the necessity for repeated injections, and the potential for anti-drug immune responses. To address these challenges, Treg cells have been engineered, including those with transgenic TCRs or CARs, which have shown improved efficacy in targeting specific antigens and suppressing effector responses compared to polyclonal Treg cell infusion.

3.2 MSCs

MSCs are pluripotent progenitor cells capable of supporting hematopoiesis and differentiating into various mesodermal cell lineages, such as osteoblasts, chondrocytes, and adipocytes (77). They possess significant repair potential through self-renewal and differentiation and are increasingly recognized for their ability to modulate the immune response, exhibiting immunomodulatory properties. MSCs are believed to play a crucial role in the formation of memory T-cell and B-cell survival niches in the bone marrow and interact effectively with a variety of myeloid and innate leukocytes, including dendritic cells, monocytes, and macrophages (78). This interaction modulates immune memory size, stability, and plasticity. Initially, MSCs were found to inhibit mitogen-induced T-cell proliferation *in vitro* and evade immune surveillance *in vivo*. Subsequent studies have demonstrated that MSCs can modulate immune responses during chronic inflammation by regulating cell recruitment, function, and fate within the innate and adaptive immune systems (79). Following extensive *in vitro* and *in vivo* preclinical trials, both autologous and allogeneic MSCs have been utilized in the treatment of various immune-mediated diseases, including graft-versus-host disease, Crohn's disease, multiple sclerosis, refractory systemic lupus erythematosus, and systemic sclerosis.

It is now established that autoreactive T lymphocytes, particularly CD4/helper T cells, play a crucial role in the development of autoimmune diseases. A significant finding across most autoimmune conditions is the imbalance between the effector T-cell subsets Th1/Th17 and Tregs, which produce the immunomodulatory cytokine IL-10. This imbalance ultimately leads to inflammation and damage in target tissues. Given that the immunomodulatory effects of MSCs have been shown to be most effective on CD4+ T cells, it is not surprising that MSCs have been utilized in the highest number of clinical trials aimed at treating autoimmune diseases. Currently, over 25% of MSC-related trials focus on autoimmune conditions; these 129 trials include 34 for inflammatory bowel disease (IBD), 25 for multiple sclerosis, 18 for type 1 diabetes, 16 for systemic lupus erythematosus, 12 for RA, 9 for psoriasis, and 15 for other autoimmune diseases. The majority of these trials are in the early phases, with 29 in phase I, 15 in phase II, and 73 in combined phases I/II. Additionally, there are seven ongoing trials assessing efficacy, including three in combined phases II/III, three in phase III, and one in phase IV. Furthermore, five trials have not yet been assigned a phase (80).

In a comprehensive analysis by Zeng et al (81), the therapeutic efficacy and safety of MSC transplantation were evaluated across five autoimmune diseases, encompassing 18 randomized controlled trials. Among patients with RA, a trio of trials by Yang et al (82) indicated that MSC treatment led to reduced disease activity, enhanced standing time over 50% alleviation in knee pain, and decreased medication reliance, with benefits persisting up to 12 months in the majority of cases. In the context of SLE, four randomized controlled clinical trials assessed parameters such as urinary protein levels, serum complement C3, and adverse events following MSC intervention. These trials revealed lower urinary protein levels and alongside elevated serum C3 levels in the treatment group compared to the control group, with no significant adverse events reported across all studies (83). Six trials concerning MS demonstrated that patients who received MSC therapy exhibited superior progression-free survival (PFS), a reduced total number of episodes, and without encountering any severe adverse events (84).

3.3 HSCs

The core pathological characteristic of autoimmune diseases is the aberrant attack of the immune system on self-tissues, leading to chronic inflammation and tissue damage. This conundrum of immune dysregulation has prompted the medical community to explore alternative cellular therapies, aiming to reset or rebalance the immune system and restore tolerance to self-antigens. In this field, HSCs have emerged as ideal candidates for the treatment of autoimmune diseases due to their unique immunomodulatory properties (85, 86). HSCs are capable of repopulating the immune system, reducing the number of autoreactive lymphocytes, and by secreting cytokines such as TGF-β and IL-10, they modulate the immune microenvironment, fostering the formation of an anti-inflammatory milieu (87). Furthermore, HSCs can differentiate into Tregs and other immunomodulatory cells, which play a crucial role in suppressing autoimmune responses.

Based on these mechanisms, hematopoietic stem cell transplantation (HSCT) has been established as a standard therapeutic approach for malignant hematological disorders, other oncological conditions, and severe immunodeficiencies (88). For patients with autoimmune diseases who have an inadequate response to conventional treatments, HSCT following high-dose chemotherapy is increasingly being utilized in clinical practice. The therapeutic principle of HSCT involves initial lympholysis to reduce the immune memory repertoire, including autoreactive clones, followed by a profound immune renewal through the regeneration of hematopoiesis and the immune system (10). This process not only offers patients the opportunity to rebuild their immune system but also provides the possibility for long-term remission of autoimmune diseases. In 2012, it was estimated that approximately 3,000 patients with autoimmune diseases worldwide received HSCT treatment (89). As clinical research progresses, the application prospects of HSCT in the treatment of autoimmune diseases are expected to expand further.

HSCT has demonstrated significant potential in addressing severe, refractory SLE. Multiple research outcomes have indicated that HSCT can markedly improve the disease prognosis for SLE patients while reducing their reliance on long-term immunosuppressive drug therapy. Since its initial application in the treatment of SLE in 1997, over 300 cases of autologous hematopoietic stem cell transplantation have been reported globally (90). Furthermore, autologous HSCT has shown positive effects in patients with aggressive MS, not only facilitating the recovery of neurological functions but also successfully halting the disease's progression. For patients with RA unresponsive to conventional therapies, HSCT treatment has achieved long-term remission of the condition. In 1996, the first case of autologous HSCT for a patient with rheumatic autoimmune disease was reported (91); the patient, who faced rejection for lung transplantation due to connective tissue disease and severe pulmonary arterial hypertension, ultimately benefited from HSCT. To date, more than 3,000 HSCT procedures have been conducted worldwide for patients with severe rheumatic and non-rheumatic autoimmune diseases (87). These data underscore the efficacy and feasibility of HSCT in treating certain autoimmune diseases, offering new directions for future therapeutic approaches.

HSCT offers a potentially transformative approach for the treatment of autoimmune diseases but still faces challenges such as GVHD, infection risks, donor matching difficulties, conditioning regimen toxicity, graft failure, and long-term complications. However, with the advent of non-myeloablative conditioning regimens, improved HLA typing techniques, gene-editing technologies, the development of new anti-rejection drugs, the expansion of indications, and the emergence of "off-the-shelf" stem cell products, the prospects for HSCT are continuously improving. This evolution provides patients with safer, more effective, and more accessible treatment options, expected to enhance long-term survival rates and quality of life.

3.4 CAR-T cells

With the inherent ability of T cells to infiltrate tissues, their high-affinity binding to specific targets, and their antitumor effector functions, CAR-T cell therapy is predicated on the precise targeting of tumor antigens, resulting in the lysis and destruction of tumor cells. CAR-T cells offer several advantages over monoclonal antibodies, as T cells are long-lived, capable of proliferation, can be transported to lymphoid tissues or target organs, and can develop memory populations that help prevent the recurrence of disease-causing lymphocytes (92). Zelig Eshhar first demonstrated a CAR prototype in 1982, which shares many conceptual similarities with the CAR structures used today (93). The cytoplasmic portion of the CAR includes signaling domains (the CD3ζ chain of the T-cell receptor) and costimulatory domains (such as 4-1BB or CD28) to ensure the proper expansion and activation of CAR-T cells, as well as the proliferation of target cells. The CD19 antigen, expressed by B-cell-derived malignancies such as lymphomas and leukemias, was the first clinically applicable target for cancer immunotherapy using autologous CAR-T cells (94).

CAR-T cells demonstrate significant potential in the treatment of autoimmune diseases due to their ability to eliminate pathologically activated immune cells and to reestablish immune tolerance in organs affected by immune dysregulation. The number of studies investigating the use of CARs for treating autoimmune diseases is rapidly increasing (Table 1), with a particular focus on CAR-T cells and T cells (95). In 2021, CAR-T cells targeting CD19 were first utilized to treat a 20-year-old woman with severe treatment-refractory SLE (96). This approach confirmed the feasibility of producing CAR-T cells from patients with autoimmune diseases, and patient compliance with CAR-T cell infusions like this is high without any serious toxic reactions. This marked the first CAR-T cell-based treatment for an autoimmune disease, and additional clinical trials using anti-CD19/BCMA CAR-T cells or anti-CD19 CAR-T cells for relapsed/refractory SLE are currently ongoing (97). Using CD19-targeted CAR-T cell therapy, some researchers successfully treated a patient with anti-synthetase antibody syndrome, who fully recovered from the autoimmune disease without the need for immunosuppressive medications six months after CAR-T cell therapy (98). Over the past two years, CAR-T cells have also been employed in preclinical trials for MS, type 1 diabetes mellitus, inflammatory bowel disease, SLE, and pemphigus vulgaris (PV) suggesting new hope for therapeutic options in autoimmune diseases (99). In RA, one study designed CAR-T cells to eliminate specific autoreactive B cells by citrullinating antigenic epitopes, resulting in the lysis of B-cell subsets (100). Additionally, another study constructed CD8 T cells expressing the *HLA-DR1* CAR, which led to a reduced CD4 T cell response and inhibition of autoantibody production, indicating the potential for a highly specific therapeutic approach in the treatment of autoimmune diseases (101).

The expression of CAR in Tregs represents a promising strategy to enhance the efficacy and specificity of Treg therapy. Researchers have developed CAR-Tregs that target carcinoembryonic antigen (CEA) for the treatment of ulcerative colitis (UC). These anti-CEA CAR-Tregs specifically inhibited colitis symptoms in various experimental UC models using the colons of CEA transgenic mice (102). In a landmark study, researchers engineered CAR-Tregs against 2,4,6-trinitrobenzene sulfonic acid (TNBS) in a mouse model of colitis (103). CAR-Tregs secrete inhibitory factors, proliferate, and ameliorate disease symptoms in an antigen-specific manner. Similar findings have been reported in mouse models of multiple sclerosis and transplant rejection (104–106). CAR-Tregs demonstrate therapeutic efficacy at doses comparable to those of non-engineered Tregs, indicating that CAR expression not only enhances the efficacy of Treg therapy but also increases its specificity. Overall, these studies provide a robust theoretical foundation for clinical trials of CAR-Treg therapy.

Transient mRNA-based CAR-T cell therapy has emerged as a promising approach for the treatment of autoimmune diseases. Conventional CAR-T cell therapies utilize lentiviral or γ-retroviral vectors to introduce permanent genetic modifications to T cells,

TABLE 1 The status of CAR-based therapies in autoimmune diseases (until 05/2024).

Target antigen	Cell type	Type of disease	Clinical Trials	Research status
CD19	CAR T	Systemic lupus erythematosus	NCT03030976	Phase 1
CD19, BCMA	CAR T	Systemic lupus erythematosus	NCT05030779	Phase 1
BCMA	mRNA CAR T	Generalized myasthenia gravis	NCT04146051	Phase 2
CD19/BCMA/CD138/BAFF-R	CAR T	Autoimmune diseases	NCT05459870	Phase 2
PD-1	CAR T	Primary biliary cholangitis	——	Preclinical study
BCMA/CD19	CAR T	Relapsed/Refractory systemic lupus erythematosus	NCT05474885	Phase 1
CD7	CAR T	Crohn diseases; Ulcerative colitis	NCT05239702	Phase 1
CD19/CD20	CAR T	Neuromyelitis optica spectrum disorder	NCT03605238	Phase 1
BCMA	CAR T	Neuromyelitis optica spectrum disorder	NCT04561557	Phase 1/2
HLA-A2 antigens	CAR Treg	HLA-A2 mismatched liver transplantation	NCT05234190	Phase 1/2
MuSK	CAAR T	MuSK myasthenia gravis	NCT05451212	Phase 1
DSG3	CAAR T	Mucosal-dominant pemphigus vulgaris	NCT04422912	Phase 1

which carry risks of genotoxicity and regulatory challenges. Additionally, CAR-T cells may persist for a lifetime (107). Given that the safety standards for autoimmune trials are exceptionally high —often exceeding those for cancer trials—mRNA-based CAR-T cell therapies present an alternative strategy for delivering CAR-encoded mRNAs to T cells without permanently altering their genomes. This method allows for transient, time-limited expression of CARs, providing a controlled and reversible therapeutic approach. In a phase 1b/2a clinical study, mRNA-based CAR-T cell therapy demonstrated the potential for more durable symptomatic relief of myasthenia gravis and was well tolerated, with no significant adverse effects reported in patients (108). However, the potential immune response to CAR-T cells, the need for precise dosing and treatment timing, and the requirement for Good Manufacturing Practice (GMP)-compliant drug manufacturing have yet to be fully addressed in this rCAR-T cell study.

In the context of malignant diseases, the risk of immune escape, along with tumor recurrence and toxic effects, represents one of the most significant limitations of CAR-T cell therapy Cytokine release syndrome is particularly concerning as a toxic effect of CAR-T cell therapy; mild cases may present with symptoms such as fever, headache, arthralgia, and myalgia, but severe cases can lead to hypotension and even cytokine storm. Another common side effect is immune effector cell-associated neurotoxicity syndrome, which can manifest as fine motor deficits, resulting in writing difficulties and altered speech. Additional symptoms associated with immune effector cell-associated neurotoxicity syndrome include headache, confusion, seizures, and behavioral changes. Therefore, further basic and preclinical studies are essential to evaluate CAR-T cell-based therapies.

3.5 CAAR-T cells

Significant success has been reported in the use of anti-CD19/anti-CD19 BCMA CAR-T cells for the treatment of cancer and SLE. However, these approaches result in the depletion of all B cells in the body (109). Consequently, B cells may be absent for weeks to months, leaving patients immunocompromised during this period. To address this widespread B-cell depletion, researchers have developed CAARs that specifically and precisely target pathogenic B-cell subsets. Therapeutic T cells are genetically modified with chimeric receptors that incorporate target antigens for autoantibodies as extracellular structural domains. Unlike CAR-T cells, which express molecular receptors found on pathological cells, CAAR-T cells express extracellular autoantigens recognized by the B-cell receptor (BCR) (110). This extracellular structural domain is linked to the transmembrane structural domain, the costimulatory structural domain, and the activation structural domain, similar to the intracellular components of CARs.

Autoantigen recognition by autoreactive B cells leads Autoantigen recognition by autoreactive B cells leads to the activation of CAAR-T cells and the specific lysis of pathogenic B cells. Findings from a preclinical mouse model of atypical pemphigus suggest that CAAR-T cells expressing Desmoglein 3 may be effective in treating the rare skin disease known as pemphigus vulgaris (99). In a recent study, researchers developed a novel therapy for the most prevalent form of autoimmune encephalitis, specifically NMDA receptor encephalitis (110). Programmed CAAR-T cells are designed to recognize and eliminate anti-NMDA receptor antibody-producing B cells with high precision, and this innovative approach demonstrated its efficacy in a mouse model. Muscle-specific tyrosine kinase myasthenia gravis (MuSK MG) is an autoimmune disorder characterized by life-threatening muscle weakness due to the presence of anti-MuSK autoantibodies, which disrupt neuromuscular junction signaling. Researchers engineered T cells expressing the MuSK chimeric autoantibody receptor with a CD137-CD3ζ signaling domain (MuSK-CAART) to specifically target B cells that produce anti-MuSK autoantibodies (111). In an experimental autoimmune MG mouse model, MuSK-CAART effectively reduced anti-MuSK IgG levels without affecting overall B-cell counts or total IgG levels, indicating the selective depletion of MuSK-specific B cells. While CAAR-T cell approaches have shown promise in treating various autoimmune disorders, their development is limited to diseases caused by single autoantibody-producing monoclonal B-cell clones.

3.6 CAR-NK cells

A total of six CAR-T cell therapies have been approved to date, demonstrating significant promise primarily for the treatment of hematological malignancies. However, this approach is effective only in a subset of patients and is associated with considerable side effects, such as cytokine release syndrome and neurotoxicity, which further limits its broader application in cancer treatment (112). To address these limitations, researchers have turned their attention to NK cells, which are integral to the innate immune response—the body's first line of defense against infections—and play crucial roles in antiviral, anticancer, and anti-aging processes. NK cells, along with T and B cells, form a major subset of lymphocytes capable of nonspecific, non-MHC-restricted direct tumor cell killing (97). NK cells are pivotal in cancer immunity as they target cancer cells that downregulate HLA class I molecules or express stress markers. Furthermore, NK cells can be genetically modified to express CARs and can be utilized without the need for recipient-matched human leukocyte antigens, thereby eliminating the necessity for patient-specific production of CAR products. One study demonstrated that lupus-like mice treated with CAR-NK cells exhibited improved splenomegaly and a reduction in the number of PD-1+CD4+ T cells (113). Additionally, a clinical phase 1/2 trial involving umbilical cord blood-derived NK cells expressing an anti-CD19 chimeric antigen receptor and IL-15 (cAR19/IL-15) was conducted with 37 patients suffering from CD19+ B-cell malignancies. The results indicated that none of the patients developed neurotoxicity or graft-versus-host disease, and only one patient experienced cytokine release syndrome (grade I) (114). These findings underscore the feasibility of utilizing CAR-NK cells for cancer treatment.

According to previous studies, there exists an innate lymphocyte population derived from NK cells known as tissue-resident memory natural killer (NKRM) cells. These cells play a crucial role in regulating immune responses within tissues by preventing the immune system from mistakenly attacking its own tissues or organs, thereby helping to avert autoimmunity (115).

Researchers have preliminarily demonstrated that NKRM cells exhibit distinct immune functions compared to traditional memory cells; they can eliminate CD4+ T cells, reduce autoimmunity in a TRAIL-mediated manner, and may hold significant potential for research into the treatment of SjD (115). The results of this study revealed that four patients with severe disease achieved deep remission after treatment with these cells, transitioning from "severe" to "mild" disease, with corresponding durability. Additionally, two patients with severe disease attained deep remission after just 15 days of treatment, with SLEDAI-2K disease scores decreasing from 14 and 17 points (indicating severe disease) to 1 and 3 points (indicating mild disease), respectively. This indicates a highly positive effect of CAR-NK cell therapy. This study represents the first international clinical report on the use of CAR-NK cells for the treatment of SLE.

4 Other emerging therapies

Gene therapy has emerged as a promising approach for the treatment of autoimmune diseases, with the CRISPR-associated protein 9 (CRISPR-Cas9) system being one of the most prominent methods. The CRISPR gene-editing technology enables rapid and efficient generation of gene knockouts, modulation of endogenous gene expression, and replication of genomic alterations associated with cancer. The CRISPR-Cas9 technique has demonstrated potential in the therapeutic research of various autoimmune diseases, including RA, SLE, MS, type 1 diabetes and psoriasis (116). Xu et al. are exploring the use of CRISPR-Cas9 technology to modulate immune responses, including the knockout or modification of specific immune cells to reduce autoimmune reactions (117). For instance, by knocking out certain genes in T cells, the function of Tregs can be enhanced, thereby suppressing excessive immune responses. A research team, after constructing CAR-T cells targeting CD19, used the CRISPR-Cas9 gene-editing tool to knockout five genes in the CAR-T cells (HLA-A, HLA-B, CIITA, TRAC, and PD-1) to avoid the graft-versus-host disease caused by allogeneic T cells. This led to the development of a new generation of off-the-shelf CAR-T therapy (TyU19), which successfully treated one patient with refractory immune-mediated necrotizing myopathy (IMNM) and two patients with diffuse cutaneous systemic sclerosis (dcSSc) (118). During the 6-month follow-up after treatment, all three patients experienced profound symptom relief, significant improvement in disease clinical response index scores, and reversal of inflammation and organ fibrosis. The entire treatment process for the three patients was well-tolerated, with no observed CRS, GvHD, or immune effector cell-associated neurotoxicity syndrome (ICANS) commonly seen in cancer patients receiving CAR-T cell therapy. These findings highlight the potential of this novel treatment approach to provide safer and more effective therapeutic options for patients with autoimmune diseases. With continuous technological advancements and the conduct of clinical trials, it is anticipated that more applications of CRISPR-Cas9 will be developed in the future to improve the therapeutic outcomes for autoimmune diseases.

In the quest for alternative immunotherapies for autoimmune diseases, the Proteolysis-targeting chimera (PROTAC) technology has emerged as a promising new therapeutic strategy. This technique harnesses the ubiquitin-proteasome system (UPS) and small molecules to achieve the degradation of specific target proteins, offering a novel approach to treatment (119). Bruton's tyrosine kinase (BTK), a crucial regulator of B cell development, proliferation, activation, and differentiation, has become a focal point for therapies targeting B cell malignancies and autoimmune diseases. The role of BTK in B cell-related conditions positions it as a key target for intervention (120). Although BTK inhibitors show potential in treating autoimmune diseases, the clinical outcomes have been mixed due to challenges in efficacy and safety. To address these issues, Liu et al. have developed a new generation of BTK-PROTAC degraders, such as L18I, which demonstrate significant efficacy in autoimmune disease mouse models (121). L18I effectively alleviates symptoms of lupus and diffuse alveolar hemorrhage (DAH) by reducing autoantibody production and mitigating inflammatory responses. Moreover, L18I exhibits high selectivity for proteins that Ibrutinib, a BTK inhibitor, struggles to target, such as ITK, EGFR, and HER2. This indicates that L18I can effectively degrade BTK in various tissues, making it a strong candidate for the treatment of autoimmune diseases. These findings underscore the potential of PROTAC technology in developing innovative therapeutic strategies for autoimmune diseases and highlight the importance of degraders targeting key signaling molecules like BTK in modulating immune responses and alleviating disease symptoms. With further research and optimization of these emerging therapies, we anticipate they will offer more effective and safer treatment options for patients with autoimmune diseases.

5 Outlook

Current strategies for the treatment of autoimmune diseases primarily involve the use of immunosuppressive drugs and biologically targeted therapies; however, none have yielded satisfactory clinical outcomes. The rapid advancement of cell-based therapies and synthetic immunology approaches has expanded their potential to treat human diseases. In this paper, we present a comprehensive overview of three prevalent autoimmune diseases and six research advancements in cellular therapy for these conditions.

Cellular therapy represents a novel approach for treating tumors and one FDA-approved engineered immune cell therapy, known as CAR-T cell therapy, has demonstrated success in treating specific B-cell malignancies. In recent years, the application of immune cell therapies has expanded to include the treatment of autoimmune diseases, yielding several promising results. However, despite the potential indicated by preclinical studies, no immune cell therapies have yet received approval for the treatment of autoimmune diseases, and only a limited number of approaches have progressed to phase 1 or phase 2 clinical trials.

As immunotherapy for autoimmune diseases is often not initiated until the disease has progressed significantly, resulting in tissue damage and inflammation, the identification and validation of biomarkers that can predict therapeutic response and monitor disease progression will enable a more precise cellular therapy strategy. The

current approach of long-term immunosuppression in treating autoimmune diseases could be transformed by cellular therapy into a strategy that induces an immune reset without the need for ongoing treatment. A deeper understanding of the pathological mechanisms underlying autoimmune diseases, coupled with recent advances in cell manufacturing technologies, will facilitate the development of novel and potent therapies that fundamentally alter cell–cell interactions and improve clinical outcomes.

Cellular therapy, as an emerging treatment modality, offers new avenues for the management of autoimmune diseases, yet it also presents challenges related to safety, ethics, cost, and technical expertise. In terms of safety, vigilant monitoring is required for potential side effects such as CRS and neurotoxicity associated with CAR-T cell therapy, while the stability and durability of Tregs and MSCs therapies must also be assessed. The uncertainty surrounding long-term efficacy and disease recurrence rates necessitates long-term follow-up studies to evaluate the persistence of treatments and the potential for relapse. On the ethical front, since cellular therapy technologies are often in experimental stages, it is crucial to ensure that patients fully comprehend the risks of treatment, possible adverse reactions, and the uncertainty of therapeutic outcomes. Moreover, the high cost and complexity of cellular therapy preparation demand sophisticated technical skills, posing a challenge for many medical institutions and limiting the broad application of these therapies. Therefore, optimizing manufacturing processes, enhancing the stability of cell products, and fostering international collaboration to share data and harmonize clinical trial standards are essential for reducing costs, increasing accessibility, and advancing the field.

Autologous cell therapies, such as autologous CAR-T cell therapy, utilizes the patient's own cells for modification to avoid rejection reactions. However, the preparation process is complex and time-consuming. Allogeneic cell therapies, which utilize native immune cells or cells derived from induced pluripotent stem cells (iPSCs), show broad application prospects due to the potential for mass production and cost-effectiveness. Allogeneic iPSC technology has shown tremendous potential in treating a variety of diseases, including neurological disorders, cardiovascular diseases, and autoimmune diseases. The world's first off-the-shelf CAR-T therapy (TyU19) for the treatment of autoimmune diseases has demonstrated significant efficacy and safety in clinical trials (122). Concurrently, in the treatment of autoimmune diseases, Tregs derived from allogeneic iPSCs have also shown therapeutic potential. Professor Shin Kaneko and his team from Kyoto University, have successfully induced Treg-like cells from iPSCs-derived conventional helper T cells (Tconvs) and confirmed their ability to suppress xenogeneic GvHD (123). Both autologous and allogeneic cell therapies have shown their respective advantages and challenges in the treatment of autoimmune diseases. As technology continues to advance and clinical trials progress, the future application prospects of iPSC in the field of cell therapy will be even broader.

In conclusion, cellular therapy demonstrates significant potential and promise in the treatment of autoimmune diseases, but they still require further research and validation in terms of efficacy, safety and cost-effectiveness. Future research aims to enhance the efficacy of immunomodulatory treatments while avoiding adverse reactions and to develop personalized immunocyte therapy strategies using patients' specific genetic and immunological information to improve treatment success rates. Additionally, exploring the integration of cell therapy with traditional drug treatments, physical therapies, and other treatment modalities to form comprehensive treatment strategies is an important direction for enhancing therapeutic outcomes. With continued research and technological advancements, we anticipate that cellular therapy will improve both the quality of life and long-term health outcomes for patients.

Author contributions

YF: Writing – original draft. CF: Writing – original draft. SQ: Writing – review & editing. ZX: Writing – review & editing. CL: Writing – review & editing. ZL: Writing – review & editing, Conceptualization, Supervision. HY: Writing – review & editing, Conceptualization, Supervision.

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Conflict of interest

Author CF was employed by the company Jiangxi Health-Biotech Stem Cell Technology Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

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Prognostic significance of programmed cell death 1 expression on CD8+T cells in various cancers: a systematic review and meta-analysis

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Background: Increased PD-1 expression on CD8+ T cells is considered as a hallmark for T-cell exhaustion, and is thought to be related to the prognosis of cancer patients. However, discrepant results have made it difficult to apply PD-1 +CD8+T cells and tumor prognosis to clinical practice. Therefore, we conducted a meta-analysis to evaluate its prognostic value in human cancers.

Methods: PRISMA reporting guidelines were strictly followed for conducting the current meta-analysis. The PubMed, Web of Science, Embase databases were searched from inception to November 2024. The pooled Hazard Ratio (HR) along with 95% confidence intervals (CIs) of each article were combined for the associations of PD-1+CD8+ T cells with overall survival (OS), progression- free survival (PFS) and disease-free survival(DFS). Subgroup analyses were performed for area, specimen type, cancer type, treatment, detected method and cancer stage.

Results: A total of 20 studies (23 cohorts, 3086 cancer patients) were included in our study. The expression PD-1+CD8+ T cells in cancer patients tended to predict poor overall survival (OS) (HR: 1.379, 95%CI: 1.084-1.753, p= 0.009), and unfavorable disease-free survival(DFS) (HR: 1.468, 95%CI: 0.931-2.316, p=0.099), though it did not reach statistical significance. Begg's and Egger's test demonstrated that no obvious publication bias was exist.

Conclusions: High PD-1 expression on CD8+ T cells is associated with worse survival outcomes, which can be potentially used as a prognostic marker of malignant tumor.

KEYWORDS

PD-1+CD8+ T cells, overall survival, progression-free survival, disease-free survival, cancer

1 Introduction

Immune cells are known to be determinants of tumorigenesis, progression and metastasis, which play an important role in tumor elimination, surveillance and escape (1). Among these immune cells, cytotoxic CD8 + T cells represent the main anti-tumor TIL population and are considered as a positive prognostic factor in the majority of tumors. However, some studies found that the successful clearance of cancer cells by tumor-infiltrating lymphocytes (TILs) is impeded by a series of immune inhibitory mechanisms that are active in the tumor microenvironment (2–4), including the upregulation of immune checkpoint proteins such as PD-1, CTLA-4, Tim-3 and Lag-3 on TILs (5).

Programmed death-1 (PD-1) is a surface receptor expressed by lymphocytes that suppresses its proliferation and effector function by binding to PD-1 ligands such as B7-H1 (also known as PD-L1) and B7-DC (also known as PD-L2) expressed on other cells (6). Recently, some studies have showed T cells expressing these checkpoint molecules are considered to be characterized by a state of dysfunction, accompanied with loss the ability of cytokine production (IL-2, TNF-α and IFN-γ) and killing capacity, termed T cell exhaustion (7–9). A study pointed out that the tumor-infiltrating effector CD8+ T cells showed a drastic increase in PD-1 expression, and PD-1 upregulation promoted CD8+ T-cell apoptosis and postoperative recurrence in hepatocellular carcinoma patients (10). Although PD-1 can be expressed on any T cell during activation, it is frequently linked to the exhaustion of CD8 + T cells (11). Therefore, increasing researchers focused their attention on the role and activation status of infiltrating CD8+ T-cells including PD-1 expression in these immune cells.

Whether the expression of PD-1 on CD8+T cells can be used as a prognostic indicator has been explored. Hsu and his colleagues (12) found that increasing of programmed death-1-expressing intratumoral CD8+ T cells predicted a poor prognosis for nasopharyngeal carcinoma. In addition, according to Ma's research, CD8 + T cells were classified intoPD1 Hi, PD1 Int and PD1-, PD1 Hi CD8 + T cells highly expressed exhaustion-related inhibitory receptors (TIM3, CTLA-4, etc.) and transcription factors (Eomes, BATF, etc.), and PD1 Hi CD8 + T cells were significantly correlated with poor prognosis (13). While some objections have been raised, they suggested that high proportion of PD-1 in CD8+ tumor-infiltrating T-cells improved survival outcomes in some cancers. Pokrývková *et* (14) indicated that increased expression of PD-1 in CD8+ T cells conferred improved survival outcomes, and PD1 + CD8 + Cells are an independent prognostic marker in patients with head and neck cancer. These results indicated a controversial prognostic value of PD-1+CD8+ T cells in human cancers.

The inconsistent results may be due to the different area, specimen type, cancer type, anti- cancer therapy, detected method and cancer stage. Hence, we conducted a meta-analysis to systematically assess the prognostic role of PD-1+CD8+ T cells in various cancers.

2 Materials and methods

2.1 Literature search and search strategy

The preferred reporting items of the Systematic Review and Meta-Analysis (PRISMA) Reporting Guidelines are strictly followed to document current meta-analyses. To verify compliance with established guidelines, the PRISMA checklist has been incorporated into Supplementary Table S1 as a key tool for determining compliance. The protocol for the meta-analysis was registered at the International Platform of Registered Systematic Review and Meta-analysis Protocols (INPLASY) and assigned the registration number INPLASY2024110075. We systematically searched the PubMed, Web of Science, Embase databases prior to November 2024. The search key words were as follows: (((((((cancer [Title/Abstract]) OR (carcinoma[Title/Abstract])) OR (neoplasm [Title/Abstract])) OR (tumor[Title/Abstract])) OR (tumor[Title/Abstract])) AND ((pd-1[Title/Abstract]) OR (programmed cell death 1[Title/Abstract]))) AND (CD8+ T cell[Title/Abstract])) AND (prognosis[Title/Abstract]). Studies investigating the association between PD-1 expression on CD8+ T cells and cancer patient survival is currently a candidate for our meta-analysis. Besides, manual review was conducted on the references of relevant articles for additional candidate studies. The search procedures and related keyword combinations are documented in Supplementary Table S2.

The PICOS framework was as follows:

Population: This study involved patients who were diagnosed with cancer.

Intervention: The intervention under consideration was the presence of programmed cell death 1 expression on CD8+ T cells.

Comparison: The comparison was conducted to assess the presence of programmed cell death 1 expression on CD8+ T cells on survival.

Outcomes: OS and/or PFS and/or DFS in the presence of programmed cell death 1 expression on CD8+ T cells.

Study design: Case–control.

2.2 Study selection and inclusion-exclusion criteria

In order to search for relevant articles, two independent reviewers first screened the title and abstract of the article, and then conducted further access to the entire article. Differences among reviewers are resolved through discussion or negotiation with a third researcher until consensus is reached. Articles were included if they met the following inclusion criteria: (1) the researcher population must be diagnosed cancer patients; (2) at least one of these patient groups must detect PD-1 expression on CD8+T cells; (3) the study population should include the hazard ratio (HR) and 95% confidence interval (95% CI) of PD-1+CD8+T cells associated with OS and/or PFS and/or DFS, or provide

sufficient data to calculate HR and 95% CI. Besides, the exclusion criteria were as follows: (1) reviews, letters or case reports; (2) animal or cell-line articles; (3) accompanied by other detection markers; (4) inefficient in providing data for calculate HR and 95% CI.

2.3 Data retrieval and quality assessment

Two researchers extracted information from eligible studies, and the information was as follows: first author, publication year, country, number of samples, specimen type, anti-cancer therapy, cancer stage, HR, and 95% CI of survival outcomes. In addition, to evaluate the quality of the included articles, the Newcastle–Ottawa scale (NOS), which was used to assess the quality of cohort studies and case–control studies. The highest score is 9 points, and studies with a score of six or more are considered to be high-quality (15). Quality assessment was presented in Supplementary Table S3.

2.4 Statistical analysis

Stata 12.0 was adopted for the meta-analysis, and $p<0.05$ was considered statistically significant. HR for OS, PFS and DFS and 95% CIs were pooled to measure the relationship between time and events. Heterogeneity was assessed by Cochran's Q and I-squared (I^2) tests. $I^2>50\%$ with p value of Q test <0.1 indicated significant heterogeneity, and then a random- effect model was applied to combine HR and 95% CI of survival outcomes. Otherwise, a fixed-effect model was used. In addition, to find the source of significant heterogeneity, sensitivity analysis and subgroup analysis were performed. Begg's and Egger's test were used to assess publication bias.

3 Results

3.1 Literature retrieval and selection

Initially, 282 articles were obtained through literature screening in PubMed (n=105), Web of Science (n=136), and Embase (n=41) databases. Of these, 35 articles were excluded due to duplication. After filtering through titles and abstracts, 224 articles were deleted for the following reasons: reviews, case reports, meeting, animal or cell-line articles and literatures were not relevant to our study. Then, a review of the entire article was conducted. Owing to report the ratio of PD-1+/CD8+ T cells and insufficient data, 6 articles were eliminated. Finally, 20 eligible studies (12, 13, 16–33) were included in our meta-analysis. The flow chart of the whole selection process is shown in Figure 1.

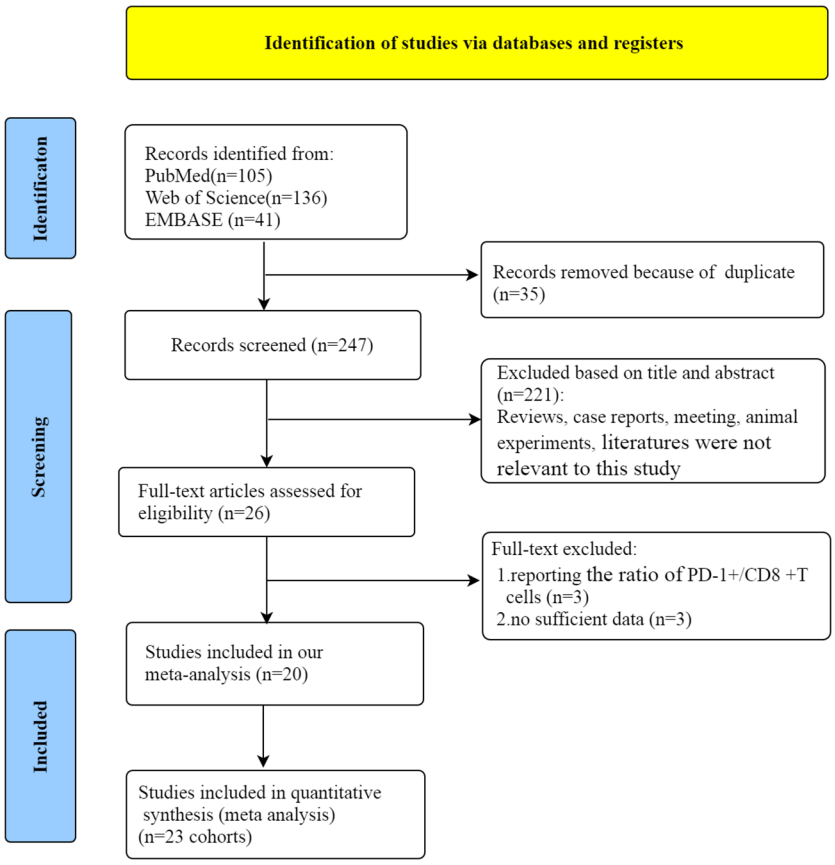

FIGURE 1
Flow chart for the literature search and study selection.

3.2 Study characteristics

A total of 20 studies with 3086 patients were included in our meta-analysis. They mainly come from China, Japan, South Korea, Singapore, Italy, the United States, and Switzerland, Greece. These studies included a variety of tumor types such as gastric cancer, non-small cell lung cancer, triple-negative breast cancer, pancreatic cancer, acute myeloid leukemia, hepatocellular carcinoma, head and neck cancer, mesothelioma. The specimen type of 14 articles was tissue, while 6 articles was peripheral blood. Five articles received immune checkpoint inhibitor therapy, 9 articles adopted other therapies(including surgical resection, chemotherapy, curative surgery+chemotherapy, and allo-SCT and so on) and 6 articles did not receive treatment. PD-1+CD8+ T cells was detected for protein expression by dual immunohistochemistry in 14 studies, and for flow cytometry in 6 studies. Specifically, three studies (13, 19, 20) each had two cohorts of cancer patients and then included each cohort in the quantitative analysis as an individual study. The characteristics of 20 studies are summarized in Table 1.

Seventeen studies comprising 2849 patients evaluated the association of PD-1+CD8+ T cells with OS. An obvious heterogeneity (I^2 = 113.51%, p < 0.001) was observed, so a random-effect model was applied. Patients with high PD-1+CD8+ T cells had significantly worse OS (HR = 1.379, 95% CI 1.084-1.753, p = 0.009, Figure 2A). The association between PD-1+CD8+ T cells and PFS was evaluated in 5 studies comprising 306 patients. Pooled analysis using a random-effect model demonstrated that patients with elevated PD-1+CD8+ T cells were not associated with PFS (HR = 1.006, 95% CI 0.417-2.430, p = 0.989, Figure 2B). The association between PD-1+CD8+ T cells and DFS was evaluated in 8 studies comprising 1359 patients. A random-effect model indicated that patients with elevated PD-1+CD8+ T cells had a borderline association with DFS (HR = 1.468, 95% CI 0.931-2.316, p = 0.099, Figure 2C).

3.3 Subgroup analyses of PD-1+CD8+ T cells in association with survival

Due to significant heterogeneity in the pooled HRs of OS in cancer patients and moderate heterogeneity in the pooled HRs of DFS in cancer patients, we conducted subgroup analysis on the selected literature to find out the sources of heterogeneity. Therefore, a subgroup analysis based on study region, cancer type, specimen type, tumor treatment, stage and detect method was carried out. The results of subgroup analyses for OS and DFS are shown in Tables 2, 3, respectively.

There was heterogeneity between research in different regions. The pooled HRs for OS in the Asian subgroup were 1.479 (95%CI: 1.160-1.887, p= 0.002), the pooled HRs for OS in the Europe subgroup were 0.654 (95%CI: 0.152-2.814, p=0.568). The pooled HRs for DFS in the Asian subgroup were 1.422 (95%CI: 0.730-2.772, p=0.301), the pooled HRs for DFS in the Europe subgroup were 1.653 (95%CI: 0.919-2.973, p=0.093). Subgroup analysis based on sample types showed that tumor tissues as sample types had poor OS (HR = 1.497, 95% CI 1.078-2.079, p = 0.016), while peripheral blood were not associated with OS (HR = 1.08, 95% CI 0.559-2.088, p = 0.819).

When the included studies were analyzed by subgroups based on cancer type. PD-1+CD8+T cells predict significantly worse OS in pancreatic cancer (HR=3.304, 95%CI 1.167-9.357, p=0.024), and significant worsening of DFS in gastric cancer and head and neck cancer (HR = 2.228, 95% CI 1.748-2.839 p<0.0001; HR = 2.972, 95% CI 1.194-7.395, p=0.019, respectively), but was associated with improved OS and DFS in triple negative breast cancer (OS: HR = 0.544, 95% CI 0.302-0.982, p= 0.043; DFS: HR =0.46, 95% CI 0.303-0.699, p<0.0001). No association was found between PD- L1 + CTCs and survival in other cancers. Due to the small number of studies, further studies were needed to confirm our results in the future.

When stratified for treatment, PD-1+CD8+ T cells were significantly associated with worse OS and DFS for other therapies (OS: HR = 1.507, 95% CI 1.126-2.017, p =0.006; DFS: HR=1.871,95%CI 1.330-2.630, p<0.0001, respectively). Surprisingly, PD-1+CD8+ T cells seemed to predict a better OS (HR = 0.421, 95% CI 0.063- 2.827, p = 0.374) for ICI treatment, though not reach statistically significant.

PD-1+CD8+ T cells predicted poor OS in double-stained IHC (HR = 1.384, 95% CI 1.079-1.776, p = 0.011). but it was not found to be related to the patient's prognosis in flow cytometric. It is possible that there are too few studies using the same method. What's more, patients with all stage had a worse OS(HR = 1.589, 95% CI 1.245-2.029, p <0.0001), while were not associated with OS in advanced patients (HR = 0.626, 95% CI 0.225-1.738, p =0.368).

3.4 Sensitivity analyses

Due to significant heterogeneity was observed. In order to find the potential source of this heterogeneity, we carried out sensitivity analysis. Sensitivity analysis indicated that the results of our meta-analysis were robust and not significantly influenced by any single study (Figure 3).

3.5 Publication bias

Publication bias is considered the main factor affecting predictive value, so we conducted Egger and Begg tests to assess the presence of publication bias and the funnel plot symmetry was examined. Based on the shape of the funnel plot and no significant publication bias was not observed and P values in Egger and Begg tests. (p > 0.05) (Figure 4).

4 Discussion

We conducted the first meta- analysis to evaluate the clinical application of PD-1 expression on CD8+ T cells in predicting the survivals of cancer patients and to identify factors that modulate

TABLE 1 Characteristics of studies included in the meta-analysis.

Author	Year	Country	Cacer type	Number	Specimen type	Treatment	Detect method	Stage	Outcome	NOS
Thommen (16)	2018	Switzerland	NSCLC	21	tissue	PD-1 blockade	Immunohistochemical double stains	advanced	OS	7
Choo (17)	2023	Singapore	gastric cancer	350	tissue	gastrectomy	mIHC/IF	all stage	OS	7
Shin (18)	2023	Korea	gastric cancer	68	peripheral blood	Chemotherapy	fluorescence-activated cell sorting	avanced	OS,PFS	7
Hsu (12)	2010	Taiwan	nasopharyngeal carcinoma	46	tissue	untreat	double immunofluorescence staining	all stage	OS,DFS	9
Yu (19)	2022	China	gastric cancer	200	tissue	Adjuvant chemotherapy	double-stained IHC	all stage	OS,DFS	9
Yu (19)	2022	China	gastric cancer	241	tissue	Adjuvant chemotherapy	double-stained IHC	all stage	OS,DFS	9
Mazzaschi (20)	2017	Italy	NSCLC	100	tissue	rescected	double immunofluorescence	all stage	OS,DFS	6
Mazzaschi (20)	2017	Italy	NSCLC	26	tissue	nivolumab	double immunofluorescence	advanced	OS	6
SAITO (21)	2019	Japan	Gastric Cancer	72	peripheral blood	untreat	multicolor flow cytometry.	all stage	OS,PFS	7
Waki (22)	2014	Japan	NSCLC	78	peripheral blood	personalized peptide vaccine	Flow cytometric	advanced	OS	6
Ma (13)	2019	China	hepatocellular carcinoma	358	tissue	untreat	multiplex immunohis-tochemistry	all stage	OS	7
Ma (13)	2019	China	hepatocellular carcinoma	254	tissue	untreat	multiplex immunohis-tochemistry	all stage	OS	7
Kansy (23)	2017	America	head and neck cancer	56	tissue	untreat	flow cytometry	advanced	DFS	6
Yang (32)	2023	China	pancreatic ductal adenocarcinoma	84	tissue	unclear	multiplex IHC	all stage	OS	7
Homicsko (31)	2023	Switzerland	mesothelioma	144	tissue	pembrolizumab	multiplex IHC	advanced	PFS	7
Yeong (30)	2019	Singapore	triple negative breast cancer	269	tissue	untreat	multiplex immunofluorescent (mIF) staining	all stage	OS,DFS	7
Shen (29)	2017	China	pancreatic cancer	94	tissue	surgical resection	double immunofuorescence staining	all stage	OS	9
Guo (27)	2020	China	triple-negative breast cancer	328	tissue	untreat	multiplexed immunohistochemistry	all stage	OS,DFS	6
You (24)	2024	Korea	acute myeloid leukaemia	60	tissue	allo-SCT	Flow cytometric analyses	all stage	OS	9
Tang (25)	2020	China	acute myeloid leukemia	50	peripheral blood	chemotherapy	multiparametric flow cytometry	all stage	OS	6
Mazzaschi (26)	2019	Italy	NSCLC	31	peripheral blood	nivolumab	Flow Mass Cytometry	advanced	OS	7
Gao (28)	2017	China	gastric adenocarcinoma	119	tissue	adjuvant chemotherapy	Immunofluorescence	all stage	OS,DFS	9
Kotsakis (33)	2019	Greece	NSCLC	37	peripheral blood	chemotherapy-naïve	Flow cytometric analyses	advanced	PFS	6

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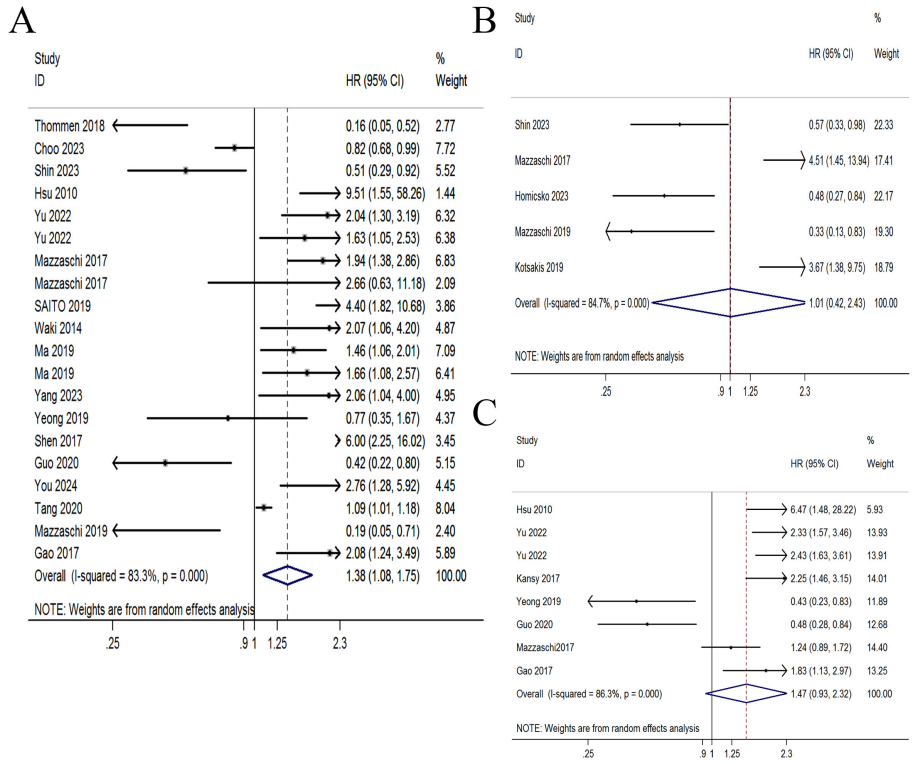

FIGURE 2
Forest plot of PD-1+CD8+ T cells with survival time. **(A)** Forest plot of PD-1+CD8+ T cells with overall survival. **(B)** Forest plot of PD-1+CD8+ T cells with progression-free survival. **(C)** Forest plot of PD-1+CD8+ T cells with disease-free survival.

TABLE 2 Association between PD-1+CD8+ T cells and overall survival in cancers.

				Heterogeneity		
	no of the studies	HR	95%CI	I^2	*P*	*P*
	17	1.379	1.084-1.753	113.51	<0.0001	0.009
Area						
Asian	14	1.479	1.160-1.887	85.97	<0.0001	0.002
Europe	3	0.654	0.152-2.814	25.89	<0.0001	0.568
Cancer type						
gastric cancer	5	1.453	0.867-2.433	41.39	<0.0001	0.156
non-small cell lung cancer	4	0.868	0.325-2.324	26.88	<0.0001	0.779
Pancreatic cancer	2	3.304	1.167-9.357	3.1	0.078	0.024
triple negative breast cancer	2	0.544	0.302-0.982	1.39	0.238	0.043
acute myeloid leukaemia	2	1.6	0.654 3.914	5.58	0.018	0.303
Specimen type						
tissue	12	1.497	1.078-2.079	83.14	<0.0001	0.016

(Continued)

TABLE 2 Continued

				Heterogeneity		
	no of the studies	HR	95%CI	I^2	*P*	*P*
Specimen type						
blood	5	1.08	0.559-2.088	26.29	<0.0001	0.819
Treatment						
untreat	6	1.525	0.906-2.568	28.56	<0.0001	0.112
other treatments	9	1.507	1.126-2.017	53.8	<0.0001	0.006
ICIs	3	0.421	0.063- 2.827	25.09	<0.0001	0.374
Stage						
all stage	13	1.589	1.245-2.029	83.8	<0.0001	<0.0001
advanced	5	0.626	0.225-1.738	23.82	0.001	0.368
Detect method						
double-stained IHC	12	1.384	1.079-1.776	81.96	<0.0001	0.011
Flow cytometric	4	1.662	0.607-4.547	16.02	0.001	0.323

prognostic value. Overall, high PD-1 expression on CD8+ T cells is associated with worse survival outcomes, which can be potentially used as a prognostic marker of malignant tumor.

CD8+T cells that express PD-1 thought to be characterized by a state of T cell exhaustion, which is accompanied with loss the ability of cytokine production (IL-2, TNF-α and IFN-γ) and killing capacity (13, 34, 35). Recent years, researchers have studied on the relationship between the expression of PD-1 on CD8+ Tcells and the prognosis of cancer patients. Lim et al (36) indicated that higher ratio of PD-1 +/CD8 + TILs was associated with poorer overall survival, relapse-free survival and distant metastasis-free survival, besides, the ratio of PD-1 +/CD8 + TILs was the independent prognostic factor in OS, RFS and DMFS after adjusting for other significant clinicopathologic variables. However, conflicting views have been raised and no consensus has been reached. Shen et al (37) showed that PD-1 + CD8 + T cells showed equivalent function to their PD-1 - CD8 +T cells counterparts and they did not predict tumor progression in gastric cancer. Due to the discrepant results among these studies, it is difficult to apply PD-1 + CD8 + T cells to clinical applicability. Therefore, our work emphasizes the predictive value of PD-1 expression on CD8+ T cells for cancer prognosis. We reviewed 20 studies of PD-1 + CD8 + T cells, and performed a systematic meta-

TABLE 3 Association between PD-1+CD8+ T cells and disease-free survival in cancers.

			DFS	Heterogeneity		
	no of the studies	HR	95%CI	I2	*P*	*P*
	8	1.468	0.931-2.316	50.98	<0.0001	0.099
Asian	6	1.422	0.730-2.772	45.55	<0.0001	0.301
Europe	2	1.653	0.919-2.973	5.39	0.02	0.093
Cancer type						
gastric cancer	2	2.228	1.748-2.839	0.86	0.651	<0.0001
triple negative breast cancer	2	0.46	0.303-0.699	0.08	0.784	<0.0001
head and neck cancer	2	2.972	1.194-7.395	1.85	0.174	0.019
Treatment						
untreat	4	1.174	0.399-3.450	35.48	<0.0001	0.771
other treatments	3	1.871	1.330-2.630	8.87	0.031	<0.0001
ICIs	–	–	–	–	–	–

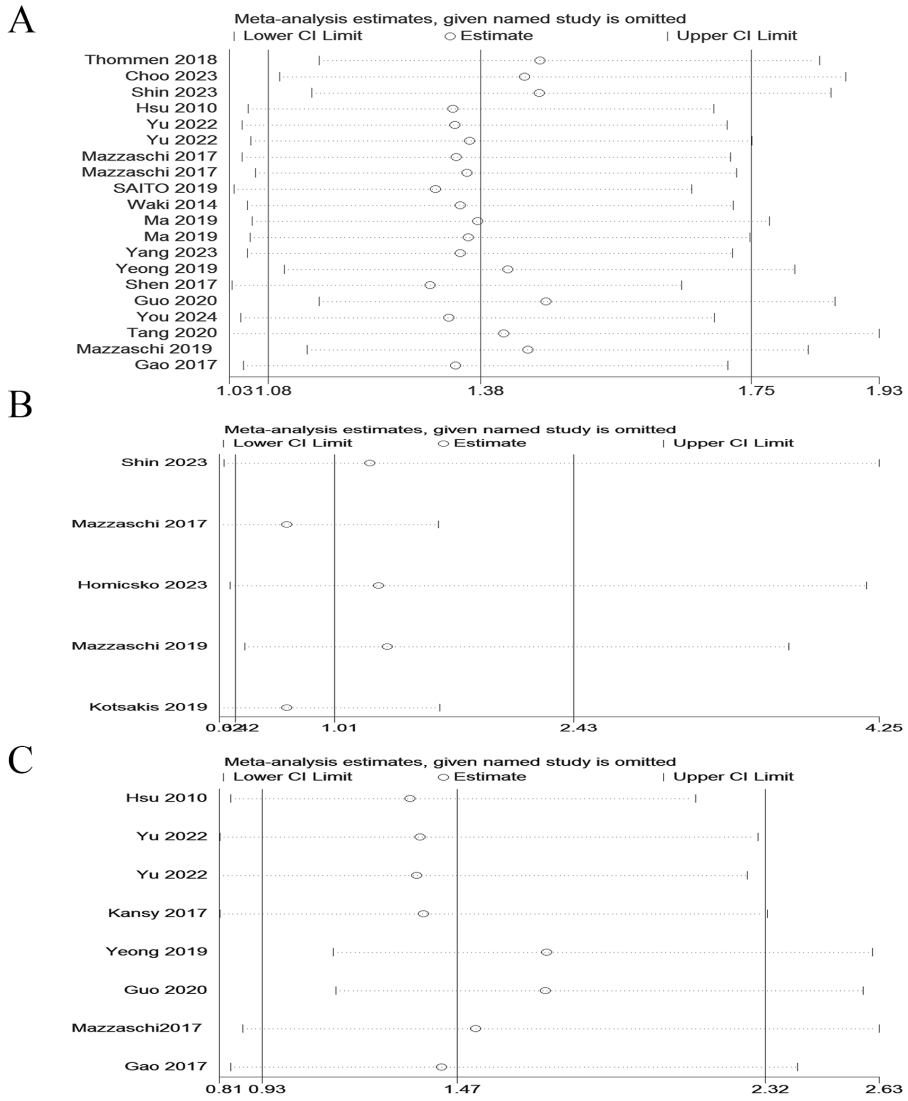

FIGURE 3
Sensitivity analyses of the pooled HRs for OS, PFS and DFS of cancer patients with PD-1+CD8+ T cells. **(A)** Sensitivity analyses of the pooled HRs for OS. **(B)** Sensitivity analyses of the pooled HRs for PFS. **(C)** Sensitivity analyses of the pooled HRs for DFS.

analysis to evaluate the expression of PD-1 on CD8+ T cells in predicting the survivals of cancer patients.

Our study indicates elevating PD-1+CD8+ T cells may predict worse survival time for cancer patients. Consistent with previous studies, researchers indicated that no matter inhibitory receptors, transcription factors or functional molecules, PD1-Hi-CD8+TIL was a exhausted T cell, thus promoting tumor growth, avoiding immune surveillance and promoting immune escape. However, significant heterogeneity was observed in our study. To find the source of heterogeneity, we analyzed the factors that may affect heterogeneity. we stratified the meta-analysis by cancer type and found PD-1+CD8+ T cells predicted significantly worse OS in pancreatic cancer and poor DFS in gastric cancer and head and neck cancer while it was associated with improved OS and DFS in triple negative breast cancer. This may be due to the heterogeneity of PD-1 CD8+ T cells in their roles in different tumors. Odorizzi et al. (38) demonstrated that T cells can be differentiated to reach terminal exhaustion in the genetic absence of PD-1. Moreover, a recent breast cancer study also revealed that there is no significant reduction in cytokine production in PD-1 + T cells compared with PD-1- T cells (39). The immune microenvironment in TNBC may not be as suppressed as in other tumors. However, the exact mechanism of PD-1 CD8+ T cells in triple negative cancer requires further research.

PD-1 is a cytotoxic T cell immune checkpoint receptor that has inhibitory effects when bound to its ligand(PD-L1). The use of checkpoint blocking antibodies to block PD-1 immunotherapy can restore and enhance the response of cytotoxic T cells to chemotherapy resistant tumors, resulting in sustained response and tolerable toxicity, and prolonging overall survival. Studies

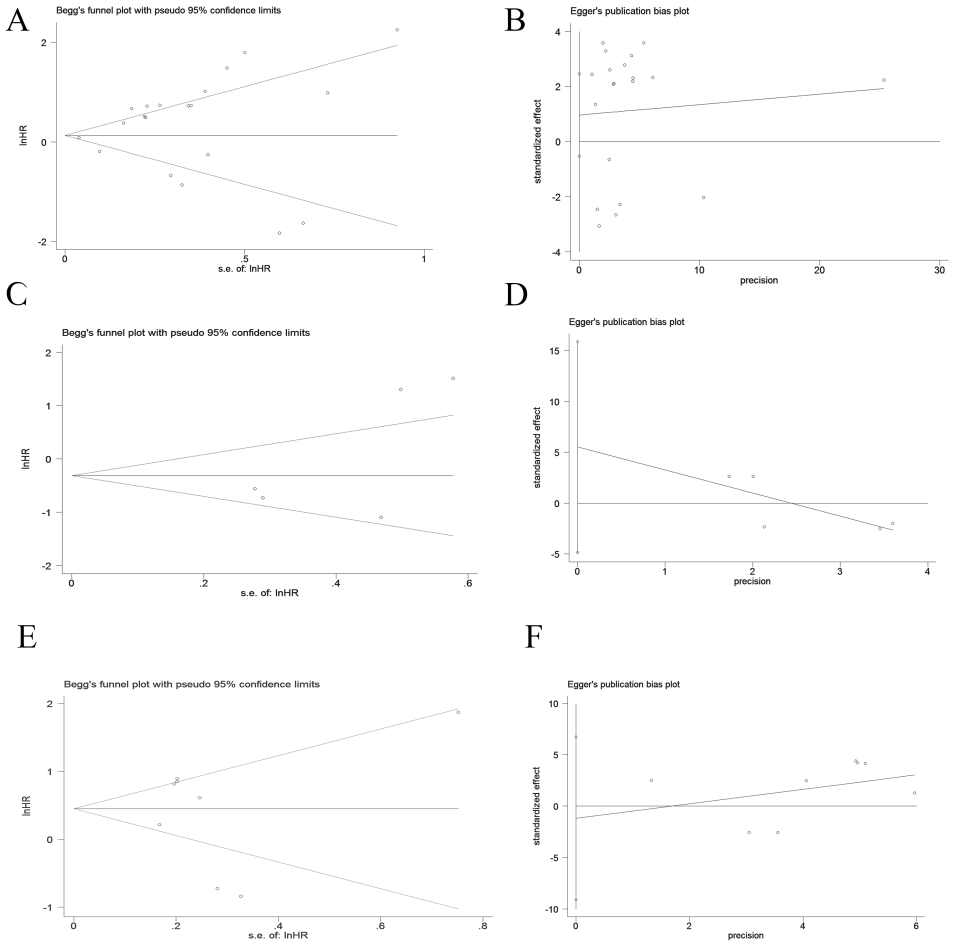

FIGURE 4
Publication bias of OS, PFS and DFS in PD-1+CD8+T cell cancer patients by Begg and Egger scatter plots. **(A, B)** Publication bias for OS. **(C, D)** Publication bias for PFS. **(E, F)** Publication bias for DFS.

have shown that PD-L1 expression responds better to ICI treatment and have a longer survival period in patients receiving immune checkpoint inhibitor therapy (40, 41). Due to this mechanism, the PD-L1/PD-1 axis has been found to be an crucial mechanism by which tumor cells evade T cell immunity, PD-1 inhibitors revitalize CD8+T cells by blocking the PD-1/PD-L1 pathway, promoting their proliferation and functional recovery (42–44). In addition, PD-1/PD-L1 axis inhibitors disrupt the interaction between PD-1 and PD-L1 and subsequently restore the immune response to tumor cells, ultimately improving survival outcomes for cancer patients (45). Homicsko et al (31) pointed out that PD-1 expression of CD8 T cells is an independent predictive factor for the clinical benefits of PD-1 inhibition in patients with advanced mesothelioma. In advanced NSCLC treated with anti-PD1 therapy, Mazzaschi et al (26) found high circulating PD-1+CD8+ cells provided a significantly prolonged progression-free survival. While some studies did not find a significant association between PD-1+CD8+ and survival for ICI treatment (20). Subgroup meta-analysis that gathered these studies showed that patients with high levels of PD-1 +CD8+T cells and treated with PD- 1/PD- L1 inhibitors may have prolonged OS, though it not reach the significance. Nevertheless, the expression of PD-1+ CD8+ T cells is a potential prognostic marker for ICI treatment, which needs to be verified by larger studies in the future. More importantly, compared to ICI treatment, our meta-analysis showed a significant association between PD-1+ CD8+T cells and survival in patients receiving non-ICI treatment, with significantly shorter overall survival in patients with PD-1+ CD8+T cells.

According to the above analysis, high PD-1 expression on CD8+ T cells had a adverse survival time, which can be potentially used as a prognostic marker of malignant tumor. However, some limitations are exist in our study. First, although we tried to collect all the articles, some data were lost because our study was restricted to articles published only in English or Chinese, and it is hoped that future research efforts will include publications in other languages to ensure a more comprehensive analysis. Second, there was obvious heterogeneity in our study, which may be caused by the study region, cancer type, treatments, specimen type and detect method. Third, most of the

included studies had very small sample sizes. More large-scale studies with PD-1expression on CD8+ T cells are needed in the future to validate the findings of our meta-analysis.

5 Conclusion

Our analysis demonstrated that high PD-1 expression on CD8+ T cells had a adverse survival time, which could be potentially used as a prognostic marker of malignant tumor.

Author contributions

ZW: Writing – original draft, Writing – review & editing. MC: Writing – original draft, Writing – review & editing, Funding acquisition. JY: Data curation, Writing – review & editing. DL: Data curation, Writing – review & editing. JC: Data curation, Writing – review & editing. FL: Supervision, Writing – review & editing. YX: Supervision, Writing – review & editing. ZC: Supervision, Writing – review & editing. YY: Visualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2024.1531219/full#supplementary-material

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Advancements in cellular immunotherapy: overcoming resistance in lung and colorectal cancer

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Immunotherapy has revolutionized cancer treatment, offering hope for patients with otherwise treatment-resistant tumors. Among the most promising approaches are cellular therapies, particularly chimeric antigen receptor T-cell (CAR-T) therapy, which has shown remarkable success in hematologic malignancies. However, the application of these therapies to solid tumors, such as lung and colorectal cancers, has faced significant challenges. Tumor resistance mechanisms—ranging from immune evasion, antigen loss, and immune checkpoint upregulation, to tumor microenvironment immunosuppression—remain major obstacles. This mini-review highlights the latest advancements in tumor immunotherapy, with a focus on cellular therapies, and addresses the resistance mechanisms that hinder their effectiveness in lung and colorectal cancers. We examine the evolution of CAR-T cell therapy, as well as the potential of engineered natural killer (NK) cells and macrophages in solid tumor treatment. The review also explores cutting-edge strategies aimed at overcoming resistance, including combination therapies, gene editing technologies, and nanotechnology for targeted drug delivery. By discussing the molecular, cellular, and microenvironmental factors contributing to resistance, we aim to provide a comprehensive overview of how these challenges can be overcome, paving the way for more effective, personalized immunotherapies in lung and colorectal cancer treatment.

KEYWORDS

immunotherapy, CAR-T cells, lung cancer, colorectal cancer, resistance mechanisms

1 Introduction

Immunotherapy has revolutionized cancer treatment, providing novel approaches that harness the body's immune system to fight cancer. Among these, cellular immunotherapies, particularly chimeric antigen receptor T-cell (CAR-T) therapy, have shown remarkable success, particularly in hematologic malignancies. The ability to genetically modify T-cells to express receptors that recognize specific tumor antigens has enabled significant advances, leading to durable responses in cancers such as leukemia and lymphoma (1). However, the application of CAR-T therapy to solid tumors, including lung and colorectal cancers, has faced considerable challenges. Despite these challenges, recent innovations in cellular therapies have opened new avenues for overcoming resistance and improving the effectiveness of treatments for solid tumors.

Resistance to immunotherapies in solid tumors is multifactorial and presents a significant hurdle in the successful application of CAR-T therapy. The tumor microenvironment (TME) in solid cancers is often immunosuppressive, consisting of regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), and tumor-associated macrophages that inhibit immune responses (2). These immune cells, along with the presence of immunosuppressive factors like transforming growth factor-beta (TGF-β), create a hostile environment that limits the ability of T-cells to effectively target and kill tumor cells. Moreover, tumors often exhibit antigen loss or downregulation, which prevent CAR-T cells from recognizing their targets. Immune checkpoint molecules, such as PD-L1, are also upregulated in these tumors, allowing them to evade immune detection by inhibiting T-cell function (3).

Despite these challenges, significant strides have been made in enhancing cellular therapies to overcome the resistance mechanisms that hinder their effectiveness in solid tumors. Development of dual-CAR-T cell and the application of "off-the-shelf" cell therapies, such as allogeneic CAR-T cells or natural killer (NK) cells provide an alternative to autologous cell therapies, thereby improving accessibility and reducing the time required for treatment preparation. NK cell-based therapies are gaining traction due to the innate ability of NK cells to recognize and kill tumor cells without prior sensitization. NK cells can be genetically engineered to enhance their tumor-killing properties and, when combined with immune checkpoint inhibitors, have shown potential for overcoming the immunosuppressive TME. Similarly, macrophages, when reprogrammed into anti-tumor phenotypes, play a crucial role in targeting and eradicating solid tumors. These alternative cellular therapies offer promising strategies for addressing the limitations faced by CAR-T cells in treating lung and colorectal cancers.

This review explores the latest advancements in cellular immunotherapies, particularly CAR-T cells, NK cells, and macrophage-based therapies, with a focus on overcoming the resistance mechanisms in solid tumors. By understanding and addressing the unique challenges posed by lung and colorectal cancers, these emerging therapies offer new hope for improving the efficacy of immunotherapy and providing long-lasting responses for patients with advanced or resistant cancers.

2 Resistance mechanisms in tumor immunotherapy

Resistance to immunotherapy remains a significant challenge, particularly in solid tumors. The TME, immune escape mechanisms, and cellular dysfunctions are key factors contributing to the failure of immunotherapies (Figure 1). In this section, we will explore seven key resistance mechanisms that hinder the effectiveness of tumor immunotherapy, providing examples from both lung and colorectal cancers to illustrate how these mechanisms manifest in different tumor types.

2.1 Tumor microenvironment as a barrier to immunotherapy

TME plays a critical role in mediating resistance to immunotherapies (4). The TME is often characterized by factors that suppress immune activity and promote tumor survival. Hypoxia is a common feature of solid tumors, which leads to a lack of oxygen in the TME and induces the production of immunosuppressive cytokines (e.g., TGF-β, IL-10) and upregulation of immune checkpoint molecules (5). This hypoxic condition not only supports tumor growth but also impairs the function of immune cells. Additionally, the TME harbors a variety of immunosuppressive cell populations such as Tregs and MDSCs, which actively suppress the anti-tumor immune responses. These cells inhibit the activation of effector T-cells, promote tumor cell survival, and prevent immune cell infiltration into the tumor. The presence of these immune-suppressive cells and factors creates a protective niche for the tumor, hindering immune-based therapies to succeed.

The TME in non-small cell lung cancer (NSCLC) is often hypoxic, hypoxia promote cancer cell stemness and invasion by promoting glycolysis via lactylation of SOX9 (6), and it increased PD-L1 to suppress T-cell activation and function, Metabolic intervention that alleviates hypoxia and reduces PD-L1 expression enhances lung cancer radio-immunotherapy (7). Furthermore, NSCLC tumors are typically infiltrated with immunosuppressive cells like Tregs and MDSCs, which contribute to immune evasion by preventing effective anti-tumor immunity (8). High PD-L1 expression in NSCLC correlates with resistance to immune checkpoint inhibitors, such as pembrolizumab or nivolumab, highlighting the suppressive effects of the TME (9). In colorectal cancer (CRC), especially in microsatellite stable (MSS) tumors and metastatic tumors, the TME is also immunosuppressive (10). Tumors secrete TGF-β and IL-10, which promote immune suppression and contribute to therapy resistance. The presence of MDSCs and Tregs in CRC tumors prevents the activation of anti-tumor T-cells and inhibits the effectiveness of immunotherapies

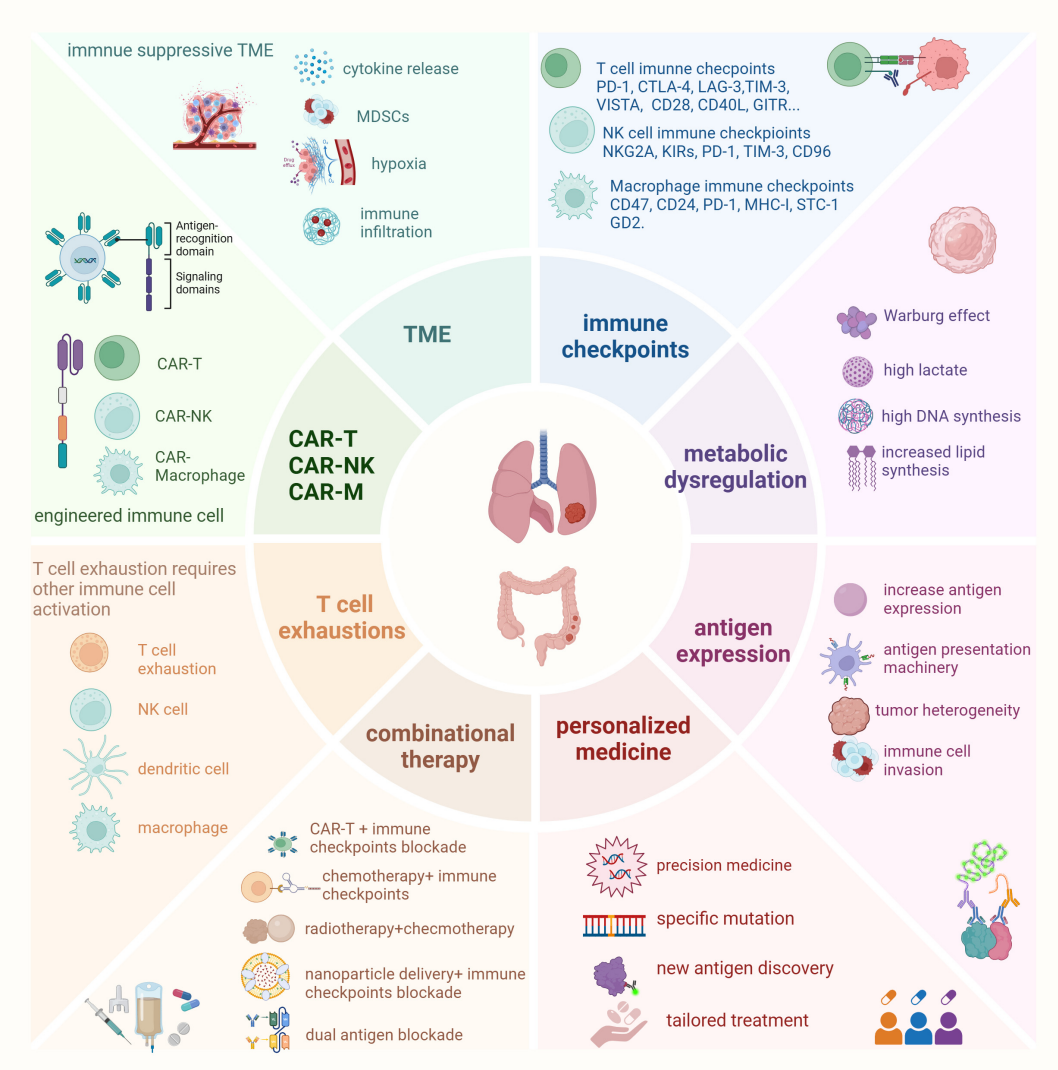

FIGURE 1
Main factors affect immune cell therapy in lung cancer and colorectal cancer Resistance to immunotherapy remains a significant challenge in lung cancer and colorectal cancer. Key factors contributing to the failure of immunotherapies include the tumor microenvironment (TME), immune escape mechanisms, and cellular dysfunctions. One emerging approach to overcome these challenges is targeting immune checkpoints on different immune cells, which has led to the development of new immunotherapies. In addition to CAR-T therapy, CAR-NK (chimeric antigen receptor natural killer cells) and CAR-Macrophages have garnered attention in preclinical research due to their specific advantages over CAR-T cells. However, T cell exhaustion and antigen loss represent new obstacles to cancer immunotherapy. Reversing T cell exhaustion requires not only the direct activation of T cells but also the activation of other immune cells, such as dendritic cells, NK cells, and macrophages, to modulate the overall immune response effectively. Moreover, metabolic dysfunctions in both immune cells and tumor cells play a critical role in affecting the efficacy of immune cell therapies. To address these challenges, combinational therapies and personalized medicine strategies are increasingly being encouraged. These approaches have shown promising results, offering hope for improving therapeutic outcomes in cancer immunotherapy.

such as immune checkpoint inhibitors (11). MSS CRC tumors show poor response to anti-PD-1 therapy due to an immune-suppressive microenvironment, blocking IL-17A potentiates tumor response to anti-PD-1 immunotherapy in MSS CRC (12).

2.2 Antigen loss or downregulation

Tumor cells evade immune detection through antigen loss or downregulation. The genetic instability leads to the generation of new mutant antigens, many of which are immunogenic. However, tumors often adapt by downregulating or losing the expression of tumor-associated antigens (TAAs) or major histocompatibility complex (MHC) molecules, which are essential for the presentation of these antigens to immune cells. Loss of antigen expression enables the tumor to escape immune surveillance. In CAR-T therapy, where the engineered T-cells target a specific antigen on tumor cells, the loss of that antigen lead to therapeutic resistance, as the T-cells no longer recognize the tumor cells as targets (13). The alteration of surface antigens or clonal evolution in tumor cells complicates treatment, as therapies targeting a single antigen may become ineffective after these changes.

In NSCLC, tumor antigen loss is a significant issue, particularly for EGFR-targeted therapies. Tumors may lose or downregulate

EGFR expression after prolonged treatment with EGFR tyrosine kinase inhibitors (TKIs) (14), leading to resistance. Additionally, PD-L1 downregulation hinders the effectiveness of checkpoint inhibitors. In NSCLC patients, the loss of HER2 or EGFR expression following targeted therapy leads to acquired resistance to treatments and targeting HER2 and EGFR exhibits positive immunotherapy results (15). CRC tumors with KRAS mutations or loss of MHC expression fail to present antigens effectively, reducing the ability of T-cells to recognize and attack tumor cells (16).

2.3 Immune checkpoint upregulation

Tumor cells use immune checkpoint molecules to inhibit immune function, effectively "braking" immune responses (17). Checkpoints like PD-1, CTLA-4, CD47 have been studied a lot in different cancers (18). In NSCLC, tumors often increase PD-L1 expression in response to inflammation, helping them evade immune detection by binding to PD-1 receptors on T-cells. Other checkpoint molecules, such as CTLA-4 and TIM-3, also contribute to resistance to PD-1/PD-L1 therapies. High PD-L1 levels in NSCLC are linked to resistance to PD-1/PD-L1 blockers, with relapse occurring after initial response. In CRC, especially MSS tumors, upregulated PD-L1 and CTLA-4 limit checkpoint inhibitor effectiveness. Even in microsatellite instability-high (MSI-H) CRC, resistance can arise if PD-L1 expression doesn't increase, hindering an immune response.

2.4 T cell exhaustion and dysfunction

T-cell exhaustion is another critical mechanism of resistance to immunotherapy, particularly in chronic or persistent tumor environments. As T-cells persistently encounter tumor antigens in the TME, they undergo a process of functional decline, characterized by the upregulation of inhibitory receptors like PD-1, TIM-3, and LAG-3 (19). Exhausted T-cells exhibit reduced cytokine production, impaired proliferative capacity, and diminished cytotoxic function, making them less effective at killing tumor cells. This phenomenon is particularly pronounced in tumors that have a high degree of immune infiltration and antigen persistence. In advanced stages of NSCLC, T-cells often show exhaustion markers such as PD-1, TIM-3, and LAG-3, impairing their function (20). This is particularly problematic for patients treated with checkpoint inhibitors, as exhausted T-cells fail to mount an effective anti-tumor response. In NSCLC, patients treated with anti-PD-1 therapy who later relapse often exhibit increased PD-1 expression on T-cells, signaling exhaustion and reduced therapeutic efficacy. Similarly, in CRC, T-cell exhaustion is driven by chronic antigen exposure, particularly in tumors that are immune-infiltrated. The presence of exhausted T-cells in the TME contributes to resistance to therapies, including immune checkpoint inhibitors (21). T cell exhaustion in CRC is regulated by many infactors, cholesterol induces $CD8^+$ T cell exhaustions by regulating endoplasmic reticulum-mitochondria contact (22); MGP promotes $CD8^+$ T cell exhaustion by activating the NF-kB pathway and leading to cancer metastasis (23).

2.5 Metabolic dysregulation in tumors

The metabolic landscape of tumors plays a significant role in immune resistance. Tumor cells often undergo metabolic reprogramming, such as enhanced glycolysis (the Warburg effect), to support their rapid growth. This reprogramming not only promotes tumor progression but also affects immune cell function. Tumor metabolism in NSCLC often involves aerobic glycolysis, which not only provides energy for rapid tumor growth but also generates byproducts like lactate that acidify the TME. The Warburg effect enhanced by AKR1B10 promotes acquired resistance to pemetrexed in lung cancer-derived brain metastasis (24). High lactate level induces tumor-associated fibroblast activation and IL-8 mediated macrophage recruitment to potentiate lung cancer progression and compromise the immunotherapy (25). MCT-4-mediated lactate secretion inhibits antitumor immunity in LKB1-deficient lung cancer (26). This acidic environment suppresses T-cell function and promotes immune evasion. In addition, in lung adenocarcinoma, harnessing lipid metabolism modulation indicates improved immunotherapy outcomes (27). Mitochondrial networks and biogenetics also influence cancer cell immunotherapy in lung cancer (28). H3K18 lactylation enhances immune escape by activating the POM121/MYC/PD-L1 pathway in NSCLC (29). In CRC, lactate production from glycolysis and arginine depletion inhibit the function of effector T-cells, making tumors less responsive to immunotherapies. High lactate upregulates lactylation, the lactylation-driven METTL3-mediated RNA m6A modification promotes immunosuppression of tumor-infiltrating myeloid cells in colon cancer (30). Circulating L-arginine predicts the survival of cancer patients treated with immune checkpoint inhibitors in colon cancer (31). Moreover, the fusobacterium nucleatum-derived succinic acid induces tumor resistance to immunotherapy in colorectal cancer (32).

3 Approaches to overcoming immunotherapy resistance

Immunotherapy resistance remains a major obstacle in the effective treatment of cancers, especially in solid tumors like lung and colorectal cancer. Overcoming this resistance requires a multifaceted approach, involving a combination of strategies targeting different mechanisms of immune evasion. Key strategies include enhancing immune cell efficacy, overcoming immune suppressive factors in TME, and leveraging combination therapies.

3.1 Utilizing alternative cell therapies (NK cells and macrophages)

NK cells and macrophages are emerging as valuable alternatives to T-cell-based therapies like CAR-T (33, 34). NK cells target tumors without prior sensitization, they are less vulnerable to antigen loss and TME immunosuppression (35, 36). NK cell-based immunotherapies including combined cytokine, CDC and ADCC, NK-92, KIR

mismatch and CAR approaches. CAR-NK cell therapy results in reduced toxicity, lower cost, and broader accessibility compared to CAR-T cells (37). Specifically, CAR-NK cells have a lower risk of graft-versus-host disease (GVHD), enabling "off-the-shelf" allogeneic therapies, and possess both CAR-mediated and innate antitumor activity, making them effective against heterogeneous tumors. They have a better safety profile with reduced risks of cytokine release syndrome (CRS) and neurotoxicity and a shorter lifespan, minimizing long-term side effects. Their scalability from various sources, like cord blood or iPSCs, further enhances accessibility. In a phase I/II trial, CD19 CAR-NK cells achieved a 73% response rate in patients with relapsed/refractory B-cell malignancies, without CRS or neurotoxicity (38). NK cells prime cancer cells for mt-apoptosis and combining them with BH3 mimetics enhances cancer cell death and tumor suppression. BH3 profiling helps identify the most effective mimetic, offering a precision strategy to improve NK-based and T cell-based immunotherapies (39). Rocaglamide enhances NK cell infiltration and antitumor immunity by activating the cGAS-STING signaling pathway in non-small cell lung cancer (40). NK-cell mediated therapy in lung cancer and CRC has been reviewed (41, 42).

Similarly, macrophages can be reprogrammed to exhibit anti-tumor properties, helping to eliminate resistant tumor cells and reprogram the TME for enhanced immune responses (43). CAR-macrophages have emerged as a new cancer immunotherapy to target solid tumors (44), offering several advantages over CAR-T cells. Unlike CAR-T cells, which rely primarily on direct cytotoxicity, CAR-macrophages utilize their natural phagocytic ability to engulf tumor cells and present tumor antigens, thereby activating downstream immune responses (45, 46). Their ability to infiltrate dense tumor microenvironments makes them particularly effective against solid tumors, a challenge for CAR-T therapies. The tandem CD3ζ-TIR dual signaling CAR design enables induced pluripotent stem cell-derived macrophages (iMACs) to engage targets, promote M1 polarization, resist immunosuppressive M2 polarization, and modulate the tumor microenvironment through an NF-κB-dependent mechanism (47). Furthermore, CAR-macrophages are less prone to exhaustion and can reprogram the immunosuppressive TME, enhancing the recruitment and activation of T cells and other immune effectors (44, 48). Preclinical studies of anti-HER2 CAR-macrophages demonstrated significant tumor growth inhibition in $HER2^{+}$ xenograft models, emphasizing their ability to overcome antigen escape and address TME immunosuppression (49). These properties make CAR-macrophages a powerful alternative to CAR-T cells in tackling the unique challenges of solid tumors.

3.2 Improving antigen expression and new generation CAR-T design

Enhancing antigen targeting or using alternative antigens overcome this challenge. In NSCLC, novel antigen targets such as mesothelin or specific mutations like EGFR are being explored for CAR-T therapy, providing potential solutions to antigen loss and improving the targeting of resistant tumors (9, 50). Engineering CAR-T cells to target multiple antigens or incorporate novel features, such as cytokine secretion or checkpoint inhibition, could overcome issues of antigen loss. Moreover, IL-10-expressing CAR T cells maintain mitochondrial structure and function in the tumor microenvironment, IL-10 secretion boosted CAR T cell proliferation and effector functions, resulting in the complete regression of established solid tumors and metastatic cancers in multiple cancer types including colon in both syngeneic and xenograft mouse models (51). Dual-targeted CAR-T cells and "armored" CAR-T cells that resist the immunosuppressive TME represent exciting new avenues to enhance efficacy in solid tumors (52). In lung cancer, CAR-T cells with dual targets on HER2 and HLA-A02 enhance tumor specificity and address on-target off-tumor toxicity in HER2+ lung cancer cell lines with HLA-A02 loss of heterozygosity (53). Clinical trials of lung cancer targeting different antigens are listed in Table 1.

3.3 Immune checkpoint Inhibition and CAR-T cell synergy

Tumor cells often exploit immune checkpoints such as PD-1/PD-L1, CTLA-4, and others to inhibit T-cell function. Combining checkpoint inhibitors with CAR-T therapy overcomes resistance by blocking these inhibitory signals, restoring immune cell function, and enhancing the anti-tumor response. Immunogenic chemotherapy enhances the recruitment of CAR-T cells to lung tumors, thereby improving the overall antitumor efficacy (54). When combined with checkpoint blockade therapy (anti-PD-1), this approach further boosts the therapeutic response, potentially leading to more effective treatment outcomes (55). In CRC, combining PD-1/PD-L1 inhibitors with CAR-T cells or NK cell therapy has demonstrated enhanced anti-tumor effects, particularly in tumors that express immune checkpoint molecules. The dual CAR-T cells with anti-PD-L1 scFv were capable of eradicating established tumors in mouse models of peritoneal metastasis of colorectal cancer (56).

Some of the advanced clinical trials have exhibited good results of chemotherapy in lung cancer. For instance, the phase III RATIONALE 303 trial (NCT03358875) demonstrated that Tislelizumab significantly extended overall survival in patients with advanced NSCLC who had progressed after platinum-based chemotherapy (57). Additionally, the ADRIATIC study showed that Duvatinib as a consolidation therapy after chemoradiotherapy significantly improved three-year survival rates in patients with limited-stage small cell lung cancer (SCLC) (58). However, for the cell therapies, they are still in the process.

3.4 Ameliorating immune cell exhaustion and senescence

T-cell exhaustion is a major cause of immune resistance (59). Immune checkpoint blockade has been used to prevent T-cell exhaustion, thereby enhancing CAR-T cell function. Additionally, NK cell therapy has shown potential to circumvent T-cell exhaustion, providing an alternative for overcoming resistance.

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TABLE 1 Targeting antigens of lung cancer for immunotherapy registered in clinical trials.

NO.	Target	First posted time	Stage	Clinical trial ID number
1	PD-1 and TIL	10/2024	recruiting	NCT06538012
2	KK-LC-1	07/2024	recruiting	NCT05483491
3	GD2	10/2024	recruiting	NCT05620342
4	DLL3	02/2024	recruiting	NCT05680922
5	GPC3	12/2024	recruiting	NCT05120271
6	CEA, HLA-A*02	11/2024	recruiting	NCT05736731
7	MSLN	11/2024	recruiting	NCT06051695
8	MUCI	09/2024	recruiting	NCT05239143
9	GPC3	06/2024	recruiting	NCT06196294
10	EGFR/B7H3	06/2024	recruiting	NCT05341492
11	EGFR	06/2024	recruiting	NCT05060796
12	CEA	09/2023	recruiting	NCT06043466
13	GPC3	06/2024	recruiting	NCT03198546
14	CEA	11/2023	recruiting	NCT06126406
15	CEA	11/2023	recruiting	NCT06010862
16	CEA	08/2024	recruiting	NCT06006390
17	EGFR	10/2023	II	NCT05299125
18	PD-L1	06/2023	II	NCT05904015
19	CEA	04/2020	I/II	NCT04348643
20	CEA	01/2015	I	NCT02349724
21	CD276	04/2021	early phase I	NCT04864821
22	EGFR	11/2019	I	NCT05060796
23	EGFR	09/2021	early phase I	NCT05060796
24	HER2	11/2018	I	NCT03740256
25	HER2	12/2020	I	NCT04660929
26	HER2	09/2013	I/II	NCT01935843
27	MSLN	04/2012	I/II	NCT01583686
28	MSLN	02/2017	I	NCT03054298
29	MUCI	10/2015	I/II	NCT02587689
30	MUCI	05/2018	I/II	NCT03525782

CEA, carcinoembryonic antigen; EGFR, epidermal growth receptor; GPC3, glypican 3. MUCI, mucin 1; PD-L1, programmed death ligand 1; HER2, human epidermal growth factor 2; MSLN, mesothelin. TIL, tumor infiltrating lymphocytes.

Senescent T cells require NK cell to promote antitumor immunity (60). Combining anti-PD-1 inhibitors with CAR-T therapy or NK cells helps rejuvenate exhausted T cells and enhance immune responses against CRC. Additionally, improving the persistence of engineered immune cells is a focus of ongoing research in CRC. Clearance of senescent macrophages ameliorates tumorigenesis in KRAS-driven lung cancer (61).

3.5 Combination therapies: a multi-pronged approach

Combination therapies have emerged as one of the most effective strategies to overcome resistance to immunotherapy (62). By simultaneously targeting different immune pathways, combination approaches address the multiple layers of resistance mechanisms that tumors use to evade immune attacks. This multi-pronged strategy aims to enhance the overall efficacy of treatment and improve patient outcomes. In NSCLC, combinations of CAR-T cells, NK cells, and immune checkpoint inhibitors have demonstrated synergistic effects, enhancing anti-tumor activity (58). This approach is particularly valuable in addressing various resistance mechanisms, such as immune evasion and immunosuppression within TME. In CRC, combining CAR-T cells with immune checkpoint inhibitors or chemotherapy has shown promising potential to improve responses, particularly in patients who are resistant to monotherapies. For instance, the combination of nivolumab and ipilimumab, with or without chemotherapy, has been shown to offer long-term, durable clinical benefits in metastatic NSCLC patients with tumor PD-L1 expression below 1% (63). This combination supports its use as a first-line treatment option, especially in populations with high unmet needs. Furthermore, adding toripalimab to perioperative chemotherapy significantly improved event-free survival in patients with resectable stage III NSCLC, while maintaining a manageable safety profile (64). These findings underscore the potential of combination therapies to offer substantial clinical benefits by overcoming resistance mechanisms and providing lasting therapeutic effects in cancers.

3.6 Personalized and adaptive immunotherapies

Given the significant variability in immune responses across patients, personalized and adaptive immunotherapies are increasingly recognized as essential for optimizing cancer treatment (65). Precision medicine tailors treatments to the specific genetic and molecular characteristics of a patient's cancer (66). Identifying tumor-specific neoantigens, which are unique to cancer cells and not present in normal tissues, can be used to develop vaccines or engineered immune cells, such as CAR-T,CAR-NK, CAR-Macrophage therapies (67). Moreover, tailoring therapies based on genetic and immune profiling has the potential to significantly enhance treatment outcomes. By analyzing both the genetic mutations of the tumor and the immune system's response to it, specific targets will be discovered. This allows for more targeted therapies that minimize side effects while maximizing antitumor efficacy (68). For instance, lung cancer patients with specific genetic alterations, like EGFR mutations, may benefit from targeted therapies combined with immunotherapy, whereas others

may respond better to conventional treatments or personalized CAR-T therapies. Ultimately, integrating these personalized and adaptive strategies into clinical practice will be crucial for improving the success of cellular therapies, especially in complex cancers like lung and colorectal cancer, where variability in patient responses is high.

Lung cancer and colorectal cancer exhibit distinct resistance mechanisms to immunotherapy and cell-based therapies due to differences in their tumor microenvironments and molecular characteristics. In lung cancer, particularly NSCLC, resistance often arises through the upregulation of immune checkpoint pathways like PD-1/PD-L1, which suppresses T-cell activity. Despite a high tumor mutational burden (TMB) in smokers' lung cancers, resistance can still occur due to antigen loss or altered antigen presentation (9). Additionally, CAR-T therapies face resistance from poor T-cell infiltration or downregulation of target antigens like EGFR. In contrast, CRC often involves mismatch repair deficiency (dMMR) and microsatellite instability (MSI), leading to an elevated mutational burden. However, resistance in CRC is marked by immune exclusion, where T-cells fail to infiltrate the tumor. This is particularly evident in mismatch repair-proficient (pMMR) tumors. CRC also has an immunosuppressive tumor microenvironment, which limits the effectiveness of immunotherapies. Resistance to cell-based therapies in CRC arises from antigen heterogeneity and downregulation of target antigens. While both cancers share immune evasion and antigen loss mechanisms, lung cancer is more affected by checkpoint resistance and poor immune cell penetration, whereas CRC faces challenges with immune exclusion and an immunosuppressive microenvironment. These differences underscore the need for tailored treatment strategies.

4 Conclusion and perspective

Significant advancements have been made in the field of immunotherapy, particularly in the application of cellular therapies like CAR-T, NK cells, and macrophages for treating solid tumors, including lung and colorectal cancers. Despite the promising results, resistance to immunotherapies remains a major challenge. The complexity of TME, antigen escape, immune checkpoint overexpression, and T-cell exhaustion are key mechanisms of resistance. While CAR-T cell therapies have shown notable success in hematologic malignancies, their application in solid tumors like lung and colorectal cancers is still limited due to the unique obstacles posed by these cancers. Additionally, the development of alternative therapies, such as NK cell and macrophage-based approaches, offers exciting new avenues to overcome these barriers and broaden the scope of cellular immunotherapies.

Looking forward, a multi-pronged approach combining different immunotherapeutic strategies will likely be essential for overcoming resistance in solid tumors. Enhancing the efficacy of CAR-T cells through better tumor targeting, engineering of immune cells, and modulation of the TME holds significant promise. Moreover, leveraging combination therapies, which include immune checkpoint inhibitors, cytokines, and alternative cell-based therapies like NK cells and reprogrammed macrophages, may provide synergistic effects to overcome resistance mechanisms. Understanding the mechanisms of resistance at a molecular level and further optimizing therapeutic designs will be key to improving outcomes for lung and colorectal cancer patients. The future of cancer immunotherapy depends on combining these new approaches, which could transform treatment and greatly improve patient survival and quality of life.

Author contributions

LQ: Writing – original draft, Writing – review & editing, Resources. YL: Resources, Visualization, Writing – original draft. JL: Conceptualization, Investigation, Supervision, Writing – review & editing. XA: Conceptualization, Supervision, Validation, Visualization, Writing – review & editing.

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Advancing biliary tract malignancy treatment: emerging frontiers in cell-based therapies

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Biliary tract malignancies, including intrahepatic cholangiocarcinoma, extrahepatic cholangiocarcinoma, and gallbladder cancer, represent a group of aggressive cancers with poor prognosis due to late-stage diagnosis, limited treatment options, and resistance to conventional therapies like chemotherapy and radiotherapy. These challenges emphasize the urgent need for innovative therapeutic approaches. In recent years, cell-based therapies have emerged as a promising avenue, offering potential solutions through immune modulation, genetic engineering, and targeted intervention in the tumor microenvironment. This Mini-review provides an overview of current advancements in cell-based therapies for biliary malignancies, encompassing immune cell-based strategies such as CAR-T cells, NK cells, dendritic cell vaccines, and tumor-infiltrating lymphocytes. We also examine strategies to overcome the immunosuppressive tumor microenvironment and discuss the integration of cell therapies into multimodal treatment regimens. By synthesizing preclinical and clinical findings, this review highlights key insights and future directions, aiming to assist researchers and clinicians in translating these approaches into effective treatments. The transformative potential of cell-based therapies discussed here makes this review a valuable resource for advancing biliary malignancy research and clinical applications.

KEYWORDS

biliary tract malignancies, cell therapy, immunotherapy, tumor microenvironment, combined therapy

1 Introduction

Biliary tract malignancies, including intrahepatic cholangiocarcinoma(ICC), extrahepatic cholangiocarcinoma(ECC), and gallbladder cancer (GBC), rank among the most lethal cancers, with dismal five-year survival rates and limited progress in improving outcomes over the past decades (1–3). The incidence of biliary tract cancer varies by region, with cholangiocarcinoma having a low incidence in high-income countries (0.35 to 2 cases per 100,000 annually) and much higher rates in endemic areas like Thailand and China, where it can be up to 40 times higher. For patients with advanced biliary tract cancer, survival remains poor, with median overall survival ranging from 2.5 to 4.5 months, as

shown in randomized controlled trials (1). Biliary tract malignancies often present asymptomatically in early stages, leading to late-stage diagnoses where curative surgical options are no longer feasible. Moreover, biliary tumors are inherently resistant to conventional therapies, including chemotherapy and radiotherapy, which are further hampered by the tumor's dense stromal barrier and immunosuppressive microenvironment (4). The standard treatment regimens for biliary malignancies, including Gemcitabine-based chemotherapy or Cisplatin and Gemcitabine combinations, provide only modest survival benefits and are frequently associated with significant toxicity (5). Targeted therapies aimed at genetic alterations such as FGFR2 fusions, IDH1 mutations (6), and HER2 overexpression have shown promise in a subset of patients but remain far from curative (7–9). Immunotherapy, a breakthrough in treating other cancers, has faced significant challenges in biliary malignancies due to the highly immunosuppressive tumor microenvironment (TME) (10, 11), characterized by regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), and cancer-associated fibroblasts (CAFs).

Cell-based therapies have emerged as a revolutionary approach, leveraging the body's immune system or engineered cellular systems to combat malignancies (12, 13). Unlike conventional treatments, these therapies are designed to specifically target tumor cells, potentially overcoming the limitations of the TME and achieving durable responses. Among the most promising strategies are chimeric antigen receptor (CAR)-T cells, which have demonstrated success in hematological cancers and are now being adapted for solid tumors like biliary malignancies (14, 15). Natural killer (NK) cell therapies and dendritic cell (DC)-based vaccines are also being explored for their potential to activate innate and adaptive immune responses against these tumors (16, 17). Tumor-infiltrating lymphocytes (TILs), another emerging avenue, exploit the natural immune infiltration of tumors to enhance therapeutic outcomes (18).

Recent advancements in genetic engineering and synthetic biology, have significantly improved the precision and efficacy of cell-based therapies (19). These technologies allow for the modification of immune cells to enhance their tumor-targeting capabilities while minimizing off-target effects. Additionally, efforts to modulate the TME—such as targeting desmoplasia, reprogramming stromal components, and reducing immunosuppressive cytokines—are creating a more favorable environment for therapeutic cells to function effectively (20). This review explores the rapidly evolving landscape of cell-based therapies for biliary malignancies. By synthesizing insights from preclinical studies and clinical trials, we aim to highlight key challenges and opportunities, providing a comprehensive perspective on how cell-based strategies may redefine treatment paradigms for these challenging cancers.

Abbreviations: CAFs, cancer-associated fibroblasts; CCA, cholangiocarcinoma; CIK, cytokine-induced killer; CRS, cytokine release syndrome; CR-T, chimeric antigen receptor T cells; DC, dendritic cell; ECC, extrahepatic cholangiocarcinoma; ECM, extracellular matrix; GBC, gallbladder cancer; ICC, intrahepatic cholangiocarcinoma; IDO, Indoleamine 2,3-dioxygenase; IL-2, interleukin-2; MAGE, melanoma-associated antigen; MDSCs, myeloid-derived suppressor cells; MMPs, Matrix metalloproteinases; MUC1, Mucin 1; NK, Natural killer; TAAs, tumor-associated antigens; TILs, Tumor-infiltrating lymphocytes; TLR, Toll-like receptor; TME, tumor microenvironment; Tregs, regulatory T cells; Trm, tissue-resident memory T; TSAs, tumor-specific antigens; WT1, Wilm's Tumor protein 1.

2 Immune cell-based therapies

2.1 CAR-T cell therapy

CAR-T cell therapy has emerged as a promising immunotherapeutic strategy for biliary malignancies. By engineering T cells to express CARs that specifically target tumor-associated antigens, CAR-T cells overcome the limitations of conventional therapies. In biliary cancers, targets such as mesothelin, HER2, and EGFR have been identified as promising candidates for CAR-T cell therapy. Mesothelin, frequently overexpressed in biliary cancers, is implicated in tumor progression through its role in cell adhesion, migration, and immune evasion (21). By interacting with MUC16, mesothelin promotes metastasis and suppresses immune responses, contributing to therapy resistance. Overexpression of mesothelin predicts malignant progression of cholangiocarcinoma (22). CAR-T cells targeting mesothelin have shown potential in reducing tumor burden, though their success has been hindered by the highly immunosuppressive TME and off-target toxicity. Mechanistically, mesothelin CAR-T cells secreting anti-CD3 molecules efficiently targeting pancreatic cancer and ovarian cancer (23, 24). The affinity-tuned mesothelin CAR-T cells indicate the potentiated targeting specificity and reduced off-tumor toxicity (25). Moreover, gallbladder cancer tumors frequently express carcinoembryonic antigen (CEA),CEA-specific CAR-T cells effectively recognize and respond to CEA, even in the presence of immune-suppressive factors like PD-L1. CAR-T cells showed strong activation, cytokine production (IFN-γ, TNF-α), and cytotoxicity, reducing tumor growth *in vitro* and *in vivo* in gallbladder cancer (26). In addition, chronic cholangitis is a significant risk factor for cholangiocarcinoma due to prolonged inflammation and bile duct damage. PD-1-targeting CAR-T cells effectively treat autoimmune cholangitis by selectively depleting pathogenic $CD8^+$ tissue-resident memory T (Trm) cells in the liver, which alleviates biliary immunopathology and highlights the therapeutic potential of CAR-T cell therapy biliary cholangitis (27). Recent research has indicated that EGFR and B7H3 antigens are highly expressed in biliary tract cancer, EGFR-CAR-T and B7H3-CAR-T cells demonstrated specific anti-tumor activity (28). Similarly, HER2, a receptor tyrosine kinase overexpressed in a subset of biliary cancers, drives oncogenic signaling through pathways like PI3K/AKT and MAPK (29). These pathways enhance tumor cell proliferation, survival, and resistance to therapies, including chemotherapy and immune checkpoint inhibitors. HER2 is overexpressed in a subset of biliary cancers, is being explored as a target, with clinical trials evaluating its therapeutic potential by pertuzumab and trastuzumab (30, 31), and Zanidatamab (32). HER2- targeting CAR-T cells has been successful in pre-clinical research in glioblastoma (33), gastric

cancer (34), ovarian cancer (35), breast cancer (36), and in clinical trial for targeting sarcoma (37). Therefore, CAR-T cells targeting HER2 will probably have promising result for biliary malignancies (Figure 1). Currently, there is insufficient evidence to support the clinical use of CAR T-cell therapy for advanced cholangiocarcinoma (CCA). Further clinical trials are essential to establish its safety and efficacy for routine clinical application. Notably, the safety and effectiveness of MUC-1 CAR T cells are being evaluated in a Phase I/II clinical trial in China for patients with ICC (NCT03633773) (38).

Despite encouraging preclinical results, CAR-T cell therapy faces significant challenges in biliary malignancies. The dense stromal barrier, composed of CAFs and extracellular matrix components, impedes CAR-T cell infiltration. Additionally, the immunosuppressive TME, characterized by Tregs and MDSCs, further limits efficacy. Moreover, the high expression of immune checkpoint molecules like PD-L1 within the TME suppresses CAR-T cell function, contributing to treatment resistance (39). through knock down immune checkpoints and immunosuppressive molecular receptors. Strategies to overcome these barriers include combining CAR-T therapy with immune checkpoint inhibitors and modulating the TME using stromal-targeting agents or cytokines.

2.2 Natural killer cell therapy

NK cells, integral to the innate immune system, possess unique advantages over T cells in cancer therapy, including their ability to target tumor cells without prior antigen sensitization. NK cell-based therapies for biliary malignancies encompass allogeneic NK cells, cytokine-induced killer (CIK) cells, and engineered NK cells. Allogeneic NK cells derived from healthy donors offer an off-the-shelf therapeutic option. Preclinical models of biliary cancers have demonstrated their efficacy, particularly when combined with cytokines like IL-2 or IL-15 to enhance their cytotoxic activity. Engineered NK cells, modified to express CARs or other tumor-specific receptors, represent an exciting avenue for enhancing specificity and potency.

Targeting PD-1 in cholangiocarcinoma by nanovesicle-based immunotherapy has exhibited good result in mouse modal (40). The biliary epithelium presents antigens to activate NK cells and NK T cells (41). Checkpoint inhibitors have shown synergistic effects when combined with NK cell therapies. By alleviating inhibitory signals within the TME, these combinations restore NK cell functionality and improve therapeutic outcomes. Current clinical trials are exploring the integration of NK cell therapies with checkpoint inhibitors in biliary malignancies.

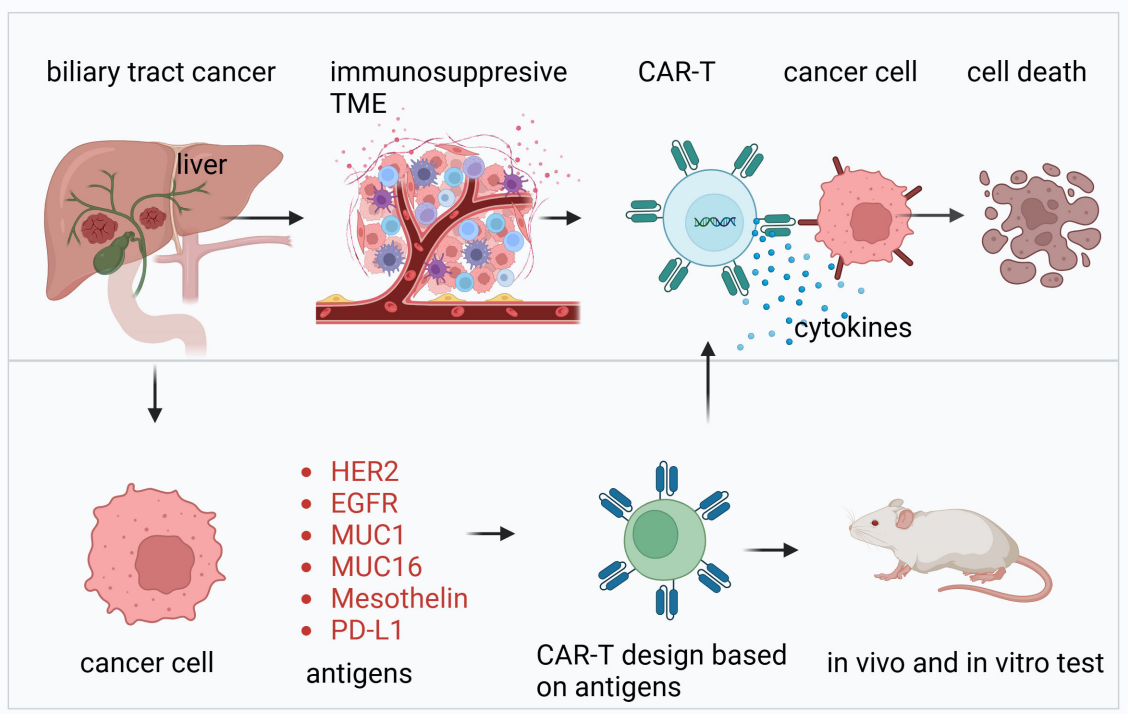

FIGURE 1
Overview of CAR-T cell therapy targeting biliary tract cancer. This figure illustrates the development and application of CAR-T cell therapy for biliary tract cancer. The upper panel highlights the immunosuppressive TME characteristic of biliary tract cancers, which hinders immune response. CAR-T cells, engineered with antigen-specific receptors, are shown to recognize and bind to cancer cells, leading to the release of cytokines and subsequent tumor cell death. The lower panel details the design and validation process of CAR-T cells. Antigens such as HER2, EGFR, MUC1, MUC16, mesothelin, and PD-L1, commonly overexpressed in biliary tract cancers, are selected as targets for CAR-T design. These CAR-T cells are tested for efficacy and safety through in vitro and in vivo experiments, including animal models, to ensure their therapeutic potential.

2.3 Dendritic cell -based vaccines

DCs, as potent antigen-presenting cells, play a pivotal role in initiating robust T cell responses (42). DC-based vaccines, designed to present tumor-specific antigens, have shown promise in biliary malignancies (17, 43). Personalized DC vaccines using neoantigens derived from individual tumors are at the forefront of this approach.

Clinical trials have demonstrated the safety and immunogenicity of DC-based vaccines in melanoma-associated antigen (MAGE)-positive gallbladder carcinoma (44). For example, vaccines loaded with peptides from commonly mutated genes, such as KRAS (45), or tumor lysates have induced tumor-specific immune responses. Combining DC vaccines with immune checkpoint blockade has further enhanced efficacy by enabling sustained T cell activation and memory formation.

The success of vaccination depends on both the immune system's capabilities and the choice of an appropriate target antigen. Ideally, the target should be tumor-specific and conserved within the cancer cells to minimize collateral damage to healthy tissues and reduce the risk of antigen-negative tumor cells that might evade immune detection. Wilm's Tumor protein 1 (WT1), a transcription factor that plays a role in urogenital development and tumor suppression, is a prime candidate for cancer immunotherapy (46). WT1 interacts with various growth factor receptors and regulatory proteins, such as PDGF-R, EGFR, c-MYC, and Bcl-2 (47). WT1 mutations are present in 68%-80% of biliary tract cancers, and while the clinical relevance in biliary tract cancer is still under investigation, similar mutations have been associated with poor prognosis in other cancers like testicular, breast, and head and neck cancers (48). Another promising antigen is Mucin 1 (MUC1) (46), a heavily glycosylated glycoprotein found on the surface of many tumor cells. MUC1 helps form a protective barrier around tumors, preventing the penetration of chemotherapy and immune cells. Overexpression of MUC1 is observed in 90% of gallbladder carcinoma cases and 59%-77% of cholangiocarcinoma cases, often correlating with more advanced disease and poor overall survival (49). The effectiveness of peptide-based vaccines can be limited by the tumor heterogeneity within cancers like biliary cancer. Although antigens such as WT1 and MUC1 are often overexpressed in biliary tumors, their distribution is not uniform. Some cancer cells may lack these antigens entirely, or express them at low levels. Additionally, individual variations in immune response, even among patients with similar HLA types, can affect the success of these vaccines. This is due, in part, to differences in the number of lymphocytes that are sensitive to these specific antigens. Approaches to improve efficacy include incorporating adjuvants like Toll-like receptor (TLR) agonists and leveraging advanced delivery platforms, such as nanoparticles, to enhance antigen presentation and immune activation.

2.4 Tumor-infiltrating lymphocytes

TILs represent a highly promising approach to harness the body's natural immune response against biliary malignancies (18, 50). These immune cells, which naturally migrate into tumor tissues, can be isolated and expanded from patient tumor specimens, generating autologous T cell products capable of recognizing and targeting tumor-associated antigens. Preclinical research has demonstrated the potential of TIL therapy to mediate tumor regression in models of biliary tract cancer (51, 52). In these studies, TILs were shown to infiltrate the TME, attack cancer cells, and lead to significant tumor reduction (53, 54). These results underscore the promise of TIL-based therapies in targeting cancers that are traditionally resistant to standard treatments. The combination of TIL together with other therapies often include cytokines such as interleukin-2 (IL-2), which can enhance TIL persistence and function, or immune checkpoint inhibitors (e.g., anti-PD-1/PD-L1), which may reverse immune suppression within the TME and enhance TIL survival and effectiveness. The aim of these combination therapies is to improve the persistence and functionality of TILs in the immunosuppressive and hostile TME often found in biliary tract cancer.

One of the major challenges in TIL therapy for biliary tract cancer is the difficulty in obtaining sufficient TILs from tumor tissues (55). Biliary tumors often exhibit low immunogenicity, which means that fewer T cells are naturally infiltrating the tumor (56, 57). This, combined with the immunosuppressive environment present in the TME, limits the availability and effectiveness of TILs. As a result, strategies to improve TIL efficacy have focused on several key areas: (1) preconditioning regimens: These involve manipulating the TME before TIL infusion to reduce the presence of immunosuppressive cells such as Tregs and MDSCs. By depleting these cells, TILs are more likely to survive and function effectively. (2) Genetic modifications: enhancements such as chimeric antigen receptor (CAR) T cell therapy and TCR modifications have been used to increase TIL survival, improve their cytotoxic potential, and enhance their resistance to the hostile TME. CAR-T cells, for example, can be engineered to specifically target tumor antigens present on cancer cells (58). (3) Combination therapies: clinical trials exploring combinations of TIL therapy with cytokines and immune checkpoint inhibitors have shown promise in improving outcomes (59). These combinations aim to create a more favorable TME, enhancing TIL expansion, persistence, and tumor-killing capacity.

While TIL-based therapies for biliary tract cancer remain in their infancy, the ongoing research and pre-clinical research hold great potential for improving outcomes with this difficult-to-treat cancer. The ability to harness and enhance the immune system through TIL therapy may represent a major breakthrough in addressing the immune escape mechanisms that often render traditional therapies ineffective in biliary tract cancer. Continued advancements in understanding the biology of TILs, the challenges of the TME, and the development of personalized approaches will be essential to maximize the therapeutic potential of TILs in biliary cancers.

3 Other immunotherapies targeting biliary tract malignancies

3.1 Targeting immune checkpoints

Immune checkpoint expression plays a critical role in cholangiocarcinoma and is strongly associated with poor prognosis.

PD-L1 is expressed in 49–94% of biliary tract cancer cases, and its presence correlates with worse survival outcomes, including a 60% reduction in survival for patients with high PD-L1 expression at the tumor front (60, 61). PD-1 and PD-L1 upregulation have been observed in biliary tract cancer subtypes, such as ECC and ICC, where they are linked to decreased $CD8^+$ T-cell infiltration, poorer overall survival, and increased metastasis (62). Elevated soluble PD-L1 levels in the serum of advanced biliary cancer patients further predict worse survival. Other checkpoints, such as B7-H4 and FOXP3, are associated with poor prognosis, while CTLA-4 expression in certain biliary tract cancer subtypes has shown mixed outcomes, including improved disease-free intervals in hilar biliary tract cancer (63). Gene expression studies indicate that hypermutated biliary tract cancer tumors frequently exhibit increased checkpoint molecule expression, such as CTLA-4, PD-L1, and LAG3, actively suppressing the immune response (64, 65). These findings underscore the potential of targeting immune checkpoints, particularly PD-1/PD-L1, as a therapeutic strategy for biliary tract cancer, providing a foundation for ongoing clinical trials to evaluate their efficacy and safety.

3.2 Synthetic approaches

Synthetic biology has paved the way for designing immune cells with enhanced therapeutic capabilities. By incorporating synthetic genetic circuits, researchers create "smart" immune cells that precisely target tumors while minimizing off-target effects. One such innovation involves the use of AND-gate circuits, which require the presence of multiple tumor-associated antigens to activate the therapeutic response, thus improving specificity in targeting biliary tumors (66, 67). Safety switches represent another critical advancement in synthetic biology. These switches, often based on inducible suicide genes, allow clinicians to terminate cell therapy in case of severe adverse effects. For instance, synthetic circuits incorporating small-molecule-inducible caspase systems rapidly eliminate engineered cells in response to toxicity, ensuring patient safety (68).

Synthetic biology has also enabled the development of "armored" CAR-T cells, which are engineered to secrete cytokines such as IL-12 within the TME. These cytokines boost local immune activation and counteract immunosuppressive signals, amplifying the therapeutic impact on biliary malignancies. CAFs in the TME inhibit T cell infiltration and induce dysfunction, while the limited availability of tumor-specific antigens (TSAs) and expression of tumor-associated antigens (TAAs) on normal tissues can cause "on-target, off-tumor" toxicity. A TALEN-based strategy to engineer allogeneic "Smart CAR T cells" were developed (66). These cells express a CAR targeting FAP+ CAFs in solid tumors and a second CAR, directed at a TAA like mesothelin, integrated into a TCR signaling-inducible locus such as PDCD1 (66). A library of multi-receptor cell-cell recognition circuits was created using synthetic Notch receptors to link multiple molecular recognition events. These circuits enable engineered T cells to recognize both extracellular and intracellular antigens, showing robustness to heterogeneity and achieving precise recognition of up to three antigens with positive or negative logic (69). Additionally, synthetic biology approaches are being employed to design NK cells with enhanced migratory and cytotoxic properties, expanding their potential applications in treating solid tumors like biliary cancers (70).

Preclinical and early clinical studies are beginning to demonstrate the potential of combining ICIs with cell-based therapies in biliary tract cancer. For example, CAR-T cells targeting PD-L1 or other tumor-specific antigens could directly lyse tumor cells while benefiting from the additional immune activation provided by ICIs. Similarly, ICIs can enhance the persistence and function of adoptively transferred TILs or NK cells, improving their efficacy against biliary tract cancer. Despite these advances, challenges remain, including the heterogeneity of biliary cancers, the complexity of the TME, and potential toxicities from combining immune-based therapies. Strategies such as optimizing antigen selection for cell therapies, using novel immune checkpoint targets, and leveraging biomarkers to stratify patients will be crucial to maximizing the benefits of these combinations.

4 Modulating the tumor microenvironment in biliary tract cancers

Biliary malignancies are characterized by a unique and challenging TME that presents significant barriers to effective therapy. The TME of these tumors is composed of a dense extracellular matrix (ECM), stromal cells, and various immunosuppressive elements, which not only limit the delivery of therapeutic agents but also actively support tumor progression and immune evasion. TME reprogramming by the combination of chemotherapy and CTLA-4 immune checkpoint block enhances anti-PD-1 therapy (71).

4.1 Cancer-associated fibroblasts and stroma cells confer immuno-suppressive TME

A defining feature of biliary cancers is their pronounced desmoplastic reaction, marked by extensive fibrosis and the accumulation of CAFs (72). CAFs secrete ECM components, such as collagen, hyaluronan, and fibronectin, creating a physical barrier that restricts the infiltration of therapeutic immune cells, including CAR-T cells, NK cells, and TILs (73). Moreover, CAFs produce immunosuppressive cytokines like transforming growth factor-beta (TGF-β) and interleukin-6 (IL-6), which inhibit the activity of cytotoxic T cells and NK cells, while promoting the recruitment of Tregs and MDSCs (74, 75). CAF promoted cholangiocarcinoma growth by secreting hyaluronan synthase 2 instead of type 1 collagen (76). In addition, Stromal cells also contribute to metabolic reprogramming within the TME, depriving immune cells of vital nutrients like glucose and amino acids, while enriching the environment with immunosuppressive metabolites such as adenosine and lactate (77). These factors create a hostile

microenvironment for immune cell therapies, necessitating strategies to target stromal components directly.

4.2 Approaches to reprogram the TME

4.2.1 Stromal cell targeting

One promising approach involves directly targeting CAFs to disrupt their tumor-supportive roles. Agents such as fibroblast activation protein (FAP)-specific inhibitors have demonstrated preclinical efficacy in depleting CAF populations, resulting in reduced fibrosis and enhanced immune cell infiltration. Additionally, efforts are underway to reprogram CAFs into a tumor-restraining phenotype using agents like vitamin D analogs, which have the potential to convert activated CAFs into a quiescent state.

4.2.2 Enzyme-based desmoplasia modulation

Another strategy focuses on enzymatic degradation of the ECM to improve immune cell penetration. Enzyme-based desmoplasia modulation aims to degrade or remodel the ECM, reduce stromal density, and enhance the accessibility of therapeutic agents. The following enzymes have shown potential in biliary tract tumor models: (1)Hyaluronidase: Hyaluronidase degrades hyaluronic acid, a major component of the ECM that contributes to elevated interstitial fluid pressure (78). By breaking down hyaluronic acid, hyaluronidase reduces tumor stiffness, improves vascular perfusion, and facilitates the delivery of chemotherapeutics and immune cells. PEGPH20, a pegylated form of hyaluronidase, has shown the ability to degrade hyaluronan, a major ECM component, thereby facilitating the delivery of immune and chemotherapeutic agents (79). In preclinical models of biliary cancers, PEGPH20 has been shown to synergize with CAR-T cell therapy, enhancing T cell infiltration and efficacy (80). More of its roles have been studied in pancreatic cancer (81). They also enhance the efficacy of gemcitabine and other systemic therapies in desmoplastic tumors (82). (2) Collagenase: Collagenase targets collagen, the most abundant structural protein in the ECM, thereby weakening the fibrotic barrier (83). Collagenase treatment has been shown to improve T cell infiltration in desmoplastic tumors, making it a promising adjunct to immunotherapies such as immune checkpoint inhibitors or CAR-T cells. (3) Matrix metalloproteinases (MMPs): MMPs are endogenous enzymes capable of degrading various ECM components. Recombinant MMPs or MMP-inducing strategies have been explored to modulate the TME and enhance the penetration of therapeutic agents. MMP2 induces COL1A1 synthesis by integrin alpha V to promote cholangiocarcinoma metastasis (84). A selective NOTCH1 inhibitor Crenigacestat reduces ICC progression by blocking VEGFA/DLL4/MMP13 axis (85). By breaking down the desmoplastic stroma, Enzyme-based modulation improve drug delivery, alleviate hypoxia, and promote immune cell infiltration. Additionally, combining enzyme therapy with chemotherapy or immunotherapy may achieve synergistic effects, enhancing overall efficacy. However, challenges remain. The nonspecific activity of enzymes leads to off-target effects and toxicity in normal tissues. Moreover, the dynamic nature of the TME means that stromal remodeling must be carefully timed and dosed to avoid adverse effects, such as tumor promotion due to excessive ECM degradation.

4.2.3 Immunomodulatory agents

TGF-β inhibitors reverse T cell exhaustion and reprogram the TME by alleviating fibrosis and immune suppression, making these inhibitors especially relevant for cholangiocarcinoma and other biliary tract cancers. Indoleamine 2,3-dioxygenase(IDO) inhibitors, which block the enzyme responsible for depleting tryptophan and suppressing T cell activity, are being tested in combination with NK cell therapies to enhance immune effector functions in these tumors (86, 87). Additionally, targeting adenosine signaling using A2A receptor antagonists helps counteract the immunosuppressive effects of adenosine, a metabolite enriched in the hypoxic and nutrient-deprived TME of biliary tumors (88). These strategies address the unique challenges of biliary tract cancers, where immune evasion and stromal barriers limit the efficacy of current treatments, offering new hope for improving therapeutic outcomes through combination approaches.

4.3 Combining cell-based therapies with TME modulators

Integrating cell-based therapies with TME-modulating agents has demonstrated potential in overcoming the challenges presented by the TME in biliary malignancies. For example, the combination of PEGPH20 and immune checkpoint inhibition has shown enhanced tumor regression in preclinical models of colon cancer (89). Similarly, using CAF-targeting agents alongside NK cell therapy has improved NK cell infiltration and cytotoxicity (90). Innovative approaches involving dual-function engineered cells are also under investigation. For example, CAR-T cells engineered to secrete ECM-degrading enzymes or immunostimulatory cytokines could simultaneously target tumor cells and modulate the TME (91). Additionally, NK cells equipped with synthetic receptors targeting stromal components like FAP or integrins may selectively disrupt the tumor stroma while maintaining anti-tumor activity, offering a promising strategy for tackling the complex TME of biliary malignancies (92).

5 Conclusions and perspectives

Recent advancements in cell-based therapies have introduced promising avenues for the treatment of biliary tract cancers. Immune cell-based strategies, including CAR-T cells, NK cells, and dendritic cell vaccines, alongside innovative genetic engineering techniques and synthetic biology, offer significant potential in addressing the challenges posed by these aggressive malignancies (Figure 2). However, the application of these therapies is still hindered by several critical challenges that must be addressed to improve their clinical utility. One major hurdle is the immunosuppressive TME in biliary tract cancers, which limits the efficacy of cell-based therapies by suppressing anti-tumor immune responses. Additionally, safety concerns such as cytokine release syndrome (CRS) and off-target

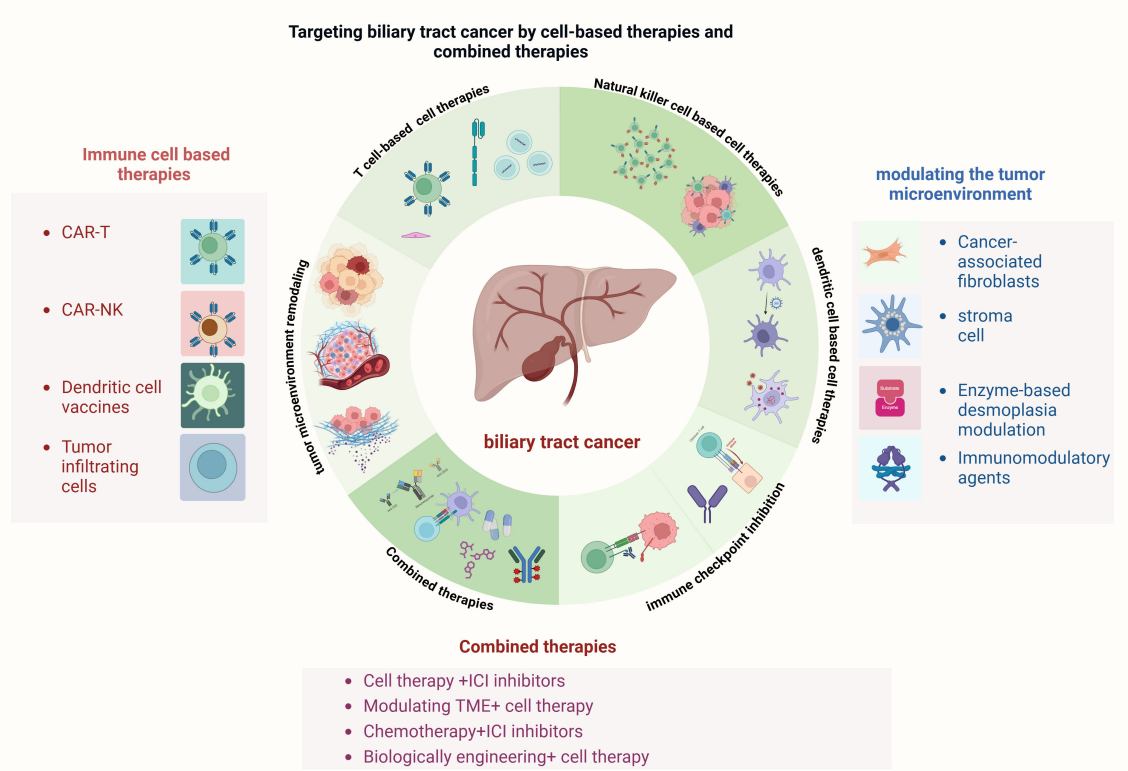

FIGURE 2
Targeting biliary tract cancer by cell-based therapies and combined therapies.

effects present significant barriers to broader clinical application. CRS, a frequent complication in CAR-T therapy, results from excessive immune activation and can lead to life-threatening systemic inflammation. To address this, mitigation strategies such as the use of IL-6 receptor antagonists (e.g., tocilizumab), corticosteroids, and dose titration protocols have been developed. Off-target effects, caused by the unintended targeting of healthy tissues due to shared antigen expression, further complicate treatment. Advanced strategies, including dual-antigen targeting systems, tumor-restricted promoters, and engineered safety switches, are being explored to enhance therapeutic precision and minimize collateral damage. Furthermore, optimizing manufacturing processes and integrating emerging technologies, such as CRISPR-Cas9 and synthetic biology, are critical steps to enhance both the efficacy and safety of these therapies. Collectively, overcoming these challenges will be essential to unlocking the full potential of cell-based therapies and improving outcomes for patients with biliary malignancies. Current main open questions in targeting biliary tract cancer by cell therapy are as below:

- How to effectively overcome the immunosuppressive tumor microenvironment to enhance the efficacy of cell-based therapies for biliary tract cancers?
- What are the next critical steps in optimizing the manufacturing processes of cell-based therapies to ensure their scalability and accessibility for a broader patient population?
- In what ways can the integration of immune-modulating agents with cell-based therapies potentially synergize to improve treatment outcomes in biliary tract cancers?
- How might advancements in genetic engineering techniques and synthetic biology further enhance the precision and effectiveness of personalized cell therapies for individual patients with advanced biliary cancers?

The immune cell-based therapies section highlights approach such as CAR-T cells, CAR-NK cells, dendritic cell vaccines, and tumor-infiltrating lymphocytes, which aim to harness or engineer immune cells to combat cancer. The modulation of the tumor microenvironment includes targeting cancer-associated fibroblasts (CAFs), stromal cells, and using enzyme-based desmoplasia modulation or immunomodulatory agents to overcome the immunosuppressive environment. Combined therapies integrate cell therapies with immune checkpoint inhibitors (ICIs), chemotherapy, or biologically engineered therapies to enhance efficacy. These approaches aim to overcome the challenges of biliary tract cancer by improving immune recognition, tumor microenvironment remodeling, and therapeutic synergy.

The future of biliary tract cancer treatment lies in continued innovation and cross-disciplinary collaboration. By combining cell-based therapies with immune-modulating agents, advanced genetic tools, and personalized treatment strategies, there is a tangible potential to significantly improve outcomes for patients with biliary tract cancers. As research progresses, the clinical translation of these therapies will mark a transformative step in

combating these challenging malignancies, offering renewed hope to patients who currently have limited effective treatment options.

To further advance biliary tract cancer treatment, several key research directions need to be explored. First, identifying and validating novel tumor-associated antigens is crucial. By leveraging high-throughput screening and bioinformatics approaches, researchers can uncover new targets for immunotherapy, potentially improving the specificity and efficacy of treatments. Second, enhancing delivery systems is essential to ensure the precise targeting of therapies to tumor sites. Developing advanced systems, such as nanoparticles or viral vectors, could significantly improve the efficiency and precision of cell-based treatments. Lastly, addressing the heterogeneity of TME is vital for overcoming resistance to therapies. The TME's immunosuppressive nature hinders effective treatment; thus, targeting TME components like tumor-associated macrophages or regulatory T cells could help overcome these barriers and potentiate the effects of immunotherapies. By focusing on these research avenues, the therapeutic landscape for BTC can be dramatically improved, leading to more effective, personalized treatment options and better outcomes for patients.

Author contributions

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The author(s) declare that no Generative AI was used in the creation of this manuscript.

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New player in CAR-T manufacture field: comparison of umbilical cord to peripheral blood strategies

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One of the most successful treatments in hematologic cancer is chimeric antigen receptor (CAR)-T cell-based immunotherapy. However, CAR-T therapy is not without challenges like the costly manufacturing process required to personalize each treatment for individual patients or graft-versus-host disease. Umbilical cord blood (UCB) has been most commonly used for hematopoietic cell transplant as it offers several advantages, including its rich source of hematopoietic stem cells, lower risk of graft-versus-host disease, and easier matching for recipients due to less stringent HLA requirements compared to bone marrow or peripheral blood stem cells. In this review, we have discussed the advantages and disadvantages of different CAR-T cell manufacturing strategies with the use of allogeneic and autologous peripheral blood cells. We compare them to the UCB approach and discuss ongoing pre-clinical and clinical trials in the field. Finally, we propose a cord blood bank as a readily available source of CAR-T cells.

KEYWORDS

CAR-T cells, umbilical cord blood, cord blood bank, allogeneic therapy, autologous therapy, off-the-shelf CAR-T cells

1 Introduction

One of the most widespread approaches in cancer immunotherapies is chimeric antigen receptor (CAR)-T cell therapy, in which genetically modified allogeneic or autologous T cells or natural killer (NK) cells are redirected against the tumor cells armored with CAR construct (1). The classic CAR combines antigen-binding domains, most commonly a single-chain variable fragment derived from antibody variable domains, with the signaling domains of the T cell receptor 3ζ chain and additional costimulatory domains from receptors such as 4-1BB, OX40, or CD28 (2). In recent years, CAR-T cell therapy has revolutionized the treatment of relapsed or refractory hematological

malignancies, resulting in Food and Drug Agency (FDA) approval of six products targeting non-Hodgkin lymphomas (NHLs), acute lymphoblastic leukemia (ALL) and multiple myeloma (MM).

Despite the enormous progress in the treatment, the therapies are associated with several limitations, such as manufacturing difficulties, risk of relapse, or the impossibility of generating clinically applicable doses of CAR-T cells from previously treated patients. Moreover, there are two main risks associated with the use of autologous CAR T cell therapy: cytokine release syndrome (CRS) and neurotoxicity (3). On the other hand, allogeneic CAR-T cells raise safety concerns, as the infusion of donor-derived cells may cause graft-versus-host disease (GvHD) or trigger graft rejection (4).

An appealing strategy to generate "off-the-shelf" allogeneic CAR-T cells with limited potential of triggering GvHD is to utilize umbilical cord blood (UCB) as a source of T/NK cells. To date, UCB has been successfully used as a hematopoietic stem cell (HSC) source in allogeneic HSC transplantations (alloHSCT) with lower rates of GvHD in transplant recipients compared to conventional sources (bone marrow or mobilized stem cells) (5). Crucially, the UCB alloHSCT procedure permits higher human leukocyte antigen (HLA) disparity between a donor and a recipient (6). These qualities are attributed to the naïve phenotype of cord blood T/NK cells (5, 7).

In this review, we discuss the pros and cons of different cell sources for adoptive CAR-T cell immunotherapy. We focus on the therapeutic application and summarize the results from the ongoing clinical trials regarding the use of UCB to provide the perspective for future research teams and clinicians interested in this field. Finally, we propose a novel approach to utilizing CB banks for CAR-T cell manufacturing.

2 T cell characteristics

One of the key aspects determining CAR-T cell therapy's effectiveness and long-term remission is the *in vivo* stability of transferred cells. It has been shown that the persistence of CAR-T cells is correlated with the phenotype of the modified T cells and that prolonged detection of CAR-T cells is associated with a better response even in patients with high-grade cancer (8). Moreover, the differentiation stage of adoptively transferred T cells affects their proliferation and survival which strongly correlates with their antitumor activity (9). Depending on the stage of cell differentiation, T cells can be divided into naïve T cells (TN), stem cell memory T cells (TSCM), central memory T cells (TCM), memory effector T cells (TEM), and effector T cells (TEF) (10).

Naïve T lymphocytes are characterized by high proliferative ability with the surface expression of CD62L, CCR7, and CD45RA isoform and a lack of activation markers like CD25, CD44, CD69, or CD45RO isoform (11). Lymphocytes are considered naïve until they are presented with an antigen that activates TN lymphocytes to proliferate and differentiate into (memory T cells) TCM and/or TEM lymphocytes (12). TSCM is a subpopulation of the least differentiated of the memory T cell subset. Surface markers expressed by TSCM include both naïve T cell markers, such as CD45RA, high levels of CD27, CD28, CD127, CD62L, and CCR7, as well as markers of memory T cells, such as CD122, CD95, or CXCR3 but do not include CD45RO (13, 14). They have stem cell-like self-renewal capacity and can regenerate all TCM and TEF cell populations (15, 16). Compared to other subpopulations, the T subset of TSCM develops a faster response to antigenic stimulation and may persist for a long time (12). TCM cells guard lymph nodes, providing central immunosurveillance against known pathogens. Compared to naïve T cells, memory T cells respond more quickly and deliver an early batch of cytokines when stimulated by a specific antigen (17). TCM secrete tumor necrosis factor α (TNF-α), interleukin 2 (IL-2), and co-express L-selectin and CCR7, as well as CD45RO, CD62L, CD27, but not CD45RA (18). Next population - TEMs produce many inflammatory cytokines such as TNF-α and interferon γ (IFN-γ) as their most important role is related to the activation of the immune response taking place in peripheral lymphoid organs (19). Phenotypically, TEM cells are positive for activation markers CD38, CD69, and CD25, they sometimes co-express CD45RA and CD45RO at high levels, however, are negative for CD62L, CCR7, and CD31 (20). Finally, the TEF subpopulation contains fully differentiated T cells, responsible for strong effector functions (12). TEF overexpress several homing receptors to migrate to inflammation sites like CCR5, LFA-1, as well as CD45RA, CD95, CD122, and KLGR1 but do not express CD45RO, CCR7, CD27, CD62L or CD28 (21, 22). Additionally, TEFs exhibit limited expansion, low self-renewal, and survival capacity as a consequence become exhausted quickly (23) (Figure 1).

Naïve CD4+ T cells differentiate into several effector T cell subsets characterized by the ability to produce specific cytokines, namely helper T cells (Th) Th1, Th2, Th9, Th17, Th22, follicular helper T cells (Tfh) and regulatory T cells (Treg). Cell differentiation is regulated by the cytokines present in the environment and induced upon T cell receptor (TCR) signaling (24). Foreign antigens from infected or unknown cells are presented to T cells in the context of specific major histocompatibility complex I (MHC class I) called HLAs. Each cell subset has particular characteristics and releases cytokines that determine its function, such as Th1 and Th2 cells which produce IFN-γ and IL-4, respectively (25). CD4+ T cells are differentiated through the cytokines upon induction by master transcription factors such as T-bet, GATA-3, RORγt, Bcl6, or Foxp3 (26).

Studies showed that both CD4+ and CD8+ CAR-T cells derived from TCM and TM populations can be involved in killing malignant cells and achieve better results than those from TEM (27). Results from the clinical trials indicate that CAR-T cell products rich in TN, TSCM, or TCM are required for the sustained *in vivo* persistence of adoptively transferred CAR-T cells due to their ability to proliferate (28, 29). Moreover, the combination of the most potent CD4+ and CD8+ CAR-T cell subsets has a synergistic antitumor effect *in vivo* (30). These results demonstrate that naïve and TSCM/TCM cells are the most important players in CAR-T cell therapy due to their sustained proliferation and persistence *in vivo*.

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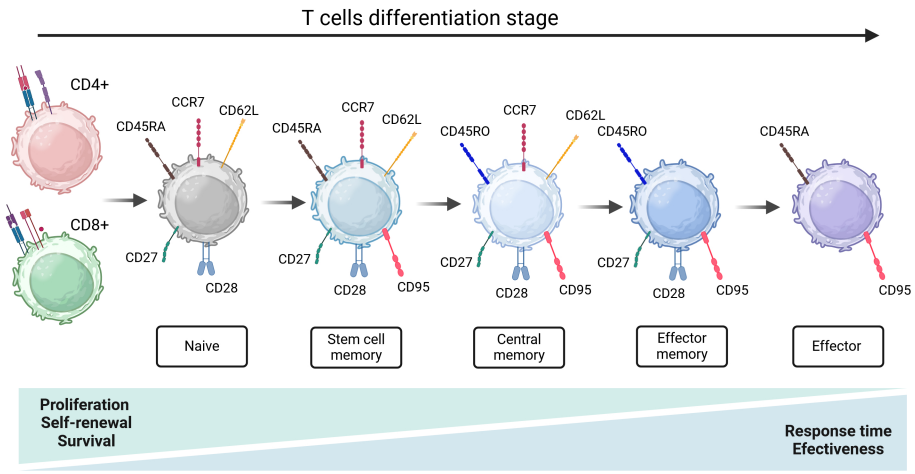

FIGURE 1
Linear model of stages and characteristics of T cells maturation. Following activation, naïve T cells differentiate into memory and effector cells. This differentiation is marked by the dynamic changes in the expression of CD45RA/RO, CCR7, CD28, CD62L, and CD95 antigens. Created in BioRender.

Comprehending the phenotype of manufactured CAR-T products is critical to mitigate adverse events, as well as to improve antitumor effectiveness, such as CAR-T dosing or activation. The phenotypic profile of infused CAR-T cells has not only been linked to the cells' persistence but also to the long-term outcomes of the treatment (18, 31).

3 CAR molecular constructs

CARs are created using the main signaling components of the T cell receptor and costimulatory molecules. In general, CAR consists of the extracellular single-chain variable fragment (scFv) specific for a cancer marker, and three main domains: an extracellular antigen-binding domain derived from a tumor-specific monoclonal antibody, a transmembrane domain that anchors the CAR to the T cell (derived from CD3, CD4, CD8 or CD28 proteins) and an intracellular T cell activation domain of CD3ζ with one or more costimulatory domains (usually from 4–1BB or CD28) that is required for full T cell activation (32–35).

Since the introduction, several CAR-T cell generations have been developed. First-generation CARs had only one signaling domain, typically the CD3ζ that proved not to be sufficient due to the limited persistence and signaling ability of the cells (36). To improve that, the second generation of CARs included a co-stimulatory signaling domain (usually CD28 or 4-1BB) localized proximal to the membrane to enhance activation, and survival, and promote the expansion of the modified T cells (37). To achieve greater antitumor activity and increase CAR-T cell persistence, a second co-stimulatory signaling domain (e.g. CD28 or OX40) was incorporated into third-generation CARs (38). The fourth generation of CARs also known as "TRUCKs" (T cells redirected for universal cytokine killing) was based on the structure of the second-generation CARs. TRUCKs contain a transgenic expression cassette coding for a synthetic nuclear factor of activated T cell response elements to be produced and secreted upon antigen recognition, such as IL-12 (39). The secretion of cytokine enhances the immune response against cancer cells and recruits other immune cells to the tumor site (40). The next generations of CARs are often based on the second generation and contain several modifications including, truncated intracellular domains of cytokine receptors such as IL-2 with the addition of STAT3/STAT5 transcription factors binding motifs (41). These CAR constructs induce cytokine secretion through the activation of the JAK/STAT signaling pathway therefore driving CAR-T cells to remain active and generate TM cells (42).

Novel developments of CAR constructs are still being extensively studied and include logic gating with the example of tandem CAR-T cells targeting two antigens simultaneously or SynNotch-engineered CAR-T cells that use a synthetic Notch receptor to sense a specific antigen, which then triggers the expression of the CAR and many others CAR variants and augmentations (43, 44) (Figure 2). On the other hand, extensive clinical experience showed that overactivation of the peripheral T cells leads to extensive exhaustion and might not be the only possible way of CAR therapy development in the future. Genome editing is also being explored to improve next-generation CAR-T cells to overcome some of the current limitations of the therapy. To improve CAR-T cells' persistence and function both knock-out/knock-in genes as well as epigenetic modifications are being extensively studied (45).

While scientists and industry continue their work on novel generations of more persistent and capable CAR-T cells, the genetic modification of constructs involves certain risks and challenges, both in the laboratory during the genetic engineering process and in the patient's body after the CAR-T cell transfer. One of the concerns relates to the safety of viral vectors. The use of viral vectors (lentiviruses or retroviruses) to deliver the CAR gene into T cells carries a risk of unintended genetic mutations or insertional oncogenesis, where genetic modification leads to the development

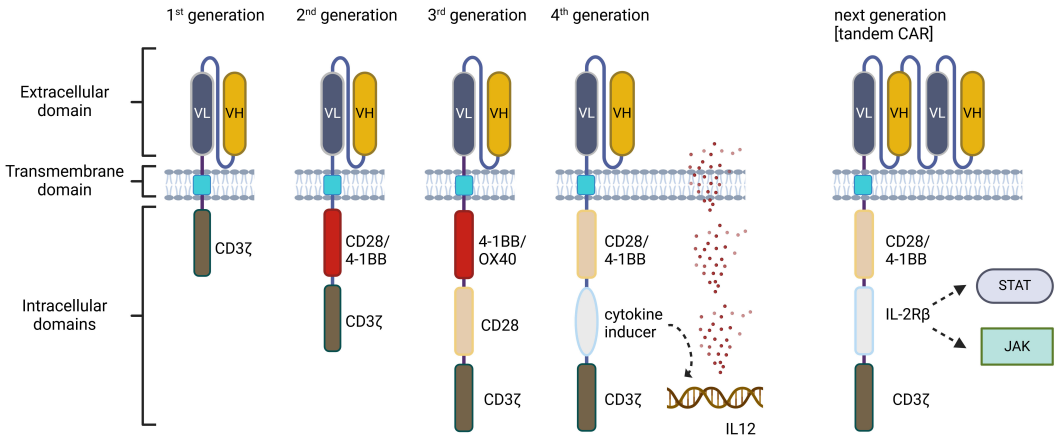

FIGURE 2
Generations of chimeric antigen receptors (CARs). The first generation of CARs is equipped with a single CD3ζ intracellular domain. The second generation includes additional costimulatory molecules, such as CD28 or 4-1BB. The third generation combines multiple co-stimulatory domains, like CD28-4-1BB or CD28-OX40. The fourth generation builds upon second-generation CARs, incorporating either constitutively or inducible expressed chemokines. The next generation of CARs retains the structure of the second generation but also incorporates cytokine receptors in the intracellular domain, such as the IL-2Rβ chain fragment. VL, variable region of light chain; VH, variable region of heavy chain. Created in BioRender.

of cancer (46). Although the FDA has called to label CAR-T cell products with a warning about secondary T cell mutagenesis, insertional mutagenesis was never proven to be a cause (47). Moreover, a recent study analyzed retrospective data from 61 centers worldwide including 3038 B-cell and non–B-cell malignancies treated with commercial or investigational CAR-T cell products in children, adolescents, and young adults. Remarkably, no cases of T-cell malignancies were reported following CAR-T cell treatment in this cohort (48).

Another risk applies to the off-target effect. Despite CAR-T cells being designed to target specific antigens on cancer cells, there is a risk of unintended attack of an antigen other than the intended one or activation of the CAR-T cells independently from their specificity (49).

4 Autologous CAR-T therapy

Autologous CAR-T cell therapy, where the patient's T cells are genetically engineered *in vitro* to express the CAR has proved its importance in clinical applications (50). Since the therapy uses the patient's T cells, it is highly personalized and tailored to the person's immune system. This reduces the risk of GvHD, a common complication of allogeneic stem cell therapy or CAR-T cell therapy that uses donor cells (51). Additionally, very high response rates, even in patients with limited treatment options followed by highly durable remissions have been observed after CD19-targeted CAR-T cell therapy (52–54).

However, this approach is not without drawbacks. Firstly, as it relies on autologous T cells, it depends on the nature and quality of T cells in the peripheral blood (PB) of the patient at the time of treatment. Frequently, patients have compromised immune systems and suffer from severe lymphopenia as a result of previous treatments which affects the quality and quantity of T cells harvested during apheresis (55). Besides, harvested T cells may have impaired functionality, reduced proliferative capacity, and altered phenotypes impacting their usefulness for CAR-T cell manufacturing (56).

A therapeutic dose of 10^7 – 10^9 cells is required for the treatment, hence too low T cell count or cell activation failure during the production process may be the cause of treatment failure. What is more, patient cells can be contaminated with malignant tumor cells (57). Autologous CAR-T cells may be also exposed to the patient's tumor microenvironment before infusion. This leads to immune exhaustion, where CAR-T cells display decreased proliferative capacity, diminished anti-tumor activity, and attenuated persistence (58). Another issue is the high manufacturing cost of CARs due to the complex and personalized manufacturing process as autologous cell therapy is "tailor-made" meaning that that CAR-T cells must be generated *de novo* for each patient (59). Factors such as the time, materials, infrastructure, and skilled manpower required to generate CAR-T cells for each patient contribute to the astounding therapy cost. Additionally, as one batch is custom-made for one patient, the economy of scale cannot be applied (60). Time is another very important issue. In many cases, CAR-T cell therapy is the last treatment option for severely ill patients. In the case of autologous treatment, the production of personalized CARs requires a long manufacturing process. To successfully carry out the entire process of transporting a patient to the manufacturing facility and back to the treatment facility requires a thorough complex transport network, planning, and a skilled workforce that is highly prone to delays. This can result in an increase in treatment time of three to five weeks, which may be intolerable due to the progression of the patient's disease (61). Recent pre-clinical studies developed a new platform called FasT CAR-T with a shortened next-day manufacturing time. The procedure was reported successfully with a median of 14 days from apheresis driven by 7 days of rapid sterility testing, which is a

significant improvement from the median of 45 days. Although the first-in-human clinical study showed early promising efficacy in B-ALL patients, more data on additional patients and longer follow-up are needed to further evaluate the efficacy of this novel CAR-T cell therapy (62, 63). Lastly, while CAR-T cell therapy has demonstrated clinical efficacy in hematologic malignancies, severe toxicities such as CRS have also been reported (64). CRS is characterized by the release of cytokines (mainly IFN-γ, IL-6, IL-10, TNF-α, and IL-2) in response to the therapy. That causes systemic inflammatory responses resulting in flu-like symptoms and, in severe cases, organ dysfunction (65). The precise characterization and understanding of the composition of the infused T cells both in terms of T-cell memory differentiation and CD4/CD8 ratio are fundamental aspects of CAR-T cell therapy development and implementation. Collectively, directly impacts the antitumor activity, safety, and consistency, ultimately leading to better therapeutic outcomes (30, 66).

5 Allogeneic CAR-T therapy

Donor-derived allogeneic CAR-T cell therapies offer many advantages compared with autologous treatments. One of the benefits of the allogeneic approach is the possibility of obtaining a higher quality of donor cells, which directly translates into the quality of the treatment (67). Healthy donors can be pre-screened for desirable characteristics of T cells like an appropriate number, phenotype, or optimal CD4:CD8 ratio that will minimize manufacturing errors. Selection of donors that have a high percentage of T cells is recommended, as contamination with non-T cell populations may affect downstream production (68). In contrast to autologous products, a large number of allogeneic CAR-T cells can be generated in a single manufacturing run making the production process cost-effective. Moreover, during production, standardized T-cell products from healthy donors are being created ahead of time, making it possible to generate many therapeutic doses from one manufacturing lot making it readily available for patients (69). Allogeneic CAR-T cells as "off-the-shelf" can reduce "vein-to-vein" time, decrease the risk of manufacturing failures, and alleviate logistical challenges (69). Eliminating treatment delays by reviving pre-frozen allogeneic CAR-T cells whenever the patient needs them may also improve the clinical outcomes of the therapy (70). Moreover, allogeneic CAR-T cell therapy does not carry the risk of contamination with cancer cells as T cells are manufactured from the healthy donor's blood. Allogeneic CAR-T cells may also have a lower risk of immune exhaustion as they are not exposed to the patient's tumor microenvironment or cytotoxic therapies prior to infusion (71).

Although allogeneic CAR-T cell therapy has numerous advantages, one of the most significant drawbacks is the risk of the development of GvHD (64). A condition where the T cell αβ receptor (TCRαβ) on the infused CAR-T cells recognizes cell surface HLA class I and class II molecules on the recipient's cells and attacks them. Therefore, ensuring compatibility between the donor and recipient's HLA types is essential to reduce the risk of GvHD, which may limit the availability of suitable donors (72). Until now, it has been established that for successful HLA-matching, the patient and potential donor should have high-resolution HLA typing using DNA-based methods for HLA-A, B, C, DRB1, and DQB1 (73).

Recently studies proposed a feasible alternative for HLA match assessment in terms of CAR-T cells derived from PB T cells. The higher efficacy of HLA-matched CAR-T cells (minimum of a 4/6 match to the patient) compared to HLA-haploidentical (sharing one haplotype) CAR-T cells has been observed (74). Additionally, results showed that haploidentical PB CAR-T cells induced only transient or no reduction in PB leukemia cell number without significant CAR-T cells expansion, which suggests rejection (74).

Achieving HLA compatibility is crucial not only to reduce the risk of GvHD but also to prevent CAR-T cell rejection. For that reason, patients receiving allogeneic CAR-T cells often require immunosuppressive drugs, which can weaken the anti-cancer immune response and increase susceptibility to infections (69). Hence, genome editing has been applied to prevent GvHD. Studies showed that the elimination of expression of the endogenous TCRαβ receptors using either zinc finger nucleases (ZFNs) or transcription activator-like effector nucleases (TALENs) successfully prevented GvHD without compromising CAR-dependent effector functions (75, 76). In two phase 1 clinical trials in pediatric and adult patients with late-stage relapsed or refractory B-cell acute lymphoblastic leukemia (NCT02808442 and NCT02746952), TALENs were used to simultaneously disrupt cell surface expression of TCRαβ and CD52. Results proved that genetically modified CAR-T cells caused only minimal GvHD with no detrimental expansion of unedited TCRαβ-positive T cells (77). Another phase 1 study (NCT04227015) has utilized the alternative genome editing technique CRISPR/Cas9 to disrupt the TCRα subunit constant (TRAC) region previously shown to lead to loss of TCRαβ expression (78). Obtained data demonstrated that CD19/CD22-targeting CAR-T cells with a CRISPR/Cas9-disrupted TRAC region and CD52 gene infused in patients with relapsed/refractory acute lymphocytic leukemia showed manageable safety profile and notable antileukemia activity, with no GvHD observed (79).

Nevertheless, complications and toxicities may arise as a result of gene editing. This may include on- and off-target effects and issues such as CRS or neurotoxicity, especially when editing inadvertently causes the CAR-T cells to become more aggressive or activate faster than desired (80). Another challenge of allogeneic therapy combined with more extensive genetic modification is the risk of chromosomal aberrations. No currently available manufacturing process can provide 100% certainty that no aberrations are present in a therapy's potentially hundreds of millions of cells. In 2021, the FDA put a 4-month hold on clinical trials of all allogeneic projects due to chromosomal abnormality found in a lymphoma patient in the Alpha-2 trial of the CD19-directed allogeneic CAR-T trial. Although the investigation has not shown any clinical consequences, such as aberrant proliferation or leukemogenesis of engineered cells, the issue of chromosomal aberrations risk is to be addressed (81).

CAR-NK cells are being investigated as an alternative cell source in allogeneic cell therapies as they are crucial in immune surveillance of invading viruses and killing cancer cells without the need for tumor-specific antigen presentation (82). Except for the CD3 signaling domain, as well as CD28 or 4-1BB as a co-stimulatory domain, CAR-NK cells enhance their cytotoxic capacity and cytokine production through additional co-stimulatory molecules, that is: NKG2D and CD244 (2B4) (83). The initial success of NK cell therapies has prompted further investigation into current allogeneic NK cell products, yielding encouraging outcomes in clinical trials. First, since NK cells do not recognize targets presented by the HLA system, therapeutic CAR-NK cells can be cultivated from donors with various genetic backgrounds and applied in diverse recipients without the concern of inducing GvHD (84). Second, due to a different spectrum of secreted cytokines, CAR-NK therapy does not cause CRS, therefore it is considered safer than CAR-T cells treatment (85). Third, NK cells are characterized by the abundance of CD16 (FcγRIIIA), which serves as a receptor for IgG1 and IgG3 and is essential for NK cell-mediated antibody-dependent cell-mediated cytotoxicity ADCC (86). Hence, they can also productively eliminate tumor cells in a CAR-independent manner through their stimulatory and inhibitory receptors which form the basis for the dual anti-tumor activity of CAR-NK cells.

In recent years several studies explored different approaches to genetically engineer CAR-NK cells. Especially in the treatment of T-ALL CAR-NK cell therapies targeting CD3, CD5, and CD7 have shown significant anti-tumor cytotoxicity both *in vitro* and *in vivo* (87–89). As of January 2025 currently ongoing clinical trials on CAR-NK focus not only on CD19 target but also on CD123 *clinical trials.gov; ID* (NCT05574608) and *clinical trials.gov; ID* CLL-1 (NCT06307054) in acute myeloid leukemia (AML) or dual targeting of CD19 and CD70 *clinical trials.gov; ID* (NCT05842707) in B cell Non-Hodgkin lymphoma and CD33 and/or FLT3 in AML *clinical trials.gov; ID* (NCT06325748).

As pre-clinical and clinical data support the potential of CAR-NK cell therapies, still several challenges need to be addressed before clinical application. One of the main barriers to CAR-NK cell therapy is the lack of persistence *in vivo* in the cytokine support deficiency (90). Additionally, due to the inhibitory tumor microenvironment, CAR-NK ability of homing and infiltration is limited (91).

6 UCB as a source for CAR-T cells

6.1 Characteristics of UCB as cells source

Over the past years, UCB transplantation has become an alternative therapeutic option for patients with cancer or genetic diseases for whom an HLA-matched family or unrelated adult PB stem cells or bone marrow donor has not been found (92). UCB therapy is currently used for the treatment of such hematological diseases as myelodysplastic syndrome (MDS), AML, aplastic anemia (AA), or ALL (93–96). It is the unique qualities of UCB compared to PB or bone marrow that make the therapy encouraging. In contrast to PB cells, UCB cells are characterized by higher proliferation, and expansion due to the higher percentage of hematopoietic stem cells, with the less mature phenotype of T cells and NK cells (97, 98). Crucially, the UCB T-cell population is characterized by a more naïve phenotype as it comprises approximately 85% naïve $CD45RA^{+}/RO^{-}$ T cells and only 6% memory T cells in comparison to 39% and 50% in PB, respectively (99) (Figure 3). Remarkably, naïve T cells of UCB differ from PB naïve T cells (100, 101). UCB naïve T cells have higher proliferative and activation capacities, as well as delayed exhaustion compared to PB (100). They do display comparable activation levels with reduced secretion of cytokines such as IL-2, IFN-γ, and TNF-α upon CD3/CD28 targeted stimulation. Moreover, naïve UCB T cells are characterized by lower expression of CD40L and perforin thus, they exhibit lower cytotoxicity than PB cells. Furthermore, compared to PB, UCB contains a relatively high frequency of distinct CD4+CD25 bright subset of regulatory T cells. Thus, the likelihood of co-purification of activated or memory CD4 + 25+ T cells in UCB compared with PB is decreased. Last but not least, UCB cells display elevated self-renewal potential as well as multiple cell divisions attributed to longer telomeres and overexpression of transcription factors like NF- κB (102).

Therefore, one of the major advantages of UCB treatment is its safety. Compared to other donor cell resources, UCB collection not only painless but also carries a lower risk of viral infections and somatic mutations, which are the primary causes of morbidity following transplantation (103). Moreover, in the case of allogeneic transplantation, UCB allows for less stringent HLA matching criteria (4 of 6 HLA loci, considering a low resolution for HLA-A and HLA-B and high resolution for HLA-DR), which may be especially important for members of racial and ethnic minorities, increasing the inclusiveness of the therapies (104). UCB grafts could also reduce the mortality of transplant as due to its low immunogenicity decrease in both acute and chronic rates of GvHD are observed (105). Studies showed that PB consists higher concentration of CD8+ T cells comparing to UCB (97, 106). Given that GvHD is primarily driven by alloreactive donor CD8+ T cells, this difference in composition may help to explain the higher frequency of the chronic GvHD observed for example when the PB is used as a stem cell source for hematopoietic stem cell transplantation (107). Additionally, PB contains more mature monocytes, lower counts of plasmacytoid dendritic cells (pDCs), and reduced numbers of NK 56bright16– cells, further contributing to increased GvHD risk, when compared to UCB (108). At a molecular level, UCB T cells exhibit impaired nuclear factor of activated T cells (NFAT) signaling as well as lower activation of the NF-κB pathway that leads to reduced production of several pro-inflammatory cytokines, further decreasing GvHD frequency and intensity (109–111). On the other hand, there are several drawbacks to UCB cell usage. To begin with, in

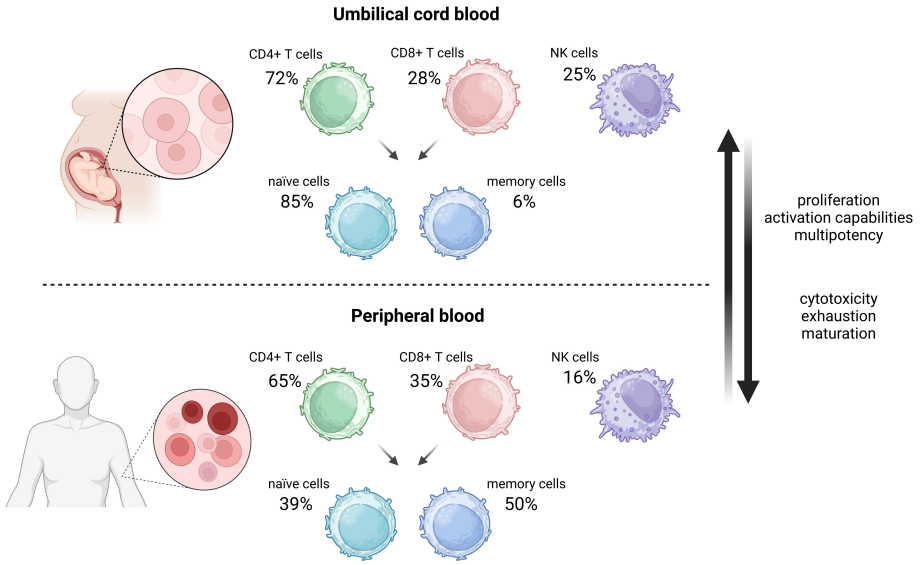

FIGURE 3
Comparison of umbilical cord blood and peripheral blood cells composition in terms of CAR-T cells application. Created in BioRender.

the case of autologous transplant and treatment of cancer or genetic disorders, stem cells derived from UCB may not be suitable as they might encompass the same abnormal cells or genetic variants that caused the onset of the disease at first (112). Furthermore, very often the amount of stem cells harvested from UCB is insufficient for the treatment of adults (113, 114). The last concern is the longer engraftment of CB stem cells (21-25 days) compared with adult donor cells (20 days) associated with higher hospital costs (115). To overcome the obstacles mainly related to the limited number of cells available in a single unit, several approaches have been investigated, including double UCB transplantation, *in vitro* UCB culture expansion, or direct intrabone transplantation of UCB cells (116–118).

Nowadays, haploidentical (haplo)-cord transplantation represents a promising approach to allogeneic transplantation. This technique involves combining two stem cell sources: UCB and $CD34^+$ cells from haploidentical donors (119). Haplo-cord transplantation has shown several benefits such as rapid availability and less stringent HLA-matching requirements compared to unrelated traditional adult grafts as it combines the benefits of both haploidentical and UCB transplants (120). Since the haploidentical component is derived from a partially matched family member and the UCB component relies less on perfect HLA matches because of its naïve immune cells, the two stem cell sources complement each other and yield better outcomes than using either source alone. Furthermore, this approach is associated with accelerated neutrophil and platelet recovery, lower risks of acute and chronic GVHD, and excellent graft-versus-leukemia (GVL) effects (121). Additionally, when combined with reduced-intensity conditioning (RIC), haplo-cord transplantation has shown decreased susceptibility to delayed opportunistic infections, shortened hospital stays, and favorable long-term outcomes (122).

6.2 Hurdles in UCB use as a CAR-T cell source

As UCB T cells exhibit distinct naïve profiles, they recently became an attractive source for CAR-T cells as an alternative to autologous cell treatment, potentially broadening access to the therapy. UCB as a CAR-T cell source offers advantages such as the shift of $CD4^+25^+$ T cells toward a Treg suppressor cell phenotype that may contribute to a lower incidence of GvHD after CAR-T cell infusion (123). Furthermore, UCB cells express significantly lower markers of exhaustion such as programmed cell death-1 (PD-1), T-cell immunoglobulin, and mucin domain-containing protein-3 (TIM-3) and lymphocyte activation gene-3 (LAG-3) compared to PB T cells, allowing for long-term persistence and efficiency (124, 125). However, the use of UCB cells for CAR-T therapy raises several challenges. Firstly, due to their naïve state, UCB-derived CAR-T cells may require longer expansion time. While studies have reported successful generation of CAR-PB-derived CAR-T cell products in as little as 8 days, UCB-derived CAR-T cells may need more time to achieve comparable therapeutic dose (126, 127). It is unclear for now how the "fast-CAR" approaches would be working in UCB case as well. What is more, specific phenotype distribution plays a significant role in establishing the proliferative and expansive potential of UCB-derived cells (126). Therefore, the specific phenotype of naïve UCB-derived T cells must be considered when designing and interpreting UCB CAR-T studies. These include protocols for activation and cytokine stimulation in the manufacturing process, the ability to differentiate into effector cells, and cytotoxicity. Current manufacturing of CAR-T products involves induction of cell activation and expansion by targeting CD3/CD28 molecules and coincubation with interleukin (IL) 2 or IL-7 and IL-15. Notably,

it is reported that IL-2 stimulation may cause cellular exhaustion, whereas IL-7 and IL-15 contribute to a higher percentage of memory cells. However, this evidence applies to conventional, PB-derived T cells so a more tailored and detailed study of UCB-derived T-cell activation pathways should be performed (128). Additionally, there is a question of whether the manufacturing process could deprive UCB T cells of their naivety features while providing them with sufficient anti-tumor cytotoxicity. According to studies, including our data, cord blood T cells retain a phenotype similar to T cells derived from peripheral blood upon CD3/CD28 stimulation and cytokine priming (129). Some differences could be attributed to the lower expression of exhaustion markers, nevertheless, the cytokine repertoire used in the manufacturing process significantly contributes to the phenotype of the resulting CAR-T cells (126).

Another concern is whether UCB-derived CAR-T cells could perform effector functions. Since T cells from UCB are more naive and less experienced in fighting infections or cancer compared to T cells from adult donors, it may take longer to adapt and respond effectively to cancer cells, potentially delaying the therapeutic effect. The seminal study completed by Serrano et al. proved that these cells are capable of CD19-specific cytotoxicity and was followed by results from other research groups (126, 130). However, in parallel with reports of satisfactory anti-tumor efficacy, concerns regarding adverse effects in allogeneic settings arise. Moreover, as UCB contains a relatively small number of T cells compared to PB, this limited T cell quantity may be insufficient for generating a therapeutic dose of CAR-T cells, especially for adult patients or those with high tumor burdens. Therefore, *ex vivo* expansion may be required to proceed with cellular transfer (131). The efficiency of the viral transduction, a crucial step in CAR gene introduction is yet another concern. Studies have shown variable transduction rates between units (15-85%), possibly due to T cell differentiation profiles (126, 132).

Besides the hurdles, several studies showed the feasibility and great therapeutic potential of UCB-derived CAR-T as such. Augmentation of attributes with targeted reduction of defective features should be in focus of new adoptive therapy approaches. Table 1 provides a comprehensive comparison of PB-derived and UCB-derived CAR-T therapies, highlighting key differences in aspects such as time, cost, cell quality, and risk profiles across autologous and allogeneic approaches.

6.3 Preclinical reports on UCB CAR-T safety and efficacy

As the immunology of UCB-T cells supports their use as a source for CAR-T manufacturing, several both *in vitro* and *in vivo* strategies have been tested in preclinical settings worldwide.

First of all, it has been proven that UCB-T cells cells can be successfully engineered to express CAR receptors capable of recognizing specific markers (133). Serrano et al. first reported that naïve UCB-T cells cells could be engineered into CD19 CAR-T cells (130). Encouraging results showed a reduction in tumor bulk in the *in vivo* arm of the study, with 60% of mice achieving complete remission with no adverse reactions following CAR-T infusion observed. Later, Huang et al. engineered 2nd generation anti-CD19 product from UCB with what seems to be an exquisite transposon-based mechanism (134). *In vitro* assays on B-cell

TABLE 1 Comparison of different approaches to generate CAR-T cells.

	PB-DERIVED THERAPY		UCB-DERIVED THERAPY
	AUTOLOGOUS THERAPY	ALLOGENEIC THERAPY	
TIME	3-5 weeks; cells generated *de novo*	"off-the-shelf" product; can be generated in advance	can be generated in advance
COST	more expensive	cost-effective	depends on availability of suitable cord blood or tissue samples and the cost of processing and expansion
QUALITY OF CELLS	T cell count may not be enough, cell activation failure, contamination with malignant tumor cells	cells pre-screened for appropriate number, phenotype, or optimal CD4:CD8 ratio	readily available, high quality, and immunologically compatible hematopoietic cells
CUSTOMIZATION	highly personalized and tailored to the person's immune system	"universal" donor cell lines	broad application
QUALITY CONTROL	variability in T cell quality and function based on individual patient factors	manufactured under controlled conditions, ensuring consistency and quality across multiple batches	FDA regulated product
APPLICABILITY	not applicable in patients with poor T-cell parameters	broad application; can be used in patients with poor T-cell parameters	broad application both in autologous and allogeneic therapy
T-CELL EXHAUSTION RISK	common	lower risk of immune exhaustion	lower than with autologous cells
GRAFT VERSUS HOST DISEASE RISK	none	common	minimal

PB, peripheral blood; UCB, umbilical cord blood; FDA, Food and Drug Administration.

lymphoma and B-cell ALL cell lines showed the efficient killing of tumor cells by UCB-derived CAR-T cells. The researchers also showed that UCB-derived CAR-T cells were capable of short-term (10 days) anti-tumor efficacy *in vivo* regardless of IL-2 injections. Nevertheless, there was no mention of off-target toxicities (134). Notably, in a direct comparison between CAR-T cells manufactured from PB, UCB-derived CAR-T cells presented similar anti-tumor responses completely eradicating B-cell ALL blasts over 5 days of coculture in the *in vitro* assay (135).

Another study of UCB CAR-T cells targeting B-cell ALL was conducted by Liu et al. The researchers compared the efficacy of autologous CAR-T cells and donor-derived, either PB or UCB CAR-T cells (136). Unsurprisingly, both donor-derived PB and UCB CAR-T cells showed increased proportions of cells displaying an immature phenotype (including naïve T cells and central memory T cells) as well as induced better responses *in vivo* than ALL patient-derived CAR-T cells. The survival analysis showed that the mice in the CB CAR-T and PB CAR-T groups survived longer than those in the PB (patient) CAR-T group with the median survival times of the CB CAR-T, PB CAR-T, and PB (patient) CAR-T groups 51 (39–51) days, 51 (46–51) days, and 32 (25–33) days ($p < 0.05$), respectively. Moreover, CAR-T and tumor cell proportions in PB revealed that on days 14 and 28, mice in the UCB CAR-T and PB CAR-T groups had higher CAR-T cell expansion and lower tumor burden compared to those in the ALL CAR-T group, making it a promising source for allogeneic CAR-T cells.

Addressing the choice of the most suitable costimulatory domain for UCB-derived CAR, the study Yu et al. elucidated the anti-tumor capacity of UCB-derived CAR with 4-1BB co-stimulatory domain (137). Obtained results showed the target-specific killing of CD19+ T cell lymphoma cells *in vitro* (over 50% dead cells in 1:1 ratio up to 85% dead cells in 10:1 ratio) as well as inhibited tumor progression *in vivo* (<1000 mm3 vs >1500 mm3 tumor volume in controls). UCB CD19-CAR- T cells were also associated with minimal GvHD as no diarrhea, rash, or jaundice, which are common symptoms of GvHD, were observed during the observation period. Furthermore, Tammana et al. indicated that incorporating the 4-1BB domain yielded better responses *in vivo* than CD28. The results showed that the construction of 3rd generation CAR-T cells with both (4-1BB and CD28) domains was associated with even more potent anti-tumor response compared to CAR-T cells with single 4-1BB construct in mice CD19(+) leukemia and lymphoma tumor models, suggesting a synergistic role costimulation in engineering antileukemia UCB effector cells. A systemic NOD/SCID mouse model with established Raji tumors showed that UCB T cells expressing both CAR constructs exhibited significantly improved tumor control and reduced bioluminescence intensity compared with 4-1BB CAR and GFP controls, with bioluminescence intensity in the 4-1BB and CD28 accounting for one-third of the value in the 4-1BB group on day 8 (132). A unique approach was adopted by Pegram et al. The researchers constructed a novel anti-CD19 CAR construct (armored CAR), programmed to secrete IL-12 to interfere with the immunosuppressive cytokine profile within the tumor (138). Obtained results suggested that IL-12 is an important factor for phenotypic changes, such as increased CD62L, CD28, GzmB, and IFNγ. In accordance with previous studies, the UCB-derived CAR-T cells showed promising anti-tumor efficacy both *in vitro* and *in vivo* as they enhanced anti-tumor efficacy compared to the CD19 CAR alone. Notably, the transfer of UCB-T cells secreting IL-12 resulted in increased survival of CD19+ tumor-bearing mice (>40 days of survival vs <40 days of survival compared to controls; $^{*}P < 0.05$) without a need for pretreatment (irradiation) or IL-2 support. Similar results were obtained while analyzing UCB-derived CAR-NK cells. The study by Herrera et al. highlighted the higher anticancer activity of UCB-derived CAR-NK cells in the treatment of primary chronic lymphocytic leukemia (CLL) compared to CAR-NK from adult cell sources. The study showed that UCB CAR-NK cells exhibited a more stable cell count per unit and demonstrated responsiveness to various interleukins to enhance their *in vitro* expansion, tumor cell killing activity, and promote prolonged cellular survival (139).

To date, four studies investigated the efficacy of UCB-derived CAR-T cells against cell lines other than B-cell lineage. Ma et al. investigated *in vitro* efficacy of uncommon HLA-A-targeting CAR T cells in AML (140). The group developed TCR-like monoclonal antibody (8F4) UCB-derived CAR-T cells that specifically recognized the PR1/HLA-A2 on the surface of AML cells and were capable of killing leukemia cell lines and primary AML blasts in an HLA-A2-dependent manner (>60% of killing and >40% of killing respectively in 4:1 effector: target ratio). A more advanced study was completed by Caël et al., who generated anti-CD123 UCB CAR-T cells, compared them with PB counterparts *in vitro*, and assessed the efficacy *in vivo* (126). Using blastic plasmacytoid dendritic cell neoplasm models they proved that UCB-derived CAR-T cell product retains the pool of less differentiated cells after nine days of expansion using IL-7 and IL-15. Notably, UCB CAR-T $CD4^{+}$ T cells exhibited a less differentiated phenotype compared to PB CAR-T cells, with a higher proportion of TSCM and TCM (68.1% vs. 31.8%, $p < 0.001$) and a lower proportion of TEM and TEMRA (31.8% vs. 68.2%, $p < 0.001$). While UCB and PB $CD8^{+}$ T cells showed no significant differences, UCB CAR-T cells trended toward a less differentiated profile, with 49.2% vs. 32.8% TSCM + TCM ($p = 0.055$) and 51.0% vs. 67.1% TEM + TEMRA ($p = 0.064$). Additionally, UCB-derived CAR-T cells presented comparable efficacy to PB product (94.3 ± 3.8% and 93.8 ± 3.9% cytotoxicity at E:T ratio 1:1 respectively) *in vitro* and contributed to significantly better overall survival (>120 days vs around 40 days in controls; P=0.004) in leukemia model*in vivo*. Moreover, they demonstrated that thawed or fresh UCB as a source for CAR-T manufacturing does not affect the product's functionality. In a study conducted by Pinz et al., 3rd generation UCB CAR-T cells were used to target peripheral T-cell lymphomas (PTCLs) *in vitro* (141). Although it was shown that UCB-derived CD4 CAR-T cells efficiently suppressed the growth of lymphoma cells *in vitro* (with the overnight elimination rate of 38, 62 and 85% at E:T ratios of 2:1, 5:1 and 10:1 respectively) while also significantly prolonging mouse survival (>30 days vs ~20 days in controls), unfortunately, the study provided little information concerning UCB efficacy. Finally, Olbrich et al. tested the effectiveness of UCB CAR-T cells against human cytomegalovirus (HCMV)-infected cells with promising

results showing high on-target effect (~30- 40% of dead target cells) and cytotoxicity *in vitro* (142). In the *in vivo* model, one week after administration, response to CAR-T cell therapy was observed in five out of eight mice, defined by significant reduction of the bioluminescent signal in relation to untreated controls. More importantly, none of all the treated mice showed adverse clinical symptoms such as loss of body weight, observable change in behavior, eczema or GvHD.

In summary, all the above-mentioned studies provided evidence of the anti-tumor efficacy of allogeneic CAR-T cells generated from UCB. However, although the feasibility of the manufacturing process as well as the anti-tumor cytotoxicity of CAR-T cells has been proved, few efficacy comparison between PB and UCB cells has been made. Table 2 summarizes the clinical trials results, presenting information on CAR constructs as well as manufacturing details.

6.4 Clinical reports of UCB applications in CAR-based therapies

Following the limited but encouraging preclinical studies, there is a clear demand for additional advancements in CAR-T cell therapies utilizing UCB. We have searched the ClinicalTrials.gov database and found several registered clinical trials evaluating UCB CAR T cells (January 2025). A group from Henan Cancer Hospital is investigating the safety and efficacy of UCB CAR-T cells redirected against relapsed/refractory B-cell leukemia or lymphoma (clinical trials.gov; ID NCT03881774) (143). This phase 1 study involves patients with disease relapse following autologous CAR-T therapy and those unsuitable for autologous therapy due to the low quality of lymphocytes. Although the estimated completion date was January 2022, no results have been published yet. The group from the University College of London aims to investigate the manufacturability of allogeneic cord-blood derived T cells in a laboratory setting, evaluate their safety and efficacy of treatment in individuals with high-risk, relapsed/refractory B cell malignancies (clinical trials.gov; ID NCT05391490). Several studies are also evaluating the safety and efficacy of CB-derived CAR-NK therapies for treating various malignancies (clinical trials.gov; NCT05922930, NCT06066424, NCT05008536, NCT05703854, NCT05020015, NCT06358430, NCT05092451, and NCT05110742). For instance, the Xinqiao Hospital of Chongqing group is examining UCB-derived CAR-engineered NK cells in the treatment of patients with relapsed and refractory MM. The CAR incorporated in the NK cells facilitates the identification and elimination of MM cells by specifically targeting BCMA, a protein present on the surface of malignant plasma cells (clinicaltrials.gov; ID NCT05008536). Following the promising results of the phase 1 trial, Takeda Pharmaceuticals is currently conducting a phase 2 study of the safety and efficacy of UCB-derived NK CAR-T cells in adult patients with relapsed or refractory B-cell Non-Hodgkin Lymphoma (clinical trials.gov; ID

TABLE 2 Summary of preclinical studies investigating UCB-derived CAR T cells.

Study	Disease model	Type of study	CAR construct	Cytokines used in manufacturing process	Reference
Serrano et al., 2006	B-cell lymphoma	*In vitro* *In vivo*	CD19scFv -CD3ζ	IL-2	(130)
Huang et al., 2008	B-cell leukemia, B-cell lymphoma	*In vitro* *In vivo*	CD19scFv-4-lBB-CD3ζ	IL-2, IL-7	(134)
Micklethwaite et al., 2010	B-cell ALL	*In vitro*	CD19scFv -CD28-CD3ζ	IL-7, IL-12, IL-15	(135)
Tammana et al., 2010	B-cell leukemia, B-cell lymphoma	*In vitro* *In vivo*	CD19scFv -CD28-CD3ζ; CD19scFv-4-lBB-CD3ζ; CD19scFv-CD28-4-lBB-CD3ζ	IL-2, IL-7	(132)
Pegram et al., 2015	B-cell ALL	*In vitro* *In vivo*	CD19scFv -CD28-CD3ζ; CD19scFv -CD28-CD3ζ/IL-12; CD19scFv-4-lBB- CD28-CD3ζ; CD19scFv-4-lBB- CD28-CD3ζ/ IL-12	IL-2, IL-7, IL-12, IL-15	(138)
Ma et al., 2016	AML	*In vitro*	PR1/HLA-A2scFv -CD28-CD3ζ (h8F4-CAR)	IL-2	(140)
Pinz et al., 2016	PTCL	*In vitro*	CD4scFv-CD28-4-lBB-CD3ζ	IL-2	(141)
Olbrich et al., 2020	HCMV-infected cells	*In vitro* *In vivo*	gBscFv -CD28-CD3ζ; gBscFv-4-lBB-CD3ζ;	IL-7, IL-15	(142)
Caël et al., 2022	BPDCN	*In vitro* *In vivo*	CD123scFv-CD28-4-lBB-CD3ζ	IL-7, IL-15	(126)
Liu et al., 2022	B-cell ALL	*In vitro* *In vivo*	CD19scFv -CD28-CD3ζ-T2	IL-2	(136)

ALL, acute lymphoblastic leukemia; AML, acute myeloblastic leukemia; BPDCN, blastic plasmacytoid dendritic cell neoplasm; CD, cluster of differentiation; gB, glycoprotein B; HCMV, human cytomegalovirus; IL, interleukin; PTCL, peripheral T-cell lymphoma; scFv, single-chain variable fragment; T2, Toll/interleukin-1 receptor domain of TLR2.

NCT05020015). With several clinical trials ongoing, only a limited number of studies have demonstrated the safety and efficacy of CAR-NK therapy. Qian et al. evaluated the therapeutic potential and safety profile of CB-derived CAR-NK cells targeting CD19 in patients with relapsed or refractory B-cell cell lymphoma. The results demonstrated that CAR-NK cell therapy was well-tolerated, with no major adverse events such as CRS, neurotoxicity, or GvHD in none of the 9 treated patients. Moreover, the median progression-free survival was 9 months, and the complete responses were achieved in 55% of the cases, with a 58.33% overall response rate at the end of the study (clinicaltrials.gov; ID NCT05472558). Second study conducted by MD Anderson Cancer Centre has also been completed (clinical trials.gov; ID NCT03056339). The investigators enrolled 11 patients with CD-19-positive malignancies and treated them with next-generation anti-CD19 CAR NK cells engineered to express IL-15 and inducible caspase 9 safety switch (85). Of the 11 treated patients, eight (73%) had a response; of these patients, seven (four with lymphoma and three with CLL) had a complete remission, and one had remission of the Richter's transformation component but had persistent CLL. Seven out of 11 patients achieved complete response, whereas none of the patients developed adverse events such as CRS or neurotoxicity despite HLA-mismatch (4/6 match in 9 patients). The study proved that UCB could be used as the allogeneic source for cellular therapies without requiring full HLA matching. Following the success of the Phase 1/2 results, the study progressed to an expansion phase (n = 26) and included 37 heavily pretreated patients with relapsed or refractory B cell malignancies demonstrating an ORR of 48.6% at both day 30 and day 100, with 1-year overall survival at 68% and progression-free survival at 32%. Rapid responses were observed at all dose levels: 100% of patients with low-grade NHL, 67% of patients with CLL without transformation and 41% of patients with diffuse large B cell lymphoma (DLBCL) achieved an OR. Most responses were complete responses (CRs), with 1-year cumulative CR rates of 83%, 50% and 29% for patients with NHL, CLL and DLBCL, respectively. Notably, CAR19/IL-15 cord blood units (CBU)-NK cells exhibited a comparable efficacy profile to autologous CAR19 T cells, while their safety profile was superior, showing no significant toxicities such as neurotoxicity, or GvHD and only one developed CRS (grade I). Although CAR-NK cells cannot be directly compared with CAR-T cells as NK cells possess distinguished immunogenic properties that decrease GvHD rates, CAR-NK cells present a promising, safe, and effective therapeutic option for patients with challenging B-cell malignancies (144, 145).

7 Cord blood bank as a source for CAR-T cell therapy

Stored in cord blood banks, UCB has established its role as an alternative source of hematopoietic stem cells for alloHSCT (146). Meanwhile, there is an increasing interest in using cord blood cells for new clinical and research applications. UCB has been regarded as an allogeneic and off-the-shelf source of NK cells as it contains relatively young and naïve NK cells, which may have greater potency and versatility in attacking target cells compared to those from adult donors (147) UCB is a rich source of progenitors and stem cells like mesenchymal stem cells (MSC), therefore UCB-derived MSCs have gained much interest for the use of potential therapeutic reasons (103, 148). Recently, it has been also proposed that a cord blood bank may function as a source for allogeneic CAR-T cell therapies (7, 126, 149).

The manufacturing of UCB CAR-T cells, as well as PB-derived CAR-T cells, consists of several key steps including T cell isolation, activation, gene modification and *ex vivo* CAR-T cell expansion (150). However, UCB-derived CAR-T cells face unique challenges due to the limited volume of UCB collections and their high nucleated red cell and mononuclear cell content which may complicate T cell isolation and processing (151). Additionally, because UCB is restricted by the small volume and low number of hematopoietic cells, *ex vivo* expansion is often required before adoptive cellular transfer (131). One remarkable advantage of UCB-derived CAR-T cells is their off-the-shelf availability, as UCB units are typically cryopreserved in UCB bank, easily accessible when needed. In contrast, autologous PB- derived T cells require patient-specific leukapheresis, leading to prolonged manufacturing and waiting time (110). What is more, allogeneic PB-derived CAR-T cells require additional genetic modifications such as elimination of the TCR receptor in order to prevent GvHD or immune rejection, a step that is not necessary in case of autologous CAR-T cells (152).

The quality of the starting material is crucial for successful CAR-T cell manufacturing, as demonstrated in autologous CAR-T therapies where baseline T cell characteristics, such as polyfunctionality, increased stemness, and reduced exhaustion, significantly impact clinical outcomes (153, 154). Similarly, for allogeneic CAR-NK cell production, donor-specific predictors of response and criteria for donor selection are vital. A study by Rezvani et al. investigated the safety and efficacy of CB-derived CAR19/IL-15 NK cells in a first-in-human phase 1/2 trial (155). The study reported day 30 overall response (OR) as the primary endpoint, with secondary objectives including day 100 response, progression-free survival, OS, and CAR19/IL-15 NK cell persistence. Among various UCB characteristics, multivariate analyses identified two key predictors of 1-year progression-free survival: a collection-to-cryopreservation time of ≤24 hours and a nucleated red blood cell (NRBC) content of $\leq 8 \times 10^7$ cells per CBU.

Cord blood banks offer three potential approaches for CAR-T cell manufacturing. Firstly, UCB could be used to generate genome-edited next-generation CAR-T cells, as gene editing in UCB-derived CAR-T cells offers significant advantages. Due to the naïve phenotype, UCB-derived T cells are highly sensitive to gene edits that enhance persistence and proliferation, such as upregulation of memory markers like CCR7 (156). Additionally, gene editing may improve their antitumor function through increasing of cytokine production (e.g., IL-7, IL-15) and elimination of exhaustion markers like PD-1, therefore prolonging their activity (157–159).

However, due to the smaller number of CD3-positive cells compared to PB and the elimination of alloreactivity-inducing molecules in genome editing, UCB seems to be a second choice rather than the first option. The second strategy is to use UCB-

banked cells as an autologous source for individuals who had their cells banked and need higher quality T cells for conventional CAR-T cell therapy. However, in this case, although T cells in the autologous cord blood unit could be expanded, overcoming technical limitations, the likelihood of an individual banking their cells (less than a few percent of the population), makes this proposal unlikely to materialize. Finally, another approach exploits the fact that in the setting of HSCT, UCB transplant requires a lower donor-recipient HLA match due to the naivety of T cells (160). In this situation, HLA-matching requires 4/6 allele complementarity (with at least one match at HLA-A, -B, and DRB1) (160). The fact that UCB units stored in the banks are HLA-typed supports the idea of generating "off-the-shelf" HLA-matched products (146). Accordingly, designing allogeneic CAR-T cells emerges as a possible application of this strategy. A population-wide bank of allogeneic CAR-T cells could be established provided that UCB-derived CAR-T cells demonstrated an acceptable safety profile in a clinical trial. Combining conventional CAR constructs with large-scale inventory would reduce costs significantly and enable product availability at request. However, such a proposal implies the calculation of a sustainable bank size. As UCB units are HLA-typed, we can estimate the required size of UCB CAR-T bank that would cover the proper population fraction at the 4/6 or higher HLA-match level. According to the analysis of the UK cord bank performed by Querol et al., a bank size of 50,000 units provides at least one donor for as much as 98% of patients (4/6 HLA match) (161). Nevertheless, decreasing the bank size has little impact on the probability of finding a 4/6 HLA-matched donor, with 10,000 unit banks still providing more than a 90% probability of donor finding (161). In the Finnish population, a bank size as small as approximately 200 units is associated with a 90% probability of finding a suitable 4/6 donor, whereas 1700 units are enough to provide 80% coverage in a 5/6 HLA setting (162). In the Korean population, a UCB donor pool required for a 95% probability of a 4/6 HLA match is estimated to be 2150 (163). For the 5/6 HLA match, the number is approximately 51,000 (163). It seems reasonable that the higher the homogeneity of the population, the lower the required size of the allogeneic CAR products. However, HLA allele and haplotype distribution within cord blood banks also play an important role, as uniformly distributed cord blood bank provides appropriate coverage while retaining a relatively small bank size (162).

Apart from biological arguments, more logistic and technical factors should be taken into account when considering cord blood banks for CAR-T production. The acquisition, processing, and depository of UCB are costly, thus relatively small percentage of the population decided on CB banking thus far, which may result in low utilization of UCB units from UCB banks (164). Additionally, the effect of long-term cryopreservation on UCB cell functionality remains unclear. Although transplantation results appear unchanged if UCB cells were cryopreserved for up to 10 years, it is yet to unravel whether longer preservation may impact UCB cell viability (165).

Moreover, both ethnic and economic challenges must be addressed to ensure fair access to UCB as a cell bank for CAR-T therapies. One of the main issues constitute lack of ethnic diversity as some of the groups are underrepresented. For instance, study by Akyurekli et al. showed that non-Caucasian ethnicity of the cord blood donor was associated with a higher risk of failing to meet banking criteria often due to lower collected volumes and reduced cell counts. This disparity can be attributed to a variety of factors, including socioeconomic barriers and lack of awareness of donation options (166). In addition, the high costs associated with collecting, processing, and storing UCB pose economic barriers, especially for lower-income families (167). Investing in cost-effective manufacturing techniques, subsidizing public UCB banking, and promoting nonprofit partnerships can help reduce the financial burden. Ethical considerations such as informed consent, donor rights, and equitable distribution of UCB-derived therapies also require clear rules to ensure transparency and trust. Addressing these issues through education, policy reform, and financial support programs will be essential to making UCB-derived CAR-T cell therapies accessible and beneficial to all patients, regardless of ethnic or economic background.

8 Discussion

In recent years, the field of CAR-T cell therapies has attracted the attention of researchers worldwide. It is now well-known that conventional autologous regimens have several disadvantages attributed to high costs, long time of the manufacture leading to the extended length of the "vein-to-vein" time, and unavailability for patients with low-quality T cells. Thus, numerous studies have investigated the possibilities of harnessing donor-derived CAR-T cells to address these hurdles. The allogeneic CAR-T cells gained much interest and are perceived as a promising solution to the shortcomings of the current therapies. Still, the vast majority of studies utilize the concept of genome editing that provides potent CAR-T products deprived of TCR or MHC molecules.

UCB provides multiple advantages over PB allogeneic or autologous sources for CAR-T cells. The use of UCB as a source of CAR-T cells offers accessibility as they could be transduced with conventional CAR constructs and be available at request from the CAR-T bank. The immunology of UCB T cells favors them as they are mostly composed of TSCM and TCM and therefore retained less differentiated phenotype than PB CAR-T cells (126). Additionally, UCB CAR-T cells express exhaustion markers like PD1, LAG3, or TIM3 on significantly lower levels compared to allogeneic PB-derived CAR, which translates into the capability of longer persistence *in vivo* and decreased potential risk of GVHD (125). UCB-derived CAR-T cells could be transferred both autologously to reduce post-transplant recurrence and in an allogeneic setting with fewer HLA restrictions that enable more accessible donor-recipient matching. Finally, UCB-derived CAR-T cells provide the cost-effective and sustainable strategy for utilizing cord blood banks. On the other hand, UCB poses challenges related to T cell numbers, maturity, and antigen recognition.

Yet, as the field of CAR-T cell therapy continues to advance, ongoing research is aimed at addressing these challenges and

optimizing the use of UCB as a source for CAR-T cells that will overcome the challenges associated with conventional autologous or allogeneic PB-derived therapies. Although current knowledge regarding the safety and efficacy of UCB CAR-T cells is limited to preclinical and few early clinical studies, reports from the ongoing research are optimistic, and further advancements are highly awaited.

Author contributions

KR: Writing – original draft, Writing – review & editing. JM: Writing – review & editing. TO: Writing – review & editing. NR: Supervision, Writing – review & editing. GB: Writing – review & editing. TK: Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

JM and TO are employees of FamiCordTx S.A. company developing CAR & TCR based gene therapies, including cord blood-derived cells; NR is an employee and a scientific advisory board of FamiCordTx S.A.; TK is a Chief Scientific Officer and Board Member of FamiCordTx S.A.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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γδT cells, a key subset of T cell for cancer immunotherapy

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γδT cells represent a unique and versatile subset of T cells characterized by the expression of T-cell receptors (TCRs) composed of γ and δ chains. Unlike conventional αβT cells, γδT cells do not require major histocompatibility complex (MHC)-dependent antigen presentation for activation, enabling them to recognize and respond to a wide array of antigens, including phosphoantigens, stress-induced ligands, and tumor-associated antigens. While γδT cells are relatively rare in peripheral blood, they are enriched in peripheral tissues such as the skin, intestine, and lung. These cells play a crucial role in tumor immunotherapy by exerting direct cytotoxicity through the production of inflammatory cytokines (e.g., interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), and interleukin-17 (IL-17)) and cytotoxic molecules (e.g., perforin and granzyme). Recent advances in γδT cell research have elucidated their mechanisms of tumor recognition, including the detection of phosphoantigens and stress-induced ligands like MICA (MHC class I polypeptide-related sequence A), MICB (MHC class I polypeptide-related sequence B), and ULBP (UL16-binding protein). Furthermore, various strategies to enhance γδT cell-based tumor immunotherapy have been developed, such as *in vitro* expansion using phosphoantigen-based therapies, cytokine stimulation, and chimeric antigen receptor (CAR)-γδT cell engineering. These advancements have shown promising results in both preclinical and clinical settings, paving the way for γδT cells to become a powerful tool in cancer immunotherapy. This review highlights the key mechanisms, functions, and strategies to harness the potential of γδT cells for effective tumor immunotherapy.

KEYWORDS

γδT cells, tumor immunotherapy, phosphoantigens, chimeric antigen receptor (CAR), cytokine production, stress-induced ligands, adoptive cell therapy, cancer treatment

1 Introduction

A unique subset of T cells, γδT cells, are characterized by expressing a T-cell receptor (TCR) composed of γ and δ chains, instead of the α and β chains of conventional αβT cells (1). For the activation of γδT cells, they do not need major histocompatibility complex (MHC)-dependent antigen presentation (2). This unique characterization allows γδT cells to recognize and respond to a wide range of antigens without antigen presentation cells (APC). However, γδT cells are relatively rare in the circulation of peripheral blood. The majority of γδT cells are resident in peripheral tissues, including the skin, intestine, and lung (3). As a promising immune cell subset in tumor immunotherapy, γδT cells possess innate-like properties, including the recognition of a variety of tumor-associated antigens without the help of APC (4). Further, γδT cells are cytotoxic by producing inflammatory cytokine attacking the tumor cells (5). The target of γδT cells includes a broad range of solid tumors and hematological malignancies by exploiting stress signals on tumor cells, tumor-associated antigens, and altered metabolic products such as phosphoantigens (pAgs) (6). Therefore, we updated the recent research on the γδT cells, focusing on its tumor recognition to adoptive transfer potential for tumor immunotherapy.

2 Mechanisms of tumor recognition by γδT cells and cytokine production

γδT cells could recognize non-peptide antigens such as pAgs, especially recognized by a subset of γδT cells named Vγ9Vδ2 T cells (7). It could also recognize stress-induced ligands, such as MICA (MHC class I polypeptide-related sequence A), MICB (MHC class I polypeptide-related sequence B), ULBP (UL16-binding protein), and heat shock proteins (HSPs), without the need for MHC.

2.1 Phosphoantigens recognition by Vγ9Vδ2 T cells

In human peripheral blood, the most abundant subset of γδT cells are Vγ9Vδ2 T cells. This subset of cells is activated by pAgs, including isopentenyl pyrophosphate (IPP) or dimethylallyl pyrophosphate (DMAPP) (8–10). These pAgs have been documented to be generated during microbial infections or tumorigenesis. Through the recognition of these pAgs by the TCR (T-cell receptor) of Vγ9Vδ2, γδT cells are activated thereby producing cytokines and immune attack on tumor cells (11).

Activated Vγ9Vδ2 γδT cells can produce and secrete pro-inflammatory cytokines, including interferon-γ (IFN-γ) (12), tumor necrosis factor-α (TNF-α) (13), interleukin-17 (IL-17) (14), and interleukin-22 (IL-22) (15); immune-regulatory cytokines, such as interleukin-10 (IL-10) (16, 17); cytotoxic molecules, including granzyme and perforin (18); and other cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-4 (IL-4), and interleukin-5 (IL-5) (16, 19).

Among these, IFN-γ is a major cytokine that plays a crucial role in modulating the immune response by activating macrophages, enhancing antigen presentation, and promoting the differentiation of Th1 cells for antitumor function (12). TNF-α is another important pro-inflammatory cytokine produced by γδT cells (13). It could induce tumor cell apoptosis via immune activation. TNF-α can also contribute to the elimination of infected or cancerous cells. IL-17 is produced by a subset of γδT cells, particularly Vγ9Vδ2 cells (14). IL-17 promotes inflammation and can recruit neutrophils and other immune cells to sites of infection or tumor growth. However, its role in the context of cancer can be more complex, as it can have both tumor-promoting and tumor-suppressing effects depending on the tumor microenvironment (TME). IL-22 is another cytokine that can be produced by activated γδT cells, particularly in mucosal immunity (15). It plays a key role in tissue repair and protection from infection, but it can also have a dual role in promoting tumor growth in certain contexts. GM-CSF is also produced by γδT cells and can promote the differentiation and activation of macrophages and dendritic cells, thereby enhancing the overall immune response and antigen presentation (19). Under certain conditions, γδT cells can produce IL-4 and IL-5, which are typically associated with the regulation of humoral immunity and eosinophil activation (16). IL-10 is an anti-inflammatory cytokine that can be produced by γδT cells during the regulation of immune responses or inflammation (20). However, its production is generally lower compared to other cytokines, such as IFN-γ or TNF-α. Granzyme and perforin are key cytotoxic molecules produced by activated γδT cells that contribute to the direct killing of infected or cancerous cells. The release of these molecules is an essential mechanism of γδT cell-mediated cytotoxicity (18) (Figure 1).

Meanwhile, tumor cells can overexpress mevalonate pathway metabolites, including mevalonate, isopentenyl pyrophosphate (IPP) (21), dimethylallyl pyrophosphate (DMAPP) (22), geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), geranylgeranyl pyrophosphate (GGPP), cholesterol, coenzyme Q, dolichol, prenylated proteins, steroid hormones (23, 24). These mevalonate pathway metabolites play a crucial role in the function of γδT cells (25). IPP is a potent stimulatory antigen for γδT cells, particularly the Vγ9Vδ2 subset (26). However, the presentation of small pAg, including IPP and DMAPP, is dependent on butyrophilin (BTN) family members, specifically BTN3A1 and BTN2A1 (27). BTN3A1, also known as CD277, is an intracellular sensor that binds to pAgs, while BTN2A1 facilitates their recognition by γδTCR (28, 29). Together, BTN3A1 and BTN2A1 form a molecular complex that enables γδT cell activation upon pAg accumulation.

In normal cells, IPP concentrations are low, but in cancer cells, the mevalonate pathway is often upregulated, leading to IPP accumulation (21). BTN3A1-mediated sensing of IPP leads to a conformational change that allows BTN2A1 to interact with the Vγ9Vδ2 TCR, triggering γδT cell proliferation and activation (30). This activation results in tumor cell recognition and cytotoxic response γδT cells a promising candidate for cancer immunotherapy (31). Further, IPP secreted from zoledronic acid

(ZOL)-stimulated myeloma cells can activate the chemotaxis of γδT cells (32). ZOL, a nitrogen-containing bisphosphonate, inhibits farnesyl pyrophosphate synthase in the mevalonate pathway, resulting in IPP accumulation, which enhanced γδT cells recruitment and activation (33). Besides, the activation of γδT cells by IPP can lead to the secretion of pro-inflammatory cytokines such as TNF-α, which can further increase the immune responses (34). Additionally, the mevalonate pathway metabolites can influence the differentiation and effector functions of γδT cells, contributing to their role in immune surveillance and anti-tumor immunity (35). Therefore, the application potential of nitrogen-containing bisphosphonates as ZOL to expand γδT cells *ex vivo* has been successful in various cancer types (36, 37). These expanded cells can then be used in adoptive cell transfer therapy.

2.2 Stress-induced ligands and heat shock proteins

MICA, MICB, and ULBP are members of the NKG2D (Natural Killer Group 2, Member D) ligand family, which play a crucial role in the immune response, particularly in the activation of natural killer (NK) cells and γδT cells (38, 39). MICA, MICB, and ULBP are typically expressed at low levels in healthy cells (40). However, their expression is significantly upregulated under conditions of cellular stress, such as infection, DNA damage, or transformation in cancer cells (41, 42). This upregulation recruits the NK cells and γδT cells to these abnormal cells under stress. During tumor immunosurveillance, the expression of MICA, MICB, and ULBP on tumor cells can be recognized by NKG2D, a NK-like activating receptor (NKR) expressed on γδT cells, particularly in Vγ9Vδ2 subset (43). Subsequently, NKG2D binds to these ligands to form NKG2D–NKG2D ligand (NKG2D–NKG2DL) axis, leading to the activation of γδT cells, triggering cytotoxicity and cytokine production (IFN-γ, TNF-α, etc) (44).

HSPs are a family of highly conserved and immunogenic proteins that are expressed in response to various stress conditions. In γδT cells, HSP60 helps maintain the integrity and function of cellular proteins, especially under stress conditions. HSP60 can also act as an immunomodulatory molecule. It can be recognized by γδT cells and other immune cells, leading to the activation of immune responses (45). Also, in γδT cells, HSP70 can enhance cell survival and function during stress. HSP70 can bind to antigens and present them to immune cells, including γδT cells, thereby activating the immune response (46, 47). HSP72 by LPS-stimulated neutrophils facilitates γδT cell-mediated killing (48). HSP90 is overexpressed in cancer cells, contributing to survival and growth. Therefore, HSP90 plays a role in the activation of γδT cells by targeting this molecule (49). Overall, these heat shock proteins can be recognized via the γδT cells and NKG2D, further contributing to cytotoxicity and immune response against cancer cells.

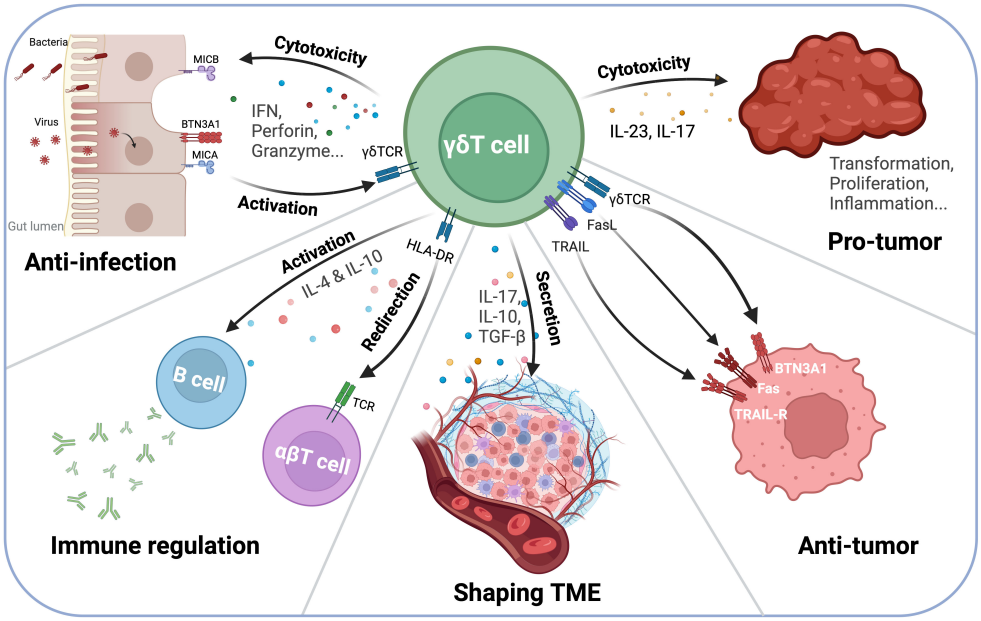

FIGURE 1
γδT Cell interactions with the immune system and Tumor Microenvironment (TME). γδT cells play a dual role in tumor immunity, exhibiting both anti-tumor and pro-tumor functions depending on the microenvironment. On one hand, γδT cells secrete IFN-γ, perforin, and granzyme, mediating direct tumor cell lysis. Additionally, they interact with antigen-presenting cells (APCs) and B cells via HLA-DR, influencing B cell class switching and antibody production. On the other hand, γδT cells can contribute to a pro-tumor environment through IL-17 and IL-23 secretion, which promotes inflammation and tumor progression. Their ability to shape the TME highlights their potential as both therapeutic targets and immunotherapy agents.

3 γδT cells in tumor immunotherapy

Upon activation, γδT cells exhibit three major functions for tumor immunotherapy, which are cytotoxicity, cytokine production, tissue-resident and memory responses.

3.1 Cytotoxicity

3.1.1 Mechanisms

Activated γδT cells eliminate tumor cells through multiple mechanisms (Figure 2). They target and eliminate tumor cells independently of MHC restriction, making them particularly advantageous for immunotherapy applications.

3.1.1.1 Granule exocytosis pathway

One of the primary mechanisms by which γδT cells eliminate tumor cells is through granule exocytosis, a process that relies on the release of cytotoxic molecules. Upon activation, γδT cells degranulate and release perforin, which forms pores in tumor cell membrane, allowing granzyme B to enter the target cell (50). Once inside, granzyme B activates caspase-dependent apoptotic pathways, leading to tumor cell death. This mechanism is rapid and highly effective, making it a crucial cytotoxic pathway utilized by γδT cells in anti-tumor immunity.

3.1.1.2 Death receptor-mediated apoptosis

γδT cells also induce apoptosis through death receptor pathways, including Fas-FasL, TRAIL (TNF-related apoptosis-inducing ligand), and TNF-α-mediated signaling. Fas ligand (FasL) expressed on activated γδT cells can bind to Fas (CD95) on tumor cells, triggering caspase-dependent apoptosis of the target cells (51). Additionally, γδT cells also produce TNF-α, which binds to TNF receptors on tumor cells, further promoting apoptosis via extrinsic signaling cascades (52). Moreover, γδT cells can engage in TRAIL signaling, where TRAIL binds to TRAIL receptors (DR4/DR5) on tumor cells, initiating apoptosis. Preclinical studies in lung and breast cancer models have shown that TRAIL-dependent cytotoxicity effectively eliminates tumor cells (53).

3.1.1.3 NK-like activating receptors-mediated cytotoxicity

In addition to their TCR-mediated responses, γδT cells express NKRs, which enhance their ability to recognize and eliminate tumor cells (54). One of the most well-characterized NKRs expressed on

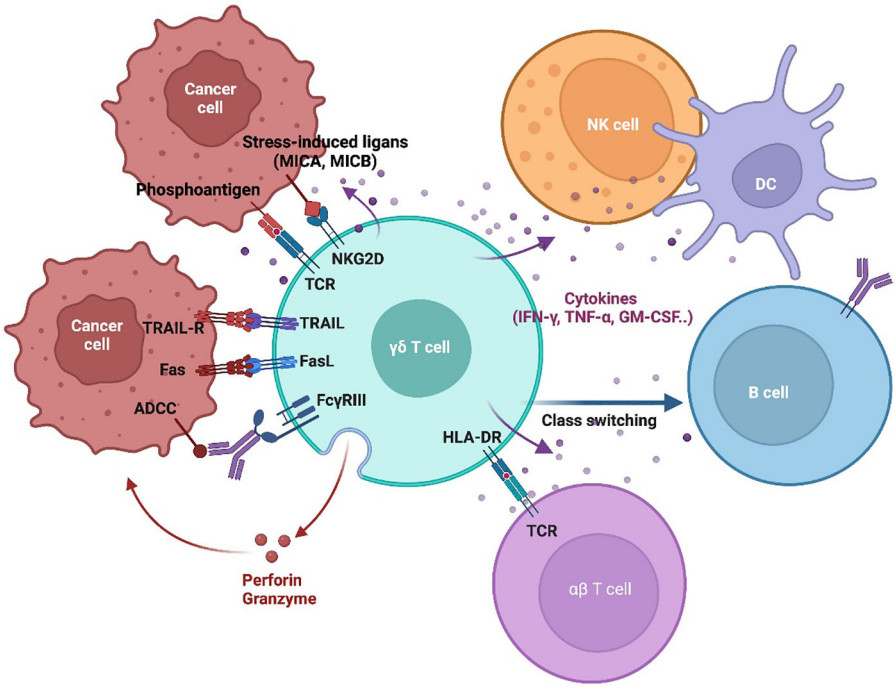

FIGURE 2
γδT Cell tumor recognition and cytotoxic mechanisms. This schematic illustrates the key mechanisms by which γδT cells recognize and eliminate tumor cells. γδT cell activation is initiated through TCR engagement with phosphoantigens and stress-induced ligands (MICA/MICB) on tumor cells. Additionally, γδT cells express NK-like activating receptors (NKRs), such as NKG2D, which enhance recognition and activation. γδT cell-mediated cytotoxicity through multiple mechanisms, including the TRAIL/TRAIL-R and Fas/FasL pathways, which induce the tumor cell apoptosis. They also release perforin and granzyme, leading to direct cytolysis. Engagement of NKG2D with tumor-expressed ligands further enhances tumor cell destruction. Moreover, γδT cell participate in antibody-dependent cellular cytotoxicity (ADCC) via FcγRIII, enabling them to target opsonized tumor cells. Beyond direct cytotoxicity, γδT cells interact with other immune cells, including NK cells, dendritic cells (DCs), B cells, and αβ T cells, playing a role in immune modulation and influencing the broader anti-tumor response.

γδT cells is NKG2D, which binds to MICA, MICB, and ULBPs, stress-induced ligands commonly upregulated in tumor cells (55). This interaction enhances γδT cell cytotoxicity by promoting activation and degranulation. Additionally, γδT cells express DNAM-1 (CD226), which binds to CD155 (PVR) and CD112 (Nectin-2), further strengthening tumor recognition and immune activation (56).

γδT cells also express natural cytotoxicity receptors (NCRs), including NKp30, NKp44, and NKp46, which interact with ligands such as B7-H6, PCNA, and Heparan sulfate (57, 58). These receptors, typically associated with NK cells, contribute to the ability of γδT cells to recognize and eliminate tumor cells that evade classical T cell surveillance (59). The presence of NKRs on γδT cells highlights their hybrid functional profile, bridging innate and adaptive immune responses for effective tumor control (60).

3.1.1.4 Antibody-dependent cellular cytotoxicity

Beyond direct killing, γδT cells mediate ADCC, an essential mechanism for monoclonal antibody (mAb)-based cancer therapies (61). γδT cells express Fc receptors (FcγRIII/CD16), allowing them to recognize and kill tumor cells opsonized by therapeutic antibodies (62). Clinical evidence suggests that γδT cells enhance the efficacy of mAb therapies, such as rituximab (anti-CD20) in B-cell malignancies and trastuzumab (anti-HER2) in breast cancer (63, 64). A Phase I/II trial evaluating the combination of ex vivo-expanded γδT cells with trastuzumab demonstrated enhanced tumor regression in $HER2^+$ breast cancer patients, indicating the clinical potential of ADCC-mediated γδT cell therapy (65).

3.1.2 Preclinical and clinical findings

γδT cells have been tested across various preclinical and clinical models, demonstrating potent tumor cytotoxicity in both solid and hematologic malignancies. Preclinical studies in ovarian and lung cancer have shown that adoptively transferred γδT cells efficiently infiltrate tumors, secrete IFN-γ, and kill tumor cells, leading to significant tumor growth inhibition (66). Clinically, several trials have demonstrated the feasibility of γδT cell-based therapies. A Phase I clinical trial (NCT03183232) demonstrated the safety and feasibility of autologous γδ T cell therapy in patients with advanced hepatocellular carcinoma (HCC), showing prolonged disease stabilization. Another clinic trial in glioblastoma showed that γδT cells, when combined with low-dose chemotherapy, led to tumor shrinkage and prolonged survival in patients with recurrent disease (67). In multiple myeloma, γδT cells expanded with ZOL and IL-2 have been investigated, demonstrating safe administration and tumor regression in patients resistant to conventional therapies (6).

3.1.2.1 Cytokine production

The cytokines generated and released by γδT cells have been addressed in detail in the previous section. IFN-γ, TNF-α, and IL-17 produced by γδT cells could enhance the immune response, promote tumor inflammation, and recruit other immune cells (68). IFN-γ can have direct anti-tumor effects by upregulating MHC class I molecules on tumor cells, enhancing their recognition by other immune cells like cytotoxic T cells ($CD8^+$ T cells) and NK cells.

In glioblastoma, a highly aggressive brain tumor, γδT cells have been shown to secrete IFN-γ, which sensitizes tumor cells to immune checkpoint blockade therapy. Additionally, in colorectal cancer, γδ T cell-derived IL-17 has been implicated in shaping the tumor microenvironment by recruiting neutrophils and enhancing immune responses.

3.1.2.2 Tissue-resident and memory responses

As the majority of γδT cells are resident in tissues, they serve as tissue-resident lymphocytes, providing long-term surveillance and response to tumor recurrence. The migration and tissue colonization of γδT cells in specific tissues, such as the small intestine, are regulated by chemotactic signals, adhesion molecules, and signaling pathways, including CCR9/CCL25 pathway (69). Meanwhile, different subsets of γδT cells express distinct chemokine receptors that determine their homing properties. The Vδ2 subset expresses CCR5 and CXCR3 (70, 71), which are associated with Th1 cell functions, while the Vδ1 subset expresses CXCR1 and CCR2 (70).

For the adaptive immunity, γδT cells also exhibit memory-like properties, similar to conventional αβT cells. They can undergo clonal expansion and differentiation upon antigen encounter, leading to the formation of memory cells. Therefore, CAR-γδT cells have been designed to enhance the cytotoxicity of γδT cells against lymphoid malignancies (72, 73). For example, a preclinical study demonstrated that CAR-γδT cells engineered to target CD19 effectively eliminated B-cell acute lymphoblastic leukemia (B-ALL). Another recent study reported that γδ T cell-based immunotherapy enhanced responses to standard chemotherapy in patients with ovarian cancer, highlighting their potential in solid tumor treatment.

4 Challenges and strategies for enhancing γδT cell-based tumor immunotherapy

Despite the promising therapeutic potential of γδT cells in cancer immunotherapy, several challenges remain in their clinical application. These challenges include optimizing ex vivo expansion protocols, enhancing persistence after infusion, addressing functional heterogeneity, and overcoming immunosuppressive tumor microenvironments (TME). Addressing these limitations is crucial for improving the efficacy and durability of γδT cell-based therapies.

4.1 Challenges and limitations in γδT cell-based therapies

4.1.1 Expansion protocols and manufacturing challenges

A major challenge in γδT cell-based immunotherapy is the variability and inefficiency of ex vivo expansion protocols, which are

critical for generating sufficient cell numbers for clinical applications (74). The expansion of γδT cells, particularly the Vγ9Vδ2 subset, relies on pAg-based activation using compounds such as zoledronate or synthetic pAgs (e.g., IPP, DMAPP) (75). While effective, this approach suffers from inconsistencies across different culture conditions and donor-dependent variability, leading to difficulties in standardization and scalability. Furthermore, prolonged ex vivo expansion can lead to functional exhaustion, reducing the cytotoxic potential of γδT cells before infusion (76). In contrast, Vδ1 γδT cells, which have shown greater efficacy against solid tumors, are more challenging to expand using conventional methods (77). Current protocols for expanding Vδ1 cells are inefficient and often yield a heterogeneous population, making it difficult to achieve reproducible therapeutic effects. Moreover, the transition from research-grade expansion to Good Manufacturing Practice (GMP)-compliant protocols poses an additional hurdle, requiring refined methods that ensure both clinical efficacy and regulatory approval.

4.1.2 Limited *in vivo* persistence after infusion

Unlike αβT cells, γδT cells exhibit limited proliferation and persistence *in vivo* following adoptive transfer, which significantly restricts their long-term anti-tumor effects (4). One of the primary reasons for this limitation is insufficient cytokine support in the host environment, which fails to sustain γδT cell survival and function. In particular, γδT cells rely on cytokines such as IL-2, IL-15, and IL-21 for survival, but their availability *in vivo* is often inadequate for robust expansion post-infusion (78). Additionally, poor metabolic fitness and an inability to efficiently utilize energy sources in the tumor microenvironment further hinder γδT cell longevity. Another major concern is the emergence of exhaustion markers such as PD-1, TIM-3, and LAG-3 on γδT cells after repeated stimulation, leading to a progressive decline in their cytotoxic activity (79). This exhaustion phenotype is exacerbated in solid tumors, where γδT cells encounter persistent antigenic stimulation and an immunosuppressive milieu that dampens their efficacy (79). Furthermore, suboptimal engraftment in the tumor microenvironment, due to poor homing signals and competition with other immune cells, limits the ability of γδT cells to accumulate and exert sustained anti-tumor effects (66). Overcoming these barriers is crucial for improving the therapeutic durability of γδT cell-based therapies.

4.1.3 Functional heterogeneity of γδT cells

γδT cells represent a highly heterogeneous immune population, with different subsets exhibiting distinct functional properties and tumor-targeting capabilities. The Vγ9Vδ2 subset, which is predominantly found in peripheral blood, has shown robust cytotoxic activity, particularly in hematologic malignancies, where these cells efficiently target tumor cells. In contrast, Vδ1 γδT cells, which are enriched in epithelial tissues, play a more prominent role in targeting solid tumors due to their tissue-resident nature and ability to interact with the TME. However, this variability complicates the application of γδT cell-based therapies, as the most effective subset for tumor elimination depends not only on the tumor type but also on the specific TME characteristics, such as cytokine milieu and immune cell composition (77).

In hematologic cancers, where γδT cells have better accessibility and can easily encounter tumor cells circulating in the bloodstream or bone marrow, Vγ9Vδ2 cells are particularly effective (80). However, in solid tumors, the limited infiltration of γδT cells into the dense tumor stroma, compounded by the immunosuppressive TME, restricts their functionality. Furthermore, while many γδT cells exert potent anti-tumor effects through the production of pro-inflammatory cytokines like IFN-γ and TNF-α, certain subsets, including IL-10-producing γδT cells, have been identified as immunosuppressive, particularly in solid tumors. These IL-10-producing γδT cells contribute to immune suppression and tumor immune evasion, raising concerns that γδT cell therapies, if not properly controlled, could inadvertently promote tumor progression (81). This highlights the critical need to develop strategies that selectively expand the most cytotoxic γδT cell subsets (such as those producing IFN-γ) and minimize the expansion of regulatory subsets that may hinder therapeutic efficacy, especially in solid tumor settings. Moreover, the differentiation and plasticity of γδT cells are influenced by factors such as cytokine exposure and metabolic signals, which further contribute to their heterogeneity across different tumor types.

4.1.4 Immunosuppression of the TME

The immunosuppressive nature of the TME represents a significant barrier to the effectiveness of γδT cell-based immunotherapies, with solid tumors posing a particularly challenging environment for γδT cell infiltration and function. Tumors evade γδT cell-mediated cytotoxicity through several mechanisms, including the downregulation of NKG2D ligands (e.g., MICA, MICB, ULBPs), which are crucial for γδT cells to efficiently recognize and attack tumor cells (82). In solid tumors, the lack of these activating ligands prevents γδT cells from initiating robust anti-tumor responses, allowing tumors to escape immune surveillance (83). Additionally, solid tumors often secrete immunosuppressive factors, such as TGF-β, IL-10, and adenosine, which inhibit γδT cell activation, proliferation, and function (84). TGF-β, in particular, has been shown to drive the conversion of cytotoxic γδT cells into regulatory subsets, further compromising the effectiveness of γδT cell therapy, particularly in solid tumor contexts (85).

While hematologic tumors are more readily accessible to γδT cells in circulation, where they do not face the same physical barriers as solid tumors, they still present challenges (86). Tumor cells in the bloodstream can evade recognition by γδT cells through mechanisms such as immune checkpoint molecule expression (e.g., PD-L1) or cytokine-driven suppression (87). In solid tumors, however, the physical barriers, including the dense extracellular matrix and abnormal vasculature, impede immune cell trafficking, and hypoxic conditions can further reduce γδT cell efficacy (88). Once γδT cells infiltrate the tumor, chronic exposure to inhibitory signals such as PD-1/PD-L1 interactions induces T cell exhaustion, which limits their functional capacity and persistence. The challenge in solid tumors, therefore, lies not only in the tumor's

ability to block γδT cell activity but also in the difficulties γδT cells face in infiltrating and surviving in these environments. Strategies to overcome these suppressive mechanisms are essential for improving the infiltration, activation, and persistence of γδT cells in the TME (53). These strategies include combining γδT cell therapy with immune checkpoint inhibitors (e.g., anti-PD-1, anti-CTLA-4), engineering γδT cells to resist TME-induced exhaustion, and utilizing approaches such as local tumor irradiation or metabolic reprogramming to enhance γδT cell recognition and infiltration in solid tumor settings.

4.2 Optimization of γδT cell expansion and activation

Efficient expansion and activation of γδT cells are critical for their clinical application, yet several challenges still remain, which necessitate refined strategies to improve γδT cell proliferation, maintain functionality, and standardize large-scale production.

4.2.1 pAgs based activation

ZOL and synthetic pAgs-based therapies are one of the most prominent ones for *in vitro* expansion and activation of γδT cells (89). Synthetic or modified pAgs, such as IPP or DMAPP analogs can be used to activate Vγ9Vδ2 T cells in patients (22). Bromohydrin pyrophosphate (BrHPP), ZOL, and 2-methyl-3-butenyl-1-pyrophosphate (2M3B1PP) are used to increase the concentration of pAgs in tumor cells, directly activating and expanding γδT cells, and thereby promoting their cytotoxicity (89–91). High concentrations of ZOL (100 μM) stimulation at a short period could induce Vδ2T cell expansion. Besides, zoledronic acid and other bisphosphonates can upregulate pAgs levels in the mevalonate pathway, indirectly activating Vγ9Vδ2 T cells (92).

4.2.2 Cytokine-based expansion

The expansion of γδT cells can be greatly increased by specific cytokines, i.e., IL-15, IL-12, IL-18, and IL-23. IL-15 is critical for the activation, survival, and expansion of γδT cells. IL-15 has been investigated in clinical trials as an adjuvant therapy to enhance γδT cell function and boost their numbers *in vivo* (11). IL-12 (8, 12, 33) and IL-18 (11) could promote the differentiation and activation of γδT cells into effector cells that produce IFN-γ and TNF-α, enhancing their anti-tumor activity. IL-23 could promote IL-17 production by γδT cells, which can contribute to tumor rejection and immune modulation (14, 15). Meanwhile, the addition of vitamin C and its more stable derivative, L-ascorbic acid 2-phosphate (pVC), could also significantly increase the proliferation and cytotoxic activity of Vδ2T cells (93).

4.2.3 Artificial APCs

Along with the cytokines and pAgs, the application of artificial APCs to express costimulatory molecules and antigens can also improve the expansion and activation of γδT cells. NKG2D-NKG2DL axis plays a crucial role in γδT cell activation and expansion (94). Therefore, KG2D agonists could enhance NKG2D signaling and increase the activation and cytotoxicity of NKG2D$^+$ γδT cells. CD20-specific immune-ligands engaging NKG2D could improve the cytotoxicity of γδT cells in lymphoma (95). This strategy can be used to target stress-induced ligands like MICA and MICB on tumor cells (40, 96). Monoclonal antibodies against MICA and MICB can enhance the activation of NK cells and γδT cells, leading to the cytotoxicity of the tumor cells (97–99). To date, CLN619, the anti-MICA/B antibody, has shown promise in preclinical models and early clinical trials (100). ULBP is another family of NKG2DLs that can be targeted. ULBP1, ULBP2, and ULBP3 are expressed on various tumor cells and can be recognized by NKG2D. Targeting ULBP with specific antibodies as 23ME-01473 is undergoing a clinical trial, too. γδT cells cultured with artificial antigen-presenting cells and IL-2 show long-term proliferation (101).

4.3 Improving γδT cell persistence and *in vivo* expansion

One of the key limitations of γδT cell-based therapies is their limited *in vivo* persistence following infusion. Improving γδT cell persistence is critical for maximizing the therapeutic potential of γδT cell-based treatments.

4.3.1 Cytokine support

The administration of cytokines such as IL-2, IL-15, and IL-21 has shown promising results in enhancing γδT cell persistence in clinical trials. For example, IL-15 has been extensively studied due to its ability to promote memory formation and long-term survival of γδT cells (102). A clinical trial demonstrated that IL-15 administration significantly improved the survival of infused γδT cells in patients with cancer and boosted their anti-tumor activity (103). IL-2 and IL-21 have also been used in combination with γδT cells, leading to enhanced proliferation and effector functions, which are essential for sustained immune responses (104). The clinical success of these cytokines in boosting γδT cell persistence highlights their importance in ensuring long-term anti-tumor effects in the clinical setting.

4.3.2 Genetic modifications

Genetic modifications to γδT cells, particularly the expression of IL-15 or other survival cytokines, have shown great promise in improving the persistence and effectiveness of these cells post-infusion. For instance, recent study demonstrated that IL-15-engineered γδT cells exhibited self-sustained proliferation *in vivo*, thereby reducing the need for exogenous cytokines (105). These modified cells showed increased cytotoxicity and survival rates in preclinical models of tumor-bearing mice. Additionally, CRISPR/Cas9-mediated genetic modifications of γδT cells to express anti-tumor cytokines have been explored in early-phase clinical trials, with some studies showing enhanced expansion and persistence of the modified cells after infusion into patients. These advances in genetic engineering ensure that γδT cells have a sustained presence in the body, enhancing their ability to target and eliminate tumor cells.

4.3.3 Biomaterial-based delivery

The use of biomaterial-based delivery systems, such as scaffolds or hydrogels, to encapsulate γδT cells has emerged as a promising strategy to improve their persistence and survival. Research has demonstrated that encapsulating γδT cells within biomaterials can sustain the release of cytokines, protect the cells from degradation, and promote local cell proliferation in the tumor site (106). For example, hydrogel-based delivery systems were used to enhance the persistence of immune cells, including γδT cells, in solid tumors. These materials provided a favorable microenvironment for γδT cells, leading to prolonged survival and enhanced anti-tumor responses in murine models (66, 107). Clinical research is ongoing, exploring biomaterial-based approaches for improving γδT cell function and longevity in tumor treatment, particularly for solid tumors with challenging microenvironments.

4.4 Overcoming functional heterogeneity of γδT cells

Not all γδT cells exhibit tumoricidal properties, some subsets, particularly those producing IL-10, can have immunoregulatory functions that suppress anti-tumor immunity, thus limiting their therapeutic potential (108). This heterogeneity complicates the clinical application of γδT cells and necessitates strategies to selectively expand the most cytotoxic subsets while minimizing the presence of regulatory subsets that may hinder therapeutic efficacy.

4.4.1 Single-cell transcriptomics and proteomics

A promising approach to overcoming functional heterogeneity is the use of single-cell transcriptomics and proteomics. These technologies allow for the identification of tumor-reactive γδT cell subsets by profiling the gene expression and protein markers of individual cells (109). By using these methods, researchers can identify γδT cell subsets with optimal tumor-targeting properties, enhancing the precision and effectiveness of therapies. A study utilized single-cell RNA sequencing to identify tumor-specific γδT cell subsets in melanoma patients. They found that certain subsets of Vγ9Vδ2 γδT cells were highly activated in the presence of tumor antigens and produced pro-inflammatory cytokines, leading to tumor regression (75). This approach is being incorporated into ongoing clinical trials aiming to selectively expand the most effective γδT cell subsets for adoptive cell therapies, providing a more personalized and targeted approach to immunotherapy.

4.4.2 Selective expansion of cytotoxic subsets

Selective expansion of cytotoxic subsets is a key strategy to enhance the therapeutic efficacy of γδT cells. By promoting the expansion of pro-inflammatory γδT cells that produce cytokines such as IFN-γ and TNF-α, while suppressing the expansion of regulatory subsets, this strategy focuses on maximizing cytotoxic potential while minimizing immune suppression (81). Recent research has shown that the expansion of γδT cells with cytokines such as IL-15 and IL-12 leads to the preferential growth of cytotoxic γδT cells, which exhibit superior anti-tumor effects. For example, clinical trials involving IL-12-stimulated γδT cells have demonstrated enhanced tumor killing and improved survival outcomes in patients with hematologic malignancies (110). Moreover, targeting regulatory γδT cells that produce IL-10 through specific cytokine manipulation has been shown to prevent immune suppression. The combination of IL-12 and IL-15 during γδT cell expansion selectively promoted cytotoxicity while reducing the presence of IL-10-producing regulatory subsets, significantly improving the efficacy of γδT cell therapy in solid tumor models (6).

4.5 Enhancing tumor infiltration and overcoming the immunosuppressive TME

A significant barrier to the efficacy of γδT cell-based immunotherapy is the immunosuppressive nature of the TME. To overcome this challenge, multiple strategies have been explored, with preclinical and clinical evidence supporting their potential to enhance γδT cell infiltration and function in the TME.

4.5.1 Immune checkpoint blockade

One of the most promising strategies to counteract tumor-induced immunosuppression is the combination of γδT cell therapy with immune checkpoint inhibitors (ICIs) targeting PD-1, PD-L1, or CTLA-4. Checkpoint molecules such as PD-1 are upregulated in exhausted γδT cells within tumors, leading to reduced cytotoxic activity. Preclinical models have demonstrated that blocking PD-1 or PD-L1 signaling restores γδT cell function, leading to enhanced tumor clearance. Recent clinical trials have provided encouraging evidence for immune checkpoint blockade in combination with γδT cell therapy. In one study, anti-PD-1 therapy significantly enhanced the anti-tumor activity of γδT cells in non-small cell lung cancer (NSCLC) patients, leading to prolonged survival (111). Additionally, PD-L1 inhibitors have been shown to enhance the persistence and function of γδT cells in melanoma and hepatocellular carcinoma (HCC) models (112). These findings suggest that checkpoint blockade therapy can effectively reinvigorate γδT cells, improving their function within the immunosuppressive TME.

4.5.2 Local tumor irradiation

Low-dose radiation therapy has been shown to modulate the tumor microenvironment and improve γδT cell-mediated immunity. One key mechanism involves the upregulation of NKG2D ligands, which enhance the recognition of tumor cells by γδT cells. Clinical studies have demonstrated the potential benefits of combining radiotherapy with γδT cell therapy. In patients with head and neck squamous cell carcinoma low-dose radiation can enhance NKG2D ligand expression, leading to improved γδT cell-mediated tumor clearance (83). Additionally, It has been shown that radiation therapy increases the susceptibility of tumors to γδT cell killing by inducing DNA damage and stress responses, making

them more vulnerable to immune attack (113). This combination strategy is currently being tested in clinical trials for glioblastoma and lung cancer, with promising early results suggesting enhanced γδT cell infiltration and improved patient outcomes.

4.6 Advances in γδT cell-based therapies

Recent advancements in γδT cell-based immunotherapy have focused on optimizing adoptive cell therapy (ACT), developing chimeric antigen receptor (CAR)-γδT cells, and exploring combination strategies to enhance therapeutic efficacy. These innovations aim to overcome current limitations, such as limited persistence, tumor infiltration barriers, and immune suppression, while leveraging γδT cells' unique ability to recognize stress ligands and tumor-associated antigens (Table 1).

4.6.1 Adoptive cell therapy

4.6.1.1 Autologous *vs.* allogeneic γδT cell transfer

Traditional adoptive γδT cell therapy relies on autologous γδT cells, which are expanded from a patient's own blood before reinfusion. However, autologous approaches are time-consuming and can yield inconsistent therapeutic responses due to variations in the patient's immune status. To increase availability and streamline production, researchers are exploring allogeneic γδT cell therapy, using donor-derived γδT cells or off-the-shelf γδT cell products. Recent clinical trials have showed that allogeneic Vδ2 γδT cells expanded from healthy donors exhibited high cytotoxicity against hematologic cancers and had low risk of graft-versus-host disease (GVHD) due to the unique MHC-independent recognition mechanism of γδT cells (114). Additionally, induced pluripotent stem cells (iPSCs)-derived γδT cells are being developed as an off-the-shelf product, showing promising results in preclinical models of solid tumors and leukemia (77) (Figure 3).

4.6.1.2 Introducing bispecific antibody

Bispecific antibodies represent an emerging approach to enhance γδT cell-mediated tumor targeting by bridging γδT cells with tumor cells. These antibodies are designed to bind both γδT cells and tumor antigens, directing γδT cells to tumor sites with greater specificity and cytotoxic efficiency. Bispecific Her2-Vγ9 antibody could trigger the killing of Her2-expressing tumor cells by Vγ9Vδ2 T cell lines (115, 116). Another study links the extracellular domains of tumor-reactive γ9δ2TCRs to a CD3-binding moiety, creating γδTCR anti-CD3 bispecific molecules (GABs) and it could redirect αβT cells against a broad range of tumors (117).

4.6.1.3 Genetic engineering of γδTCRs: TCR-engineered γδT cells

Another approach to enhancing γδT cell specificity is TCR engineering, in which high-affinity γδTCRs are introduced into αβT

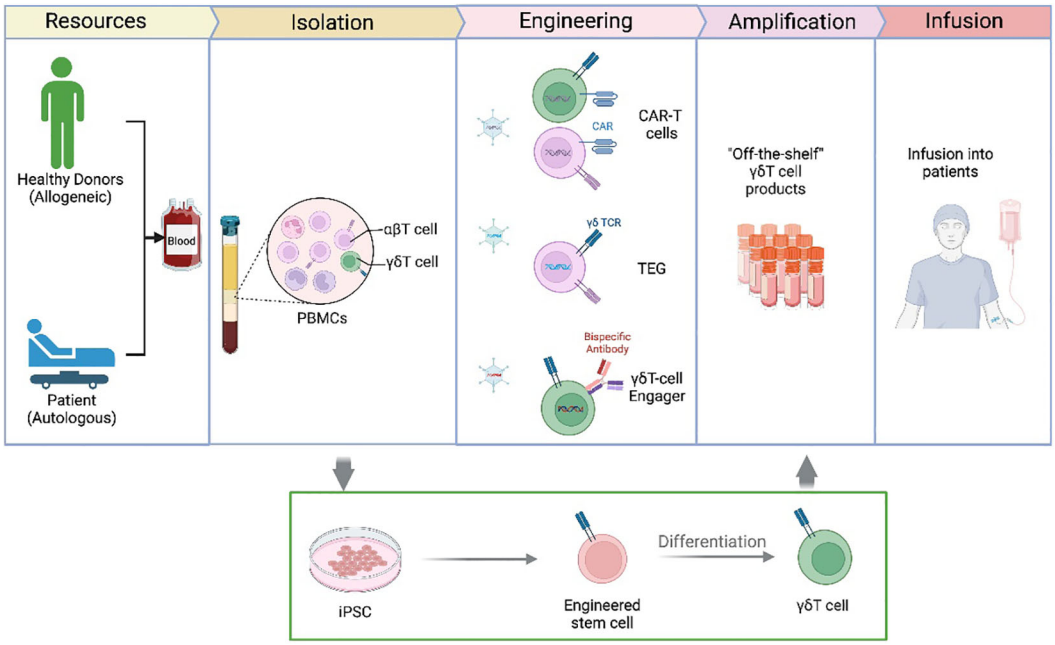

FIGURE 3
γδT Cell-based therapies and engineering approaches. This figure presents an overview of current γδ T cell-based immunotherapies, highlighting different strategies for their clinical application. γδ T cells can be isolated from peripheral blood mononuclear cells (PBMCs) from healthy donors (allogeneic) or patients (autologous). Following isolation, γδT cells can be engineered using: CAR-γδT cells – expressing chimeric antigen receptors to enhance tumor recognition; TCR-engineered γδT cells (TEG therapy) – modifying γδ TCRs for improved antigen specificity; or Bispecific antibody-based γδ T cell engagers – linking γδT cells to tumor cells via targeted antibodies. After ex vivo expansion and amplification, γδT cells are infused into patients as an "off-the-shelf" cellular therapy. Additionally, induced pluripotent stem cells (iPSCs) can be genetically engineered to generate γδT cells, offering an alternative approach for scalable production.

TABLE 1 Strategies for improving γδT cells in tumor immunotherapy.

Category	Methods	Key Points & Representative Strategies
In Vitro Expansion and Activation		
Phosphoantigen (pAg)-based Therapy	- Use of phosphoantigens (IPP, DMAPP) or their derivatives to activate γδT cells	- Zoledronic acid (ZOL), BrHPP, and 2M3B1PP promote phosphoantigen accumulation, activating Vγ9Vδ2 T cells and enhancing cytotoxicity
Cytokine Stimulation	- Use of specific cytokines to promote γδT cell expansion	- IL-15 enhances survival and expansion of γδT cells
		- IL-12 and IL-18 increase IFN-γ and TNF-α secretion
		- Vitamin C boosts proliferation and cytotoxicity
Artificial APCs (aAPCs)	- Use of artificial antigen-presenting cells expressing co-stimulatory molecules and antigens	- NKG2D signaling enhances γδT cell activation and cytotoxicity
		- Anti-MICA/MICB antibodies (e.g., CLN619) are in preclinical and clinical trials
Adoptive Cell Therapy (ACT)		
γδT Cell Adoptive Transfer	- Isolation and ex vivo expansion of γδT cells for reinfusion	- γδT cells expanded using IPP or ZOL can be reinfused into patients, showing promise in early-phase clinical trials, especially for hematological cancers
CAR-γδT Cells	- Genetic modification of γδT cells to express chimeric antigen receptors (CARs)	- CAR-γδT cells combine γδTCR and CAR specificity to target tumor antigens, providing dual functionality
High-Affinity TCRs	- Transfection of αβ T cells with γδTCRs	- High-affinity Vγ9Vδ2 TCRs enhance tumor recognition and cytotoxicity
In Vivo Activation and Targeting		
Agonistic mAbs	- Monoclonal antibodies (e.g., anti-BTN3A1/CD277) to activate γδT cells	- ICT01 (anti-BTN3A1 mAb) is in phase I/IIa clinical trials for activating Vδ2 T cells
Bispecific Antibodies	- Bispecific antibodies linking γδT cells with tumor cells	- HER2-Vγ9 bispecific antibodies trigger HER2-expressing tumor cell killing
		- γδTCR-CD3 bispecific molecules (GABs) redirect αβT cells to attack tumors
Other Strategies		
Metabolic & Epigenetic Modulation	- Modulate γδT cells through metabolic and epigenetic pathways	- Histone deacetylase inhibitors (e.g., valproic acid) and DNA demethylating agents (e.g., decitabine) enhance γδT cell cytotoxicity
Enhancing Tumor Infiltration	- Improve γδT cell infiltration into tumor tissues	- Low-dose gamma irradiation enhances γδT cell recruitment
		- Hyaluronan synthesis inhibitors promote γδT cell penetration into tumors
Targeting Tumor Microenvironment	- Reverse immunosuppression in the tumor microenvironment	- Checkpoint inhibitors (anti-PD-1, anti-PD-L1, anti-CTLA-4) enhance γδT cell function by overcoming immune suppression
Combination Therapies	- Combine γδT cell therapy with other treatments	- Valproic acid synergizes with ZOL to enhance cytotoxicity
		- PARP inhibitors increase NKG2DL expression, improving tumor cell killing by γδT cells
Combination Therapies	- Combine γδT cell therapy with other treatments	- Valproic acid synergizes with ZOL to enhance cytotoxicity
		- PARP inhibitors increase NKG2DL expression, improving tumor cell killing by γδT cells

cells to generate TEG cells. These engineered cells combine the tumor-targeting versatility of γδTCRs with the *in vivo* persistence and expansion capacity of αβT cells, resulting in a potent anti-tumor response (Figure 3). A study in acute myeloid leukemia (AML) reported that TEG cells targeting Wilms' tumor antigen (WT1) successfully controlled leukemia progression in patients without severe toxicity (118).

4.6.2 Combination therapies

4.6.2.1 γδT cell therapy + checkpoint blockade

γδT cell express immune checkpoint inhibitors, including PD-1, particularly within the TME, where chronic antigen exposure can drive T cell exhaustion. However, unlike conventional αβT cells, γδT cells exhibit a distinct pattern of PD-1 expression and regulation, making their response to immune checkpoint inhibitors (ICIs) unique.

Recent studies have shown that PD-1$^+$ γδT cells can display both exhausted and highly functional phenotypes, depending on co-stimulatory signals within the TME. While high PD-1 expression is often associated with reduced cytotoxicity, some tumor-reactive γδT cells retain effector function despite expressing PD-1. Checkpoint blockade using PD-1/PD-L1 inhibitors can reinvigorate γδT cell activity, restoring cytokine production and enhancing tumor killing (119).

A recent review highlighted that γδT cells engage with the PD-1/PD-L1 axis in a context-dependent manner, where PD-1 blockade can rescue γδT cell function in some tumors, while in others, additional co-stimulatory signals, like IL-15, NKG2D activation, may be required (119). Preclinical studies have demonstrated that blocking PD-1 in γδT cells enhances their cytotoxicity against ovarian cancer (120). Checkpoint blockade could also increase the expression of activating ligands on tumor cells, such as NKG2D ligands, making tumors more susceptible to γδT cell-mediated cytotoxicity (121–123).

4.6.2.2 γδT cells + chemotherapy or radiation therapy

Combining γδT cell therapy with chemotherapy or radiation therapy is another strategy to enhance its effectiveness. Chemotherapy can cause tumor cell stress, leading to the upregulation of stress-induced ligands like MICA and MICB, which are recognized by γδT cells (124). This synergy can increase γδT cell activation and tumor cytotoxicity, potentially overcoming chemotherapy resistance mechanisms. Similarly, radiation therapy can increase tumor antigen release and the expression of immune-stimulatory molecules, making tumor cells more vulnerable to immune-mediated killing.

4.6.2.3 γδT cells + metabolic modulation

The tumor microenvironment imposes metabolic constraints on γδT cells, including adenosine accumulation and TGF-β-mediated immune suppression, which inhibit γδT cell function (125). Targeting these pathways through metabolic modulation can enhance γδT cell survival and cytotoxicity.

Targeting adenosine A2A receptors (A2AR inhibitors) significantly boosted γδT cell cytotoxicity, leading to improved tumor regression in breast cancer models (126). Similarly, blocking TGF-β signaling was shown to prevent γδT cell exhaustion and enhance proliferation in pancreatic cancer models (107). These findings suggest that metabolic reprogramming could be a valuable adjunct to γδT cell immunotherapy, particularly for tumors with strong immunosuppressive microenvironments.

4.6.3 Chimeric antigen receptor -γδT cells

Genetic modification of γδT cells to express CARs targeting tumor-specific antigens is an emerging strategy to enhance their tumor-targeting specificity and cytotoxic potential (73). While CAR-T cell therapy is for its application to αβT cells, CAR-γδ T can be designed to target specific tumor antigens, providing dual specificity through both the endogenous γδ TCR and the CAR (72). A recent preclinical study demonstrated that CAR-γδT cells targeting EGFRvIII in glioblastoma exhibited superior tumor eradication compared to conventional CAR-αβT cells (127). This highlights the potential advantages of CAR-γδT cells in solid tumors, where antigen escape is a common resistance mechanism. Clinical trials are now evaluating CD19- and BCMA-targeting CAR-γδT cells for B-cell malignancies, with early results showing promising tumor regression and minimal off-target toxicity.

5 Conclusion and future perspective

γδT cells represent a promising immune cell subset with unique tumor-targeting properties, particularly their ability to recognize cancer cells independently of MHC restriction. This characteristic allows them to overcome tumor heterogeneity and immune evasion, making them attractive candidates for immunotherapy. However, several challenges remain in fully translating γδT cell therapy into broad clinical application. One primary challenge is the variability of therapeutic effects across different tumor types, with some patients showing limited or poor responses to therapy (67). Additionally, the heterogeneity of γδT cells itself presents a complex challenge, as different subsets may exhibit distinct functional profiles, complicating their clinical use (128). Further research into the subtypes of γδT cells and their distinct roles in cancer immunity is essential to enhance efficacy across different cancer types.

A critical area of future research involves enhancing the persistence of γδT cells within the TME. Achieving sustained activity of γδT cells in TME is crucial for long-term therapeutic success, particularly in overcoming tumor recurrence and immune evasion (129). Strategies to optimize the trafficking, homing, and infiltration of γδT cells into solid tumors will be essential for improving clinical outcomes (130). Moreover, maintaining target antigen expression on tumor cells is key to preventing immune escape and ensuring durable responses.

Another critical area for advancement is minimizing the treatment -associated adverse effects, such as cytokine release syndrome (CRS) and other immune-related toxicities, which can arise from the activation and expansion of γδT cells (131). Understanding the mechanisms underlying these adverse reactions and developing strategies to mitigate them will be vital for improving the safety profile of γδT cell-based therapies.

The feasibility of non-viral gene transfer techniques for the generation of universal CAR γδT cells represents an exciting frontier. Non-viral methods could potentially overcome some of the limitations associated with viral vectors, such as immunogenicity and safety concerns, while enabling the development of off-the-shelf CAR γδT cell therapies. Advances in genome-editing technologies, such as CRISPR/Cas9, may play a pivotal role in this regard, facilitating the generation of more efficient and safer γδT cell therapies (132).

Despite these challenges, γδT cells offer a unique advantage in cancer treatment, particularly due to their ability to recognize and kill tumor cells without MHC restriction (108). This characteristic minimizes the risk of immune escape and addresses the issue of tumor heterogeneity. Meanwhile, ongoing clinical trials are

assessing the safety and efficacy of γδT cell adoptive transfer in cancer patients, with early-phase studies demonstrating promising results, especially in combination with other immunotherapies.

In conclusion, the future of γδT cell-based cancer therapies holds great promise, with ongoing research aimed at optimizing their persistence, minimizing adverse effects, and exploring non-viral gene transfer techniques. By overcoming these hurdles, γδT cells could emerge as a transformative therapeutic approach for a wide range of cancers, offering new hope to patients who currently have limited treatment options.

Author contributions

JL: Writing – original draft, Funding acquisition. ZL: Writing – original draft. XR: Writing – original draft. SS: Investigation, Supervision, Writing – review & editing. YZ: Investigation, Validation, Writing – review & editing. YW: Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The author(s) declare that no Generative AI was used in the creation of this manuscript.

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Advancing personalized, predictive, and preventive medicine in bladder cancer: a multi-omics and machine learning approach for novel prognostic modeling, immune profiling, and therapeutic target discovery

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Objective: This study aimed to identify and analyze immunogenic cell death (ICD)-related multi-omics features in bladder cancer (BLCA) using single-cell RNA sequencing (scRNA-seq) and bulk RNA-seq data. By integrating these datasets, we sought to construct a prognostic signature (ICDRS) and explore its clinical and biological implications, including its association with immune cell infiltration, tumor microenvironment (TME), and drug sensitivity.

Methods: Publicly available datasets from TCGA and GEO, including scRNA-seq (GSE222315, 9 samples) and bulk RNA-seq (TCGA-BLCA, 403 samples; GSE13507, 160 samples), were analyzed. Single-cell data were processed using Seurat, and ICD scores were calculated using single-sample gene set enrichment analysis (ssGSEA). Weighted gene co-expression network analysis (WGCNA) identified ICD-related modules, and machine learning algorithms (Lasso, Ridge, CoxBoost) were employed to construct the ICDRS. Survival analysis, immune infiltration, pathway enrichment, and drug sensitivity were evaluated to validate the model.

Results: The ICDRS, based on eight key genes (IL32, AHNAK, ANXA5, FN1, GSN, CNN3, FXYD3, CTSS), effectively stratified BLCA patients into high- and low-risk groups with significant differences in overall survival (OS, $P < 0.001$). High ICDRS scores were associated with immune-suppressive TME, including increased infiltration of T cells CD4 memory resting ($P = 0.02$) and macrophages M0/M1/M2 ($P = 0.01$). Pathway enrichment revealed correlations with cholesterol homeostasis, epithelial-mesenchymal transition (EMT), and KRAS signaling. Drug sensitivity analysis showed high-risk groups were resistant to Cisplatin ($P = 0.003$), Mitomycin C ($P = 0.01$), and Paclitaxel ($P = 0.004$), with IC50 values significantly higher than low-risk groups.

Conclusion: The ICDRS serves as a robust prognostic biomarker for BLCA, offering insights into tumor immune evasion mechanisms and potential therapeutic targets. Its integration with clinical features enhances personalized treatment strategies, highlighting the importance of ICD in BLCA immunotherapy and precision medicine. The model's predictive accuracy and biological relevance were validated across multiple datasets, underscoring its potential for clinical application.

KEYWORDS

bladder cancer, immunogenic cell death, machine learning, multi-omics integration, immunotherapy, prognosis signature

1 Introduction

Over the past few decades, the advent of personalized, predictive, and preventive medicine (PPPM/3PM) has significantly transformed the landscape of cancer treatment and research (1). Among urological malignancies, bladder cancer (BLCA) stands as the second most prevalent, with an estimated 550,000 new cases and approximately 200,000 deaths reported annually worldwide (2). The management of BLCA continues to be a critical focus in urological oncology. Platinum-based chemotherapy remains the cornerstone of perioperative and advanced BLCA treatment. More recently, the introduction of immune checkpoint inhibitors (ICIs) has expanded therapeutic options, particularly for first-line and platinum-resistant cases (3). Despite these advancements, the proportion of patients experiencing long-term remission through immunotherapy remains limited. The overall response rate of ICIs in BLCA is no more than 24% (4). To address this, future research must prioritize the exploration of novel immunotherapeutic strategies, the optimization of therapeutic sequencing and combinations, the precise selection of therapies tailored to individual patients, and the identification of new molecular targets.

Within the realm of BLCA immunotherapy, the investigation of immunogenic cell death (ICD) within the tumor microenvironment (TME) has emerged as a pivotal research avenue. ICD is characterized by the release of damage-associated molecular patterns (DAMPs), which activate the immune system to combat tumors. This process involves the recruitment of antigen-presenting cells (APCs) to damaged or infected cells, leading to the presentation of captured antigens via major histocompatibility complex proteins to primary T cells. Consequently, ICD enhances the antigenicity of tumor cells and promotes robust anti-tumor immune responses (5–7). As a novel biomarker, ICD holds considerable promise. ICD has been extensively studied in various cancer types, demonstrating its central role in antitumor immunity. Conventional chemotherapeutics like anthracyclines, oxaliplatin, and taxanes have been shown to induce ICD in multiple malignancies, enhancing immunogenicity and correlating with improved patient outcomes (8). However, its clinical applications, such as prognostic stratification and the prediction of responses to immunotherapy and chemotherapy, remain underexplored (9). A comprehensive understanding of ICD at the molecular level, coupled with advanced analytical techniques, is essential for its accurate identification and effective utilization.

Recognizing the potential of ICD in BLCA treatment, the systematic identification of its associated multi-omics features is imperative for the development of innovative therapeutic strategies. Furthermore, the application of diverse machine learning frameworks to analyze these complex datasets not only enhances the robustness of the research but also uncovers the intricate interactions and integration mechanisms among different data types. This approach is instrumental in identifying key biomarkers and molecular pathways associated with BLCA (10).

In light of these considerations, this study aims to employ a range of machine learning computational frameworks to systematically identify and analyze ICD-related multi-omics features in BLCA. By integrating genomics, transcriptomics, and proteomics data, we seek to elucidate key molecules and pathways that influence BLCA immune responses. Additionally, we aim to provide new targets for precision therapy and construct risk models using follow-up data. This research not only offers a novel perspective on understanding the immunoregulatory mechanisms of BLCA and advancing its precision treatment but also underscores the significant potential of machine learning in the analysis of complex diseases within the 3PM framework.

2 Materials and methods

2.1 Data source

In this study, we utilized publicly available genomic data from two primary sources: The Cancer Genome Atlas (TCGA) and the Gene Expression Omnibus (GEO) database. From the TCGA database (https://portal.gdc.cancer.gov/), we obtained gene expression profiles and corresponding survival data for 403 BLCA samples,

providing a robust dataset for analysis (11). Additionally, we accessed two BLCA-specific datasets from the GEO database (https://www.ncbi.nlm.nih.gov/geo/): GSE222315, which includes single-cell RNA sequencing (scRNA-seq) data from 9 bladder cancer samples, and GSE13507, which contains tissue-based RNA sequencing data and survival information from 160 bladder cancer samples (12).

For the TCGA dataset, we extracted gene expression data in Transcripts Per Million (TPM) format from STAR count data. The data were normalized using a log2(TPM+1) transformation to stabilize variance and approximate a normal distribution. To ensure the reliability of downstream analyses, we retained only those samples with both RNA sequencing data and complete clinical information. This approach allowed us to integrate diverse data types while maintaining methodological consistency and analytical rigor.

2.2 Single-cell RNA sequencing analysis

2.2.1 Data preprocessing

The scRNA-seq data were processed using the Seurat package (version 5.1.0) in R (version 4.3.1) (13). Quality control was performed to remove low-quality cells, retaining those with between 200 and 6,000 detected genes and mitochondrial gene content below 5%. Red blood cell contamination was assessed using hemoglobin genes (e.g., *HBA1*, *HBB*), and samples with more than 1% hemoglobin gene expression were excluded. The data were normalized using the LogNormalize method with a scale factor of 10,000, and highly variable genes were identified using the FindVariableFeatures function, retaining the top 3,000 genes for downstream analysis.

To address batch effects, the Harmony algorithm was applied, ensuring robust integration across samples. Harmony was specifically chosen for our batch correction strategy because of its demonstrated effectiveness in single-cell data integration. The algorithm works by iteratively clustering cells and adjusting their positions in the reduced dimensional space to align shared cell populations across datasets, while preserving biologically meaningful variations. Unlike other integration methods that may overcorrect and remove important biological signals, Harmony maintains cell type-specific transcriptional signatures while minimizing technical variation. This balanced approach was critical for our analysis, as we needed to integrate multiple scRNA-seq datasets while preserving the subtle transcriptional differences that characterize distinct immune cell states and their interactions within the tumor microenvironment. Additionally, Harmony's computational efficiency allowed us to process our large-scale integrated dataset without compromising resolution or accuracy, making it particularly well-suited for our multi-source data integration approach. Principal component analysis (PCA) was conducted, and the top 30 principal components were selected for dimensionality reduction and clustering. t-distributed Stochastic Neighbor Embedding (tSNE) (14) was employed with parameters perplexity=30 and max_iter=1000 to visualize the data, and clusters were identified using the FindNeighbors and FindClusters functions with a resolution of 0.8. Cell types were annotated based on well-established marker genes, including *CD3D/CD3E* for T cells, *CD19/MS4A1* for B cells, *COL1A1/COL1A2* for fibroblasts, *PECAM1* for endothelial cells, *CD163/CD68/CD14* for macrophages, and *APC/GSTP1/CDKN2A/DAPK1* for cancer cells. Marker genes for each cluster were identified using FindAllMarkers with parameters logfc.threshold=0.35 and min.pct=0.35.

2.2.2 Immunogenic cell death analysis

To investigate the role of ICD in the tumor microenvironment, a curated list of ICD-related genes was used to compute an ICD score for each cell using single-sample gene set enrichment analysis (ssGSEA) (15). Cells were stratified into high and low ICD score groups using the median score as the cutoff. Differential expression analysis between these groups was performed using the FindAllMarkers function, with a log2 fold change threshold of 0.35 and a minimum detection rate of 35%.

Pathway enrichment analysis was conducted using the clusterProfiler package, and significant pathways were identified using gene set enrichment analysis (GSEA) on the Hallmark gene sets. The results were visualized using dot plots and enrichment plots to highlight pathways associated with high ICD scores. UMAP plots were generated to visualize the distribution of ICD scores across cell types, and violin plots were used to compare ICD scores between annotated cell types. Statistical significance was defined as $p < 0.05$.

2.2.3 Cell-cell communication analysis

The CellChat package was employed to infer cell-cell communication networks based on ligand-receptor interactions (16). The human CellChatDB database was used to identify overexpressed ligand-receptor pairs, and communication probabilities were computed using the computeCommunProb function. Significant interactions were filtered (min.cells = 10), and pathway-level communication networks were aggregated using the computeCommunProbPathway function.

Key signaling pathways, such as MIF, ITGB2, MK, and APP, were visualized using circle plots and heatmaps to illustrate their strength and specificity across cell types. Chord diagrams were generated to depict interactions between high-risk tumor cells and other cell types, providing insights into the role of cell-cell communication in the tumor microenvironment.

To further explore the impact of risk-associated genes on cell-cell communication, cells were classified into high- and low-risk groups based on a predefined set of genes (e.g., *IL32*, *AHNAK*, *ANXA5*) using ssGSEA. Differential expression analysis between these groups was performed, and enriched pathways were identified using GSEA. The results were visualized using dot plots and enrichment plots, highlighting pathways associated with high-risk cells. Statistical significance was defined as $p < 0.05$.

2.3 Weighted gene co-expression network analysis

To identify co-expression modules and explore their associations with ICD in BLCA, we performed WGCNA using the R package WGCNA (17). Gene expression data from TCGA-BLCA samples were preprocessed, and ICD-related genes were selected based on a curated gene list. The ssGSEA method was employed to calculate ICD scores for each sample, which were used as traits in the WGCNA analysis.

The expression matrix was filtered to retain genes with high variability, and a soft-thresholding power was determined to construct a scale-free network. Modules of co-expressed genes were identified using hierarchical clustering and dynamic tree cutting. Module-trait relationships were assessed by correlating module eigengenes with ICD scores. The yellow module, which showed the strongest association with ICD, was further analyzed. Gene significance and module membership were calculated to identify key genes within the yellow module.

Functional enrichment analysis of the yellow module genes was performed using the clusterProfiler package to uncover biological processes and pathways associated with ICD. Visualization included dendrograms, heatmaps, and scatterplots to illustrate module-trait relationships and gene significance. Statistical significance was defined as $p < 0.05$.

2.4 Integrating machine learning methods to construct prognostic features

We conducted an intersection analysis between ICD-DEGs and the module genes identified through WGCNA to obtain a set of genes related to immunogenic cell death (ICDRgenes). To construct a robust prognostic model with high predictive accuracy, we randomly divided the TCGA-BLCA dataset into a training set (302 samples) and an internal testing set (101 samples) in a 3:1 ratio, ensuring an even distribution of clinical characteristics between the two groups. Furthermore, the GSE13507 dataset (160 samples) served as an external testing set to ensure the robustness of the model.

During the model construction phase, our study incorporated various machine learning algorithms, including least absolute shrinkage and selection operator (Lasso) (18), stepwise multiple Cox (StepCox) (19), Ridge (20), CoxBoost (21), Survival Support Vector Machine (Survival-SVM) (22), Elastic Net (Enet) (23, 24), Partial Least Squares Regression for Cox Models and Related Techniques (plsRcox) (25, 26), Supervised Principal Components (SuperPC) (27), Random Survival Forests (RSF) (28), and Gradient Boosting Machine (GBM) (29). We arranged 100 combinations of these 10 algorithms across the TCGA-BLCA and GSE13507 datasets, employing a ten-fold cross-validation framework for variable selection and model building.

Ultimately, we selected the algorithm combination that demonstrated the best robust performance and potential for clinical translation based on its performance across the three datasets. This led to the establishment of a final feature set called the immunogenic cell death-related signature (ICDRS), which is used to predict overall survival (OS) in BLCA patients.

2.5 Survival analysis and construction of nomograms

Based on the median risk score of the ICDRS, we divided the samples in the TCGA training set, internal testing set, and external testing set into high-risk and low-risk groups. Kaplan-Meier(KM) survival curves were analyzed using the R package survminer (30), and differences in OS between the high-risk and low-risk groups were compared using the log-rank test. Additionally, the timeROC package (31) was used to perform ROC curve analysis to assess the sensitivity and specificity of ICDRS in predicting OS in BLCA patients, with the area under the curve (AUC) reflecting the robustness of the model (32, 33). Further, we stratified the ICDRS scores by clinical characteristics and analyzed the correlation of ICDRS with age, gender, tumor stage, T, M, N classification, and other clinical features.

To enhance the predictive accuracy and prognostic capability of our model, we developed a nomogram (34) that combines ICDRS with clinical features to quantify the expected survival of BLCA patients. Finally, we comprehensively evaluated the precision and accuracy of the nomogram through ROC curves, the concordance index (C-index), and calibration curves. Additionally, we used decision curve analysis (DCA) (35) to assess the clinical net benefit of the nomogram, ensuring its practicality and effectiveness in clinical decision support. These comprehensive assessments helped us validate the clinical application value of the nomogram, ensuring its contribution to the survival prediction of BLCA patients in actual medical settings. Statistical significance was defined as $p < 0.05$.

2.6 Comprehensive analysis of immune characteristics and responses to immune checkpoint inhibitor therapy

To explore the relationship between immune cell infiltration within TME of BLCA and ICDRS, we used the IOBR package (36) to assess ESTIMATE scores, CIBERSORT infiltration estimates, and the infiltration of 28 types of immune cells in BLCA samples from TCGA. Firstly, we employed the CIBERSORT algorithm to quantify the infiltration of 22 immune cell types. CIBERSORT is a computational method based on the principle of linear support vector regression (SVR) and uses a predefined reference gene expression feature matrix (LM22) to deconvolute RNA sequencing data. The LM22 matrix includes characteristic expression profiles of 22 immune cell types, allowing us to accurately estimate the relative abundance of these cell types at the RNA transcription level, facilitating the identification and quantification of cell types (37).

Following the initial analysis, we refined our results on immune cell infiltration using the immune phenotype scoring method for 28 types of immune cells, as published by Charoentong et al. (38). We then utilized the ESTIMATE algorithm to assess the correlation between immune cells and genes in tumor samples. The combined use of these methods not only provided a multidimensional perspective on immune cell infiltration but also helped us to verify and enhance the precision and robustness of the CIBERSORT estimates. This ensured that our study results accurately and deeply elucidated the relationship between immune cell infiltration and survival prognosis in BLCA. Statistical significance was defined as $p < 0.05$.

2.7 Significance of the ICDRS in drug sensitivity

We utilized the Genomics of Drug Sensitivity in Cancer (GDSC) database (https://www.cancerrxgene.org/) to predict the sensitivity of samples from high and low-risk groups to common anticancer drugs. This database is one of the largest public resources in the field of pharmacogenomics, providing rich data on drug sensitivity and related genomic information, which is crucial for identifying potential cancer treatment targets (39). We employed the pRRophetic package to construct a Ridge regression model based on cell lines, using the gene expression profiles and risk scores of ICDRS from BLCA to estimate the half-maximal inhibitory concentration (IC50) of drug samples (40). This method allows us to assess the sensitivity of different ICDRS risk groups (high risk and low risk) to anticancer drugs.

2.8 HPA validation

We validated the protein expression of relevant genes in BLCA using the Human Protein Atlas (HPA) database. The HPA database (https://www.proteinatlas.org/) is the most extensive and comprehensive resource on the spatial distribution of proteins in human tissues and cells (41). By integrating advanced transcriptomics and proteomics technologies, the HPA database provides detailed information on protein expression at both RNA and protein levels across various human tissues and organs. This approach allows for a thorough validation of the protein expression of genes of interest in BLCA, providing valuable insights into their biological relevance and potential therapeutic significance.

3 Results

All analytical processes are illustrated in the flowchart (Figure 1).

3.1 ICD features of single-cell transcriptomics

We assessed the single-cell transcriptomic landscape to characterize the features of ICD across different cell types. Utilizing t-SNE technology, we set marker genes according to previous relevant literatures (42, 43), then identified cell types defined by marker genes, annotating cells into 9 major clusters: endothelial cells, bladder epithelial cells, macrophages, monocytes, cancer cells, fibroblasts, mast cells, B cells, and T cells, thereby revealing the distribution patterns of various cell populations (Figure 2). In the heatmap, we displayed the expression of marker genes for each cell cluster, such as high expression of CD68 and CD163 in macrophages (Figure 2). We further assessed the ICD activity score in each single cell (Figure 2). Using a continuous color gradient from green (low ICD score) to red (high ICD score), we observed the distribution of ICD activity among different cell groups. Finally, we presented the distribution of ICD scores across different cell types by violin plots (Figure 2). Results indicated that immune cells like macrophages, T cells, and monocytes exhibited higher ICD scores, while non-immune cells such as fibroblasts, endothelial cells, and cancer cells showed lower scores. Based on ICD activity, we categorized cells into high ICD and low ICD groups and identified 7,233 ICD-DEGs between the two groups for further analysis.

3.2 Identifying ICDRgenes in bulk RNA sequencing

We utilized the TCGA-BLCA sample dataset and applied the WGCNA method to identify and analyze genes related to the immunogenic cell death-related modules.

By constructing a hierarchical clustering dendrogram of the samples (Figure 3), we displayed the clustering relationships among tumor samples. The heatmap at the bottom shows each sample's ICD score to illustrate the relative activity of ICD features within the samples. In WGCNA, we constructed a dendrogram of sample clustering (Figure 3) and revealed through a module-trait heatmap (Figure 3) that the brown and yellow modules are closely associated with ICD traits. In the brown module, the scatterplot of gene significance (GS) and module membership (MM) relationships (Figure 3) shows a positive correlation between them. We further displayed the gene expression differences in single-cell tumor samples classified by ICD scores through a volcano plot (Figure 3). Additionally, using a Venn diagram (Figure 3), we identified 108 intersecting genes between the two modules and bulk RNA sequencing ICD-DEGs, termed ICDRgenes, which are considered to be significantly involved in ICD at both the whole and single-cell transcriptomic levels.

Subsequently, we established a protein-protein interaction network (PPI network) composed of 108 ICDRgenes (Figure 3), revealing potential interactions among these genes. We then conducted GO and KEGG enrichment analyses (Figure 3) to explore the distribution of these ICDRgenes in BP, CC, and MF, as well as their potential roles in various biological pathways. The results showed that these genes are primarily enriched in pathways related to the regulation of the actin cytoskeleton, which was further confirmed in KEGG analysis.

To further construct and validate the model, we performed a Cox cross-validation with the TCGA gene list and clinical prognosis data, identifying 31 genes with significant statistical significance. After correlation analysis, these genes were further divided into three clusters (Figure 3), which were used for subsequent analyses.

3.3 Construction of prognostic features based on integrated machine learning

We utilized an integrated machine learning approach to develop a consensus ICDRS. Within a ten-fold cross-validation framework, we evaluated 100 different predictive models by assessing the accuracy of each model across all datasets (Figure 4). The main criteria for model selection was the best balance between predictive performance and model stability across all datasets, with minimal reduction in C-index values between training and testing sets compared to other algorithms.

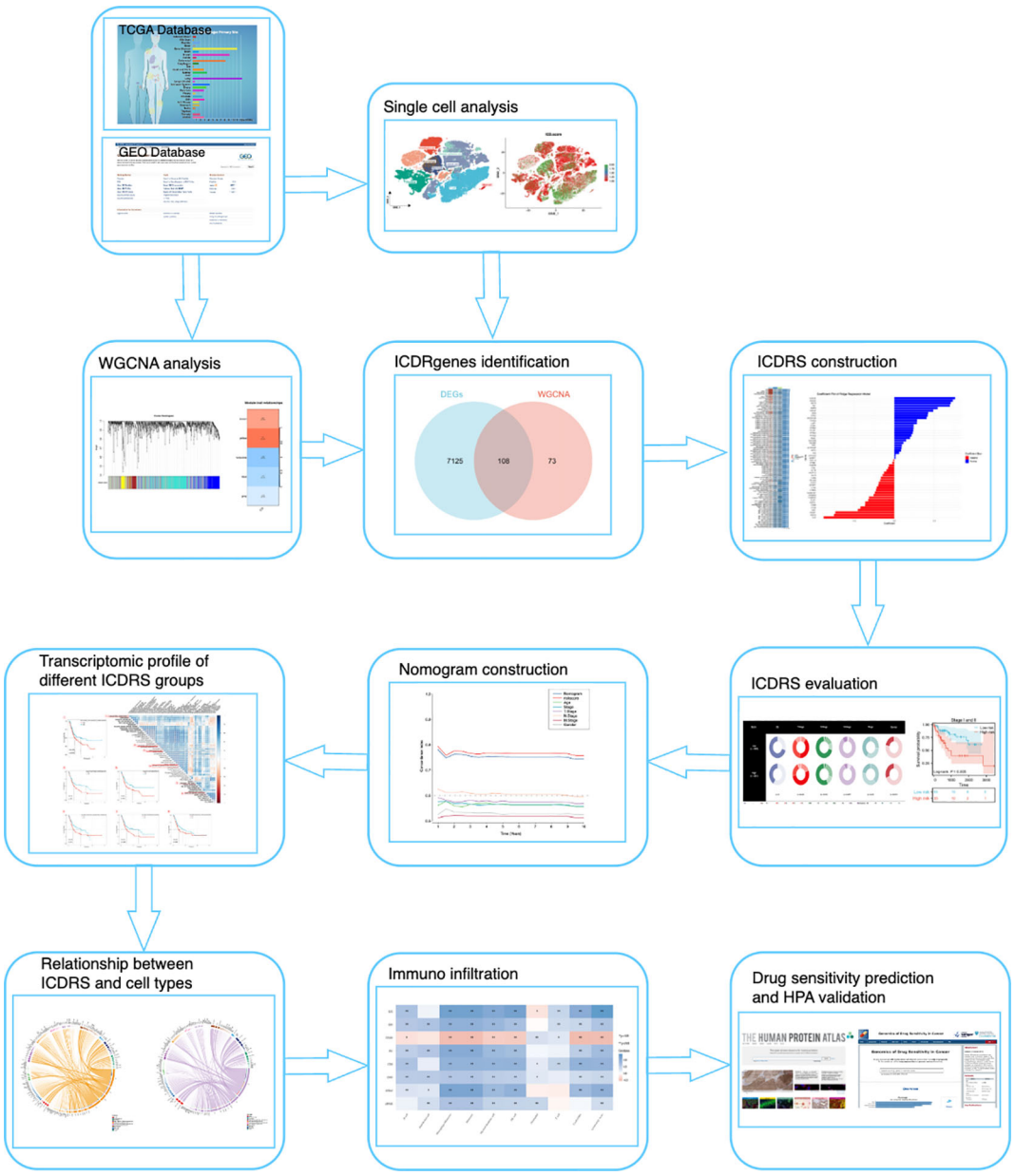

FIGURE 1
Study flowchart.

Considering the overall performance, we selected the Ridge model for constructing the ICDRS and displayed the variable weights within the Ridge model (Figure 4). These weights reflect the importance of each variable in the model.

We further validated the prognostic capabilities of the ICDRS using KM curves in three independent datasets. In the TCGA training set, patients stratified into low-risk and high-risk groups based on the ICDRS showed significant differences in survival ($P < 0.001$) (Figure 4). In the internal TCGA testing set, while there was a trend indicating survival differences, the differences did not reach statistical significance ($P = 0.096$) (Figure 4). However, in the external GSE13507 testing set, the high and low scores of the ICDRS demonstrated significant statistical differences in survival ($P = 0.007$) (Figure 4).

3.4 Assessing the performance of the ICDRS

We assessed the distribution and performance of the ICDRS across different clinical characteristic subgroups of BLCA patients. Figure 5 shows the distribution of ICDRS low-risk and high-risk groups among patients in terms of OS, T stage, N stage, M stage, clinical stage, and gender.

Using violin plots, we demonstrated the differences in risk scores among patients grouped by age, gender, M stage, clinical stage, and T stage (Figure 5). We noticed that the risk scores were significantly higher among older, female, stage III-IV, and T3-4 patients compared to younger, male, stage I-II, and T1-2 patients. These results suggest

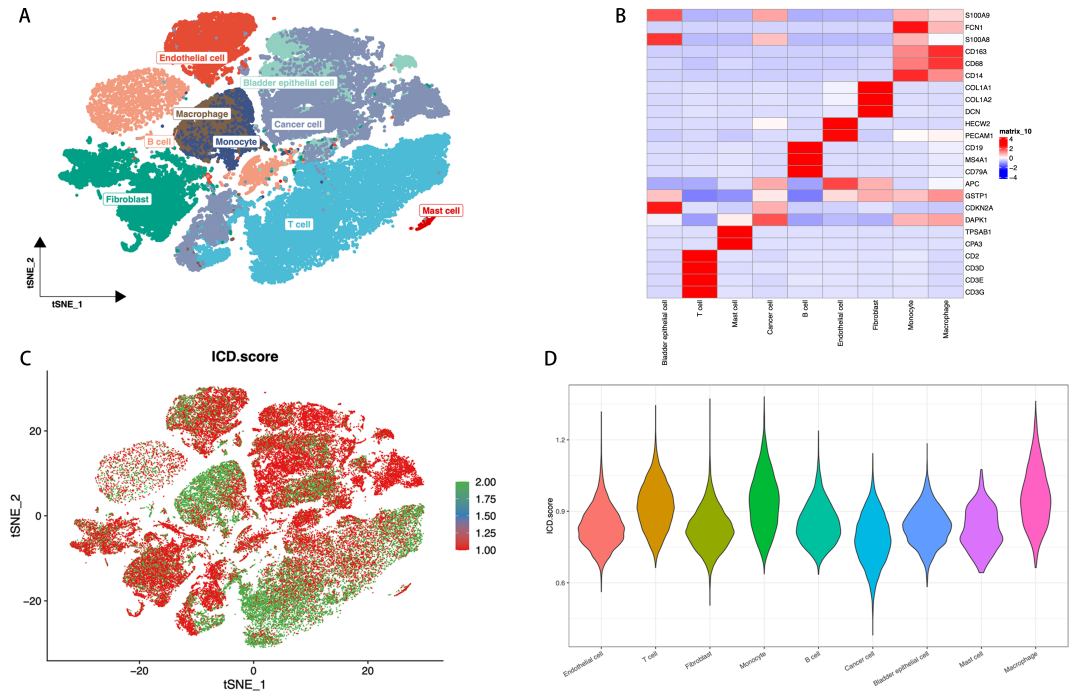

FIGURE 2
ICD Features in Single-Cell Transcriptomics. **(A)** t-SNE plot showing the cell types identified by marker genes. **(B)** Heatmap displaying the marker genes in each cell cluster, where red and blue respectively indicate high and low gene expression levels. **(C)** The activity score of ICD in each cell. **(D)** The distribution of the ICD scores in different cell types, with the width of each violin plot shape indicating the density and range of ICD scores in the corresponding cell type.

that the ICDRS is associated with poor prognosis in BLCA patients. Figure 5 reveals the proportion of T stage distribution within the ICDRS risk subgroups, highlighting the differences in early versus late stages among patients at different risk levels.

Further survival analyses demonstrated the stable performance of the ICDRS across age and stage stratifications. As shown in Figure 5, KM curves reveal the survival probabilities of low-risk and high-risk groups as defined by the ICDRS among patients aged ≥60 and <60, as well as those in clinical stages I and II versus III and IV. In all subgroups, the high-risk group exhibited poorer survival rates compared to the low-risk group, and these differences were statistically significant ($p < 0.05$).

These results indicate that the ICDRS can effectively differentiate prognostic risks among BLCA patients with varying clinical characteristics, providing robust clinical decision support for personalized treatment.

3.5 Establishment and validation of a nomogram integrating clinical features

To evaluate whether the ICDRS is an independent prognostic factor for BLCA, we assessed the impact of age, gender, TNM staging, clinical staging, and ICDRS on patient OS in two cohorts: TCGA-BLCA and GSE13507. Univariate analysis results (Figure 6C) showed that in the TCGA-BLCA cohort, age, TNM staging, clinical staging, and ICDRS were all significant prognostic factors for OS. However, in the GSE13507 cohort, only ICDRS significantly impacted OS as an independent prognostic factor. Subsequent multivariate analyses (Figure 6D) further confirmed ICDRS as an independent prognostic indicator in both cohorts. In this analysis, ICDRS significantly influenced OS even after adjusting for other clinical features.

A prognostic scoring nomogram was constructed based on ICDRS and clinical features (Figure 6), integrating age and ICDRS scores. The nomogram's predictions for 1-year, 3-year, and 5-year OS were highly consistent with the actual observations, as shown by the calibration curve (Figure 6). We also compared the nomogram's C-index with that of other individual clinical features (Figure 6), and the results showed that its predictive capability for OS was superior to that of individual clinical features alone. Decision curve analysis (Figure 6) indicated that, within a certain range of high-risk thresholds, using the nomogram could achieve a higher standardized net benefit compared to other clinical features. This means that decision-making based on the nomogram offers superior expected benefits over traditional clinical feature-based decisions.

3.6 Transcriptomic feature analysis of different ICDRS patient groups

To further investigate the molecular mechanisms underlying the correlation between ICDRS and prognosis in BLCA, we

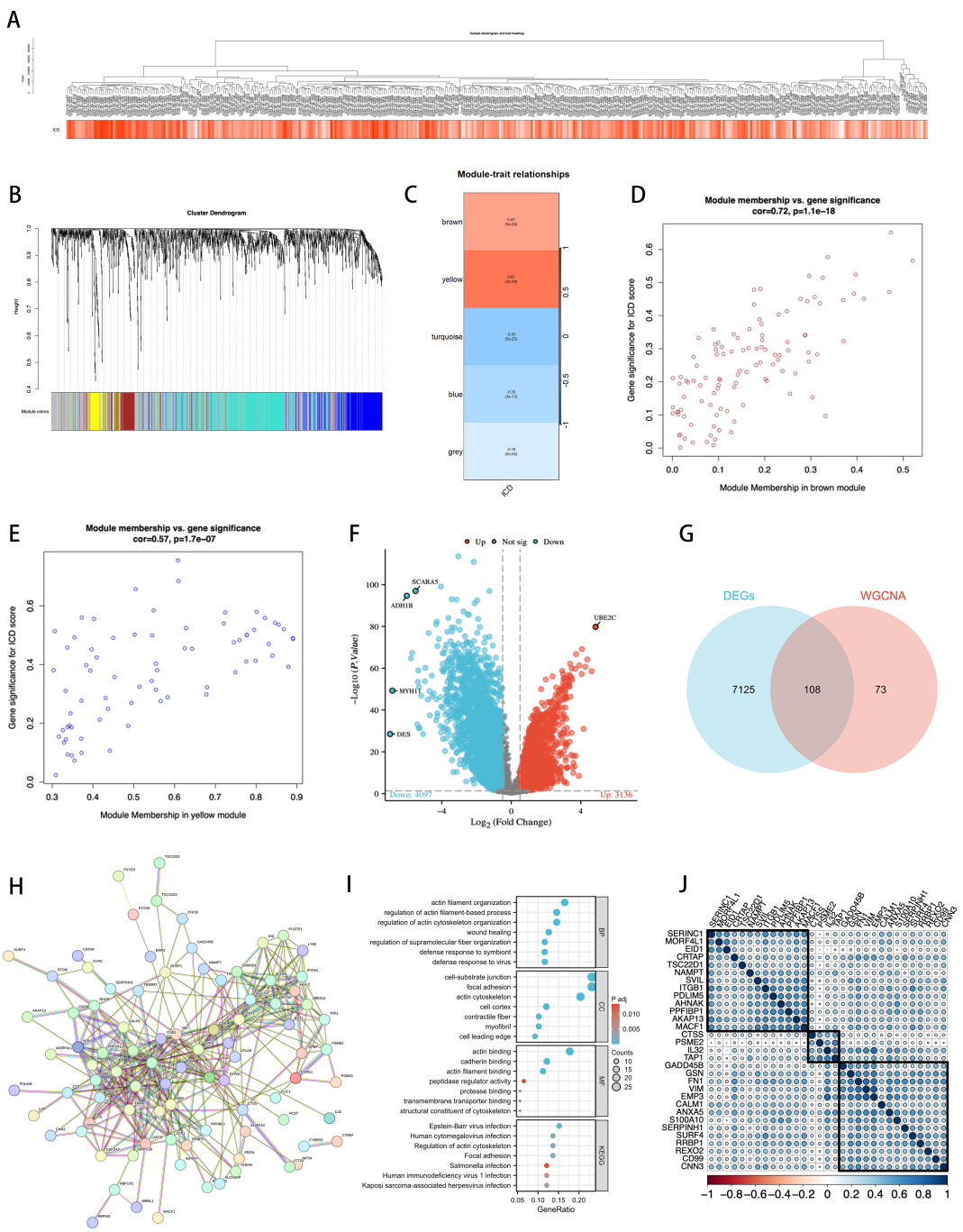

FIGURE 3
Identification of ICDRgenes. **(A)** Dendrogram showing the hierarchical clustering of TCGA-BLCA samples. The heatmap at the bottom represents the ICD scores of each sample. **(B)** Cluster dendrogram of the WGCNA analysis. **(C)** Module-trait heatmap showing that the brown and yellow modules were closely related to the ICD trait. **(D)** Scatter plot showing the relationship between GS and MM in the brown module. **(E)** The relationship between GS and MM in the yellow module. **(F)** Volcano plot showing differential analysis results between TCGA-BLCA samples and normal samples, with the top 5 most significantly different genes specially marked. **(G)** Venn plot showing the intersecting genes between the two modules and DEGs in bulk RNA-seq. **(H)** PPI Network of 108 ICD-DEGs. **(I)** GO and KEGG Analysis Results. **(J)** After using Cox regression and cross-validation, 108 genes resulted in 31 statistically significant genes related to prognosis, which were then divided into 3 clusters for correlation analysis.

conducted GSEA and GSVA. These analyses can reveal differences in biological processes and pathway activities related to patient groups with high and low ICDRS scores.

Figure 7 from the GSEA analysis reveals the GO pathways enriched in different ICDRS groups. Figure 7 shows pathways related to structural constituent of postsynaptic actin cytoskeleton, norepinephrine transport, peptide cross-linking, sequestering of metal ion, calyx of held, cornified envelope, ferrous iron binding, and modulation of processes of another organism. Figure 7B's ridge plot highlights enriched GO pathways that involve different aspects of TME.

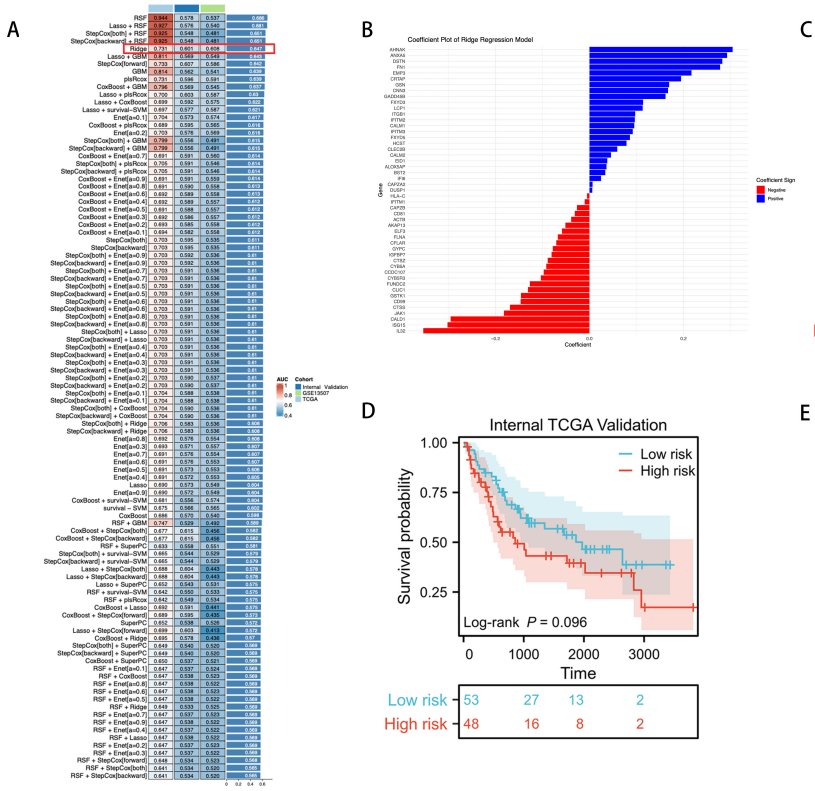

FIGURE 4
Through a machine learning-based integration process, we developed and validated a consensus ICDRS. **(A)** We evaluated 100 predictive models within a ten-fold cross-validation framework and further calculated the C-index for each model across all validation datasets. **(B)** We visualized the variable weights in the Ridge model, where red indicates negative weights, blue represents positive weights, and the size denotes the absolute value of the weight. **(C–E)** KM curves for OS of ICDRS in the TCGA training set, TCGA internal testing set, and GSE13507 external testing set based on the log-rank test.

Figure 7 provides GSVA scores for KEGG pathways, further enhancing our understanding of the pathway activities associated with ICDRS scores. Through KEGG pathway analysis, we can quantitatively compare differences in metabolism and signaling between groups with high and low ICDRS scores.

In Figure 7, using the Hallmark gene set, we evaluated GSVA scores to assess the correlation between ICDRS scores and activity of typical pathways, and through KM survival plots, we demonstrated a significant correlation between OS and GSVA scores. Integrating these data, we conclude that ICDRS scores are positively correlated with the activity of several key biological pathways, including cholesterol homeostasis, estrogen response, epithelial-mesenchymal transition (EMT), androgen response, and the unfolded protein response (UPR). Moreover, the upregulation of these pathways is significantly associated with poor prognosis in BLCA patients. Conversely, pathways negatively correlated with ICDRS, such as KRAS signaling, are associated with a better prognosis. These results suggest that the activation or inhibition of these pathways may contribute to the different prognostic outcomes observed within ICDRS subgroups. These findings underscore the importance of ICDRS as a potential prognostic biomarker in BLCA treatment and provide a scientific basis for future development of targeted therapies against these pathways.

3.7 Establishment and validation of an ICD-related gene risk scoring model based on Lasso regression

Building on the previous results, given the robustness and universality of the Lasso-Cox model across most models, we revisited the modeling of all 108 genes using the Lasso-Cox approach. The Lasso regression coefficient path plot displayed how the coefficients of the 108 genes shrink towards zero as the L1 regularization penalty (λ value) increases, revealing the model variables ultimately selected (Figure 8). In Lasso regression, the cross-validation deviation plot identified the optimal λ value that minimizes the cross-validation error, providing the best model complexity (Figure 8). Figure 8 shows the distribution of risk scores between high-risk and low-risk samples in the training set, calculated based on the selected λ value. The KM survival curves illustrate significant differences in prognosis between the high-risk and low-risk groups ($P < 0.001$), with median survival times of 1.6 years and 8.1 years, respectively (Figure 8). The risk score distribution plot shows the relationship between each sample's score and survival status (alive/dead) in the training set, with high-risk scores associated with the occurrence of death events (Figure 8). The ROC curve evaluated the accuracy of the risk scoring model in predicting survival at 1 year, 3 years, and 5 years. The

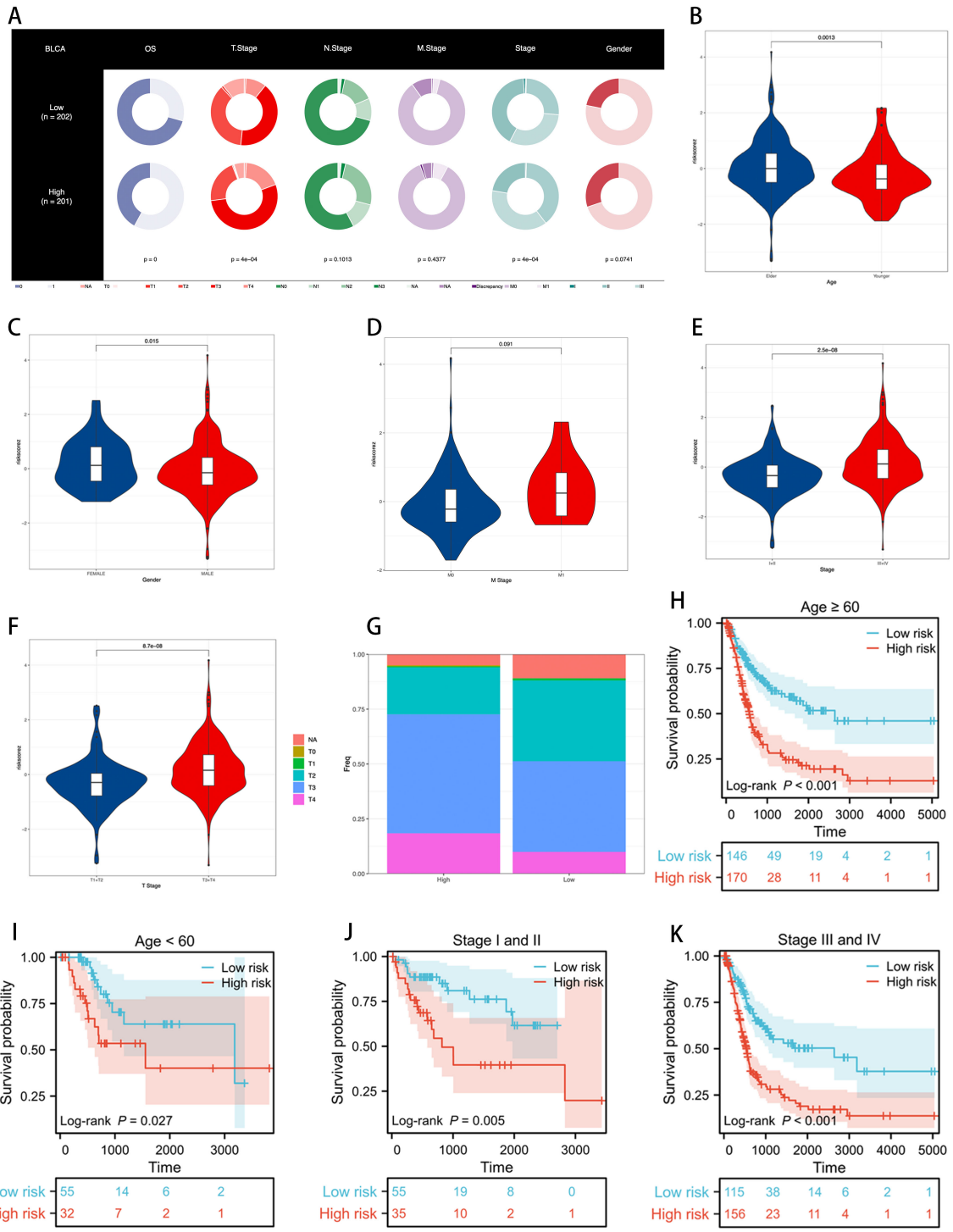

FIGURE 5
Assessing the performance of the ICDRS. **(A)** Clinical characteristics of ICDRS low-risk and high-risk groups are visualized through the proportions in each ring, illustrating the risk distribution within each subgroup, with p-values used to assess the correlation between ICDRS and these clinical features. **(B–F)** Differences in risk scores between patients grouped by age, gender, M stage, clinical stage, and T stage. **(G)** The proportion of T stage distribution in ICDRS risk subgroups, showing the variation in early versus late stages across different risk levels. **(H–K)** KM curves demonstrating the stable performance of the ICDRS in subgroups, including age and stage.

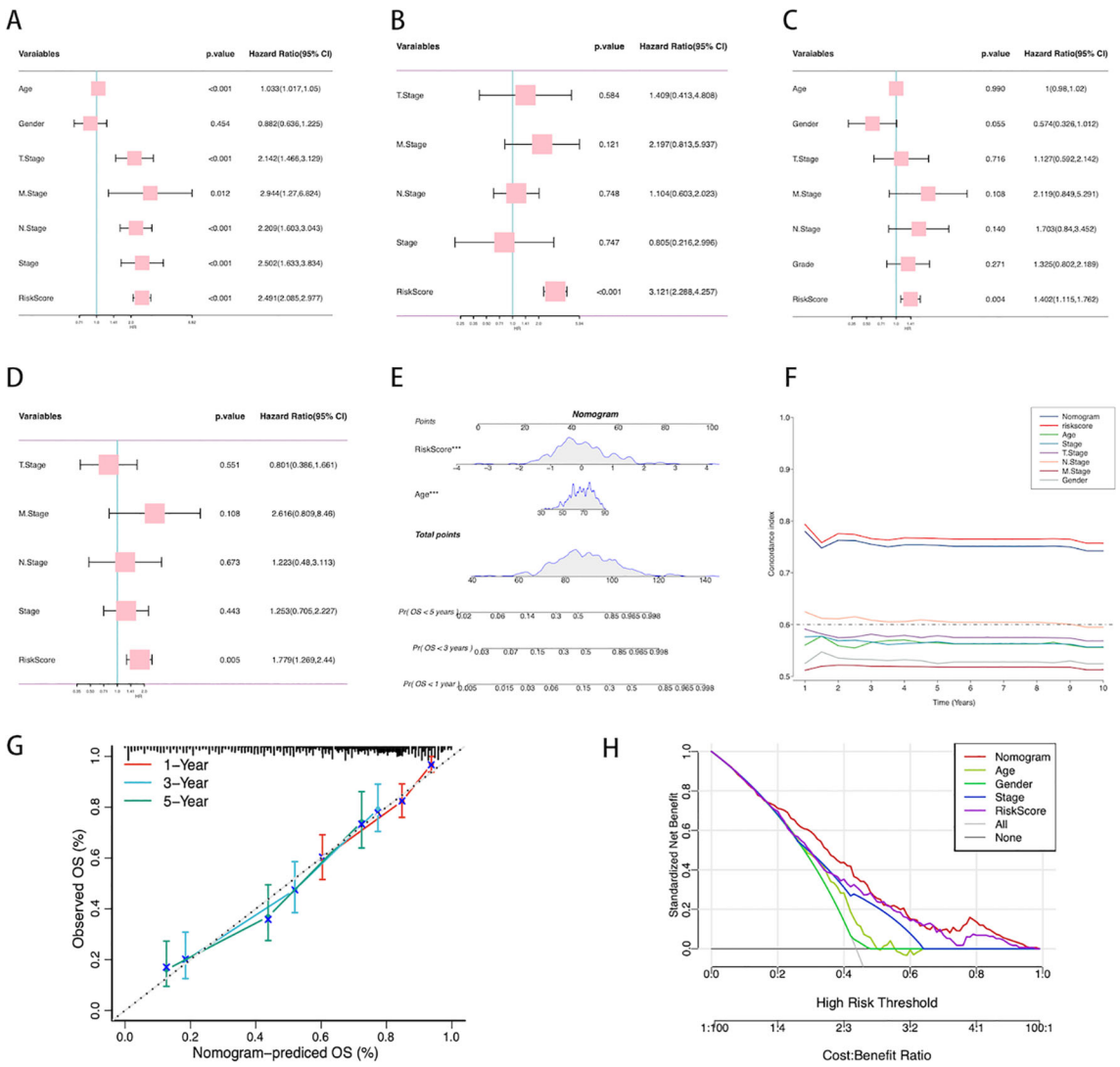

FIGURE 6
Establishment of the nomogram. **(A)** Univariate analysis of the clinical characteristics and ICDRS for OS in the TCGA-BLCA cohort. **(B)** Multivariate analysis in the TCGA-BLCA cohort. **(C)** Univariate analysis of the clinical characteristics and ICDRS for OS in the GSE13507 cohort. **(D)** Multivariate analysis in the GSE13507 cohort. **(E)** Construction of the nomogram based on ICDRS and clinical characteristics. Each variable's contribution to the predictive model is represented by a point-line plot next to it, where a larger contribution indicates a stronger association with survival prediction. **(F)** Comparison of the C-index between the nomogram and other clinical characteristics. **(G)** Calibration curve of the nomogram for 1, 3, and 5-year OS. **(H)** Decision curve analysis showing the standardized net benefit of applying the nomogram compared to other clinical characteristics.

AUC values for 1-, 3-, and 5- year OS are 0.733, 0.735, and 0.733. The AUC at different time points showed the model's strong predictive performance (Figure 8). These results indicate that the further developed ICDRS not only accurately distinguishes between high-risk and low-risk BLCA patients with powerful prognostic prediction capabilities but also significantly enhances the model's robustness.

3.8 Comprehensive correlation analysis of ICDRS with single-cell characteristics

We intersected the top 10 upregulated and downregulated genes in the Ridge model with the high-weight genes from the LASSO analysis to identify the eight most prominent genes in the ICDRS, namely IL32, AHNAK, ANXA5, FN1, GSN, CNN3, FXYD3, and CTSS. We conducted a detailed analysis of the expression of ICDRS in different single-cell types and its functional associations (Figure 9). Through single-cell RNA sequencing analysis (Figure 9), we identified the expression patterns of these eight genes across various cell types, showing that these genes are primarily expressed in bladder epithelial cells, macrophages, monocytes, cancer cells, fibroblasts, and T cells. This analysis revealed the association of ICDRS with the functions of specific cell populations.

KEGG pathway analysis further identified functionally enriched pathways in DEGs between high-risk and low-risk cells (Figure 9). Results show that various biological processes and signaling

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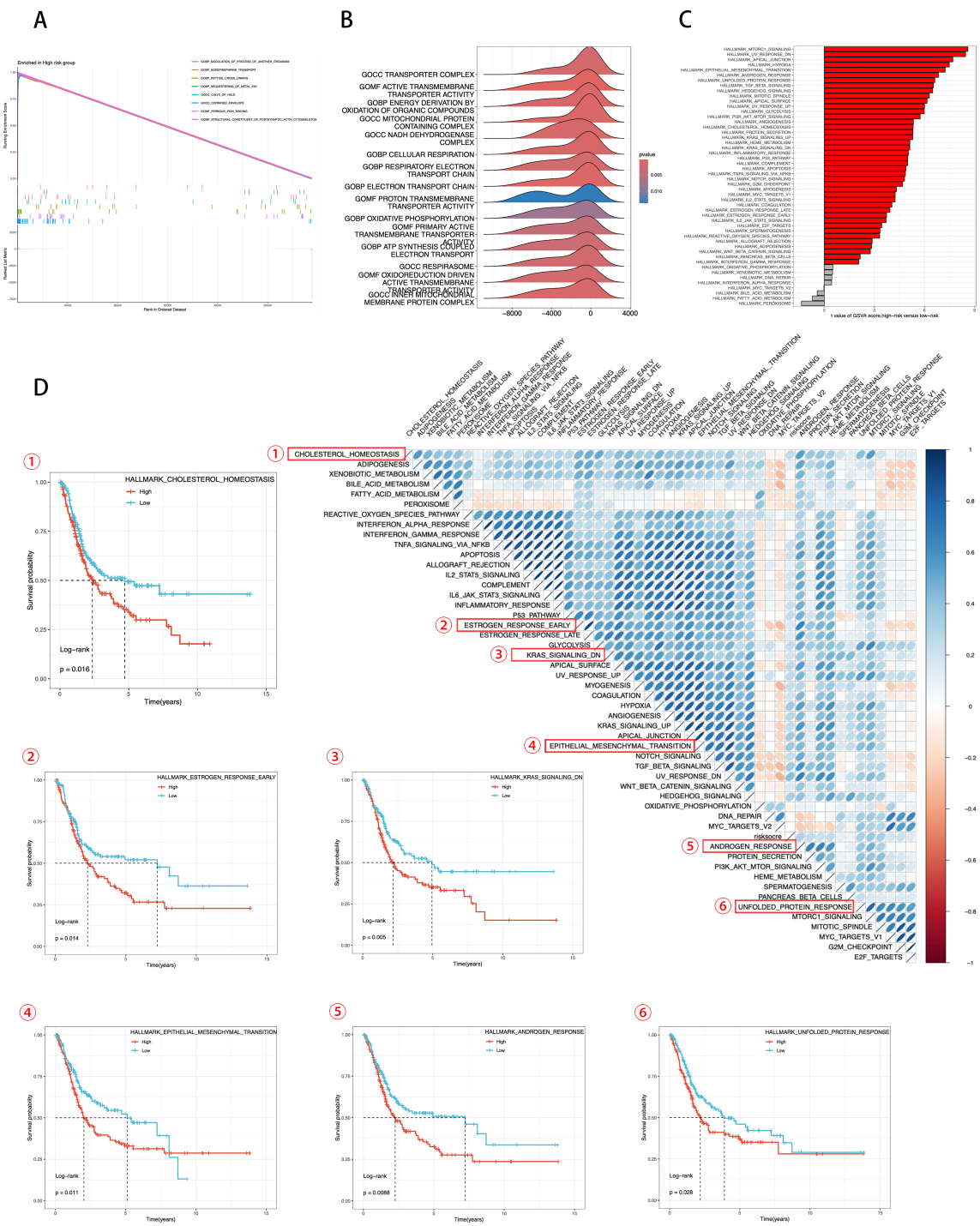

FIGURE 7
Transcriptomic features of various ICDRS patient groups. **(A, B)** GO terms enriched by GSEA analysis. **(C)** Differences in KEGG analysis between the high- and low-risk groups scored by GSVA. **(D)** Correlation between the risk score and hallmark pathway activities scored by GSVA. KM survival plots showing the significant correlations between the OS and GSVA scores.

pathways such as neurodegenerative disease pathways, the PI3K-Akt signaling pathway, and extracellular matrix (ECM)-receptor interactions are significantly enriched in high-risk cell populations. Through GSEA analysis, we identified enriched HALLMARK pathways in high-risk cells, including angiogenesis, activation of the complement system, EMT, interferon-α response, activation of MYC targets, and myogenesis, revealing the key biological processes these cells may be involved in (Figure 9).

The Cellchat diagram provides a detailed view of interactions among different cell types within specific signaling pathway networks (Figure 9), revealing the complexity and diversity of cell communication. Furthermore, we visualized the molecular

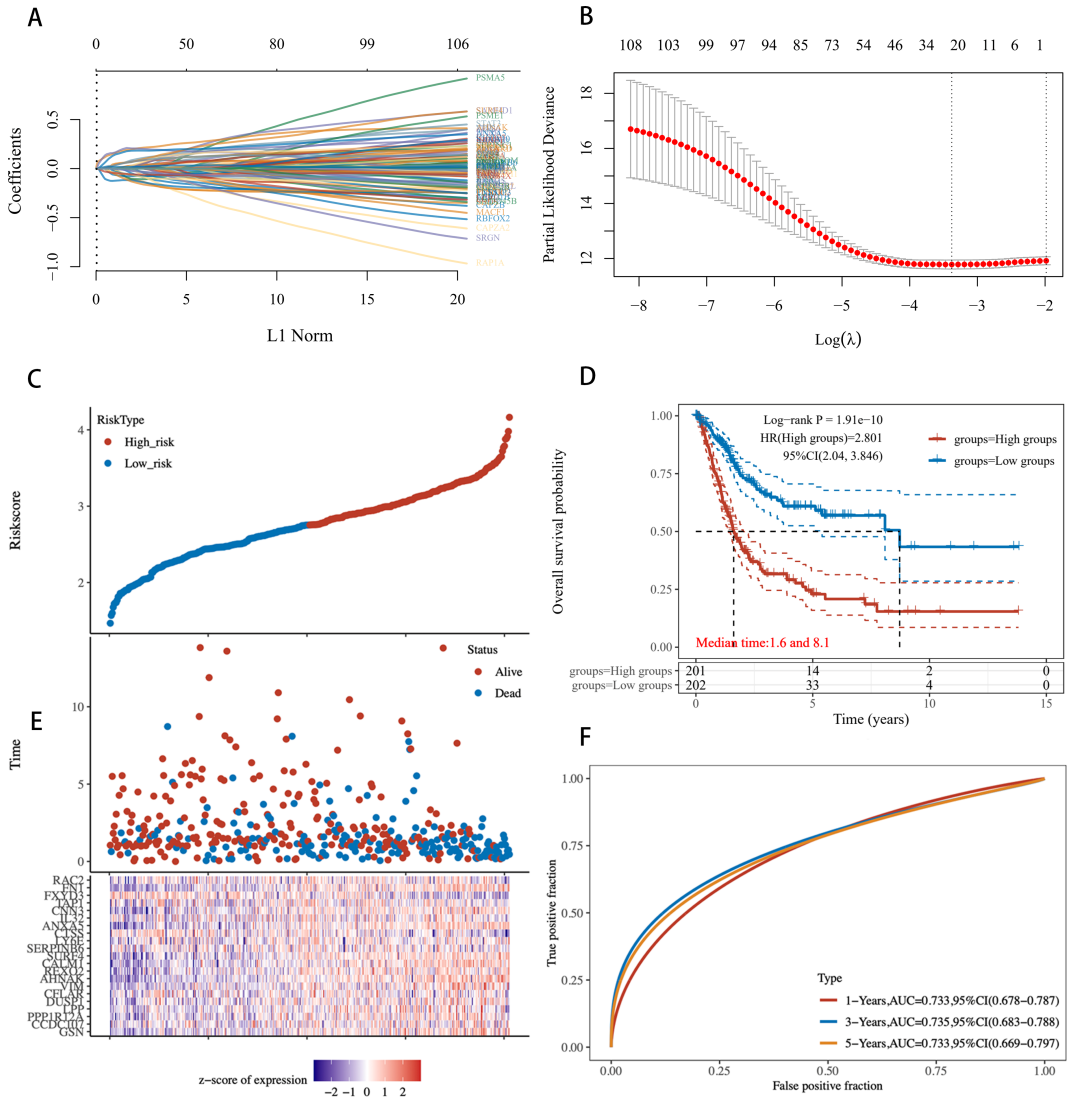

FIGURE 8
Risk scoring model based on 108 ICD-related genes constructed using Lasso regression. **(A)** Lasso Regression Coefficient Path Plot for 108 Genes. **(B)** Deviation in cross-validation for Lasso regression. The X-axis represents the logarithm of λ values, and the Y-axis represents deviation. Red points indicate the average deviation at each λ value, gray lines represent the standard error of the deviation, and the vertical line on the X-axis marks the optimal λ value. **(C)** Risk profile in the training set. **(D)** KM survival curves for high-risk and low-risk groups in the training set. **(E)** Distribution of Risk scores for each sample. **(F)** ROC curves for the training set.

interactions between tumor cells and other cell types with high and low risk scores. Figures 9, 9F respectively show the ligand-receptor interactions observed in high-risk and low-risk tumor cells. Compared to the low-risk group, the high-risk group displays specificity in the FN1 and CD99 signaling pathways. FN1, a major extracellular matrix protein, is involved in various cellular processes, including cell adhesion, migration, wound healing, and embryonic development. Increased expression of FN1 is often associated with higher malignancy, poor prognosis, and enhanced invasiveness of cancer. The active FN1 signaling in the high-risk group may indicate these cells have greater invasive capabilities and metastatic potential. CD99, a cell surface glycoprotein widely expressed in many cell types, is involved in cell adhesion, migration, and immune regulation. In high-risk tumor cells, the expression of CD99 may be associated with immune evasion, cell migration, and invasion.

To further discuss the expression and correlation of pathways across various cell types, we selected several pathways for further heatmap visualization, including the APP pathway, which influences cell proliferation and death, the MIF pathway associated with the worsening prognosis of various cancers, and the MK and ITGB2 pathways, which are linked to tumor aggressiveness and poor prognosis (Figure 9). The results indicate that in high-risk tumor cells, the APP, MIF, and MK pathways exhibited more significant expression compared to the low-risk group. Moreover, the MIF pathway showed a high correlation with

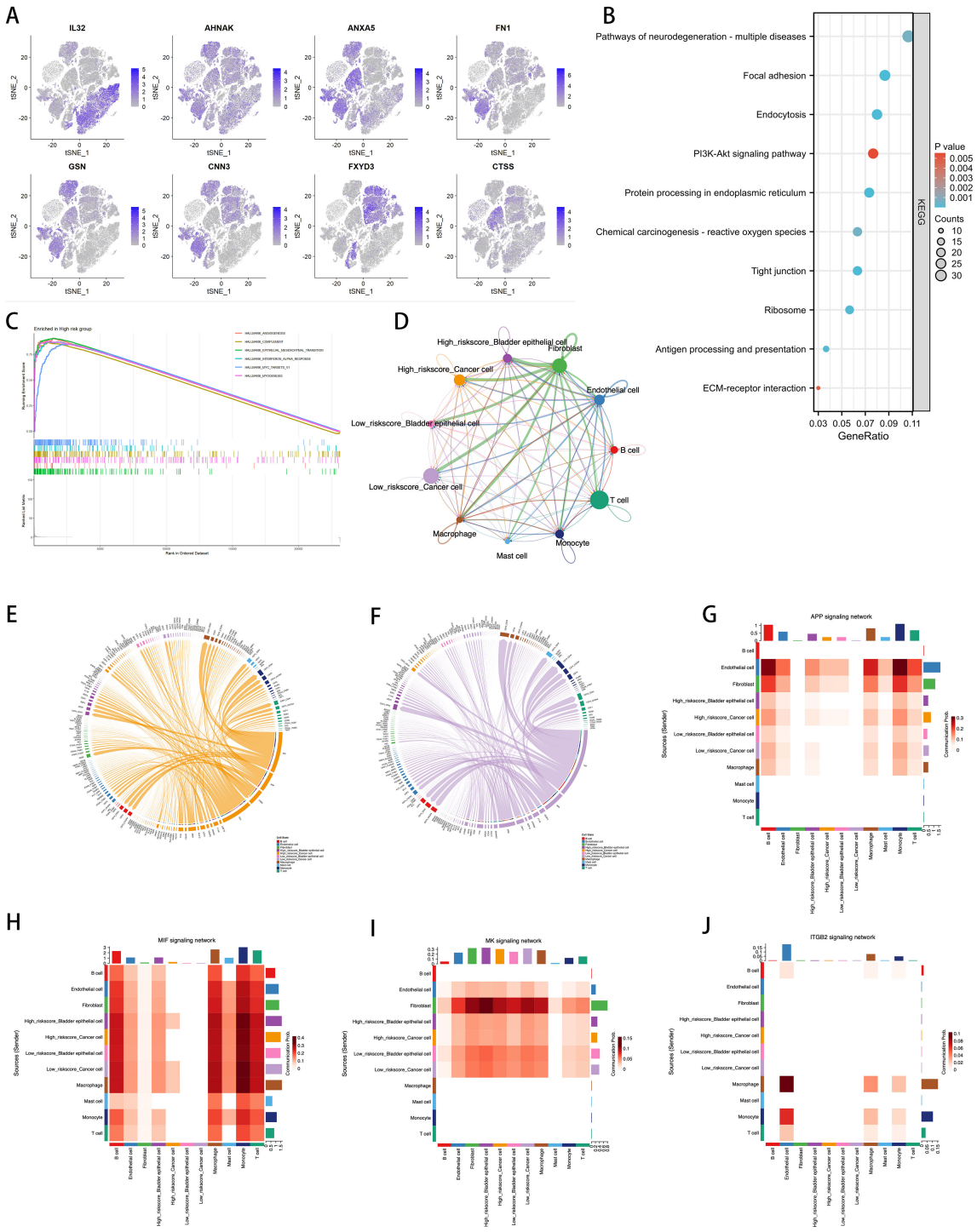

FIGURE 9
Correlation of ICDRS with Single-Cell Characteristics. **(A)** Expression of the 8 genes in various cell types by single-cell RNA-seq analysis. **(B)** KEGG analysis of the DEGs between the high- and low-risk cells. **(C)** GSEA analysis identified the GO terms enriched in the high-risk cells. **(D)** Circos plots showing the signaling pathway networks. **(E)** Ligand-receptor interactions sent from high-risk tumor cells. **(F)** Ligand-receptor interactions sent from low-risk tumor cells. **(G–J)** The heatmaps showing the roles of different cell types in the APP, MIF, MK, and ITGB2 signaling networks.

fibroblasts, corroborating the specific expression of FN1 as seen in Figure 9. However, the ITGB2 pathway did not show differences in expression in BLCA and had very low expression levels, suggesting that this pathway is less relevant to BLCA. These heatmaps provide an intuitive view for understanding the role of ICDRS across different cell types and signaling pathways, supporting the importance of ICDRS in cell communication and functional execution within TME.

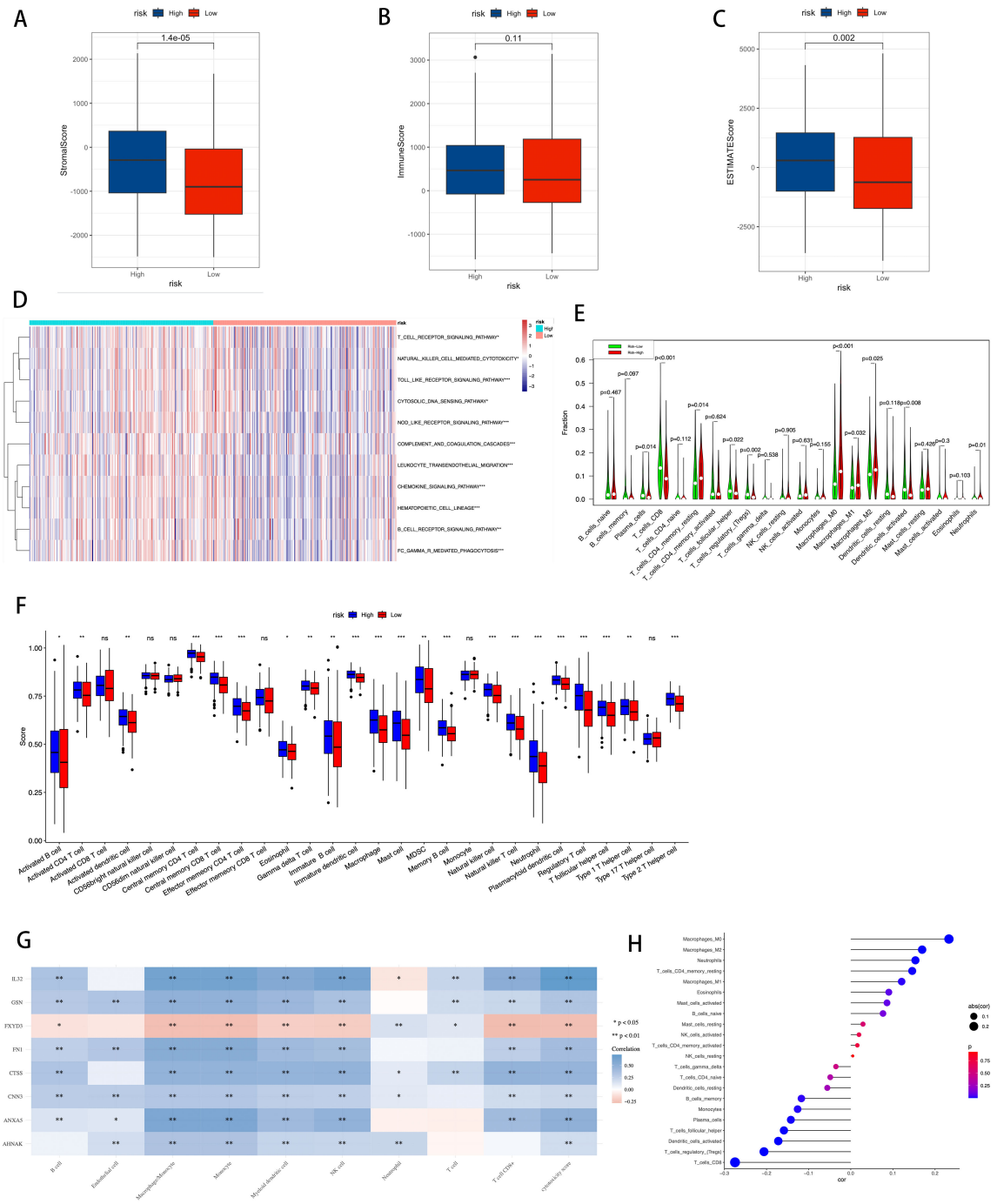

FIGURE 10
Correlation between TME, immune characteristics, and ICDRS. **(A–C)** The Stromal Score, Immune Score, and ESTIMATE Score were used to quantify the different immune statuses between the high- and low-risk groups. **(D)** Activity of immune-related pathways showed significant differences between the high- and low-risk groups. **(E)** Using the CIBERSORT algorithm to calculate the abundance of each type of TME-infiltrating cell in the high and low-risk groups. **(F)** Quantitative scoring of infiltrating cell abundance using the scheme for 28 types of immune phenotype scores. **(G)** The association between TME-infiltrated cells and genes built into the ICDRS. **(H)** Correlation analysis between TME-infiltrated cells and the ICDRS. Statistical significance is indicated as follows: * for p-value < 0.05, ** for p-value < 0.01, *** for p-value < 0.001, and ns (not significant) for p-value ≥ 0.05.

3.9 Immune landscape associated with ICDRS in BLCA

Figure 10 provides a detailed display of the differences in the immune landscape between high-risk and low-risk groups in BLCA patients. Using the Stromal Score, Immune Score, and ESTIMATE Score, we quantified the varying immune states between the two risk groups (Figure 10), revealing that the high-risk group has higher Stromal Scores (P = 1.4e-5), Immune Scores (P = 0.11), and ESTIMATE Scores (P = 0.002).

Additionally, using the ssGSEA algorithm, differences in the activity of immune-related pathways were confirmed between the high-risk and low-risk groups, including T cell receptor signaling pathway, natural killer cell mediated cytotoxicity, Toll-like receptor signaling pathway, cytosolic DNA sensing pathway, NOD-like receptor signaling pathway, complement and coagulation cascades, leukocyte transendothelial migration, chemokine signaling pathway, hematopoietic cell lineage, B cell receptor signaling pathway, and FC gamma R mediated phagocytosis (Figure 10). This demonstrates that the activation status of these pathways varies in the TME of different risk levels. To further analyze the differences in specific immune cell infiltration between the high-risk and low-risk groups, we used the CIBERSORT algorithm to calculate the abundance of each TME infiltrating cell type in high-risk and low-risk groups (Figure 10). It was found that high-risk groups had a higher abundance of T cells CD4 memory resting, macrophages M0, macrophages M1, and macrophages M2, while the low-risk group had a higher abundance of T cells CD8, T cell follicular helper, T cells regulatory, and dendritic cells activated. Subsequently, these findings were validated using a quantitative scoring scheme for 28 types of immune cell phenotypes (Figure 10) and an analysis of the correlation between immune cells and risk scores (Figure 10), both yielding consistent results.

The study further investigated the correlation between infiltrating cells in the TME and the eight genes constituting the ICDRS (Figure 10), revealing associations between specific immune cell subpopulations and gene expression patterns in the ICDRS.

These findings collectively point to the ICDRS as an effective tool for quantifying the immune status of BLCA patients, indicating significant differences in the immune landscape characteristics of patients at different risk levels.

3.10 Drug sensitivity prediction and HPA validation

By analyzing the GDSC database, we calculated the IC50 values for commonly used drugs in the treatment of BLCA across different cancer cell lines. Specifically, significant differences were observed in the IC50 values for Cisplatin, Mitomycin C, Paclitaxel, Methotrexate, Gemcitabine, and Docetaxel between different risk groups (Figure 11). This highlights the potential value of these gene expression levels in predicting BLCA patients' responses to specific chemotherapy drugs.

In Figure 11, compared to normal tissue, IL32, FN1, FXYD3, and CTSS are significantly upregulated in BLCA tissues, ANXA5, AHNAK, GSN, and CNN3 are significantly downregulated in BLCA tissues.

4 Discussion

In the field of BLCA treatment, the current reliance on costly invasive surgeries makes it one of the most expensive cancers to treat (44). Therefore, exploring new methods to reduce treatment costs is particularly important. In the new era of immunotherapy, methods that classify, intervene, and predict cancer based on immune characteristics are increasingly becoming a research focus (45). Leveraging advancements in modern genomics and transcriptomics, this study uses single-cell analysis methods to calculate ICD scores and explore DEGs. Using the WGCNA method, gene clusters were constructed in TCGA database samples, and after integrating single-cell and TCGA data, key genes related to ICD scores were identified. These genes were then simulated through hundreds of machine learning models to predict patient long-term survival, and the predictive performance of the models was validated using external datasets. Additionally, the effectiveness and robustness of the predictive model were further confirmed through clinical data. To understand the functions of key genes in the model, GSEA and GSVA were conducted to explore related biological function pathways, and the survival impacts of these pathways were analyzed, thus affirming the importance of the new predictive model. Finally, by testing the model in multiple immune databases and scoring systems, and combining it with drug sensitivity analysis, we assessed the variability of drug therapy in model predictions, laying the foundation for further clinical application and expansion of the model.

In this study, we integrated multi-omics datasets, including single-cell and bulk data, to enhance the comprehensiveness and depth of our analysis. We divided the TCGA database into a training set and an internal testing set, adding an external testing set to validate the generalizability of the model. Notably, the internal testing set was not used in the model training process. In the comparison of various machine learning models, although the RSF model showed extremely high accuracy on the training set, its accuracy significantly decreased on the testing set, indicating potential overfitting issues. To overcome this, we focused particularly on Ridge and Lasso regression models, combining the fitting results of a hundred machine learning models. Both models employ regularization techniques to reduce the risk of overfitting but differ in how they handle variable selection and complexity adjustment. The Ridge model applies L2 regularization to shrink all variables, suitable for dealing with highly correlated variables. In contrast, the Lasso model employs L1 regularization to achieve sparse selection of variables, compressing the coefficients of unimportant variables to zero, thus demonstrating advantages in model simplification and variable selection. In our study, the Ridge model was deemed more suitable due to its advantage in maintaining variable stability. Additionally, we explored the combination of the Lasso model with the Cox proportional hazards model, particularly important in survival analysis, allowing for deeper exploration and validation of biomarkers and frequently appearing in other studies. These analyses showed that the combination of Lasso and Cox models not only provided results similar to other studies but also enhanced the predictive accuracy and interpretability of the model on clinical data. Through precise model selection and algorithm comparison, our research not only improved the accuracy of predictive models but also provided new molecular targets for future BLCA treatment strategies.

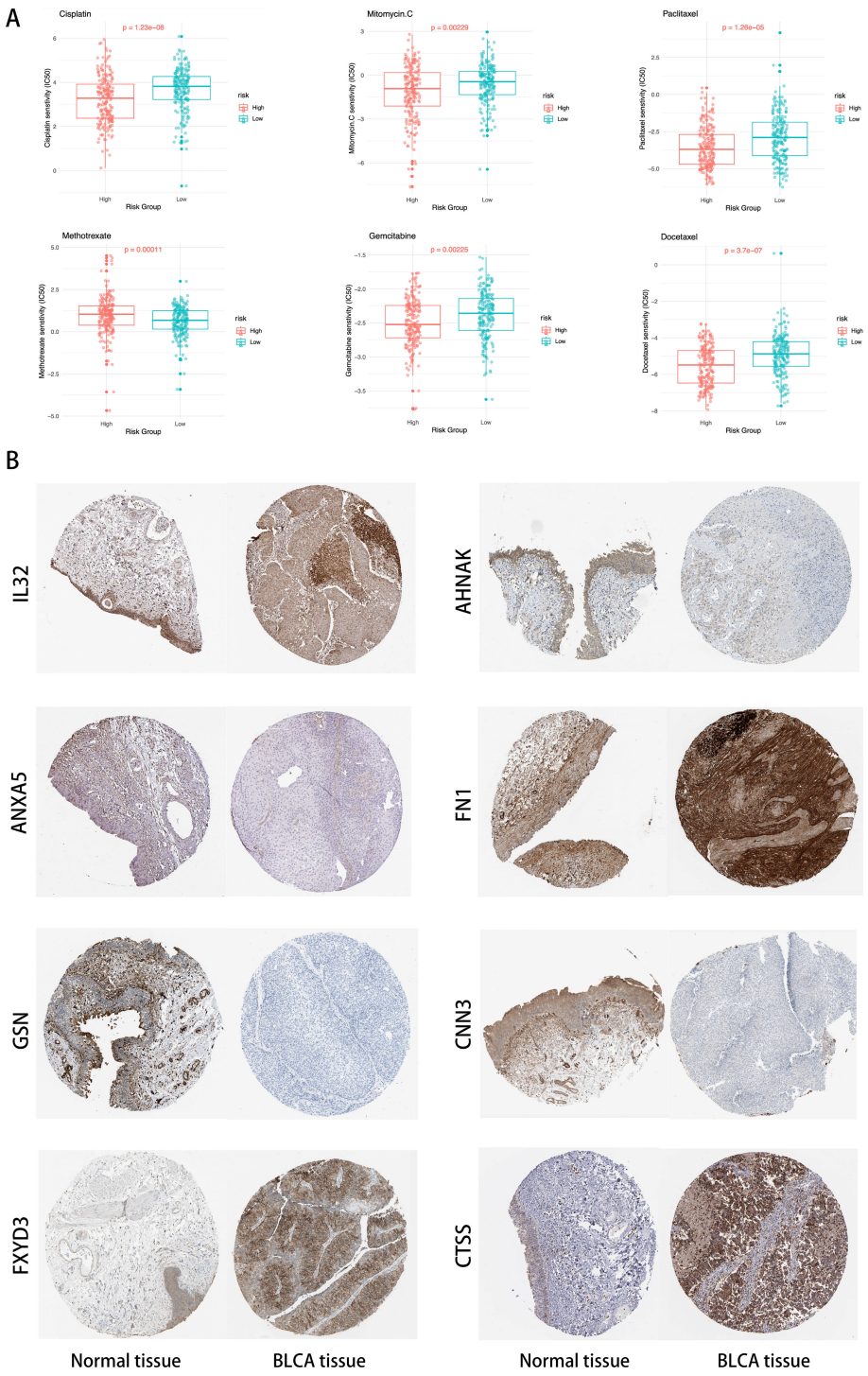

FIGURE 11
(A) Distribution of IC50 scores for drugs in high- and low-risk groups defined by ICDRS. **(B)** Validation of the eight genes expression in the HPA database.

Initially, we evaluated ICD features using single-cell transcriptomic analysis methods. The results showed that immune cells such as macrophages, T cells, and monocytes exhibited high ICD activity, further confirming ICD's critical role in stimulating anti-tumor immune responses. In contrast, non-immune cell types such as fibroblasts, endothelial cells, and cancer cells displayed lower ICD activity. Further analysis of the 108 ICDRgenes through GO and KEGG enrichment revealed that the GO analysis predominantly points to the regulation of the actin cytoskeleton pathway, closely related to the crucial role of the actin cytoskeleton in tumor cell migration and invasion in BLCA. This regulation not only affects the TME but may also impact the

interactions between tumor cells and immune cells, potentially influencing immune responses. Research indicates that the occurrence of ICD depends not only on the immune cells' recognition and elimination of tumor cells but also on the presentation of tumor cell surface antigens, which is closely linked to the regulation of the actin cytoskeleton (46–48). In the KEGG analysis, in addition to evidence related to actin cytoskeleton regulation, we found that ICDRgenes associated with viral infection pathways were enriched in BLCA, highlighting the potential for viral infections to promote tumor development through chronic inflammation or impacting immune surveillance (49, 50).

We conducted a comprehensive comparison between our model and previous gene prognostic models. Wang et al.'s risk score model based on methylation-driven genes achieved an AUC of 0.698 for 3-year OS in BLCA (51). Liang et al.'s model constructed from ferroptosis-associated genes demonstrated an AUC of 0.729 for 5-year OS (52). In contrast, our model achieved AUC values of 0.733, 0.735, and 0.733 for 1-year, 3-year, and 5-year OS, respectively. These results suggest that our ICDRS model offers superior predictive performance and greater stability across different survival timeframes compared to existing models, highlighting its potential value for clinical application in BLCA prognostication. In exploring the correlation between ICDRS and BLCA prognosis, our analysis of transcriptomes from different ICDRS risk subgroups uncovered significant differences in energy metabolism processes and TME-related pathways between groups with high and low ICDRS scores. GSVA scoring analysis using the Hallmark gene set further revealed significant associations between these scores and OS. Notably, ICDRS scores were found to positively correlate with the activation of several key biological pathways, including cholesterol homeostasis, early estrogen response, EMT, androgen response, and UPR, all of which are linked to poor prognosis. Cholesterol homeostasis is crucial for maintaining tumor cell membrane integrity, producing bioactive molecules, and providing energy, while disturbances in cholesterol metabolism may enhance tumor invasiveness and metastasis (53, 54). This finding aligns with recent studies in BLCA, where Liang et al. demonstrated that targeting cholesterol metabolism inhibited BLCA proliferation (55). Early estrogen responses might accelerate BLCA cell proliferation and progression by activating specific estrogen receptor pathways (56, 57). EMT facilitates tumor metastasis by reducing intercellular adhesion and increasing migratory and invasive capacities (58). Dysregulation of EMT has proven to drive the progression of BLCA (59–61). The androgen response impacts tumor biology by regulating the cell cycle and apoptosis (62). The androgen response pathway has particular relevance in BLCA as recent studies have revealed sex disparities in outcomes, with Chen et al. and Li et al. demonstrating that androgen receptor expression correlates with advanced stage and poor prognosis specifically in male BLCA patients (63, 64). And the enhanced UPR helps tumor cells survive under adverse conditions like hypoxia and nutrient deficiency (65, 66). In contrast, the KRAS signaling pathway's activity inversely correlates with ICDRS and aligns with a better prognosis. The activation of KRAS may decrease tumor cells' adaptability to treatments and promote apoptosis, while its upregulation might inhibit tumor-promoting pathways and enhance immune surveillance, thereby preventing tumor escape (67). Overall, the KRAS pathway potentially exerts a positive effect on BLCA prognosis by orchestrating tumor growth regulation and immune response, highlighting its role in tumor biology and as a target for therapeutic intervention.

By integrating single-cell transcriptomic data, we further unveiled the molecular mechanisms associated with ICDRS. Our ICDRS comprises eight key genes (IL32, AHNAK, ANXA5, FN1, GSN, CNN3, FXYD3, CTSS) with diverse biological functions in the tumor microenvironment. CTSS, ANXA5, GSN, AHNAK and IL-32 have established roles in ICD: CTSS inhibition has non-redundant therapeutic potential to enhance anti-tumor immune responses (68). ANXA5 acts as an immunostimulatory agent to render apoptotic tumor cells immunogenic and induce tumor regression (69, 70). Galluzzi et al. proved that cells can avoid ICD by secreting large amounts of GSN (8). IL-32 has the metastasis-promoting effect in BLCA (71). And FXYD3 is an unfavorable prognostic biomarker associated with hypoxia, pro-tumor TILs, and T cell exhaustion (72). FN1 and CNN3 are genes encoding extracellular matrix and cytoskeleton-related proteins (73). Analysis revealed that eight characteristic genes were primarily expressed in bladder epithelial cells, macrophages, monocytes, cancer cells, fibroblasts, and T cells, illustrating the complex roles of ICDRS in tumor development and immune regulation. Specifically, gene expressions in bladder epithelial cells and cancer cells may be directly related to tumor oncogenesis. M1 macrophages exhibit high anti-tumor activity, whereas M2 macrophages may suppress immune responses and support tumor growth and metastasis (74). Monocytes are crucial for initiating and maintaining anti-tumor immunity, while TME can alter their differentiation and function, sometimes promoting tumor survival and immune evasion (75, 76). Fibroblasts play a significant role in tumor fibrosis, intercellular signaling, and maintaining tumor structure (77, 78). T cells can directly recognize and kill tumor cells or enhance the attack against tumors (79). Additionally, immune cell infiltration analysis showed that in the high ICDRS group, cell types such as T cells CD4 memory resting and various macrophages were more abundant, whereas the low ICDRS group was enriched with activated immune effector cells like T cells CD8, T cell follicular helper, T cells regulatory, and activated dendritic cells.

ICD and ICDRS scores are closely related, and while tumors with high ICDRS scores would theoretically exhibit strong immune cell infiltration, this does not necessarily imply effective immune-mediated tumor control. In fact, tumors with high ICDRS may evade immune surveillance by promoting an immunosuppressive microenvironment, reflecting a poorer prognosis. For example, the enrichment of M2 macrophages creates an immunosuppressive environment that promotes chemotherapy resistance by enhancing tumor cell survival and DNA damage repair mechanisms, with previous studies showing that M2 macrophage abundance is significantly positively correlated with BLCA progression and metastasis (80, 81). Cell-cell interaction analysis demonstrated reduced effector T cell-tumor cell engagement via diminished co-stimulatory signaling and enhanced inhibitory checkpoint interactions in high ICDRS tumors, providing a

mechanistic basis for immune evasion despite the presence of immune cells. In contrast, tumors with low ICDRS scores may be more likely to stimulate effective cytotoxic T cell responses, suggesting a more favorable immune environment and better prognosis. These findings indicate that the specific conditions of the TME in BLCA play a crucial role in determining the prognostic value of ICDRS, pointing to the need for more personalized treatment strategies for BLCA patients with high ICDRS scores to optimize their prognosis. In summary, these research results not only provide new insights into the mechanisms of BLCA progression but also offer a scientific basis for developing more targeted immune-mediated treatment strategies.

The potential clinical application of ICDRS in BLCA management could significantly enhance current treatment. At initial diagnosis, ICDRS could complement conventional risk stratification, potentially identifying high-risk patients who might benefit from earlier aggressive intervention or patients requiring intensified neoadjuvant approaches. Our drug sensitivity findings suggest ICDRS could guide therapy selection, directing high-risk patients (showing resistance to conventional chemotherapeutics) toward alternative treatments like immunotherapy or targeted agents. Additionally, ICDRS could serve as a biomarker for monitoring treatment response, with changes during therapy potentially indicating resistance development. From an implementation perspective, the eight-gene signature could be assessed using RT-PCR or targeted RNA sequencing, making it feasible for integration into clinical testing workflows alongside established risk factors to guide treatment decisions throughout the BLCA care continuum.

Although this study utilized multi-omics datasets for a comprehensive analysis and explored various aspects such as clinical features, immune infiltration, and TME, there are still significant limitations to address. Firstly, despite our attempts to validate the model using external datasets, batch effects between datasets and differences in gene numbers led to significant heterogeneity across datasets. This heterogeneity not only complicates model validation but may also impact the consistency and accuracy of the model across different datasets. Additionally, since the samples might predominantly originate from specific clinical settings or geographic locations, this limits the broad applicability and generalizability of the study results. Secondly, in terms of technical choices, although Lasso and Ridge regularization models were used to reduce overfitting, these models still have limited capabilities in handling extreme data and capturing non-linear relationships. Furthermore, the selection of regularization strength is a challenge in itself and could introduce additional biases. Although we conducted gene function enrichment and pathway analysis, these analyses might not be sufficient to fully reveal the complex biological mechanisms and pathways involved. From a clinical application perspective, although our model has been statistically validated for effectiveness, its real-world application might face several challenges such as patient acceptance, treatment costs, and operational complexity. Additionally, while drug sensitivity prediction offers potential for personalized treatment, the impact of experimental conditions, drug dosages, and individual biological variations may cause fluctuations in prediction outcomes.

In summary, future efforts should focus on enhancing the generalizability of the model by incorporating samples from a broader range of ethnicities and regions to verify the model's applicability. Additionally, further research should aim to integrate more data at the biological level to strengthen the model's biological interpretability and to explore the challenges and solutions that may arise in actual clinical applications. These efforts will help to increase the practical impact and scientific value of the research.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Author contributions

HY: Conceptualization, Data curation, Formal Analysis, Methodology, Project administration, Resources, Supervision, Visualization, Writing – original draft. XJ: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Resources, Software, Visualization, Writing – review & editing. BL: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

Publisher's note

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