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Anti-tumor activity of cytotoxic immune cells: Basic research and clinical perspectives

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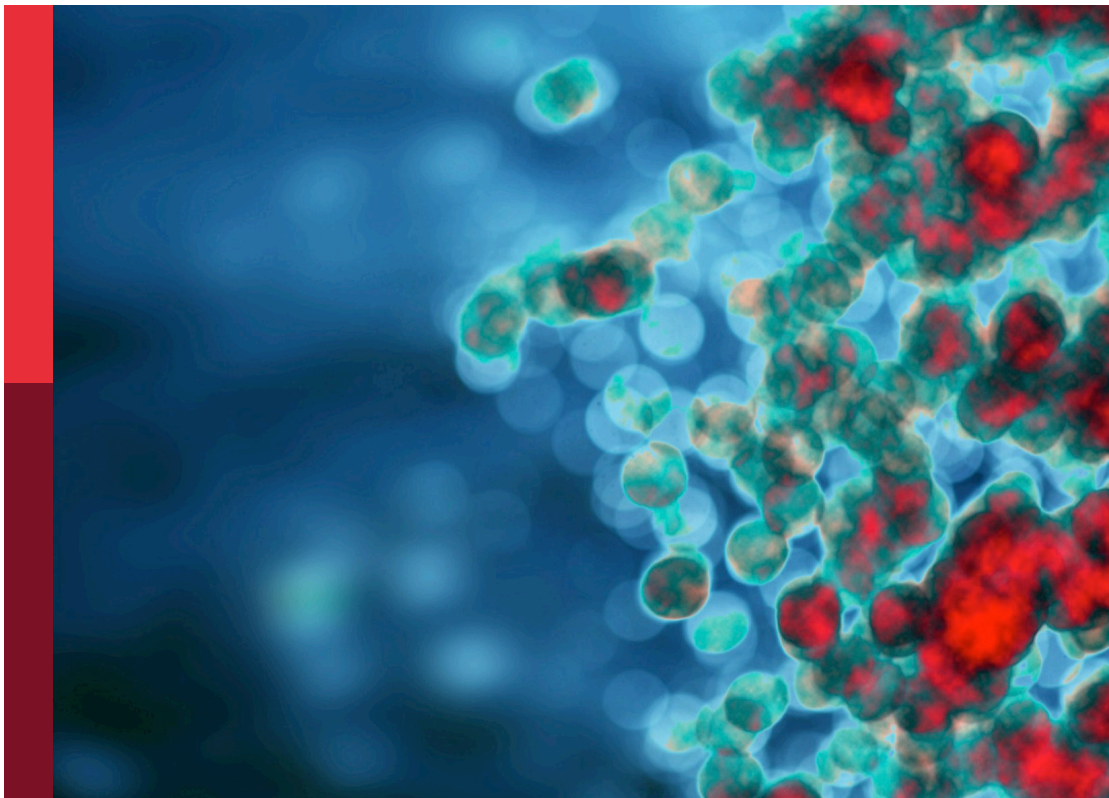

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Anti-tumor activity of cytotoxic immune cells: Basic research and clinical perspectives

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Editorial: Anti-tumor activity of cytotoxic immune cells: basic research and clinical perspectives

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KEYWORDS

cancer immunotherapy, T cell, NK cell, cytotoxic cell, CAR-T cell, monoclonal antibody, TME (tumor microenvironment), clinical trial

Editorial on the Research Topic
Anti-tumor activity of cytotoxic immune cells: basic research and clinical perspectives

Immunotherapy has become an important strategy for the treatment of cancers. Currently available cancer immunotherapies include monoclonal antibodies, bispecific T cell engagers, tumor vaccines, T cells modified with chimeric antigen receptors (CAR-T), and immune checkpoint inhibitors (ICIs). In the vast majority of cases, current immunotherapy is based on the engagement of cytotoxic cells. This Research Topic explores T cell activity regulation and the use of cytotoxic T cell and NK cell-based methods in cancer treatment through a compilation of experimental and review articles. It covers studies on pathways governing T cell activation, the mechanisms orchestrating inter and intra-cellular communication among T cells and the tumor microenvironment (TME), and the prognostic significance of distinct T cell subpopulations across diverse cancer types. Additionally, the effective utilization of cytotoxic T cells and NK cells for cancer immunotherapy, specifically by leveraging monoclonal antibodies, ICIs, and CAR-T cells is discussed. Furthermore, insights derived from clinical trials evaluating the efficacy of cancer immunotherapies are presented. These contributions furnish important perspectives to the dynamic landscape of cancer treatment.

Three articles focus on the functional aspects of T cell activity. Firstly, Monticone et al. present an original paper introducing a novel immunosuppressive pathway within T cells. This pathway, involving adenosine receptor A2AR, Cbl-b, and Notch1, acts as a distinct immune checkpoint in the TME, modulating T cell responses. The authors demonstrate that enhancing Notch1 signaling, by impeding Cbl-b-mediated degradation, substantially boosts anti-cancer T cell responses, providing an innovative immunotherapeutic strategy with potential selectivity for T cells over cancer cells. The review article by Zhou et al. focuses on T cell-derived exosomes. It explores the dual effects of CD8 and CD4 T cell-derived exosomes on tumor progression, with insights into modifying exosome surfaces for therapeutic intervention. The role of regulatory T cells (Tregs)-derived exosomes in tumor immune escape is also discussed, suggesting novel cancer immunotherapy through targeting of Treg-derived exosomes. The review also points to engineered T cell-derived exosomes as a potential drug delivery system with high stability and

low immunogenicity. Finally, the article by Moussawy et al. reviews the non-cytotoxic functions of CD8 T cells, including their role in cancer immunotherapy, in particular in anti-tumoral vaccination. Addressing weak immunogenicity of tumoral antigens, the article describes how bystander CD8 T cells enhance the anti-tumoral effect of dendritic cell-based vaccines, suggesting their role as potent adjuvants.

The two subsequent original papers focus on detecting specific tumor-infiltrating lymphocytes (TILs) subpopulations in certain types of cancer, exploring their prognostic functions and discussing their potential impact on immunotherapy efficacy. In Vlaming et al.'s article, a rare CD8 T cell subpopulation expressing CD103/TIM-3/CXCL13 markers was found to be associated with improved survival of epithelial ovarian cancer patients. TIM-3/CD8/CD103-positive T cells can serve as a prognostic marker for epithelial ovarian cancer patients and as a target population for reactivation by immunotherapeutics. The article by Zhou et al. focuses on the quantitative evaluation of Tregs in nasopharyngeal cancer. Tregs play a crucial role in suppressing antitumor immunity in the TME. The study outlines the infiltrating profile and spatial distribution of TILs in nasopharyngeal cancer, examining the prognostic value of TILs composition, spatial architecture, and PDL1 expression on TILs subpopulations in a large cohort of nasopharyngeal cancer patients. Increased infiltration of Tregs, especially PDL1+ Tregs, near tumor cells and CTLs correlates with unfavorable outcomes, highlighting the crucial role of dynamic intercellular interactions between heterogeneous T cell subtypes in disease progression. The findings suggest that PDL1+ Tregs interact with CTLs via the PD1/PDL1 axis, mediating CTL dysfunction and enhancing immune suppression, with implications for future clinical investigations and immunotherapy in nasopharyngeal cancer patients.

Finally, four articles focus on harnessing cytotoxic T and NK cells for cancer immunotherapy. The review article by Dabkowska et al. presents advancements in cancer immunotherapies targeting CD20. It highlights the revolutionary impact of CD20-targeting immunotherapies like rituximab and further discusses novel advancements such as bispecific T cell engagers and CAR-T cells. The article focuses mainly on CD20-targeting immunotherapeutics that are clinically approved or tested in clinical trials. The article by Singh et al. presents promising safety results from a phase 1 clinical trial involving non-virally modified CAR-T cells, employing the sleeping beauty transposon-based approach. This innovative T cell modification strategy, characterized by shortened manufacturing time, demonstrates both safety and cost-effectiveness, offering promising antitumor activity, particularly beneficial in the context of solid tumor immunotherapy. The article by Tomasik et al. provides an in-depth overview of FDA-approved 2nd generation CAR-T cell products and explores the advancements in 3rd and next-generation CAR constructs, summarizing initial results of clinical trials. Although 3rd-generation CAR-T cells, incorporating two costimulatory domains in their CAR constructs, exhibit improved expansion and persistence, their response rates are similar to conventional CAR-T therapies. Ongoing clinical trials investigate various additional approaches, including immune checkpoint modulation, cytokine secretion, safety-switch mechanisms, and genetically edited CAR-T cells. Some of these innovative solutions demonstrate promising potential, achieving response rates of up to 100%, however, they still await evaluation in bigger patient cohorts. Additionally, CRISPR-KO technologies contribute to the development of off-the-shelf CAR-T cells by knocking out TCR and MHC molecules, offering cost and time-saving advantages. Ongoing trials will evaluate their efficacy, safety, and potential concerns, such as susceptibility to natural killer cell-mediated cytotoxicity. As the authors suggest, the strategic combination of efficacy enhancers with safety switches emerges as a rational approach, pending in-depth analysis and comprehensive trial results. Finally, a comprehensive review article by He and Hu focuses on the progress in utilizing ICIs for small-cell lung cancer. ICIs monotherapy and combination therapy have become established as standard options for small-cell lung cancer patients, with ongoing research aiming to further improve ICI immunotherapy by investigating novel combination strategies involving chemotherapeutics and radiation treatment. The article addresses current limitations and explores prospects for future developments, marking significant progress in both first- and third-line treatments for small-cell lung cancer patients.

In conclusion, the research outlined in these articles underscores the dynamic progress and potential avenues within cytotoxic cell-based cancer immunotherapies.

Author contributions

MF: Conceptualization, Writing – original draft, Writing – review & editing. MW: 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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Targeting the Cbl-b-Notch1 axis as a novel immunotherapeutic strategy to boost CD8+ T-cell responses

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A critical feature of cancer is the ability to induce immunosuppression and evade immune responses. Tumor-induced immunosuppression diminishes the effectiveness of endogenous immune responses and decreases the efficacy of cancer immunotherapy. In this study, we describe a new immunosuppressive pathway in which adenosine promotes Casitas B-lineage lymphoma b (Cbl-b)-mediated Notch1 degradation, causing suppression of CD8+ T-cells effector functions. Genetic knockout and pharmacological inhibition of Cbl-b prevents Notch1 degradation in response to adenosine and reactivates its signaling. Reactivation of Notch1 results in enhanced CD8+ T-cell effector functions, anti-cancer response and resistance to immunosuppression. Our work provides evidence that targeting the Cbl-b-Notch1 axis is a novel promising strategy for cancer immunotherapy.

KEYWORDS

adenosine, Cbl-b, immunotherapy, immunosuppression, Notch1

Introduction

Tumor-induced immunosuppression is a hallmark feature of cancer which allows tumors to evade immune surveillance and progress (1). This is a major challenge for endogenous anti-cancer immune responses as well as for the successful application of cancer immunotherapies (2). Therefore, there is a critical need for novel cancer immunotherapies that can not only boost the immune response, but also overcome tumor-induced immunosuppression.

Tumors achieve immunosuppression in different ways, including production of immunosuppressive molecules, recruitment of suppressive immune cells and formation of physical barriers to immune infiltration (3). Among these strategies, overproduction of adenosine, an ATP metabolite, plays a major part in suppressing immune responses in the tumor microenvironment (4). Adenosine modulates the immune response by activating transmembrane G-protein coupled receptors (GPCRs) expressed on the membrane of immune cells (5, 6). Several studies have shown that adenosine suppresses CD8+ T-cells by activating the adenosine A2A receptor (A2AR) and blocking A2AR with selective antagonists results in enhanced anti-cancer immune responses (4, 7–9). Our group has shown that A2AR activation leads to downregulation of Notch1, a key regulator of T-cell effector functions (7). Notch1 signaling is triggered by T-cell receptor (TCR) activation and modulates T-cell effector functions by regulating proliferation and production of cytokines, including γ-interferon (IFN-γ) and Granzyme B (GNZB) (10–14). In line with the role of Notch1 in stimulating effector T-cell functions, exogenous expression of Notch1 in CD8+ T-cells enhances anti-cancer T-cells responses and render T-cells resistant to immunosuppression in the tumor microenvironment of lung carcinoma and thymoma (15). On the contrary, inhibition of Notch1 activation with gamma-secretase inhibitors (GSI) has been shown to suppress T-cell activation, reduce proliferation and cytokine production (16–18). These findings suggest that Notch1 is a potential target to control tumor-induced immunosuppression.

Notch receptors are heterodimeric transmembrane proteins consisting of a large extracellular domain for ligand binding and a transmembrane domain. Upon binding to ligands presented on adjacent cells, the extracellular subunit dissociates from the transmembrane subunit, which the undergoes proteolytic cleavages mediated by ADAM10 and gamma-secretase, respectively. This releases the Notch intracellular domain (NICD) which will translocate into the cell nucleus, form a complex with co-activators and activate transcription of target genes (19, 20). Notch signaling can also be regulated in a ligand-independent manner (21–23). Ligand-independent endocytic regulation of Notch is of particular importance in *Drosophila* (24–26), as well as, in mammalian T-cells, where TCR activation triggers ligand-independent activation of Notch1 (10, 12, 13). Several studies have reported that different ubiquitin ligases are involved in the ligand-independent regulation of Notch in *Drosophila* (24, 26, 27). However, it is yet unknown which and how ubiquitin ligases are involved in regulating Notch1 in T-cells.

Casitas B-lineage lymphoma b (Cbl-b) is an E3 ubiquitin ligase that has been identified as an important negative regulator of TCR signaling cascade. Cbl-b controls the threshold of T-cell activation and T-cell anergy (28, 29). Cbl-b has been implicated in the degradation of surface receptors in coordination with the Tyr-phosphatase Suppressor of T-cell receptor signaling 1 (STS-1 or UBASH3B), another negative regulator of TCR signaling (30–32). Cbl-b deficiency in T-cells has been associated with enhanced T-cell function and anti-tumor potential. Indeed, Cbl-b deficient T-cells have a lower activation threshold and can be stimulated in the absence of CD28 co-stimulation (29). Mice lacking Cbl-b reject tumors because of increased T-cell and NK cell immune responses (33–35) and depletion of Cbl-b inhibits exhaustion in CD8+ T-cells and CAR-T cells (36).

Tumor-induced immunosuppression, including adenosine-mediated suppression of T-cells, poses a major limitation to the efficacy of cancer immunotherapy. To identify new effective immunotherapeutic strategies to overcome tumor-induced immunosuppression, here we investigated the immunosuppressive pathway regulating Notch1 downstream of A2AR and strategies to target this pathway to enhance T-cell anti-tumor responses. Our results identified a new regulatory axis in which adenosine, *via* A2AR, promotes Cbl-b-mediated Notch1 degradation, causing immunosuppression in CD8+ T-cells. We showed that genetic KO and pharmacological inhibition of Cbl-b prevents Notch1 degradation in response to adenosine and reactivates its signaling, thus resulting in enhanced CD8+ T-cell effector functions, anti-cancer response and resistance to immunosuppression. Our findings indicate that the Cbl-b-Notch1 axis is a novel promising target for cancer immunotherapy.

Materials and methods

Mice

C57BL/6 and FVB mice (6-8 week-old) were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in specific pathogen-free conditions in a 12h light/12h dark cycle with food and water available ad libitum. For isolation of CD8+ T-cells, spleens and lymph nodes were aseptically harvested post-euthanasia. For the orthotopic TNBC models 2x10^6 million C0321 or M-WNT cells were injected into the 4th mammary fat pad of C57BL/6 or FVB mice, respectively. For the syngeneic colon adenocarcinoma model, 2.5x10^5 MC-38 were injected subcutaneously in C57BL/6 mice. All experiments

involving mice were approved by the Institutional Animal Care and Use Committee (IACUC) at LSUHSC (New Orleans, LA).

Cell lines

C0321 and M-WNT TNBC cell lines were developed as described in Zhang, (37), Zhang, (38) and Dunlab, (39). MC-38 colon adenocarcinoma cell line and 293T cells were purchased from ATCC (Manassas, VA). C0321 and 293T cells were cultured in DMEM and M-WNT and MC-38 cells in RPMI. Both media were supplemented with 10% fetal bovine serum, 4 mM L-Glutamine, 50 U/ml penicillin and 50 µg streptomycin (Gibco).

Primary CD8+ T-cells

CD8+ T-cells were aseptically isolated from the spleen and lymph nodes of C57BL/6 or FVB (6-8 weeks old) mice using the negative selection Easysep mouse CD8+ T-cell isolation kit (StemCell Technologies) according to the manufacturer's instructions. Spleens and lymph nodes from Cbl-b KO (*Cbl-b* $^{-/-}$) were kindly provided by Dr. J. Chiang [NCI-NIH; (29)] and STS-1/STS-2 double KO (*STS-1* $^{-/-}$*STS-2* $^{-/-}$) by Dr. N. Carpino [Stony Brook University; (31)]. CD8+ T-cells were cultured in RPMI supplemented with 10% fetal bovine serum, 4 mM L-Glutamine, 50 U/ml penicillin, 50 µg streptomycin and 50 µM 2-mercaptoethanol. The cells were activated in plates coated with anti-mouse CD3ϵ and anti-mouse CD28 antibodies (1 µg/ml, BD Biosciences) for up to 72h.

CRISPR-Cas9 Notch1 KO CD8+ T-cells

Mouse pan T-cells were isolated from C57BL/6 mouse spleens and immediately subjected to electroporation. crRNAs targeting the gene of interest were mixed with tracrRNA in 1:1 ratio and annealed at 95°C for 5min. 300pmol of crRNA: tracrRNA (IDT) duplex were combined with 100pmol Cas9 (IDT) and incubated at room temperature for at least 15min to generate Cas9/gRNA RNP. 1-5 x 10^6 mouse T cells were collected by centrifuging at 300g for 5min and re-suspended in 100ul mouse T-cell Nucleofector® Solution (Lonza). Cell suspensions were mixed with Cas9/gRNA RNP and transferred in cuvettes for electroporation with the Nucleofector 2b device (Lonza). Program X-001 was used for electroporation. 500ul Pre-warmed IL-2-containing (10ng/ml) complete T-cell medium (RPMI with 10% FBS, 2 mM GlutaMAX, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 55 µM β-mercaptoethanol, 100 U/ml penicillin,100 µg/ml streptomycin, and 10 mM Hepes) was added immediately into the cuvette after electroporation and cells were gently transferred to a 12-well plate prefilled with 1.5ml pre-warmed complete T-cell medium containing IL-2 in each well and cultured in 37°C with 5% CO2. Mouse T-cells were then activated with anti-CD3/anti-CD28 for 5h after electroporation, in complete T-cell medium supplemented with IL-2. Transfection efficiency was evaluated 3 days post-electroporation and positively edited T-cells were sorted on the basis of surface Notch expression level. Cells were then cultured in complete T-cell medium containing IL-2 and IL-7 (10ng/ml) for a week. We used the following crRNA targeting mouse Notch1, 5'-TTGAGATGCTCCCAGCCAAG-3', which was validated in Sawangarun et al. (40).

Tumor-derived organoids and imaging

Organoids were derived from C0321, M-WNT or MC-38 tumors in FVB and C57BL/6 mice. To establish organoids cultures, tumors were minced and digested at 37°C in FBS-free DMEM/F12 Glutamax containing 1mg/ml type IV collagenase (Gibco). Digested tumors were passed through a 100 µm and a 70 µm strainer to isolate organoids of 70-100 µm in size. The organoids were resuspended in type I rat tail collagen gel (Gibco) and plated in 8-well chambered coverslips (µ-slide 8 well, Ibidi). The organoids cultures were hydrated with DMEM/F12 Glutamax supplemented with 5% FBS, 50 U/ml penicillin and 50 µg streptomycin. Treatments were added directly into the medium after plating the organoids-collagen cultures. Cells in organoids were stained using CellTracker™ Red CMTPX (cytosol, Invitrogen) and Hoechst 33342 (nucleus, BD Biosciences), and dead cells were labelled using a cell membrane impermeable nucleic acid dye, NucGreen™ Dead 488 ReadyProbes™ (Invitrogen). Infiltrating CD8+ T-cells were stained in organoids using rat anti-mouse CD8α PE-conjugated antibody (Clone 53-6.7, BD biosciences). For CD8-Notch1 co-localization experiments, organoids were fixed in 2% formalin, permeabilized in 0.2% Triton and block with 2.5% goat serum and Fc block (BD Biosciences). CD8+ T-cells were stained with rat anti-mouse CD8α PE-conjugated antibody (Clone 53-6.7, BD biosciences) and Notch1 with rabbit anti-Notch1 (D6F11, Cell Signaling Technology) primary antibody, and goat anti-rat Texas Red (Invitrogen), goat anti-rabbit Alexa Fluor 488 (Invitrogen) secondary antibodies, respectively. Cell nuclei were stained using DAPI.

Organoids were imaged at day 0-6 using a BZ-x800 microscope (Keyence) with 4x, 20x or 60x objectives. To measure the size of organoids over time, bright field images of a given organoid were taken at day 0, 4 and 6 of culture using the multi-point tool of BZ-x800, which allows to save a specific position in the culture plate. The area of the organoids was measured using ImageJ. To quantify cell death in organoids, the area positive for dead cell staining was measured and normalized by the total area of organoids using the BZ-x800 analyzer software; or the fluorescence intensity of the dead cell staining was measured and normalized by the area of the

organoids using ImageJ. The same method was used to quantify the infiltration of CD8+ T-cells, by measuring the CD8+ area/fluorescence intensity and normalizing by the total organoid area. To quantify the co-localization between Notch1 and CD8, Notch1 fluorescence intensity was measured within the CD8+ areas of at least three different images per sample using ImageJ. Full co-localization fluorescence intensity was set to a value of 100 and the fluorescence intensity values obtained were expressed in percentages accordingly. Images were processed using the BZ-x800 analyzer software. For clarity, we reduced the threshold intensity of Notch1 staining in organoids to highlight Notch1 staining in CD8+ T-cells, which is more focused and intense compared to the staining in surrounding cancer and stroma cells. The threshold-adjusted images are presented in Figure 6C, whereas the original images are presented in Supplementary Figure 7B.

For flow cytometry analysis, organoids were dissociated into single cells at 37°C in FBS-free DMEM/F12 Glutamax containing 1mg/ml type IV collagenase (Gibco). Cells were stained for viability using the Fixable Viability Stain 780 (FVS780, BD biosciences) for 10min in PBS at RT, surface stained with anti-CD45 (FITC rat anti-mouse, clone 30-F11, BD biosciences), CD3 (BV421 hamster anti-mouse, clone 145-2C11 RUO, BD biosciences), CD8 (BV711 rat anti-mouse, clone 53-6.7 RUO, BD biosciences) in PBS at 4°C, fixed with 2% formaldehyde for 20min at 4°C, permeabilized using the Perm/Wash buffer of the Transcription Factor Buffer Set (BD biosciences) and intracellularly stained with anti-IFN-gamma (PE-CF594 rat anti-mouse, clone XMG1.2 RUO, BD biosciences). Flow cytometric analysis was performed using Gallios cytometer (Beckman Coulter). Results were analyzed using Kaluza software (Beckman Coulter) and CD8+ IFNg+ cells were identified by gating for live cells, CD45+ and CD3+.

Compounds and potency studies

A2AR agonist, CGS-21680, and antagonist, ZM-241385, were purchased from Tocris. Cbl-b inhibitors, NTX-512, NTX-447 and NTX-307, were produced by Nimbus Therapeutics. TR-FRET was used to assess the potency of the compounds. Briefly, recombinant human Cbl-b (aa 36-427) was expressed in *E. coli*, purified and biotinylated *in vitro*. Recombinant human Src (aa 254-536)-Zap-70 (aa 281-297) fusion protein was expressed in *E. coli* and purified. Recombinant human UBE2D2(C85K) was expressed in *E. coli*, purified, ubiquitinated and BODIPY labelled *in vitro*. The compounds were dissolved in DMSO (typically at 10-20mM), and a ten-point half log dilution series was prepared using acoustic dispensing. The assay was performed by adding Cbl-b enzyme and Src-Zap/ATP (enzyme reaction) in the presence of UBE2D2(C85K)-Ub-FL-BODIPY, Streptavidin-Tb (binding reaction). The assay signal was measured at 520nm on an Envision plate reader, with reference signal at 620nm. Data was normalized using high and low assay controls: % Inhibition =100-(100*((high control) - unknown)/(high control - low control)). A 4-parameter dose-response equation was used to fit the normalized dose-response data and derive an IC50 for the compounds.

Constructs and transfection

The constructs used were as follows: all HA-tagged Cbl-b constructs were a gift from Dr. Stanley Lipkowitz (41, 42) while the His-tagged ubiquitin construct was from Dr. Dirk Bohman (43). NTM and all Myc-tagged Notch1 deletion constructs have been previously described (44, 45). Lipofectamine 3000 (Invitrogen) was used to transfect 293T cells following the manufacturer's instructions.

Luciferase reporter assay

Luciferase assays were performed using the Dual Luciferase assay kit (Promega). 293T cells were transfected with Notch and Cbl-b constructs as indicated. All cells received a Hes-luciferase construct and a Renilla luciferase expression vector (pRL-CMV). Cells were cultured for 48 hours, harvested and lysates were made as per the manufacturer's protocol. Reporter gene transcription was measured using a luminometer.

Immunoprecipitation and western blot

Primary CD8+ T-cells were lysed in RIPA lysis buffer (Santa Cruz Biotechnology) for western blot or in Pierce IP lysis buffer (Thermo Fisher) for IP, supplemented with 1 mM Protease Inhibitor Cocktail (Thermo Fisher), 1 mM PMSF and 1 mM sodium orthovanadate. For IP, lysates were pre-cleared using Pierce Protein A/G Magnetic beads (Thermo Fisher) for 1 hour. Pre-cleared lysates were incubated over night at 4°C with end-over-end rotation with 2 µg of anti-Notch1 (D1E11, Cell Signaling) or anti-Cbl-b (G1, Santa Cruz Biotechnology) or rabbit IgG control antibody (Thermo Fisher). Pierce Protein A/G Magnetic beads (Thermo Fisher) were added and incubated for 1 hour at room temperature with end-over-end rotation. The beads were washed three times with lysis buffer and two times with deionized water at RT, resuspended in 2X Laemmli sample buffer (Biorad) and incubated at 95°C for 5 minutes to elute the immunoprecipitated proteins. For mass spectrometry, IP samples were prepared using Pierce MS-compatible Magnetic IP kit (Thermo Fisher) according to the manufacturer's instructions.

293T cells were transfected with constructs as indicated, cultured for 48 hours and lysates were prepared using a lysis buffer containing 50 mM Hepes (pH 7.8), 1% NP-40, 250 mM NaCl, Pefabloc (0.125 mM, Fluka) and approximately 1 mg of protein was used per IP. For the ubiquitination assay, the

proteosomal inhibitor lactacystin (20 μM, Kamiya Biomedical Corp.) was added to the culture media for 8 hours before preparing protein lysates with the above lysis buffer supplemented with N-Ethylmaleimide (25mM, Sigma). Lysates were pre-cleared using Protein G Sepharose beads (GE Healthcare) for 1 hour at 4°C and, depending upon the experiment, 4-6 μg of anti-Notch1 (C-20, Santa Cruz Biotechnology), anti-HA tag (Cell Signaling) or rabbit IgG control antibody (Santa Cruz Biotechnology) was added to pre-cleared lysates. After incubating on ice for 2 hours, 30 μl of Protein G Sepharose beads were added per tube and incubated for 1 hour at 4°C with end-over-end rotation to pull down antigen-antibody complexes. Beads were washed thoroughly with lysis buffer at RT, resuspended in 2X Laemmli sample buffer (Biorad) and incubated at 95°C for 5 minutes to elute the immunoprecipitated proteins.

For Western blotting, protein lysates were resolved on 4-15% or 7.5% SDS-PAGE gels (Biorad) and transferred on to PVDF membranes (Millipore). Blots were incubated overnight with primary antibody diluted in Intercept blocking buffer (Licor). The next day, blots were incubated with an appropriate HRP-conjugated secondary antibody for chemiluminescence detection, or with IRDye fluorescent secondary antibodies (Licor) for fluorescence detection, for 1 hour at RT. Proteins were visualized by developing the blots with ECL reagent (Biorad) or imaged on an Odyssey scanner (Licor). The following primary antibodies were used for Western blot in this study: anti-Notch1 (D1E11, Cell Signaling, or mN1A, Novus, or C-20, SCBT) for Notch1 full length and cleaved forms; anti-cleaved Notch1 Val1744 (D3B8, Cell Signaling or PA5-99448, Invitrogen-Thermofisher) for NICD; anti-Cbl-b (G1, Santa Cruz Biotechnology or 12781-1-AP, Proteintech); anti-STS-1 (19563-1-AP, Proteintech); anti-β-actin (AC-15, SCBT); anti-phospho-Tyr (PY99, STCB); anti-HA tag (Clone 6E2, Millipore).

Mass spectrometry

Mass spectrometry analyses was performed by Dr. Samuel Mackintosh's team as part of the IDeA National Resource for Quantitative Proteomics Voucher program. The samples were trypsin-digested and analyzed through data independent acquisition (DIA) quantitative proteomic platform in an Orbitrap Exploris 480 mass spectrometer.

ELISA assay

Cytokine production was measured in supernatants of primary CD8+ T-cells activated and treated as explained above, using ELISA kits (Invitrogen) according to the manufacturer's instructions.

Proliferation assay

Proliferation was measured by labelling primary CD8+ T-cells with 1 μM carboxyfluorescein diacetate succinimidyl ester (CFSE, Thermo Fisher) for 10 min at 37°C before activation and treatments. CFSE fluorescence was measured by flow cytometric analysis on a Gallios cytometer (Beckman Coulter) and data analyzed using Kaluza software (Beckman Coulter).

Statistics

Two-tailed unpaired Student's *t*-test was used for pairwise comparisons between two groups and one-way ANOVA with Bonferroni correction for multiple comparisons was used for comparisons between multiple groups. P values ≤ 0.05 were considered significant.

Results

A2AR modulates Notch1 degradation and T-cell functions in CD8+ T-cells

Our previous work showed that A2AR activation by adenosine downregulates Notch1 in CD8+ T-cells (7). However, how A2AR regulates Notch1 is still unknown. In our previous study, we did not observe effects of A2AR activation on Notch1 mRNA (7), suggesting that Notch1 downregulation may occur at the protein level. Therefore, we hypothesized that Notch1 downregulation by A2AR activation was the result of increased protein degradation. To test this idea, we isolated primary CD8+ T-cells from mouse spleens, activated with anti-CD3/CD28, and treated the cells with the selective A2AR agonist CGS-21680 (CGS), which mimics the effect of physiological adenosine and activates A2AR, and the selective A2AR antagonist, ZM-241395 (ZM), which blocks A2AR. We observed that Notch1 full length (N1FL), transmembrane Notch1 (N1TM) as well as the cleaved, transcriptionally active form of Notch1 (NICD) were downregulated in CD8+ T-cells treated with CGS, whereas the A2AR antagonist ZM rescued CGS-mediated downregulation (Figure 1A). To test whether Notch1 downregulation by CGS was the result of increased protein degradation, Notch1 was immunoprecipitated and ubiquitination was detected. We observed that CGS dramatically increased the ubiquitination of Notch1, compared to untreated and ZM-treated CD8+ T-cells (Figure 1B). To confirm that increased ubiquitination was related to more degradation, we treated CD8+ T-cells with the protein synthesis inhibitor Cycloheximide (CHX) and detected Notch1 protein levels at different time points. We found that Notch1 was

decreased more rapidly in CGS treated CD8+ T-cells (Figure 1C and Supplementary Figure 1), further suggesting that A2AR activation promotes Notch1 degradation. Finally, we confirmed that A2AR activation results in immunosuppression. We observed that CGS decreased proliferation and IFN-gamma production, which is a transcriptional target of Notch1 (12, 13), in CD8+ T-cells (Figure 1D).

Several studies have shown that A2AR blockade with selective antagonists results in enhanced T-cell function and anti-tumor activity (4, 8, 9). Consistently, we observed that the A2AR antagonist ZM reverses Notch1 degradation and CGS-induced suppression of T-cell functions (Figures 1A–D), indicating that rescuing Notch1 from degradation promotes activation of T-cell function including proliferation and cytokine secretion. We also asked if restoring Notch1 in CD8+ T-cells, by blocking A2AR, promotes anti-tumor immunity. To test this idea, we generated tumor-derived organoids from a syngeneic Triple-Negative-Breast Cancer (TNBC) model, C0321 (37, 38), treated with ZM or CGS. Tumor-derived organoids are clusters of cells which contain all cell types present in the original tumor, including cancer cells, infiltrating immune cells and stroma cells and are a reliable *ex vivo* system that recapitulates the features of the original tumor and its microenvironment (46, 47). Remarkably, we found that more CD8+ T-cells were positive for Notch1 in ZM-treated organoids than in controls (Figure 2A) and ZM-treated organoids displayed increased IFN-gamma production, higher infiltration and clusters of CD8+ T-cells surrounding cancer cells, instead of the dispersed organization of T-cells observed in control organoids (Figure 2A and Supplementary Figure 2A). This pattern of Notch1 positive CD8+ T-cells associated with increased anti-tumor effect, as ZM treatment significantly suppressed tumor organoid growth and increased cancer cell death compared to control and CGS-treated organoids (Figures 2B, C). In addition, ZM was only effective in organoids derived from immunocompetent mice, but not from immunocompromised athymic Nu/Nu mice, consistent with the finding that the anti-tumor effect of ZM is immune-mediated (Figure 2C and Supplementary Figure 2B; 4, 8, 9). Taken together, our results indicate that A2AR activation suppresses CD8+ T-cells function, at least in part, through promoting Notch1 degradation, and A2AR blockade restores Notch1, T-cell function and anti-tumor potential.

A2AR promotes Cbl-b-mediated Notch1 degradation *via* STS-1

To determine how Notch1 degradation is regulated by A2AR, we analyzed the protein interactome of Notch1 in activated primary CD8+ T-cells treated with CGS or ZM

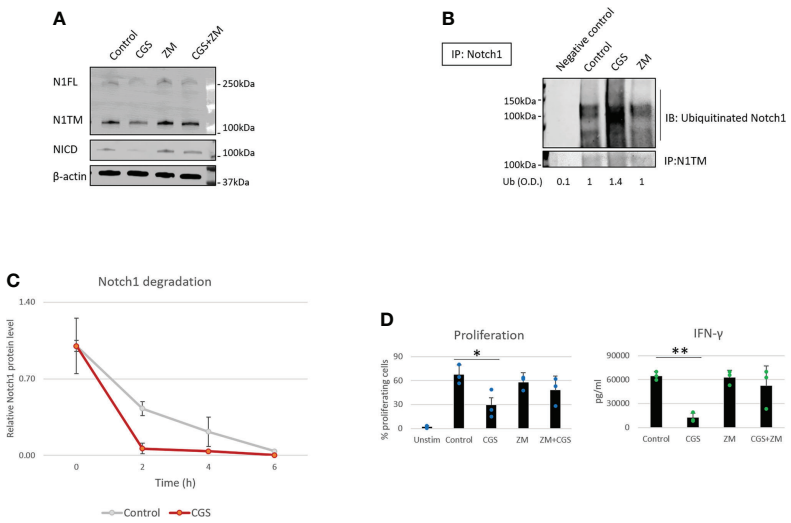

FIGURE 1
A2AR modulates Notch1 degradation and T-cell functions. Primary CD8+ T-cell isolated from the spleen and lymph nodes of C57B16 or FVB mice were stimulated with anti-CD3/CD28 antibodies and treated with vehicle (control, DMSO) or 1μM ZM-241385 (ZM) or 1μM CGS-21680 (CGS) or ZM+CGS, as indicated in the figure. **(A)** Protein levels of Notch1 full length (N1FL), Notch1 transmembrane (N1TM) and Notch1 transcriptionally active form (NICD) in primary activated CD8+ T-cells. **(B)** Ubiquitinated Notch1 protein levels from Notch1 immunoprecipitation in primary activated CD8+ T-cells. Densitometry (O.D.) results for ubiquitinated Notch1 normalized by IP:N1TM are shown below the panel. Negative control refers to samples immunoprecipitated using beads but not antibody. **(C)** Notch1 protein levels over time in primary activated CD8+ T-cells in the presence of the protein synthesis inhibitor Cycloheximide (CHX). The plot shows densitometry results of western blots. **(D)** Proliferation and production of IFN-γ in supernatants from samples of primary activated CD8+ T-cells. The graphs show averages ± standard deviation from three independent experiments. *p < 0.05, **p < 0.01, two tailed T-test with equal variance. Unstimulated cells (Unstim) and vehicle treated cells (control) were used as controls. β-actin was used to normalize densitometry values.

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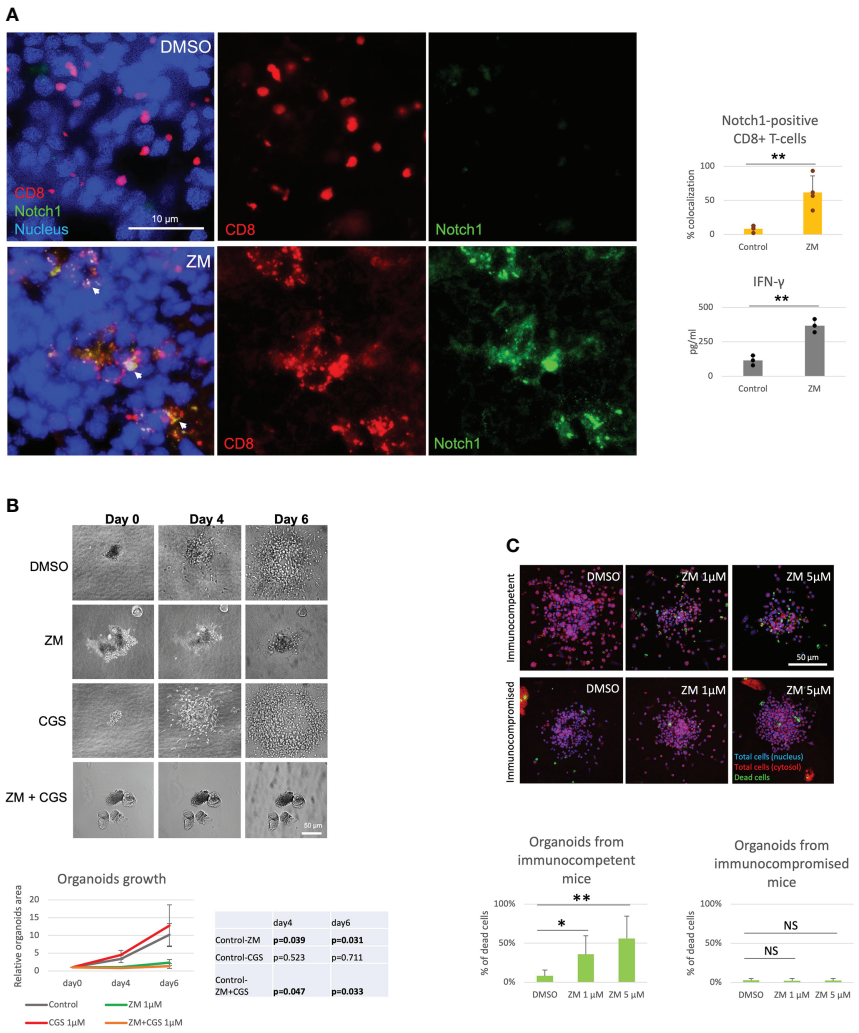

FIGURE 2
A2AR antagonist ZM enhances Notch1-positive T-cell anti-tumor activity. Organoids were derived from a syngeneic TNBC mouse model, C0321, in FVB mice or immunocompromised athymic Nu/Nu mice and treated with vehicle (control, DMSO) or 1µM ZM-241385 (ZM) or 1µM CGS-21680 (CGS) or ZM+CGS, as indicated in the figure. **(A)** Colocalization of Notch1 and CD8+ T-cells and production of IFN-gamma in organoids. **(B)** Growth of organoids measured over time. **(C)** Cancer cell death in organoids from immunocompetent *vs.* immunocompromised mice. Scale bars length is indicated above each bar (µm). The graphs show averages ± standard deviation from ≥ 10 organoids from at least three independent experiments. *p < 0.05, **p < 0.01, two tailed T-test with equal variance. NS, non-significant.

(Figure 3A). From the mass spectrometry analysis STS-1 appeared as the Notch1-interacting protein that was changed the most by the treatments and more importantly, in opposite directions: CGS increased Notch1 interaction with STS-1, whereas ZM decreased this interaction (Figures 3A, B). This finding was of particular interest because STS-1 is a Tyr-phosphatase that acts as an important negative regulator of T-cell activation, but has never been linked with immunosuppression *via* adenosine and A2AR. To test whether STS-1 may control Notch1 degradation in response to A2AR activation, we detected Notch1 in STS-1/2 KO CD8+ T-cells treated with CGS. In line with our hypothesis, we found that Notch1 was not downregulated in STS-1/2 KO T-cells in response to CGS treatment (Figure 3C), indicating that lack of STS-1 prevents A2AR-induced Notch1 degradation. We next asked how STS-1 mediates the degradation of Notch1. Since we found that A2AR modulates the ubiquitination and degradation of Notch1 (Figure 1), we wanted to test whether STS-1 regulates Notch1 in CD8+ T-cells through a ubiquitin ligase. STS-1 is known to interact with ubiquitin ligases of the Cbl-family proteins through its SH3 domain and contribute to the regulation of target proteins (30–32). We analyzed the interactome of Notch1 in activated primary CD8+ T-cells using mass spectrometry (Figure 3D) and found that the Cbl-

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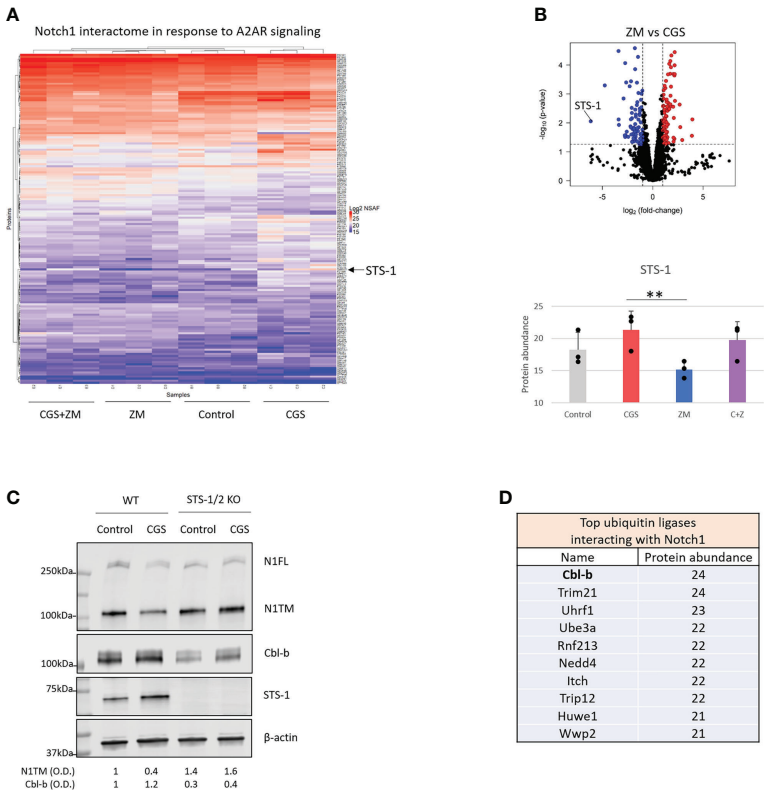

FIGURE 3
A2AR promotes Cbl-b-mediated Notch1 degradation *via* STS-1. Mass spectrometry analysis was carried out in lysates where Notch1 was immunoprecipitated from primary mouse CD8+ T-cells stimulated with anti-CD3/CD28 and treated with vehicle (control, DMSO) or 1μM ZM-241385 (ZM) or 1μM CGS-21680 (CGS) or ZM+CGS, as indicated in the figure. **(A)** Heat map of mass spectrometry analysis of Notch1 interactome. Uniprot IDs of the detected proteins are indicated on the right side of the heat map. Protein abundance is expressed as NSAF log2 fold-change on a color scale from blue to red where blue indicates the lowest and red the highest value, respectively. **(B)** Volcano plot of proteins identified in the mass spectrometry in ZM-treated *vs.* CGS-treated CD8+ T-cells. The X axis indicates fold change (log2) and Y axis the p-value (-log10). The dotted lines indicate the cut-off separating proteins with $p<0.05$ (blue and red dots) from non-significant ones (black dots). Proteins that are interacting the least with Notch1 are shifted to the left of the plot whereas the ones interacting the most are shifted to the right. The bottom graph shows the interaction between Notch1 and STS-1 (protein abundance) from the mass spectrometry analysis. **(C)** Protein levels of Notch1, STS-1 and Cbl-b in primary activated CD8+ T-cells isolated from C57BL6 (WT) or STS-1/2 -/- (STS-1/2 KO) mice and treated with vehicle (control, DMSO) or 1μM CGS. Densitometry (O.D.) results for N1TM and Cbl-b normalized by β-actin are shown below the panel. **(D)** Top 10 ubiquitin ligases interacting with Notch1 ordered by the most to the least interacting based on mass spectrometry results (protein abundance). The graphs show averages ± standard deviation from three independent experiments. **$p<0.01$, two tailed T-test with equal variance.

family protein, Cbl-b, was among the top ubiquitin ligases that interact the most with Notch1. Therefore, we asked whether STS-1 may promote Cbl-b and, in turn, Notch1 degradation. There is evidence of Cbl-b protein stability being positively regulated by Tyr-phosphatases (48) and, consistently, we found that Cbl-b was downregulated in STS-1/2 KO T-cells (Figure 3C), suggesting that STS-1 may stabilize Cbl-b and in turn, promote Cbl-b-mediated Notch1 degradation.

We then asked whether Cbl-b mediates the regulation of Notch1. To confirm the interaction between Notch1 and Cbl-b and map the interacting sites, we set up a series of immunoprecipitation assays using 293T cells transfected with various Myc-tagged Notch1 and HA-tagged Cbl-b constructs (Figures 4A, B). We tested whether Cbl-b could associate with transmembrane Notch1 (N1TM) as well as the cleaved, transcriptionally active form of Notch1 (NICD). Cbl-b was pulled down with both Notch constructs, however, N1TM always showed slightly higher affinity for Cbl-b than NICD at similar expression levels (Figure 4A). Next, we attempted to identify the regions indispensable for Notch1 - Cbl-b interaction. First, 293T cells were transfected with a series of Notch1 constructs in which an increasing amount of the C-terminal region was deleted, along with the HA-tagged full length Cbl-b construct. Among the various deletion mutants used, only Δ2095, a construct that has part of the transcriptional activation domain (TAD) deleted, did not bind Cbl-b (Figure 4A). Reciprocal experiments using HA-tagged Cbl-b deletion mutants revealed that the C-terminal region of Cbl-b is important for binding Notch1 (Figure 4B). Deletion of the protein

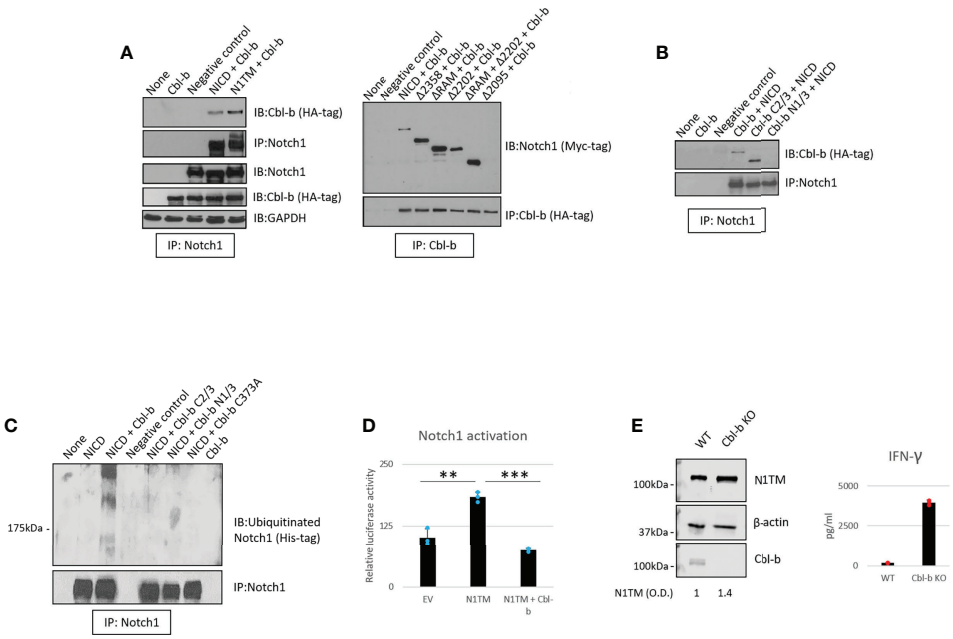

FIGURE 4
Cbl-b ubiquitinates and degrades Notch1. **(A, B)** Notch1 or Cbl-b co-immunoprecipitation in protein lysates from 293T cells were transfected with various Myc-tagged Notch1 and/or HA-tagged Cbl-b constructs, as indicated. **(C)** Ubiquitinated Notch1 protein levels detected upon immunoprecipitation of Notch1 in 293T cells co-transfected with a His-tagged NICD construct and various HA-tagged Cbl-b constructs, as indicated. **(D)** Notch1 activation in 293T cells measured using the Hes-luciferase reporter gene system. **(E)** Protein levels of Notch1 and IFN-gamma production in primary activated CD8+ T-cells isolated from C57BL6 (WT) or Cbl-b -/- (Cbl-b KO). Densitometry (O.D.) results for N1TM normalized by β-actin are shown below the panel. The graphs show averages ± standard deviation from three independent experiments. **p < 0.01, ***p < 0.001 two tailed T-test with equal variance. Negative control refers to samples immunoprecipitated using rabbit IgG instead of anti-Notch1/HA-tag/Cbl-b antibody. None, non-transfected cells. EV, empty vector.

kinase binding domain (TKB) at the N-terminus of Cbl-b in mutant Cbl-b C2/3, did not impair the binding with Notch1, whereas a mutant consisting of TKB only (Cbl-b N1/3) was unable to pull down Notch1 (Figure 4B). Lastly, we confirmed that Cbl-b could be immunoprecipitated with Notch1 and STS-1 in primary CD8+ T-cells (Supplementary Figure 3). These data showed that Cbl-b and Notch1 associate with each other and a region containing part of the TAD domain of NICD and the C-terminal region of Cbl-b are important for this interaction. This is consistent with the observations that other Notch-targeting ubiquitin ligases bind to NICD (24) and Cbl-b interacts with its targets and SH3-domain containing proteins, like STS-1, through its C-terminal region (41, 49).

As Notch1 ubiquitination is increased in CD8+ T-cells in response to A2AR activation (Figure 1), we asked if Cbl-b regulates Notch1 signaling by directly ubiquitinating Notch1 protein. To do this, 293T cells were transfected with a His-tagged ubiquitin construct along with Notch1 and Cbl-b constructs (Figure 4C). We detected the presence of ubiquitinated Notch1 only in the cells that were co-transfected with Notch1 and full-length Cbl-b. N- and C-terminal deletion mutants, as well as a point mutant of Cbl-b (C373A) which is deficient in its E3 ligase activity, were unable to ubiquitinate Notch1 (Figure 4C).

To determine whether Cbl-b-mediated Notch1 ubiquitination leads to Notch1 signaling downregulation, we used a reporter gene assay based on a reporter construct in which the luciferase gene is placed downstream of a target of Notch1, the *Hes* promoter (50). Reporter gene activity was reduced two- to three-fold when Notch1 and Cbl-b were co-transfected in 293T cells, compared to Notch1 alone (Figure 4D), indicating that Cbl-b negatively regulated Notch1 signaling. In agreement with these results, we found that Notch1 was upregulated in Cbl-b KO primary CD8+ T-cells compared to WT cells (Figure 4E) and led to increased IFN-gamma production, suggesting that lack of Cbl-b rescues Notch1 from degradation and restores the transcription of its target genes. Taken together, these results indicate that Cbl-b ubiquitinates and degrades Notch1 and lack of Cbl-b is sufficient to restore Notch1 protein levels and signaling. Our data supports a model in which A2AR controls, *via* STS-1, Cbl-b-mediated Notch1 degradation.

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Genetic KO and pharmacologic inhibition of Cbl-b rescue Notch1 and T-cell functions from A2AR-mediated immunosuppression

Our data place Cbl-b at the core of an immunosuppressive pathway connecting A2AR, Notch1 and T-cell functions. This is also consistent with previous work that showed that Cbl-b is a key negative regulator of T-cell activation (28, 29). Therefore, we asked if genetic KO or pharmacological inhibition of Cbl-b could be a strategy to enhance T-cell functions and counteract tumor-induced immunosuppression by promoting Notch1. We treated CD8+ T-cells from Cbl-b KO mice with CGS and detected Notch1 level and IFN-gamma (Figures 5A, B). In agreement with our hypothesis, we found that CGS did not reduce Notch1 and INF-gamma in Cbl-b KO CD8+ T-cells (Figures 5A, B), confirming that lack of Cbl-b prevents Notch1 downregulation and, in turn, promotes T-cell function. Considering these results, we asked if pharmacologic inhibition of Cbl-b could recapitulate what we observed in Cbl-b KO CD8+ T-cells. To accomplish this, we tested the effect of novel small molecule investigational compounds (NTX, Nimbus Therapeutics), designed to inhibit Cbl-b, in primary CD8+ T-cells. NTX compounds were designed using a structure-guided approach to inhibit Cbl-b enzymatic activity. This structure-based approach ensures exquisite selectivity towards Cbl-b and c-Cbl, another member of the

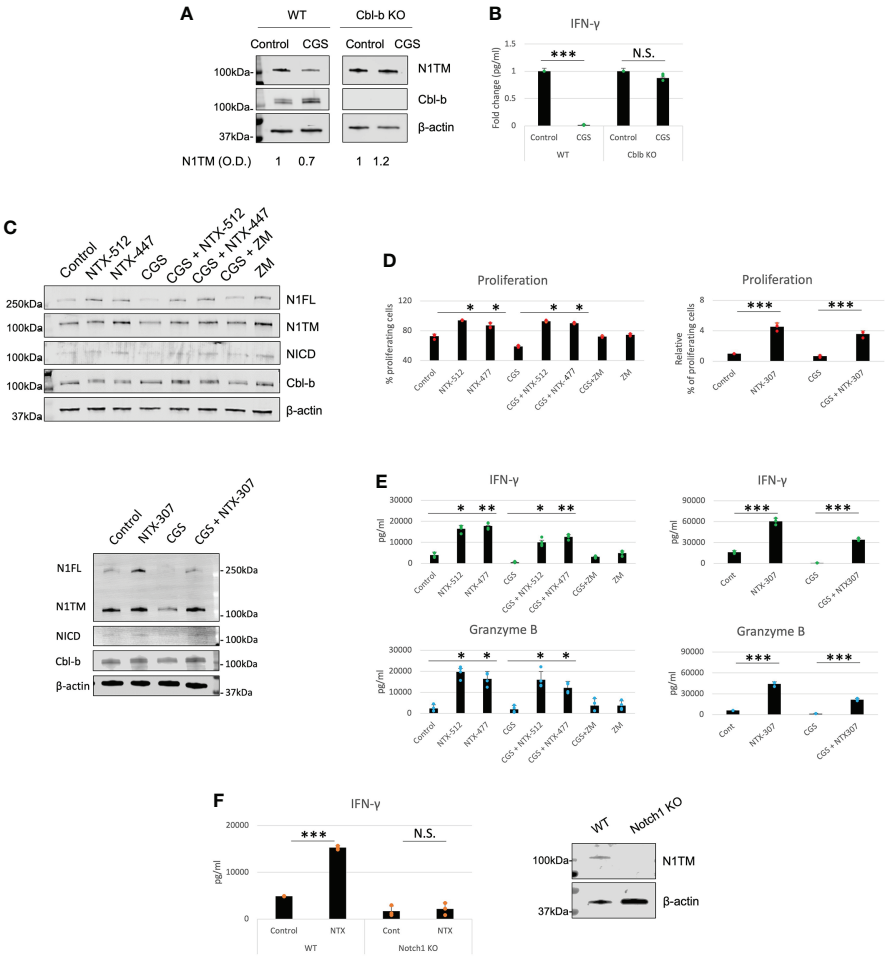

FIGURE 5
Genetic KO and pharmacologic inhibition of Cbl-b rescue Notch1 and T-cell functions from A2AR-mediated immunosuppression. **(A)** Protein levels of Notch1 and **(B)** IFN-gamma production in primary activated CD8+ T-cells isolated from C57BL6 (WT) or Cbl-b -/- (Cbl-b KO). Densitometry (O.D.) results for N1TM normalized by β-actin are shown below the panel. **(C)** Protein levels of Notch1 in primary activated CD8+ T-cells and treated with Cbl-b inhibitors (1μM NTX-512, NTX-447, and NTX-307) or 1μM ZM-241385 (ZM) or 1μM CGS-21680 (CGS) or combinations, as indicated in the figure. **(D)** Proliferation, **(E)** IFN-γ and Granzyme B in primary CD8+ T-cells treated as in **(C)**. **(F)** IFN-γ production in unmodified or CRISPR-Cas9 Notch1 KO primary activated CD8+ T-cells untreated (control) or treated with 1μM NTX-307. The panel in **(F)** shows Notch1 protein levels in unmodified *vs*. Notch1 KO cells. The graphs show averages ± standard deviation from three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001. two tailed T-test with equal variance. N.S, non-significant.

Cbl-family of ubiquitin ligases. In this study, we selected three candidate compounds, NTX-512, NTX-447 and NTX-307, based on potency. The potency of the compounds to inhibit Cbl-b was assessed by time-resolved measurement of fluorescence with fluorescence resonance energy transfer technology (TR-FRET) (Supplementary Figure 4). Consistent with our data in Cbl-b KO T-cells, we found that Cbl-b inhibitors, NTX-512, NTX-447 and NTX-307, rescue both full length and cleaved forms of Notch1 from CGS-mediated downregulation, suggesting that Cbl-b inhibition prevents Notch1 downregulation (Figure 5C). The effect of Cbl-b inhibitors on Notch1 resulted in rescue of proliferation, IFN-gamma and Granzyme B (GNZB) production from CGS-mediated suppression and increased proliferation and production of cytokines compared to vehicle-treated CD8+ T-cells (Figures 5D, E). The compounds showed dose-dependent effect on proliferation and cytokines production and remarkable potency as indicated by low EC50s both as single agents and against CGS (Supplementary Figures 5A, B). Importantly, the compounds did not increase the production of IFN-gamma in unstimulated CD8+ T-cells, indicating that they only boost the function of antigen-stimulated CD8+ T-cells (Supplementary Figure 5C). Finally, Cbl-b inhibitors failed to increase IFN-gamma production in somatic (CRISPR/Cas9-mediated) Notch1 KO primary CD8+ T-cells, suggesting that the Cbl-b inhibitor effect on T-cell function is Notch1-dependent (Figure 5F). These results strongly indicate that Cbl-b inhibition boosts Notch1 and T-cell functions and renders CD8+ T-cells resistant to A2AR-mediated immunosuppression.

Cbl-b inhibitors enhance CD8+ T-cells anti-tumor responses as single-agent and combinatorial immunotherapy with immune-checkpoint inhibitors

Since Cbl-b inhibition enhances T-cell function and resistance to A2AR-mediated-immunosuppression, we next wanted to test whether the effect of Cbl-b inhibition could translate into increased anti-tumor T-cell responses. *In vivo* experiments were not attempted as the pharmacokinetics of the novel inhibitors is still under investigation. Therefore, to answer this question, we treated TNBC C0321 tumor-derived organoids with different concentrations of Cbl-b inhibitors and analyzed several readouts for anti-tumor activity. We found that Cbl-b inhibitors significantly induced cell death in organoids in a dose-dependent manner (Figure 6A). This effect was absent in organoids derived from immunocompromised atymic Nu/Nu mice, suggesting that the anti-cancer activity of the compounds is immunologically mediated (Figure 6A). Concomitantly, we found that Cbl-b inhibitors-treatment increased the production of IFN-gamma in infiltrating CD8+ T-cells and in organoids cultures (Figure 6B, Supplementary Figure 6), a sign of increased CD8+ T-cell activation. Lastly, we labeled CD8+ T-cells and Notch1 in organoids and observed a significant infiltration of Notch1-positive CD8+ T-cells in organoids treated with Cbl-b inhibitors compare to control organoids (Figure 6C). These Notch1-positive T-cells were surrounding cancer cells, possibly establishing immunological synapses for cancer cell killing. We also confirmed that Cbl-b inhibitors did not affect Notch1 in cancer cell lines, including C0321, and cancer cells in organoids (Supplementary Figure 7). Overall, our results indicate that Cbl-b inhibition enhances CD8+ T-cell anti-cancer responses and show potential as single-agent cancer immunotherapy.

Immune-checkpoint inhibitor therapy is FDA-approved for the treatment of certain tumors, including TNBC, but only a limited number of patients benefit from it (51). Therefore, we tested if Cbl-b inhibitors could enhance the efficacy of anti-PD1/PDL1 immunotherapy. We treated organoids derived from two genetically distinct pre-clinical models of TNBC, C0321 and M-WNT (37–39), and a model of colon cancer, MC38, with Cbl-b inhibitors (Figure 7). We choose TNBC and colon cancer models since both tumors are known to have an immunosuppressive microenvironment (52, 53). The compounds were effective in inducing cell death in organoids both as single agents and in combination with anti-PD1/anti-PDL1 (Figure 7). Interestingly, a marked enhancement of anti-tumor activity was seen when Cbl-b inhibitors and anti-PDL1 were used in combination in all models (Figure 7), thus highlighting the possibility that these classes of agents could be used for combinatorial immunotherapy. Our results show that Cbl-b inhibitors, by inducing Notch1-dependent CD8+ T-cells responses, have a promising potential as anti-cancer single-agents and as combinatorial immunotherapy with checkpoint inhibitors. Overall, Cbl-b inhibition represents a new immunotherapeutic strategy that could be exploited to sensitize tumors to anti-cancer T-cell responses and treat tumors that are refractory to immunotherapy.

Discussion

Tumor-induced immunosuppression is a critical feature of cancer that allows evasion from the immune system (1). This is a major challenge for designing effective cancer immunotherapies that circumvent immunosuppression and provide significant response rates.

Our work describes a new regulatory pathway that is critical for tumor-induced immunosuppression in CD8+ T-cells and demonstrates that targeting this pathway is a promising strategy to overcome immunosuppression and enhance anti-cancer responses. We showed that activation of A2AR by adenosine, promotes Cbl-b-mediated Notch1 ubiquitination and degradation. STS-1 Tyr-phosphatase associates with Notch1 in response to A2AR activation and coordinates Cbl-b-mediated Notch1 degradation (Figure 8). Genetic KO of Cbl-b increases Notch1 levels and signaling. Similarly, pharmacological

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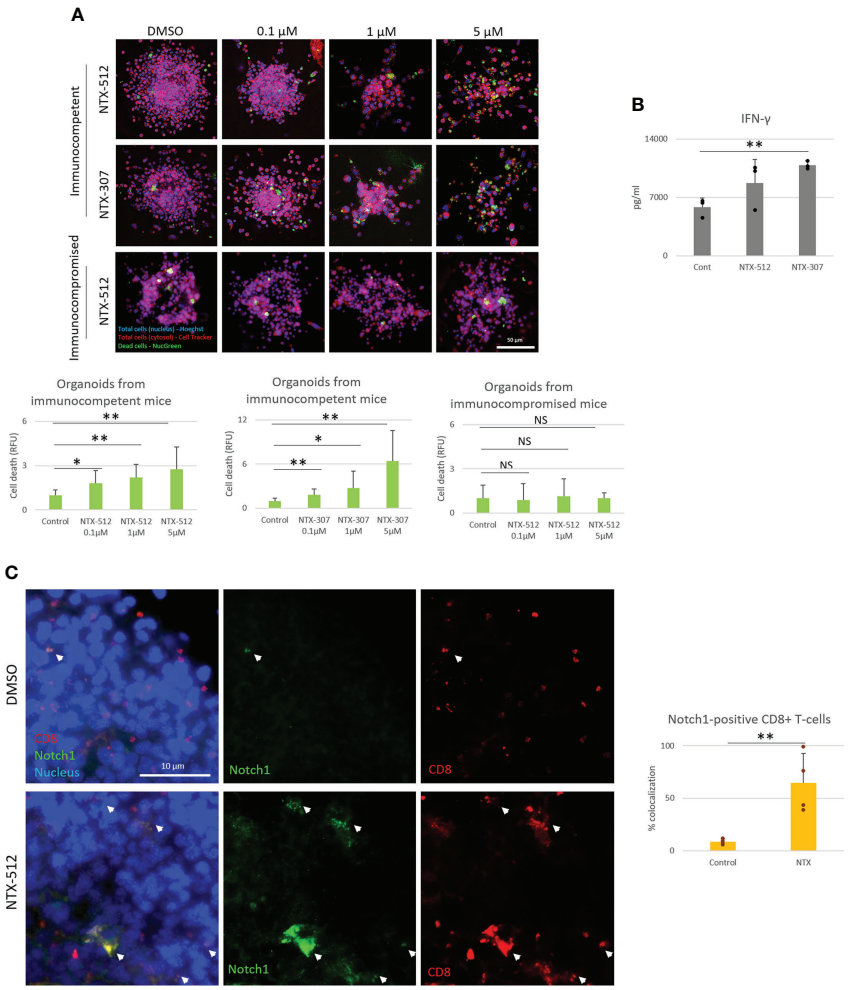

FIGURE 6
Cbl-b inhibitors enhance Notch1-positive CD8+ T-cells anti-tumor responses. Organoids were derived from a syngeneic TNBC mouse model, C0321, in FVB mice or immunocompromised athymic Nu/Nu mice and treated with vehicle (control, DMSO) 0.1, 1 and 5µM of NTX-512 or NTX-307, as indicated in the figure. **(A)** Cancer cell death, **(B)** production of IFN-gamma in organoid cultures and **(C)** colocalization of Notch1 and CD8+ T-cells in organoids indicated with white arrows in the panel and in the plot. Panel **(C)** shows threshold-adjusted images to better highlight Notch1 staining in CD8+ T-cells, whereas the original images are presented in Supplementary Figure 7B. Scale bars length is indicated above each bar (µm). The graphs show averages ± standard deviation from ≥ 10 organoids from at least three independent experiments. *p < 0.05, **p < 0.01, two tailed T-test with equal variance. NS, non-significant.

inhibition of Cbl-b results in increased Notch1, T-cell functions, anti-cancer response and resistance to immunosuppression in TNBC and colon cancer models. We also provided evidence illustrating that combination of Cbl-b inhibition and immune-checkpoint inhibition has enhanced efficacy in the same models.

By identifying the Cbl-b-Notch1 axis in CD8+ T-cells, we showed for the first time a direct link between Cbl-b and adenosine-mediated immunosuppression, and placed Cbl-b and STS-1 at the center of Notch1 regulation in CD8+ T-cells. Our findings, suggest that the Cbl-b-Notch1 axis could represent a new functional immune-checkpoint that dampens T-cell responses in the tumor microenvironment and that blockade of this pathway may be a key strategy to overcome tumor-induced immunosuppression. Future work will need to determine the significance of this pathway in the tumor microenvironment as well as under physiological conditions, and its potential applications for cancer immunotherapy.

Our work supports a model in which both immunosuppressive and activating signals may converge onto the Cbl-b-Notch1 axis and may be translated by Notch1 into transcriptional (and potentially non-transcriptional) signals to regulate T-cell activation (Figure 8). In addition, Cbl-b may mediate a constitutive degradative pathway that is switched on and off depending on whether the cell requires more or less Notch1. In T-cells Notch1 is activated in a ligand-independent manner through endocytosis (10). It is possible that Cbl-b

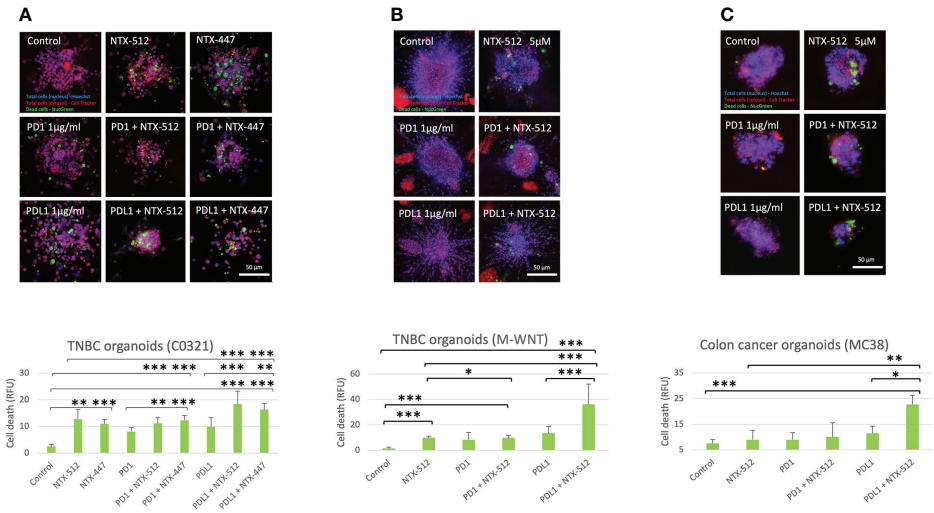

FIGURE 7
Cbl-b inhibitors enhance anti-PD1/PDL1 efficacy in tumor-derived organoids. Organoids were obtained from syngeneic TNBC, **(A)** C0321, **(B)** M-WNT, and colon cancer, **(C)** MC38, models and treated with vehicle (control, DMSO) or 5µM of NTX-512 or NTX-477, alone or in combination with 1µg/ml anti-PD1 or 1µg/ml anti-PDL1. The plots show cancer cell death in organoids. Scale bars length is indicated above each bar (µm). The graphs show averages ± standard deviation from ≥10 organoids from independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA. NS, non-significant.

degradative pathway could be part of the ligand-independent endocytic pathway of Notch1 in T-cells, since endocytic regulation of Notch can either lead to activation or degradation (10, 21, 23). In this endocytic pathway, Notch1 may be directed to degradation by Cbl-b-mediated ubiquitination, whereas other signals may instead direct Notch1 to activation, as observed in other systems (24, 26). This degradation pathway may be controlled in response to extracellular stimuli, including TCR activation and A2AR signaling, which are both known to regulate the level of

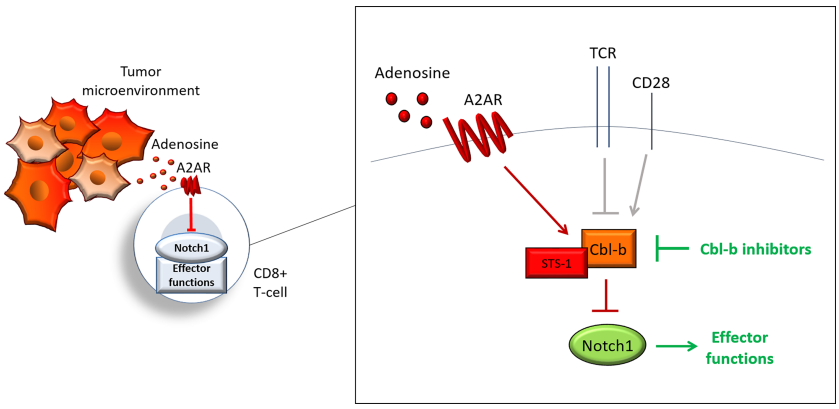

FIGURE 8
Cbl-b-Notch1 axis regulation. Adenosine induces immunosuppression in CD8+ T-cells in the tumor microenvironment by activating the adenosine A2A receptor (A2AR), thus causing downregulation of Notch1 and suppression of effector functions. A2AR controls Notch1 levels by regulating the ubiquitin ligase Casitas B-lineage lymphoma b (Cbl-b), which mediates the ubiquitination and degradation of Notch1. A2AR promotes Cbl-b *via* Suppressor of T-cell receptor signaling 1 (STS-1) Tyr-phosphatase, possibly through Tyr-dephosphorylation of Cbl-b. A2AR, together with other receptors, including T-cell receptor (TCR) and co-stimulation receptor CD28, may control Notch1 levels in T-cells and, in turn, effector functions by modulating Cbl-b. This model places Cbl-b at the core of a regulatory axis, which, by integrating positive and negative signals from different receptors, determines the fate of Notch1 and effector functions. Pharmacological inhibition of Cbl-b blocks this axis and reactivates Notch1 and effector functions.

Notch1 in T-cells (7, 10, 12, 13). For example, upon T-cell activation, signaling downstream of TCR may inhibit Cbl-b to elevate Notch1 and effector functions. Later, to avoid over-activation and exhaustion of T-cells, co-stimulatory signals (e.g. CD28) or immunosuppressive ones (e.g. A2AR) may promotes Cbl-b-mediated degradation of Notch1 and downregulation of effector functions (Figure 8). This would explain at least in part how Cbl-b controls the threshold for T-cell activation (28, 29).

Previous work and our data, suggest that Cbl-b-mediated degradation may be regulated through phosphorylation. Cbl-b is negatively regulated by Tyr-phosphorylation by SHP-1 or Ser/Thr-phosphorylation by PKC-theta in response to CD28 co-stimulation, but dephosphorylated and promoted in response to TCR stimulation (48, 54, 55). Accordingly, our results suggest that Cbl-b may be positively regulated by STS-1, possibly through Tyr-dephosphorylation, and promoted upon A2AR activation. It is very likely that the phosphorylation of different residues or a different phosphorylation status of Cbl-b may increase or decrease Cbl-b function and/or stability. If Cbl-b is controlled by phosphorylation, it is also possible that a Tyr-kinase antagonizes STS-1 function, by phosphorylating and inhibiting Cbl-b. Overall, our work supports the idea that antagonistic signals from TCR and other receptors, like A2AR, control Cbl-b and, in turn, effector functions *via* Notch1, thus regulating the threshold of T-cell activation. This regulation, including the signals it responds to, the function of STS-1 and the possible involvement of Cbl-b in the endocytic trafficking of Notch1, warrant further investigation.

Despite the successful application of immunotherapy, response rates remain limited especially for immunosuppressive solid tumors (51). New immunotherapies that are less sensitive to different forms of tumor-induced immunosuppression could greatly increase response rates among cancer patients. Our work, together with previous studies (7, 15), supports the idea that therapies that reactivate Notch1 in T-cells could be used to tune T-cells against tumor-induced immunosuppression and enhance anti-cancer immune responses (Figure 9). Specifically, we described a new pathway that is amenable to pharmacological targeting and has promising selectivity for Notch1 in T-cells versus cancer cells, a feature that is very important for Notch-targeted therapies (16). By reactivating Notch1 in T-cells we aim to lower the threshold of T-cell activation to make tumor-suppressed T-cells more responsive to tumor antigen recognition when infiltrating the tumor microenvironment. Consistently, we observed that reactivation of Notch1 increased/restored effector functions and primed T-cells to attack cancer cells in tumor-derived organoids. Our results in organoids from TNBC and colon cancer, two tumor types which can suppress immune responses (52, 53), suggest that Notch1 reactivation *via* Cbl-b inhibition could be a promising strategy to sensitize "cold" immunosuppressive tumors to cancer immunotherapy. Another promising application of this strategy, could be adoptive T-cell therapies. Resistance to immunosuppression plays a critical role in these therapies and both Notch1 expression and Cbl-b deletion were found to enhance the efficacy of adoptive T-cell transfer and CAR-T cell therapy (15, 36). Our work presents a new class of candidate immunotherapeutic compounds, Cbl-b inhibitors, that enhance anti-cancer T-cell responses and resistance to tumor-induced immunosuppression. We show that Cbl-b inhibitors are effective as single-agents or in combination with anti-PDL1/PD1 in

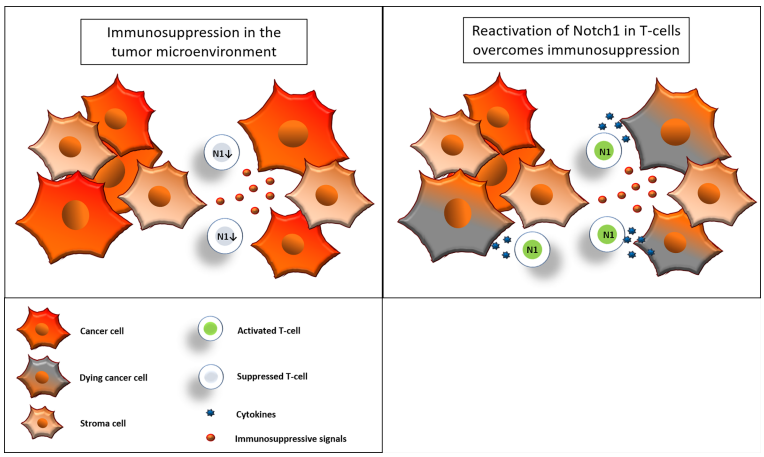

FIGURE 9
Reactivation of Notch1 in T-cells overcomes immunosuppression. Immunosuppressive signals induce suppression in CD8+ T-cells in the tumor microenvironment. Some of these signals, including adenosine, may suppress CD8+ T-cell effector functions by downregulation of Notch1. Reactivation of Notch1, with Cbl-b inhibitors or other strategies, may tune CD8+ T-cells against immunosuppression and enhance effector functions, ultimately promoting anti-cancer responses in the tumor microenvironment.

organoids derived from pre-clinical cancer models, thus these compounds also have the potential to enhance responses to other immunotherapies. The pharmacokinetic and bioavailability of Cbl-b inhibitors is currently under investigation and future work will focus on evaluating the compounds anti-tumor activity *in vivo* in pre-clinical models and their potential clinical development.

Our work described for the first time a critical immunosuppressive pathway linking A2AR, Cbl-b and Notch1, which could represent a new functional immune-checkpoint that modulates T-cell responses in the tumor microenvironment. We showed that promoting Notch1 signaling by blocking Cbl-b-mediated degradation results in a robust increase in anti-cancer T-cell responses and resistance to immunosuppression. Our findings provide evidence that targeting Cbl-b-Notch1 axis represents a promising novel immunotherapeutic strategy to boost anti-cancer T-cell responses and overcome tumor-induced immunosuppression.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at LSU Health Sciences Center - New Orleans.

Author contributions

GM, ZH, LM, CL, BO, and SM contributed to the conception and design of the study. GM, ZH, FC, SL, DC, AC, JA, DU, FH, SI, AB, NC, KX, and SM performed experiments and/or provided key materials. GM wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

Authors FC, SLt, DC, and CL were employed by company Nimbus Theraputics.

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.2022.987298/full#supplementary-material

SUPPLEMENTARY FIGURE 1
A2AR activation increases Notch1 degradation. Notch1 protein levels in primary activated CD8+ T-cells treated with the protein synthesis inhibitor Cycloheximide (CHX) and vehicle (DMSO, control) or 1μM CGS-21680 (CGS), over time.

SUPPLEMENTARY FIGURE 2
CD8+ T-cells infiltration and organoid growth. **(A)** CD8+ T-cells in TNBC C0321 organoids, from immunocompetent FVB mice, treated with vehicle (DMSO) or 1μM ZM-241385 (ZM). **(B)** Growth of organoids, from immunocompromised athymic Nu/Nu mice, treated with vehicle (DMSO) or 1μM ZM-241385 (ZM). Scale bar length is indicated above the bar (μm). The graphs show averages ± standard deviation from ≥4 independent experiments. *p<0.05, two tailed T-test with equal variance. NS, non-significant.

SUPPLEMENTARY FIGURE 3
Co-immunoprecipitation of Cbl-b, Notch1 and STS-1. Immunoprecipitation of Cbl-b and co-detection of Notch1 and STS-1 in primary activated CD8+ T-cells. Negative control refers to samples immunoprecipitated using beads but not antibody.

SUPPLEMENTARY FIGURE 4
Potency studies of NTX compounds. The potency of NTX compounds to inhibit Cbl-b was assessed by time-resolved measurement of fluorescence with fluorescence resonance energy transfer technology (TR-FRET). The figure shows dose-response curves and IC50s calculated using a 4-parameter dose-response equation. The X axis of the graphs

show concentrations (μM) in log10. The Y axis show the responses (Cbl-b inhibition) expressed as percentages. Data was normalized using high and low assay controls: % Inhibition =100-(100*[(high control) - unknown)/(high control - low control)].

SUPPLEMENTARY FIGURE 5
Cbl-b inhibitors show a dose-response effect in activated T-cells and no effect in unstimulated T-cells. Dose-response curves and EC50s of proliferation and production of IFN-γ and Granzyme B (GNZB) in primary activated CD8+ T-cells isolated treated with different concentrations of **(A)** NTX-512 alone or against **(B)** 1μM CGS-21680 (CGS). The X axis of the graphs show concentrations (μM) in log10. The Y axis show the responses (proliferation or cytokine production) expressed as percentages. For clarity, the highest response value was set to 100% and the other values were set accordingly. **(C)** Production of INF-γ in activated or unstimulated primary CD8+ T-cells and treated with 1μM NTX-512 or vehicle-treated (Control, DMSO). The graphs show averages ± standard deviation from three independent experiments. ***p<0.001. two tailed T-test with equal variance. N.S, non-significant.

SUPPLEMENTARY FIGURE 6
Cbl-b inhibitors increase the production of IFN-gamma in infiltrating CD8+ T-cells. Percentage of IFN-gamma+ CD8+ T-cells in organoids untreated (DMSO) or treated with NTX-512. IFN-gamma+ CD8+ T-cells were gated for live cells, CD45+ and CD3+.

SUPPLEMENTARY FIGURE 7
Cbl-b inhibitors do not modify Notch1 in cancer cells. **(A)** Protein levels of Notch1 in TNBC cancer cell lines, C0321, M-WNT, and colon cancer, MC38, treated with vehicle (control, DMSO) or 0.01, 0.1, 1 and 5μM of NTX-512. The graphs show the densitometry analysis of NICD normalized by β-actin. The graphs show averages ± standard deviation from three independent experiments. Two tailed T-test with equal variance identify no significant differences in NICD in the samples. **(B)** Notch1 and CD8+ T-cell staining in C0321 organoids treated with vehicle (control, DMSO) or NTX-512. White arrows indicate co-localization of Notch1 and CD8+ T-cells. This panel show the images presented in without threshold adjustment to show Notch1 staining in surrounding cancer and stroma cells in organoids.

References

1. Hanahan D, Weinberg RA. Hallmarks of cancer: The next generation. *Cell* (2011) 144:646–74. doi: 10.1016/j.cell.2011.02.013

2. Hegde PS, Chen DS. Top 10 challenges in cancer immunotherapy. *Immunity* (2020) 52:17–35. doi: 10.1016/j.immuni.2019.12.011

3. Labani-Motlagh A, Ashja-Mahdavi M, Loskog A. The tumor microenvironment: A milieu hindering and obstructing antitumor immune responses. *Front Immunol* (2020) 11:940. doi: 10.3389/fimmu.2020.00940

4. Vijayan D, Young A, Teng MWL, Smyth MJ. Targeting immunosuppressive adenosine in cancer. *Nature reviews. Cancer* (2017) 17:709–24. doi: 10.1038/nrc.2017.86

5. Ohta A, Sitkovsky M. Role of G-protein-coupled adenosine receptors in dowregulation of inflammation and protection from tissue damage. *Nature* (2001) 414:916–20. doi: 10.1038/414916a

6. Linden J. New insights into the regulation of inflammation by adenosine. *J Clin Invest* (2006) 116:1835–7. doi: 10.1172/JCI29125

7. Sorrentino C, Hossain F, Rodriguez PC, Sierra RA, Pannuti A, Osborne BA, et al. Adenosine A2A receptor stimulation inhibits TCR-induced Notch1 activation in CD8+T-cells. *Front Immunol* (2019) 10:162. doi: 10.3389/fimmu.2019.00162

8. Willingham SB, Ho PY, Hotson A, Hill C, Piccione EC, Hsieh J, et al. A2AR antagonism with CPI-444 induces antitumor responses and augments efficacy to anti-PD-(L)1 and anti-CTLA-4 in preclinical models. *Cancer Immunol Res* (2018) 6:1136–49. doi: 10.1158/2326-6066.CIR-18-0056

9. Ohta A, Gorelik E, Prasad SJ, Ronchese F, Lukashev D, Wong MK, et al. A2A adenosine receptor protects tumors from antitumor T cells. *Proc Natl Acad Sci USA* (2006) 103:13132–7. doi: 10.1073/pnas.0605251103

10. Steinbuck MP, Arakcheeva K, Winandy S. Novel TCR-mediated mechanisms of notch activation and signaling. *J Immunol (Baltimore Md.: 1950)* (2018) 200:997–1007. doi: 10.4049/jimmunol.1700070

11. Dongre A, Surampudi L, Lawlor RG, Fauq AH, Miele L, Golde TE, et al. Non-canonical notch signaling drives activation and differentiation of peripheral CD4(+) T cells. *Front Immunol* (2014) 5:54. doi: 10.3389/fimmu.2014.00054

12. Cho OH, Shin HM, Miele L, Golde TE, Fauq A, Minter LM, et al. Notch regulates cytolytic effector function in CD8+ T cells. *J Immunol (Baltimore Md.: 1950)* (2009) 182:3380–9. doi: 10.4049/jimmunol.0802598

13. Palaga T, Miele L, Golde TE, Osborne BA. TCR-mediated notch signaling regulates proliferation and IFN-gamma production in peripheral T cells. *J Immunol (Baltimore Md.: 1950)* (2003) 171:3019–24. doi: 10.4049/jimmunol.171.6.3019

14. Monticone G, Huang Z, Csibi F, Leit S, Ciccone D, Champhekar A, et al. Targeting cblb-Notch1 axis as a novel immunotherapeutic strategy to boost CD8+ T-cells responses. *Dryad Dataset* (2022). doi: 10.1101/2022.03.15.484378

15. Sierra RA, Thevenot P, Raber PL, Cui Y, Parsons C, Ochoa AC, et al. Rescue of notch-1 signaling in antigen-specific CD8+ T cells overcomes tumor-induced T-cell suppression and enhances immunotherapy in cancer. *Cancer Immunol Res* (2014) 2:800–11. doi: 10.1158/2326-6066.CIR-14-0021

16. Majumder S, Crabtree JS, Golde TE, Minter LM, Osborne BA, Miele L, et al. Targeting notch in oncology: the path forward. *Nat Rev Drug Discovery* (2021) 20:125–44. doi: 10.1038/s41573-020-00091-3

17. Golde TE, Koo EH, Felsenstein KM, Osborne BA, Miele L. γ-secretase inhibitors and modulators. *Biochim Biophys Acta (BBA) - Biomembranes* (2013) 1828:2898–907. doi: 10.1016/j.bbamem.2013.06.005

18. Kopan R, Ilagan MXG. Gamma-secretase: proteasome of the membrane? *Nat Rev Mol Cell Biol* (2004) 5:499–504. doi: 10.1038/nrm1406

19. Bray SJ. Notch signalling in context. *Nat Rev Mol Cell Biol* (2016) 17:722–35. doi: 10.1038/nrm.2016.94

20. Chillakuri CR, Sheppard D, Lea SM, Handford PA. Notch receptor-ligand binding and activation: insights from molecular studies. *Semin Cell Dev Biol* (2012) 23:421–8. doi: 10.1016/j.semcdb.2012.01.009

21. Monticone G, Miele L. Notch pathway: A journey from notching phenotypes to cancer immunotherapy. *Adv Exp Med Biol* (2021) 1287:201–22. doi: 10.1007/978-3-030-55031-8_13

22. Palmer WH, Deng WM. Ligand independent mechanisms of notch activity. *Trends Cell Biol* (2015) 25:697. doi: 10.1016/j.tcb.2015.07.010

23. Baron M. Endocytic routes to notch activation. *Semin Cell Dev Biol* (2012) 23:437–42. doi: 10.1016/j.semcdb.2012.01.008

24. Shimizu H, Woodcock SA, Wilkin MB, Trubenová B, Monk NA, Baron M. Compensatory flux changes within an endocytic trafficking network maintain thermal robustness of notch signaling. *Cell* (2014) 157:1160–74. doi: 10.1016/j.cell.2014.03.050

25. Vaccari T, Rusten TE, Menut L, Nezis IP, Brech A, Stenmark H, et al. Comparative analysis of ESCRT-I, ESCRT-II and ESCRT-III function in drosophila by efficient isolation of ESCRT mutants. *J Cell Sci* (2009) 122:2413–23. doi: 10.1242/jcs.046391

26. Wilkin MB, Carbery AM, Fostier M, Aslam H, Mazaleyrat SL, Higgs J, et al. Regulation of notch endosomal sorting and signaling by drosophila Nedd4 family proteins. *Curr Biol* (2004) 14:2237–44. doi: 10.1016/j.cub.2004.11.030

27. Dalton HE, Denton D, Foot NJ, Ho K, Mills K, Brou C, et al. Drosophila ndfip is a novel regulator of notch signaling. *Cell Death Differentiation* (2011) 18:1150. doi: 10.1038/cdd.2010.130

28. Paolino M, Thien CB, Gruber T, Hinterleitner R, Baier G, Langdon WY, et al. Essential role of E3 ubiquitin ligase activity in cbl-b–regulated T cell functions. *J Immunol* (2011) 186:2138–47. doi: 10.4049/jimmunol.1003390

29. Chiang YJ, Kole HK, Brown K, Naramura M, Fukuhara S, Hu RJ, et al. Cbl-b regulates the CD28 dependence of T-cell activation. *Nature* (2000) 403:216–20. doi: 10.1038/35003235

30. Mikhailik A, Ford B, Keller J, Chen Y, Nassar N, Carpino N. A phosphatase activity of sts-1 contributes to the suppression TCR signaling. *Mol Cell* (2007) 27:486. doi: 10.1016/j.molcel.2007.06.015

31. Carpino N, Turner S, Mekala D, Takahashi Y, Zang H, Geiger TL, et al. Regulation of ZAP-70 activation and TCR signaling by two related proteins, sts-1 and sts-2. *Immunity* (2004) 20:37–46. doi: 10.1016/S1074-7613(03)00351-0

32. Kowanetz K, Crosetto N, Haglund K, Schmidt MHH, Heldin CH, Dikic I. Suppressors of T-cell receptor signaling sts-1 and sts-2 bind to cbl and inhibit endocytosis of receptor tyrosine kinases. *J Biol Chem* (2004) 279:32786–95. doi: 10.1074/jbc.M403759200

33. Paolino M, Choidas A, Wallner S, Pranjic B, Uribesalgo I, Loeser S, et al. The E3 ligase cbl-b and TAM receptors regulate cancer metastasis *via* natural killer cells. *Nature* (2014) 507:508–12. doi: 10.1038/nature12998

34. Chiang JY, Ihn KJ, Hodes R, Gu H. Ablation of cbl-b provides protection against transplanted and spontaneous tumors. *J Clin Invest* (2007) 117:1029. doi: 10.1172/JCI29472

35. Loeser S, Loser K, Bijker MS, Rangachari M, van der Burg SH, Wada T, et al. Spontaneous tumor rejection by cbl-b-deficient CD8+ T cells. *J Exp Med* (2007) 204:879–91. doi: 10.1084/jem.20061699

36. Kumar J, Kumar R, Kumar Singh A, Tsakem EL, Kathania M, Riese MJ, et al. Deletion of cbl-b inhibits CD8 + T-cell exhaustion and promotes CAR T-cell function. *J immunotherapy Cancer* (2021) 9. doi: 10.1136/jitc-2020-001688

37. Zhang S, Chung WC, Miele L, Xu K. Targeting met and notch in the lfng-deficient, met-amplified triple-negative breast cancer. *Cancer Biol Ther* (2014) 15:633. doi: 10.4161/cbt.28180

38. Zhang S, Chung WC, Wu G, Egan SE, Miele L, Xu K. Manic fringe promotes a claudin-low breast cancer phenotype through notch-mediated PIK3CG induction. *Cancer Res* (2015) 75:1936. doi: 10.1158/0008-5472.CAN-14-3303

39. Dunlap SM, Chiao LJ, Nogueira L, Usary J, Perou CM, Varticovski L, et al. Dietary energy balance modulates epithelial-to-Mesenchymal transition and tumor progression in murine claudin-low and basal-like mammary tumor models. *Cancer Prev Res* (2012) 5:930–42. doi: 10.1158/1940-6207.CAPR-12-0034

40. Sawangarun W, Mandasari M, Aida J, Morita KI, Kayamori K, Ikeda T, et al. Loss of Notch1 predisposes oro-esophageal epithelium to tumorigenesis. *Exp Cell Res* (2018) 372(2):129–40. doi: 10.1016/j.yexcr.2018.09.019

41. Ettenberg SA, Magnifico A, Cuello M, Nau MM, Rubinstein YR, Yarden Y, et al. Cbl-b-dependent coordinated degradation of the epidermal growth factor receptor signaling complex*. *J Biol Chem* (2001) 276:27677–84. doi: 10.1074/jbc.M102641200

42. Ettenberg SA, Keane MM, Nau MM, Frankel M, Wang LM, Pierce JH, et al. Cbl-b inhibits epidermal growth factor receptor signaling. *Oncogene* (1999) 18:1855–66. doi: 10.1038/sj.onc.1202499

43. Treier M, Staszewski LM, Bohmann D. Ubiquitin-dependent c-jun degradation *in vivo* is mediated by the delta domain. *Cell* (1994) 78:787–98. doi: 10.1016/S0092-8674(94)90502-9

44. Jeffries S, Capobianco AJ. Neoplastic transformation by notch requires nuclear localization. *Mol Cell Biol* (2000) 20:3928. doi: 10.1128/MCB.20.11.3928-3941.2000

45. Aster JC, Robertson ES, Hasserjian RP, Turner JR, Kieff E, Sklar J. Oncogenic forms of NOTCH1 lacking either the primary binding site for RBP-jkappa or nuclear localization sequences retain the ability to associate with RBP-jkappa and activate transcription. *J Biol Chem* (1997) 272:11336–43. doi: 10.1074/jbc.272.17.11336

46. Jenkins RW, Aref AR, Lizotte PH, Ivanova E, Stinson S, Zhou CW, et al. *Ex vivo* profiling of PD-1 blockade using organotypic tumor spheroids. *Cancer Discovery* (2018) 8:196–215. doi: 10.1158/2159-8290.CD-17-0833

47. Puca L, Bareja R, Prandi D, Shaw R, Benelli M, Karthaus WR, et al. Patient derived organoids to model rare prostate cancer phenotypes. *Nat Commun* (2018) 9:1–10. doi: 10.1038/s41467-018-04495-z

48. Xiao Y, Qiao G, Tang J, Tang R, Guo H, Warwar S, et al. Protein tyrosine phosphatase SHP-1 modulates T cell responses by controlling cbl-b degradation. *J Immunol (Baltimore Md.: 1950)* (2015) 195:4218–27. doi: 10.4049/jimmunol.150120

49. Thien CBF, Wallace YL. C-cbl and cbl-b ubiquitin ligases: substrate diversity and the negative regulation of signalling responses. *Biochem J* (2005) 391:2. doi: 10.1042/BJ20050892

50. Jarriault S, Le Bail O, Hirsinger E, Pourquié O, Logeat F, Strong CF, et al. Delta-1 activation of notch-1 signaling results in HES-1 transactivation. *Mol Cell Biol* (1998) 18:7423–31. doi: 10.1128/MCB.18.12.7423

51. Haslam A, Prasad V. Estimation of the percentage of US patients with cancer who are eligible for and respond to checkpoint inhibitor immunotherapy drugs. *JAMA Network Open* (2019) 2:e192535–e192535. doi: 10.1001/jamanetworkopen.2019.2535

52. Hossain F, Majumder S, David J, Miele L. Precision medicine and triple-negative breast cancer: Current landscape and future directions. *Cancers* (2021) 13:3739. doi: 10.3390/cancers13153739

53. Arora SP, Mahalingam D. Immunotherapy in colorectal cancer: for the select few or all? *J Gastrointestinal Oncol* (2018) 9:170–9. doi: 10.21037/jgo.2017.06.10

54. Gruber T, Hermann-Kleiter N, Hinterleitner R, Fresser F, Schneider R, Gastl G, et al. PKC-theta modulates the strength of T cell responses by targeting cbl-b for ubiquitination and degradation. *Sci Signaling* (2009) 2. doi: 10.1126/scisignal.2000046

55. Zhang J, Bárdos T, Li D, Gál I, Vermes C, Xu J, et al. Cutting edge: regulation of T cell activation threshold by CD28 costimulation through targeting cbl-b for ubiquitination. *J Immunol (Baltimore Md.: 1950)* (2002) 169:2236–40. doi: 10.4049/jimmunol.169.5.2236

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Non-cytotoxic functions of CD8 T cells: "repentance of a serial killer"

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Cytotoxic CD8 T cells (CTLs) are classically described as the "serial killers" of the immune system, where they play a pivotal role in protective immunity against a wide spectrum of pathogens and tumors. Ironically, they are critical drivers of transplant rejection and autoimmune diseases, a scenario very similar to the famous novel "*The strange case of Dr. Jekyll and Mr. Hyde*". Until recently, it has not been well-appreciated whether CTLs can also acquire non-cytotoxic functions in health and disease. Several investigations into this question revealed their non-cytotoxic functions through interactions with various immune and non-immune cells. In this review, we will establish a new classification for CD8 T cell functions including cytotoxic and non-cytotoxic. Further, we will discuss this novel concept and speculate on how these functions could contribute to homeostasis of the immune system as well as immunological responses in transplantation, cancer, and autoimmune diseases.

KEYWORDS

non-cytotoxic, cross-talk, direct-, indirect-, CD8 T cells (CTLs)

1 A brief history of CD8 T cell cytotoxicity

The history of science is full of discoveries which usually begins with the intention to understand a specific phenomenon in a cohort of patients or preclinical disease models-so called "phenomenology"; however, most of the time, it ends with a completely different story. For instance, much of what we know about cell-mediated cytotoxicity was borne out from pioneering *in vitro* studies started in the early 1960s, which investigated graft rejection using animal models. Indeed, the first report demonstrated that cellular antibodies i.e., lymphocytes from canines transplanted with homograft kidney destroy allogeneic targets *in vitro* as observed microscopically (1). Along the same lines, lymphocytes from Balb/c mice allosensitized with C3H cells were shown to target and

induce C3H cytolysis (2). Similarly, thoracic duct or lymph node cells from allosensitized rodents were able to target and kill donor kidney cells *in vitro* (3).

The above-mentioned observations further served as an impetus to define the nature of these cells exerting cytotoxic killing. By the end of 1960s and early 1970s, a series of elegant studies demonstrated that treatment of such populations with Thy1 (CD90), Ly-2 (CD8a), and Ly-3 (CD8b) anti-sera abolished the anti-allogeneic cytotoxicity of mouse cells, suggesting the cytotoxic effect of T cells (4–6). However, the mechanisms of cytotoxicity and T cell specificity were not clear at that time. It was not until D. B. Amos hypothesized that cytotoxicity was a result of a two-step process: (1) specific recognition followed by (2) non-specific cytotoxicity, which implied that there should be a specific T cell receptor for antigen recognition (7). Later, several lines of evidence supported this notion showing that sensitized lymphocytes isolated from allo-immunized mice showed both specificity and cytotoxicity against their targets. In these studies, upon culturing these cells with macrophage monolayers expressing the allo-H2 MHC antigen, the non-adherent cells did not possess a cytotoxic activity while the adsorbed cells showed cytotoxicity when eluted from the monolayer macrophage cells. These data suggested that the cytotoxic cells were adsorbed on the monolayers because they express receptors that could recognize the H2 alloantigen (8–10). In two seminal papers, Zinkernagel and Doherty further refined the specificity of the lymphocyte receptor binding to their target cells showing MHC restriction using LCMV-infected mouse model. They proposed an "altered-self or the one receptor model" where MHC recognition occurs *via* T cell receptor rather than the "two-receptor or intimacy model" in which MHC recognition is a separate event from viral antigen recognition by the T cell receptor (11, 12).

During this period, huge strides had been achieved in understanding CD8 T cell (CTL) biology including the nature of the cytotoxic cells and antigen recognition by receptor; albeit the mechanism(s) involved in cell-mediated killing post-antigen recognition were still enigmatic. It all began with C. Sanderson's observation where the dying target cell showed morphological changes that was distinct from complement-mediated lysis but similar to recently described apoptotic cell death (13). At that time, it has become appreciated that CTLs are able to lyse several targets sequentially- so called "serial killing" (14–16).

Despite their well-documented cytotoxic capabilities, several elegant studies emerged in the past 30 years showing that CTLs are equipped with non-cytotoxic functions as well. These cytotoxic and non-cytotoxic functions can be exerted directly through the killing machinery or indirectly *via* cross-talk with other immune cells and possibly non-immune cells. Hence, we thought to classify CTLs functions into four types: (1) Direct cytotoxicity, (2) Indirect cytotoxicity, (3) Direct non-cytotoxicity, and (4) Indirect non-cytotoxicity (Figure 1).

2 Cytotoxic and non-cytotoxic functions of CD8 T cells

2.1 Direct cytotoxicity: A serial killer with many weapons

One of the cardinal features of CTLs is their potent killing capacity against target cells including virally infected cells as well as tumor cells. They perform these functions directly using a whole arsenal of effector molecules including granzymes, perforin, and FAS/FASL pathway (Figure 2A).

The cytotoxic effect of these molecules was first hinted by the observation of microscopical tubular lesions in target cell membranes (perforated cell membrane) following incubation with cytotoxic T cells or granules isolated from them (17–21). Consequently, a protein homologous to C9 of the complement system was discovered and isolated from these granules known as perforin. Both proteins can polymerize and form the membrane attacking complex resulting in membrane perforation (22–26). Along the same lines, granule exocytosis model was proposed around the same time hypothesizing that granule contents including perforin were released by exocytosis at the synaptic space between CTL and target cell. To draw a cause-and-effect relationship, several studies showed marked decrease in the cytotoxicity of CTLs isolated from perforin knockout mice or transfected with perforin siRNA, implying the importance of such molecule in cytotoxicity (27–29).

However, researchers began to realize that other effector molecules beside perforin could also induce cytotoxicity since CTLs can activate apoptosis as observed microscopically while purified perforin induced necrosis (15, 30). Hence these observations hinted that other effector molecules could work hand-in-hand with perforin resulting in cell-mediated cytotoxicity through granule exocytosis mechanism. One of the strong candidates were serine esterases since cell-mediated cytotoxicity was blocked in the presence of their inhibitors (31, 32). Later on, they were named as granzymes since they could be isolated from granules (33–35). Consequently, perforin-granzyme pathway was considered as one of the major mechanisms of cell-mediated cytotoxicity where perforin opened pores in the target cell membrane facilitating the entry of granzymes into the cytosol including granzyme B (GzmB), which in turn directly initiated apoptosis through activation of Caspase 3 or indirectly through interaction with BH3-interacting domain death agonist (BID) (30, 36, 37).

Although the perforin-granzyme pathway is considered as one of the major pathways that CTLs use in their killing process, it still does not fully account for the CTL killing

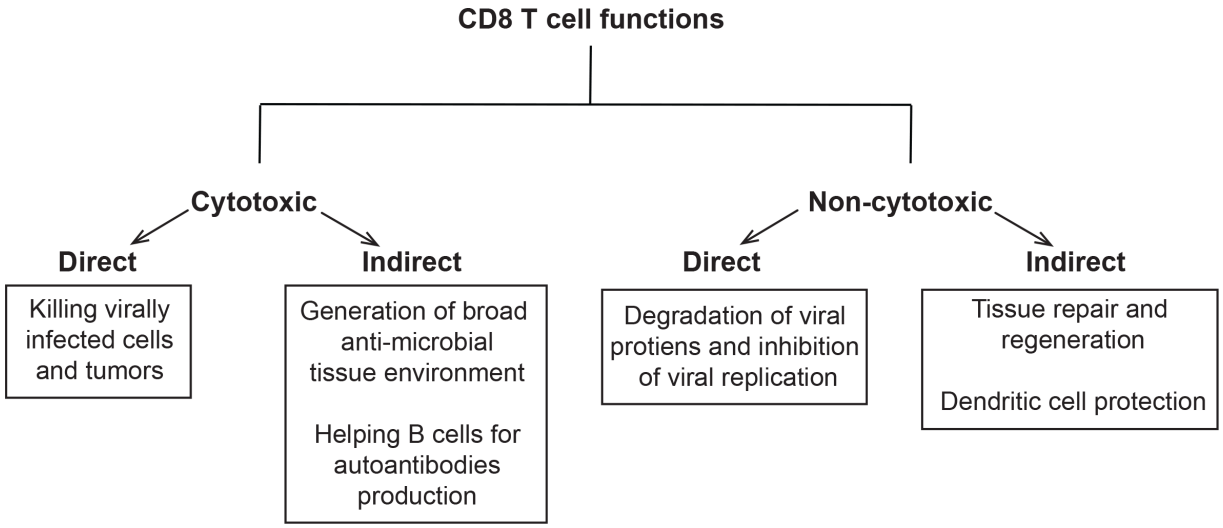

FIGURE 1
Classification of CD8 T cells functions: Schema showing classification of these functions into cytotoxic and non-cytotoxic. The cytotoxic functions were further partitioned into direct (killing tumors or virally infected cells) and indirect (recruitment of immune cells to the site of infection, tumor vaccines, and B cell help in cancer, pathogen clearance and autoimmunity). The non-cytotoxic functions were classified into direct (degradation of viral proteins and inhibition of viral replication) and indirect (tissue repair and regeneration, protection of DCs, and homeostasis).

capacity. For instance, lymphocytes that lack perforin were still cytotoxic. Furthermore, although granule exocytosis requires calcium signaling, CTLs are still capable of killing their targets in a calcium-independent manner (38–40). These observations along with other studies suggested the existence of an alternative cytotoxic killing machinery, leaving the field in a big debate (41). The discovery of the second cytotoxicity pathway started with the generation of T cell hybridoma PC-60-d10S showing calcium independent as well as non-MHC restricted killing capacity specially against thymocytes (42, 43). Around the same period, Nagata's lab reported that thymocytes isolated from wild-type mice expressed CD95 (APO-1/Fas), a known cell death containing domain receptor, while *lpr* mice did not (44) (mice with CD95 mutation leading lymphoproliferation phenotype). Later on, the same lab was successful to clone the ligand using Fas-Fc construct to select and isolate PC60-d10S clones expressing the Fas ligand (FasL/CD95L) using FACS (45). In the FAS-mediated mechanism, the binding of FASL to FAS expressed by the target cell result in activation of Caspase 8 through FAS-associated death domain protein (FADD), which ultimately results in activation of Caspase 3 and induction of apoptosis (46, 47) (Figure 2A). Thus far, the above-mentioned studies demonstrated that CTLs kill their target cell through two main pathways: (1) perforin-granzyme granule exocytosis mechanism and (2) FAS-dependent pathway. However, several studies showed that CTLs can also contribute to the process of cytotoxicity indirectly through cross-talk with other immune cell types. This type of cytotoxicity will be discussed in the following section.

2.2 Indirect cytotoxicity: Calling for help

2.2.1 Tissue-resident broad anti-microbial state

Since the immune system is constituted of multiple cell types, it is expected that different cells cross-talk to each other to perform specific functions. As discussed in the previous section, CTLs can execute their killing functions locally through direct contact with target cells in an MHC-I dependent manner using a wide-spectrum of effector molecules (Figure 2A). However, they should be present in sufficient numbers at peripheral tissues to control the pathogen, which is not the case prior to infections. To circumvent such dilemma, following infection, T cells migrate to non-lymphoid tissues and differentiate into tissue-resident non-circulating memory T cells (T_{RMs}). Both antigen presentation and cytokines are required for the differentiation of T_{RMs}. For instance, in mice, naïve cells require cross-talking to DNGR-1^+ dendritic cells (cDC1, $CD103^+$ $CD8a^+$ DCs) for the generation of T_{RMs} in response to Flu and Vaccinia viruses (48, 49). This type of communication also involves IL-12, IL-15, and CD24 co-stimulation signals as well (50–54). In humans, the cross-talk of $CD1c^+$ DCs with naïve CD8 T cells plays a pivotal role in generation of T_{RMs} in a TGF-β-dependent manner (55). Further, both effector T cells (T_{EFF}) and central memory T cells (T_{CM}) have the capacity to differentiate into T_{RMs} (56). However,

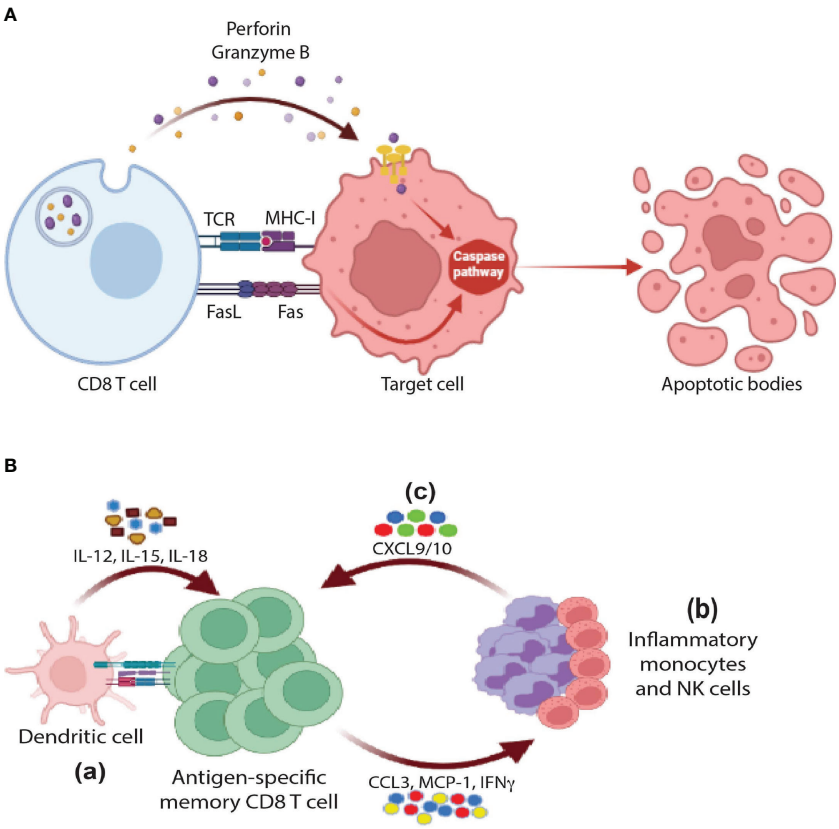

FIGURE 2
Cytotoxic functions of CD8 T cells: **(A)** Direct cytotoxicity: CD8 T cells release perforin and granzyme B through MHC-I/TCR axis in order to activate the apoptotic pathway in the target cells. Further, the interaction between FasR and FasL results in activation of caspases and eventually apoptosis **(B)** Indirect cytotoxicity: (a) In response to activation by antigen re-exposure and cytokine release, antigen-specific memory CD8 T cells release a wide range of cytokines and chemokines such as IFNγ, CCL3, and MCP1 that would help (b) recruit innate cells such monocytes and NK cells that in turn secrete CXCL9/10 in order to further (c) amplify the recruitment of memory CD8 T cells, activate of B cells, and DCs (not shown in the figure).

the question remains: "how such small numbers of T_{RMs} still can control pathogen dissemination".

One way to overcome this challenge is to start communicating with other immune cell types. As shown in Figure 2B, upon antigen and cytokine stimulation, memory T cells rapidly express IFNγ and chemokines promoting recruitment and activation of innate myeloid and lymphoid cells including monocytes and NK cells. These cells further amplify the recruitment of memory cells *via* expression of CXCL9/10 chemokines resulting in the generation of systemic and/or tissue broad anti-microbial state (Figure 2B). The Masopust lab and others spearheaded elegant studies to examine this model (57–61). For example, Schenkel et al. showed that T_{RMs} were able to recruit bystander circulating memory CD8 T cells to peripheral tissues through VCAM-1/IFNγ axis by using an OT-I or P14 chimeric mouse model. In this model, naïve OT-I or P14 CD8 T cells were adoptively transferred to B6 mice followed by VV-OVA or LCMV infection respectively. To activate T_{RMs} at the female reproductive tract (FRT), OVA or LCMV-specific peptides were injected transcervically (t.c). Concurrently, there was an upregulation of VCAM-1 (a4β1-CD49d) on vascular endothelium and IFNγ by reactivated T_{RMs}. These events were associated with recruitment of OT-I specific CD8 T cells (bystander) in response to LCMV infection, which was blocked by neutralization of VCAM-1 or IFNγ. Further, the recruitment of additional immune cell types, including B cells, to FRT as well as the activation of innate cells such as DCs and NK cells were observed (57, 58). Along the same lines, it has been shown by Soudja et al. that memory CD4 and CD8 T cells plays an essential role in orchestrating activation of splenic innate immune cells following secondary infections in an IFNγ dependent manner (60). In conclusion, T_{RMs} can deploy their cytotoxicity indirectly through recruitment of a wide-range of immune cell types acting as guardians of peripheral tissues in case of reinfection.

2.2.2 Tumor vaccines

Indirect cytotoxicity has been further supported by other studies using tumor vaccine mouse models. For example, Kalinski's lab demonstrated that CD8 T cells can act as *de fecto* helper cells supporting an effective anti-tumoral DC immunological response (62). In these studies, they showed that the adoptive transfer of autologous DCs loaded with poorly immunogenic MC38 tumor lysate to the animals bearing the same tumor was only marginally effective. However, the inclusion of OVA257–264 epitope into the vaccine supported the generation of MC38-specific CTL responses in wild-type B6 animals and tumor clearance. These data suggest that non-specific CD8 T cells could play an indirect cytotoxic role through harnessing the killing capacity of antigen-specific T cells *via* unknown mechanism(s). Similar data were also obtained in a model of wild-type mice harboring memory responses against LCMVgp33–41, a dominant epitope of a natural mouse pathogen, where the inclusion of LCMVgp33–41 peptide strongly enhanced the induction of CTLs against MC38 tumors. These vaccines not only elevated CTLs' function against the poorly immunogenic MC38 adenocarcinoma but also against the highly immunogenic OVA-expressing EG7 lymphoma (62). These studies provide a mechanistic insight into the role of memory CD8 T cells in enhancing the anti-tumoral effect, which suggest indirect cytotoxic function. However, additional studies are required to determine which T cells are responsible for the killing of the tumor, is it the tumor-specific, non-specific (bystander), or both?

2.2.3 Helper function towards B cells

The helper function assumed by T cells has been extended to encompass the role of CD8 T cells in inducing antibody production by B cells and their involvement in killing tumors, pathogen clearance and autoimmunity. More than 30 years ago, the Le Gros lab showed that polyclonal stimulation (PMA/Ionomycin + IL-2 + IL-4) of total CD8 T cells isolated from murine lymph nodes (LNs) resulted in (1) down regulation of CD8α, (2) decrease in cytotoxicity, (3) downregulation of IFNγ and perforin, (4) upregulation of T_H2 cytokines IL-4, IL-5, and IL-10, and (5) help for B cells to produce IgG antibodies (63). The authors took their analyses one step further and examined whether this phenomenon was MHC-I restricted. Indeed, stimulating CD8 T cells with MHC allo-antigens in the presence of IL-4 resulted in non-cytolytic phenotype (63). Co-culturing these activated cells with autologous B cells resulted in the secretion of IgG antibodies in the culture supernatant. This early study put CD8 T cells at a crossroad with antibody producing B cells, which underlined a possible indirect cytotoxic role of CD8 cells in the pathogenesis of autoimmunity.

Later on, extensive body of literature discussed the existence of T follicular helper CD4 cells (CD4 Tfh cells) and their role in providing help to B cells for antibody production (64–70). Similar to CXCR5$^+$ PD1$^+$ CD4 Tfh cells, CXCR5$^+$ CD8 T cells exhibit a B cell helper function, where they support antibody production either (1) through a direct interaction with B cells (71–74) or (2) *via* enhancement of CD4 T cell-B cell interaction (75). Indeed, upon TCR stimulation, these cells upregulate CD70, OX40 and ICOS molecules, which are required for T cell dependent humoral responses.

The indirect cytotoxic function of CXCR5$^+$ CD8 T cells in antibody production and B cell support had been demonstrated in various disease states. For example, in gastric cancer, the accumulation of CXCR5$^+$ CD8 T cells in the tumor is associated with better patient overall survival (OS) (76). Similarly, IL-21 producing CXCR5$^+$ CD8 T cells accumulate in the hepatocellular carcinoma tumor tissues in close proximity to CD19$^+$ B cells, which predicts better disease prognosis (71). These studies raise the question: what type of cross-talk is taking place within the tumor microenvironment. One can predict interaction between B cells and CXCR5$^+$ CD8 T cells. Indeed, co-culturing these cells with B cells resulted in enhanced *in vitro* differentiation of B cells as well as an increase in IgG and reduction in IgM production. Hence, the indirect cytotoxic role of CD8 T cells against tumors could be explained by helping B cells to produce antibodies, which in turn bind tumor cells and recruit NK cells to initiate antibody-dependent cell cytotoxicity (ADCC). In another study, CXCR5$^+$ ICOS$^+$ CD8 T cells had been shown to infiltrate tumoral lymph nodes (LNs) in Hodgkin lymphoma (HL) (77). This subpopulation was shown to upregulate the expression of IL-2, IL-4 and IL-21, key cytokines for antibody production and B cell support. However, they showed weak expression of effector molecules including GzmB, perforin, and IFNγ. Similarly, CXCR5$^+$ PD-1$^+$ ICOS$^+$ CD8 T cells isolated from nasal polyp tissue promote antibody production when co-cultured with B cells (78). Along the same lines, the Youngblood lab showed elegantly that HIV-specific CD8 T cells isolated from Elite controllers (ECs) expressed high levels of CXCR5 transcript compared to ART-suppressed non-controllers (79), which suggested the protective role of CXCR5$^+$ CD8 T cell in EC patients. Further, the *in vitro* stimulation of CD8 T cells isolated from ECs with HIV-specific peptides (gag) upregulates CXCR5 (80).

The indirect cytotoxic role of these cells had been further described in autoimmune diseases. For instance, in an autoimmune hemolytic anemia murine model, Valentine et al. demonstrated a significant increase of CXCR5$^+$ PD1$^+$ CD8 and CD4 T cells in secondary lymphoid tissue early during pathogenesis (75). The two subpopulations upregulated ICOS, IL-21 and Bcl-6. However, treating the mice with CD8 and CD4 depleting antibodies resulted in increased survival, improved anemia, reduced B cell survival and decreased anti-erythrocyte IgG autoantibodies, suggesting the pathogenic role of these cells. Thus far, these data support the potential protective indirect cytotoxic function of T cells in the context of tumor development and viral control, while pathogenic in case of autoimmune diseases. Hence, it is important whether to

harness or inhibit these indirect cytotoxic functions in the context of cancer or autoimmunity, respectively.

In line with previous studies highlighting the capability of the transcription factor Stat5 in negatively controlling CD4 Tfh cells and maintaining B cell tolerance (81, 82), Chen et al. demonstrated that the deficiency of Stat5 in CD8 T cells led to an increased autoantibody production in Ig ^{HEL} sHEL transgenic mice. This deficiency resulted in an increase in germinal center B cells and expansion of CXCR5$^+$ PD-1$^+$ CD8 T cell population after an acute viral infection. These data suggest that Stat5 negatively control CXCR5 PD1 CD8 T cell population as well (72). In conclusion, CD8 T cells can provide help to B cell resulting in enhancement of antibody production.

The specific cell surface molecules and cytokines expressed by CD8 T cells involved in B cell support and antibody production had been explored by Shen et al. (74). The authors demonstrated that CD8 T cells that localize to B cell follicles in tonsils and LNs express CXCR5 (74). Further, CXCR5$^+$ CD8 T cells upregulate CD40L and ICOS, while polyclonal stimulation of these cells resulted in increased expression of IFNγ, IL-4 and IL-21 (74). Additionally, co-culturing TCR stimulated CXCR5$^+$ CD8 T cells with autologous B cells resulted in increased production of antibodies, where this phenomenon was completely abolished by blocking either CD40L or IL-21. Finally, Loyal et al. defined a CD40L$^+$ helper CD8 memory subpopulation that expresses IL-6 receptor and lacks the cytotoxicity surface marker SLAMF7 (83). Ironically, this indirect cytotoxic mechanism seems to be a double-edged sword in a way where antibodies can protect against pathogens, or kill tumor cells but also they can precipitate autoimmunity and induce a self-damage.

2.3 Direct non-cytotoxicity: The other face of the serial killer

Besides their known direct and indirect cytotoxic roles in host protection against wide-spectrum of pathogens and tumors, CTLs surprisingly can perform an entire array of non-cytotoxic functions using their effector molecules to protect the host. We classified these novel functions as direct non-cytotoxic since CTLs can still use their effector molecules but to protect the host against viral infections in a non-cytolytic fashion. In this section, we will discuss the studies that address these functions in the context of anti-viral and alloimmune responses.

2.3.1 Anti-viral responses

2.3.1.1 Human immunodeficiency virus (HIV)

The earliest report of CD8 T cell non-cytotoxicity in anti-viral immune response was first described by Waler and colleagues in 1986. In this study, the authors showed that depletion of CD8 T cells from PBMCs *in vitro* culture resulted in an increased production of HIV viral particles (84). Interestingly, this early study showed that CD8 T cells are exerting their anti-viral effect on infected cells in a non-cytolytic manner, independent of cell death, where HIV infection was kept in a dormant phase. Further work revealed that CD8 T cells' non-cytotoxicity is mediated mostly by a secreted factor that is a protein in nature referred to as the CD8 T cell anti-viral factor (CAF) (Figure 3A). The isolation of such protein is technically challenging due to its low expression profile (85).

Later on, the non-cytotoxic role of CD8 T cells has been demonstrated in non-human primates. Castro et al. reported that *in vivo* antibody depletion of CD8 T cells in AIDS associated retrovirus 2-infected chimpanzee, whose viral load is undetectable for 8 years post inoculation, leads to HIV-viremia. Nonetheless, when the animals recovered from the antibody depletion, the viral load decreased again to its initial undetectable levels (86). This study, along with other seminal early studies, demonstrated that the infected CD4 T cells were not cleared by specific CD8 T cells but rather the pro-viral DNA residing in the infected CD4 T cells was kept stable and non-transcribed (86). For more details about non-cytotoxic functions of CD8 T cells during AIDS, please refer to this excellent review (87). In conclusion, these studies along with others provide strong evidence of a non-cytotoxic anti-viral role against HIV (86, 88, 89).

2.3.1.2 Hepatitis B and C viruses (HBV and HCV)

Hepatitis B-virus specific CD8 T cells play an indispensable role in controlling and resolving hepatitis B infection (90). For instance, treating a hepatitis B infected chimpanzee with CD8 depleting monoclonal antibodies at week 6 post-infection resulted in dramatic increase in the viral DNA (90), suggesting the essential anti-viral role of CD8 T cells. Although CD8 T cell cytotoxicity contributes to the viral control, this mechanism appears to come into play later in the course of the disease since viral DNA suppression preceded the peak of hepatic pathologic damage (90).

In 1994, Guidotti et al. demonstrated that CD8 T cells contribute to HBV control in a non-cytopathic-manner through inhibition of viral gene expression in transgenic mouse models (91). Since mice are inherently immune to hepatitis B infection, two elegant transgenic models were employed where they constitutively express HBV surface proteins either under the control of HBV regulatory element or under the control of murine albumin promoter (91). In this model, the administration of HBsAg-specific CD8 T cells into the transgenic mice resulted in significant reduction in hepatic viral mRNA content without induction of any hepatic damage (91). Additionally, both liver IFNγ and TNFα mRNA were elevated coinciding with hepatic CTL infiltration. To draw a cause-and-effect relationship, the authors either used IFNγ and

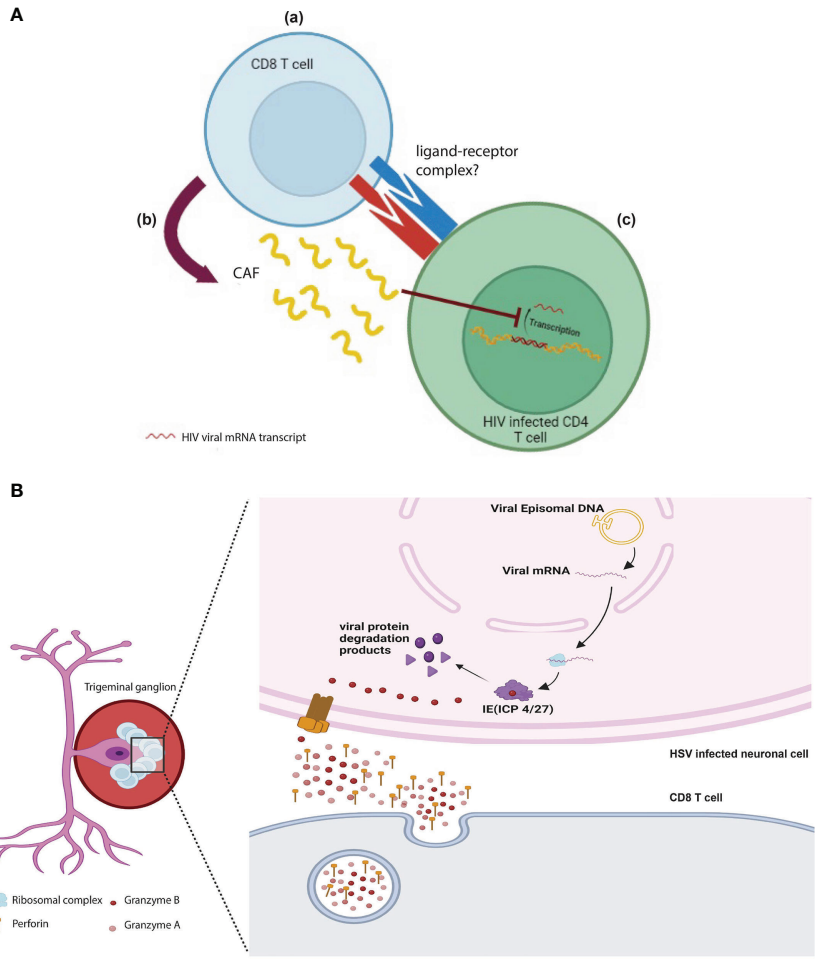

FIGURE 3
Direct non-cytotoxic effects of CD8 T cells: **(A)** HIV specific CD8 T cells (a) interact and recognize HIV infected CD4 cells *via* unknown receptor-ligand and (b) release CAF which (c) prevents the transcription and translation of HIV viral proteins and therefore prevents the propagation of viral progenies. **(B)** GzmB+ CD8 T cells cluster around the bodies of HSV-1 infected ganglionic neurons keeping the virus in latent phase by degrading viral proteins (ICP 4/27), which is essential for the viral shift to active phase. This role is mediated by perforin and granzyme B released by CD8 T cells.

TNFα knockout HBV transgenic mice or treated the mice with blocking monoclonal antibodies 24 hours prior to CTL injection (91, 92). In this approach, they observed failure to reduce viral mRNA. Additionally, the transfer of HBsAg-specific CTLs derived from perforin or FasL deficient mice into HBV transgenic mice clears the HBV DNA replicative forms along with the hepatocellular cytoplasmic HBV core antigen (HBcAg). These data highlight the pivotal role of IFNγ and TNFα in controlling HBV infection independent of perforin and FasL pathway (62), which raises the question: what are the molecular mechanisms underlying these functions?

It has been shown that both IFNγ and TNFα inhibit viral replication through three different molecular mechanisms: (1) upregulation of the nuclear deaminases APOBEC(A3)A and (A3)B, which resort to the hepatitis B virus core protein to get access to the covalently closed circular DNA(cccDNA), essential for viral persistence (92–94). Consequently, the deaminated cccDNA can be degraded by nucleases (92–94), (2) IFNγ can prevent the assembly of the viral RNA containing capsid in the hepatocellular cytoplasm in a proteosome and Kinase dependent-manner (95, 96), and (3) IFNγ induced proteases cleave SSB/La, an RNA binding protein that protects and stabilizes HBV mRNA, rendering the viral mRNA susceptible to endoribonucleolytic degradation (97, 98).

CD8 T cells can also play an essential role in the clearance of HCV and enhancement of protective immunity during acute infection. However, this cytotoxicity stunts with chronic infection where CD8 T cells frequently develop reduced cytotoxicity. To efficiently inhibit HCV progression, CD8 T cells develop a protective mechanism involving the TCR axis

against the nonstructural protein 5 (NS5A). The NS5A TCR-specific CD8 T cells, only represent a small proportion of anti-viral CD8 T cells with a relatively low affinity requiring a higher ligand burden to initiate cytotoxicity and production of effector molecules. Nevertheless, these CD8 T cells can effectively inhibit the replication of HCV in hepatocytes keeping the HCV mRNA intact inside the infected cells. This process is rather non-cytotoxic as it does not induce a change in the level of hepatocellular enzymes such as AST (99).

The direct suppression of viral replication in HCV-infected hepatocytes can be mediated by IFNγ and TNFα, independent of cell-to-cell contact with virus-specific CD8 T cells (100). IFNγ upregulates various enzymes with robust antiviral effect such as protein Kinase R, ADAR adenosine deaminases, guanylate binding protein. These enzymes phosphorylate the eukaryotic initiation factor 2 (EIF-2), which in turn inhibits viral protein synthesis and generates truncated nonfunctional viral proteins that hinder viral replication (101). Thus far, these molecular mechanisms highlight the pivotal non-classical role of CD8 T cells in anti-HBV and HCV response. Instead of killing the virally infected cells, CD8 T cells can, *via* its effector medicators such as IFNγ and TNFα, inhibit viral replication and viral protein synthesis, limiting viral spread.

2.3.1.3 Herpes simplex virus (HSV-1)

The non-cytotoxic function of CD8 T cells in anti-viral responses has been extended to involve their role in maintaining alpha herpes family infections at latency. It is widely accepted that apoptosis of virally infected or tumor cells is largely mediated by the effector molecule GzmB. However, a novel non-cytotoxic function of GzmB has been discovered by two groups in controlling the pathogenesis of human alpha herpes viruses (102, 103). In these studies, the authors showed that $GzmB^+$ CD8 T cells cluster around HSV-1 latently infected trigeminal ganglia (TG) where GzmB surprisingly degrades one of the important proteins in the lytic cycle of the virus (ICP4) without induction of apoptosis (Figure 3B). Another study further reported additional GzmB targets expressed by HSV-1 (ICP27) and the closely related virus Varicella Zoster Virus (VZV ORF4 and ORF62). These studies highlight a novel mechanism in which CTLs prevent viral reactivation in a non-cytotoxic manner using the effector molecule GzmB (102, 103). However, it is not completely clear why $GzmB^+$ CTLs did not induce apoptosis in this context. One can speculate that the viral peptides outnumbered the concentration of caspases in the cell. Hence, the peptides act as a GzmB "sponge" switching its effect away from initiation of apoptosis.

In general, the common theme in the anti-viral studies discussed in this section is the preference towards a non-cytotoxic mechanism rather than cytotoxic, which begets the question: what are the signals that drive the immune system to decide between cytotoxic vs non-cytotoxic mechanisms? The answer to this question encompasses several factors including but not limited to the type of infected tissue, degree of infection, and type of virus. For instance, the immune system might decide not to restore its cytopathic mechanisms if large number of cells are infected in the tissue specially for vital organs such as the liver or the brain. On the contrary, CTLs can eradicate virally-infected cells if they are few in number (104, 105).

2.3.2 Alloimmune responses

Alloreactive memory CD8 T cells are considered as the main drivers of allograft rejection through their cytotoxic machinery (106, 107). However, Krausnick et al. demonstrated a non-cytotoxic role of CD8 T cells in regulating the alloimmune response during lung transplantation (108). In this study, the authors showed that B6 CD8 depleted mice or B6 $CD8^{-/-}$ mice acutely reject their pulmonary allografts from Balb/c mice with a significant inflammatory infiltration in the grafted lungs. Further, the adoptive transfer of wild-type B6 CD8 T cells into immunosuppressed B6 $CD8^{-/-}$ recipients restored tolerance to BALB/c lung allografts. The authors took their analyses one step further and performed a mixed lymphocyte reaction (MLR) to further understand the role of CD8 T cells in this model. In these analyses, they observed that CD8 T lymphocytes isolated from tolerated BALB/c→B6 lung allografts, but not spleens could inhibit proliferation of B6 congenic $CD4^+$ and $CD8^+$ T lymphocytes (responders) in the presence of BALB/c splenocytes (stimulators). These findings suggest that CD8 T cells with regulatory capacity accumulate in lung allografts enhancing graft tolerance, where a large proportion of CD8 T cells infiltrating tolerated lung grafts acquire an $IFN\gamma^+$ central memory phenotype.

To further understand this phenomenon, the authors pretreated recipient mice with IFNγ–neutralizing antibody or $Ifn\gamma^{-/-}$ animals were used as hosts. Surprisingly, they observed a break in tolerance and graft rejection. Injection of $Ifn\gamma^{-/-}$ CD8 T cells into $CD8^{-/-}$ mice failed to rescue BALB/c lung allografts from rejection, despite costimulatory blockade (108). Additionally, the authors observed that trafficking of these central memory CD8 T cells was chemokine dependent. Indeed, injection of Pertusis toxin treated CD8 central memory cells (which irreversibly inactivate Gαi-coupled chemokine receptors) into immunosuppressed B6 recipient of Balb/c lung impaired migration of central memory cells to the lung. To this end, the obvious question is: How does IFNγ act to prevent rejection? Is it a signal related to the lung microenvironment or intrinsic to the T cells? The authors took their study one step further and showed that IFNγ exerted its regulatory effect *via* a Nitric oxide (NO)-pathway. In fact, inhibition of iNOS abrogated the suppressive capacity of T cells. Hence, they showed that NO was essential in allowing graft acceptance and maintaining tolerance locally. Taken

together, these data demonstrated the non-cytotoxic role of host CD8 T cells in lung allograft tolerance in an IFNγ dependent-manner. This work provided a deep insight into the role of CD8 T cells in regulating the alloimmune response in a non-cytotoxic manner, albeit such role seems to be milieu dependent as similar functions were not shown in other transplanted tissues.

2.4 Indirect non-cytotoxicity: The many faces of a protector

Far from cytotoxicity, CD8 T cells were shown to exhibit tissue protective functions and play an important role in healthy re-modeling in the face of inflammation (109–111). Indirectly, without resorting to their cytotoxic machinery, CD8 T cells can exert reparative functions by cross-talking with immune and non-immune cells to recruit different types of immune cells.

2.4.1 Tissue repair

Early after an acute myocardial ischemic attack, lymphocytes and macrophages migrate to the necrotic myocardial area (111). Infiltrating immune cells phagocytose the necrotic debris and initiate the scar tissue formation (111). Along the same lines, Curato et al. demonstrated that a subset of CTLs play a key role in this process (110). In this study, the authors showed that CTLs expressing the Angiotensin II receptor (AT2R) are protective against myocardial ischemia through upregulation of the immunomodulator cytokine IL-10 and downregulation of the proinflammatory cytokines such as IFNγ. Further, co-culturing post-ischemic $AT2R^+$ CD8 T cells with adult cardiac myocytes resulted in significantly lower apoptotic rate when compared to $AT2R^-$ CD8 T cells. Adding neutralizing IL-10 antibodies to the co-culture led to an increase in the cardiac myocyte apoptotic rate in both $AT2R^+$ and $AT2R^-$ CD8 T cells, suggesting that IL-10 is critical for the non-cytotoxic cardioprotective effect of $AT2R^+$ CD8 T cells. These findings highlighted the role of $AT2R^+$ CD8 T cells in locally regulating cytokine expression, skewing it towards a reparative profile. Furthermore, the protective effect of this population was emphasized by the adoptive transfer of $AT2R^+$ CD8 T cells in cardiac tissue, which reduced myocardial ischemia. Thus far, this process reduces the bystander inflammatory injury to the healthy myocardial tissue, maintains cardiac myocyte viability, and prevents one of the most drastic post infarctions sequalae, which is autoimmunity against cardiac proteins and possibly Dressler syndrome. Although the authors demonstrated the pivotal non-cytotoxic role CD8 T cells in this disease model, the means by which they are recruited to the necrotic area is still to be determined. A possible mechanism could be *via* the release of damage associated molecular patterns (DAMPs) by necrotic myocytes that might activate infiltrating macrophages, which in turn create a chemokine rich niche that helps recruitment of CD8 T cells to the site of injury.

Later on, interest has increased to further understand the involvement of CD8 T cells in various tissue repair mechanisms. Indeed CD8 T cells have been shown to play an important role in post-traumatic skeletal muscle regeneration. Although the muscle repair process depends namely on progenitor satellite cells and anti-inflammatory macrophages, the recruitment of CD8 T cells to the inflammatory microenvironment suggests a crucial role for these cells in the regenerative process (112). Using cardiotoxin induced mouse skeletal muscle injury model, Zhang et al. demonstrated that depletion of CD8 T cells impaired skeletal muscle regeneration and increased scar formation by excessive matrix deposition (109). Consistently, adoptive transfer of CD8 T cells into CD8 knockout mice improved myofibroblast size and inhibited matrix deposition post cardiotoxin injury. CD8 knockout mice have limited recruitment of the $Gr1^{high}$ anti-inflammatory macrophages, which are essential for skeletal muscle repair (113), into the inflammatory environment leading to a reduction in the number of satellite cells (109). CD8 T cells were further shown to play a key role in the recruitment of $Gr1^{high}$ macrophages through the secretion of MCP-1 (Figure 4A). The mechanism through which CD8 T cells are recruited to the injured skeletal muscle tissue is still to be identified to provide basis for a therapeutic regenerative model.

2.4.2 Protection of dendritic cells (DCs)

CD8 T cells can interact with various cell types of their innate counterparts, orchestrating and fine tuning the immune response. For instance, one study demonstrated that blood circulating memory CD8 T cells, as opposed to the tissue effector CD8 T cells, have a reduced cytotoxic ability towards DCs. These cells were shown to characteristically express GzmB and perforin at a lower level. They were shown to confer a helper signal to DCs mediated by IFNγ, supporting the production of IL-12p70, a key cytokine for a Th1 immune response. Memory CD8 T cells help protecting antigen presenting DCs from the cytotoxic killing by effector T cells through the upregulation of the endogenous anti-granzyme protease inhibitor-9 (PI-9) in a TNFα dependent-manner (114) (Figure 4B). This provides a feedback mechanism that optimizes an effective antigen presentation and allows for a stronger immune response where potentiation of antigen presentation has a multitude of clinical implications in the area of anti-microbial and cancer vaccines.

2.4.3 Homeostasis

Consciousness, the state of internal and external awareness of a living-being, remains a controversial topic among scientists and philosophers. Although it is not completely understood how conscious the immune system is, one way to explain it is through the cross-talk between wide-spectrum of immune and non-

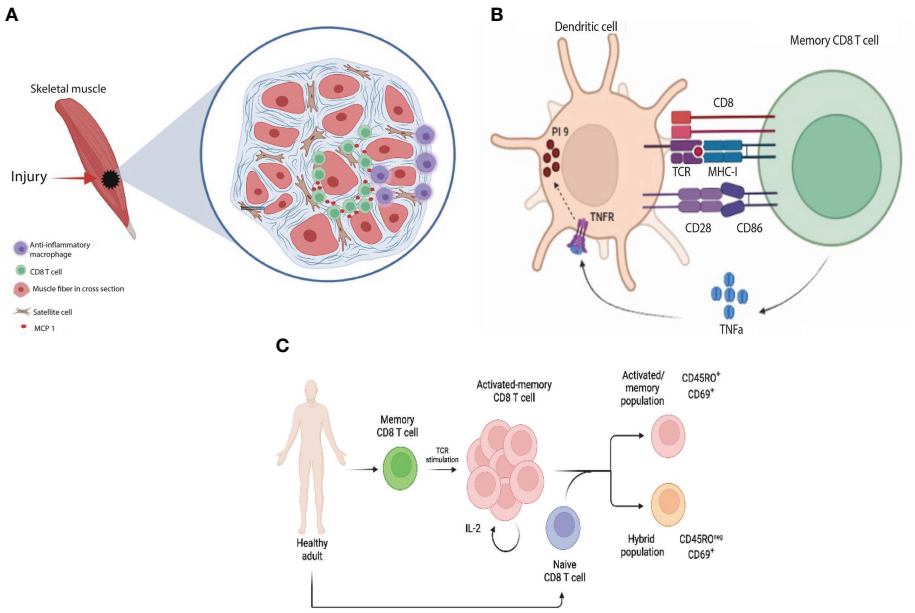

FIGURE 4
Indirect non-cytotoxic functions of CD8 T cells: **(A)** In response to skeletal muscle injury, CD8 T cells infiltrate the necrotic area and release MCP-1 which in turn helps recruit reparative macrophages to the site of injury **(B)** Upon recognition of antigen presented by DCs, memory CD8 cells release TNFα, which in turn upregulates protease inhibitor 9 (PI-9, endogenous anti-granzyme B) in DCs protecting them from CTL killing **(C)** Upon TCR stimulation, memory T cells undergo a rapid transition from a quiescent to a highly activated and proliferating state which is mediated by IL-2 cytokine downstream TCR stimulation. The cross-talk between naïve and activated memory CD8 T cells results in acquisition of two main states by naïve CD8 T cells: (1) activated/memory ($CD45RO^+$ $CD69^+$) and (2) hybrid population between naïve and effector ($CD45RO^{neg}$ $CD69^+$).

immune cells. For instance, soluble mediators such as chemokine gradients guide different immune cell types from one organ to another. Additionally, cells can crosstalk to each other through receptor-ligand interactions. Whether there is an additional means by the cells to remain conscious is yet to be discovered. Along the same lines, once memory T cells see a foreign antigen, they undergo a rapid transition to a highly activated proliferative state. Consequently, they become "conscious" of the chemokine gradient and hence, they migrate to the site of infection to clear the pathogen. For instance, TCR activation of naïve T cells results in down regulation of CCR7 and CXCR4 chemokine receptors and upregulation of inflammatory chemokine receptors including CCR3, CCR5, and CXCR3. Consequently, they acquired the capacity to migrate to inflamed tissues (115, 116). During the migration of activated memory T cells, a portion of them surprisingly migrate to antigen-free lymph nodes (117, 118) yet, the biological significance behind this route of trafficking is still enigmatic. Along the same lines, a recent study discussed the migration of unconventional T cell cells (UTCs) from peripheral tissues to draining LNs (119).

This scenario raises the question: "what is the function of memory T cells following pathogen clearance? Do they have any role(s) during homeostasis?". One possibility could be a cross-talk between activated memory CD8 T cells and naïve CD8 T cells that continuously patrol between the periphery and LNs (120–122). Indeed, recently our lab showed that activated memory CD8 T cells acquire a novel non-cytotoxic function by which they can interact and influence the phenotype and transcriptome of naïve CD8 T cells. In this scenario, they acquire two states (1) an activated/memory T cell state and (2) a unique hybrid state between naïve and effector/activated memory cells (123) (Figure 4C). Since both cell populations were sorted from the same healthy subject, we speculate that activated memory T cells are presenting self-antigen to naïve T cells generating auto-reactive T cells. These findings may explain the non-cytotoxic functions of activated memory T cells and their contribution for the rise of autoimmunity following vaccination or transplantation.

3 Potential clinical implications of CD8 T cells' non-cytotoxic functions

Multiple studies have discussed the non-cytotoxic anti-viral effect of CD8 T cells in clinically asymptomatic HIV-infected individuals (84, 124–127). CD8 T cells from these patients can suppress *in vitro* viral replication with CD8/CD4 T cell ratios as

low as 0.05:1 (128, 129). In contrast, CD8/CD4 cell ratios, as high as 4:1, are needed to suppress 90% of the HIV replication in CD4 T cells from AIDS patients (128, 129). The mechanism of HIV replication inhibition is independent of CD4 killing since the number of CD4 in coculture with CD8 were the same as the control infected CD4 T cells alone (124, 127, 129). Further, CD8 T cells from AIDS patients demonstrated lower anti-viral activity when co-cultured with autologous, naturally infected CD4 cells or with acutely infected CD4 cells (128). This might be explained by the development of an exhausted phenotype by CD8 T cells because of persistent antigen stimulation secondary to a chronic infection. Thus, substantial differences in the CD8 T cell response between different types of HIV patients were observed (124). Regarding the elite controllers (HIV positive individuals whose immune system is capable to keep the HIV viral load under 50 copies/ml), this non-cytotoxic CD8 activity can remain stable for up to 20 years or more in some subjects not receiving anti-retroviral therapy (ART). Notably as well, the levels of integrated HIV-1 pro-viral DNA are lower in the PBMCs from clinically asymptomatic HIV-1-seropositive individuals than in progressors (86, 128). This integrated pro-viral HIV DNA increases when the CD8 T cells are depleted from their cultured PBMCs. Therefore, CD8 T cells can block the virus spread by suppressing the levels of viral mRNA as well as progeny virus.

Despite the overwhelming success of ART in controlling HIV infection, HIV-specific CD8 T cells were shown to be required for such control in tandem with ART. Indeed, *in vivo* depletion of CD8 T cells using monoclonal antibodies in 13 Indian origin SIV infected Rhesus Monkeys maintained on ART resulted in a significant increase in viral loads and SIV RNA in plasma, with minimal change in the SIV DNA containing CD4 T cells between pre and post depletion (130). This study further underlines the non-cytotoxic role of CD8 T cells in controlling viral replication. Hence, harnessing the non-cytotoxicity of CD8 T cells along with ART seems a plausible and potential therapeutic approach specially in resistant patients or progressors despite ART. As discussed previously, CAF could be a potential candidate to enhance non-cytotoxic functions of CD8 T cells in the context of HIV infection. Further, additional studies are needed to draw parallels and learn for other viral models such as HBV, HCV, and HSV-1.

Another implication for the non-cytotoxic functions of CD8 T cells is in the realm of anti-tumoral vaccination. The weak immunogenicity of tumoral antigens raised the need for a stronger immunogenic adjuvant that would confer help for anti-tumoral immune response. Bystander CD8 T cells have been shown by the previously mentioned work of Kalinski to enhance the anti-tumoral effect provided by dendritic cell-based vaccines (62). This work has been recently complemented by Newman et al. who showed that active influenza vaccine improved the outcome of lung cancer in both mouse models and human patients (131). The study further showed that intratumorally vaccination with heat inactivated influenza virus significantly reduced skin melanoma in mice and improved host survival. This effect was shown to be mediated by DCs *via* a Batf3$^{-/-}$ mice and leds to increase of CD8 T cells and intra-tumoral anti-tumor CD8 T cells as well. Finally, CD8 T cells were shown to protect DCs (direct non-cytotoxicity) in a way to enhance antigen presentation and thereby augment the subsequent immune response (114).

In summary, enhancing the antigenicity of tumor vaccines by including a tumor non-specific antigen could be a potential therapy in addition to chemotherapy and immunotherapy. Despite these seminal studies showed a substantial help provided by such vaccines, whether this effect is mediated directly by anti-tumoral CD8 cells or anti-viral CD8 cells is still unclear. This begets the following question: do anti-tumor CD8 cells crosstalk and interact with non-specific CD8 cells in the tumor microenvironment in order to receive the needed help to amplify the anti-tumoral response?

The protective benefit of the non-cytotoxic effect of CD8 T cells can be extended to be medically employed for regeneration and tissue repair. As previously reported in this review, following an ischemic event, a subpopulation of CD8 T cells that expresses AT2R migrates to the injured cardiac tissue and participates in the post-ischemic reparative processes. This subpopulation produces IL-10 that enhances the reparative mechanisms and prevents the deleterious scar formation. It is crucial to understand the mechanisms involved in the recruitment of these cells into cardiac tissue post-ischemic injury. The first signal to recruit and activate these cells in order to initiate their reparative functions is still to be deciphered: whether it depends on an Angiotensin gradient post ischemia, DAMPs, or a specific chemokine. Identifying the first step in this cascade of events would allow finding a therapeutic measure to enhance post ischemic cardiac remodeling and prevent scar formation within the injured cardiac tissue.

4 Concluding remarks

CTLs are classically considered as the serial killers of the immune system. As such, they are equipped with a wide array of cytotoxic molecules such as Granzymes and perforin. They are the soldiers of the immune system that clear pathogens, and fight against tumoral growth. However, CD8 T cells assume other protective, reparative, and homeostatic roles beyond their cytotoxic capacity. Resorting to their cytotoxic molecular machinery, CD8 T cells seem to play a direct non-cytotoxic function. For instance, they were able to control infections beyond directly killing the infected cells mainly by suppressing viral replication to limit viral spread in case of HBV and HCV or maintaining viral latency (e.g., HSV-1). Further, CD8 T cells were shown to be implicated in regeneration and tissue repair especially in post-ischemic cardiac remodeling (indirect non-

cytotoxicity). Understanding the non-cytotoxic functions of CD8 T cells is a critical step to harness CD8 T cell function in cancer immunotherapy and vaccines.

5 Outstanding questions

- Are CTLs heterogenous regarding their cytotoxic functions? Or are they plastic? In other words, can the same CTL perform both cytotoxic and non-cytotoxic functions depending on their environment and signal received?
- Why do CTLs that express GzmB do not kill HSV-1 latently infected neurons?
- In tumor vaccines, what are the mechanisms responsible for enhancement of tumor clearance? Is it crosstalk and interaction between non-specific and tumor-specific CD8 T cells? if so, how?
- How can we harness recruitment of $AT2R^{+}$ CD8 T cells to the site of tissue injury during myocardial infarction to enhance repair? What signals are responsible for recruitment of these cells?
- Why do alloreactive T cells in lung transplantation play a protective role but not in other solid organ transplantation? What is so special about the lungs?
- What kind of signals can we learn from the lung microenvironment to apply to other solid organ transplants?
- Why is there differential non-cytotoxic capacity of CD8 T cells in HIV patients? Is it cell-intrinsic or microenvironment driven?

Author contributions

MA help in writing the review and created the figures. HA conceive the idea, write and edit the review main text and figures. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

References

1. Govaerts A. Cellular antibodies in kidney homotransplantation. *J Immunol* (1960) 85:516–22. Available at: https://www.jimmunol.org/content/85/5/516

2. Rosenau W, Moon HD. Lysis of homologous cells by sensitized lymphocytes in tissue culture. *J Natl Cancer Inst* (1961) 27:471–83. doi: 10.1093/jnci/27.2.471

3. Wilson DB. The reaction of immunologically activated lymphoid cells against homologous lymphoid cells against homologous target tissue cells in vitro. *J Cell Comp Physiol* (1963) 62:273–86. doi: 10.1002/jcp.1030620307

4. Cerottini JC, Nordin AA, Brunner KT. Specific *in vitro* cytotoxicity of thymus-derived lymphocytes sensitized to alloantigens. *Nature* (1970) 228:1308–9. doi: 10.1038/2281308a0

5. Golstein P, Blomgren H. Further evidence for autonomy of T cells mediating specific *in vitro* cytotoxicity: Efficiency of very small amounts of highly purified T cells. *Cell Immunol* (1973) 9:127–41. doi: 10.1016/0008-8749(73)90174-3

6. Lonai P, Clark WR, Feldman M. Participation of theta-bearing cell in an *in vitro* assay of transplantation immunity. *Nature* (1971) 229:566–7. doi: 10.1038/229566a0

7. Amos DB. The use of simplified systems as an aid to the interpretation of mechanisms of graft rejection. *Prog Allergy* (1962) 6:468–538. doi: 10.1159/000391333

8. Berke G, Levey RH. Cellular immunoabsorbents in transplantation immunity. Specific *in vitro* deletion and recovery of mouse lymphoid cells sensitized against allogeneic tumors. *J Exp Med* (1972) 135:972–84. doi: 10.1084/jem.135.4.972

9. Brondz BD. Lymphocyte receptors and mechanisms of *in vitro* cell-mediated immune reactions. *Transplant Rev* (1972) 10:112–51. doi: 10.1111/j.1600-065X.1972.tb01541.x

10. Golstein P, Erik MD, Svedmyr AJ, Wigzell H. Cells mediating specific *in vitro* cytotoxicity. i. detection of receptor-bearing lymphocytes. *J Exp Med* (1971) 134:1385–402. doi: 10.1084/jem.134.6.1385

11. Zinkernagel RM, Doherty PC. Immunological surveillance against altered self components by sensitised T lymphocytes in lymphocytic choriomeningitis. *Nature* (1974) 251:547–8. doi: 10.1038/251547a0

12. Zinkernagel RM, Doherty PC. Restriction of *in vitro* T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature* (1974) 248:701–2. doi: 10.1038/248701a0

13. Sanderson CJ, Thomas JA. The mechanism of T cell mediated cytotoxicity. III. changes in target cell susceptibility during the cell cycle. *Proc R Soc Lond B Biol Sci* (1976) 194:417–29. doi: 10.1098/rspb.1976.0086

14. Rothstein TL, Mage M, Jones G, McHugh LL. Cytotoxic T lymphocyte sequential killing of immobilized allogeneic tumor target cells measured by time-lapse microcinematography. *J Immunol* (1978) 121:1652–6. Available at: https://www.jimmunol.org/content/121/5/1652

15. Sanderson CJ. The mechanism of T cell mediated cytotoxicity. II. morphological studies of cell death by time-lapse microcinematography. *Proc R Soc Lond B Biol Sci* (1976) 192:241–55. doi: 10.1098/rspb.1976.0011

16. Zagury D, Bernard J, Dufer J, Jeannesson P. The biology of isolated immunocytes. i. isolation into a closed liquid microchamber: Application to PFC. *Ann Immunol (Paris)* (1975) 126:23–30.

17. Criado M, Lindstrom JM, Anderson CG, Dennert G. Cytotoxic granules from killer cells: Specificity of granules and insertion of channels of defined size into target membranes. *J Immunol* (1985) 135:4245–51. Available at: https://www.jimmunol.org/content/135/6/4245

18. Dennert G, Podack ER. Cytolysis by h-2-specific T killer cells. assembly of tubular complexes on target membranes. *J Exp Med* (1983) 157:1483–95. doi: 10.1084/jem.157.5.1483

19. Dourmashkin RR, Deteix P, Simone CB, Henkart P. Electron microscopic demonstration of lesions in target cell membranes associated with antibody-dependent cellular cytotoxicity. *Clin Exp Immunol* (1980) 42:554–60.

20. Henkart PA, Millard PJ, Reynolds CW, Henkart MP. Cytolytic activity of purified cytoplasmic granules from cytotoxic rat large granular lymphocyte tumors. *J Exp Med* (1984) 160:75–93. doi: 10.1084/jem.160.1.75

21. Podack ER, Konigsberg PJ. Cytolytic T cell granules. isolation, structural, biochemical, and functional characterization. *J Exp Med* (1984) 160:695–710. doi: 10.1084/jem.160.3.695

22. Henkart PA. Mechanism of lymphocyte-mediated cytotoxicity. *Annu Rev Immunol* (1985) 3:31–58. doi: 10.1146/annurev.iy.03.040185.000335

23. Masson D, Tschopp J. Isolation of a lytic, pore-forming protein (perforin) from cytolytic T-lymphocytes. *J Biol Chem* (1985) 260:9069–72. doi: 10.1016/S0021-9258(17)39328-6

24. Podack ER, Young JD, Cohn ZA. Isolation and biochemical and functional characterization of perforin 1 from cytolytic T-cell granules. *Proc Natl Acad Sci U S A* (1985) 82:8629–33. doi: 10.1073/pnas.82.24.8629

25. Shinkai Y, Takio K, Okumura K. Homology of perforin to the ninth component of complement (C9). *Nature* (1988) 334:525–7. doi: 10.1038/334525a0

26. Young JD, Hengartner H, Podack ER, Cohn ZA. Purification and characterization of a cytolytic pore-forming protein from granules of cloned lymphocytes with natural killer activity. *Cell* (1986) 44:849–59. doi: 10.1016/0092-8674(86)90007-3

27. Acha-Orbea H, Scarpellino L, Hertig S, Dupuis M, Tschopp J. Inhibition of lymphocyte mediated cytotoxicity by perforin antisense oligonucleotides. *EMBO J* (1990) 9:3815–9. doi: 10.1002/j.1460-2075.1990.tb07599.x

28. Kagi D, Ledermann B, Burki K, Seiler P, Odermatt B, Olsen KJ, et al. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature* (1994) 369:31–7. doi: 10.1038/369031a0

29. Lowin B, Beermann F, Schmidt A, Tschopp J. A null mutation in the perforin gene impairs cytolytic T lymphocyte- and natural killer cell-mediated cytotoxicity. *Proc Natl Acad Sci U S A* (1994) 91:11571–5. doi: 10.1073/pnas.91.24.11571

30. Golstein P, Griffiths GM. An early history of T cell-mediated cytotoxicity. *Nat Rev Immunol* (2018) 18:527–35. doi: 10.1038/s41577-018-0009-3

31. Chang TW, Eisen HN. Effects of n alpha-tosyl-L-lysyl-chloromethylketone on the activity of cytotoxic T lymphocytes. *J Immunol* (1980) 124:1028–33. Available at: https://www.jimmunol.org/content/124/3/1028

32. Redelman D, Hudig D. The mechanism of cell-mediated cytotoxicity. i. killing by murine cytotoxic T lymphocytes requires cell surface thiols and activated proteases. *J Immunol* (1980) 124:870–8. Available at: https://www.jimmunol.org/content/124/2/870.long

33. Masson D, Nabholz M, Estrade C, Tschopp J. Granules of cytolytic T-lymphocytes contain two serine esterases. *EMBO J* (1986) 5:1595–600. doi: 10.1002/j.1460-2075.1986.tb04401.x

34. Masson D, Tschopp J. A family of serine esterases in lytic granules of cytolytic T lymphocytes. *Cell* (1987) 49:679–85. doi: 10.1016/0092-8674(87)90544-7

35. Young JD, Leong LG, Liu CC, Damiano A, Wall DA, Cohn ZA. Isolation and characterization of a serine esterase from cytolytic T cell granules. *Cell* (1986) 47:183–94. doi: 10.1016/0092-8674(86)90441-1

36. Chowdhury D, Lieberman J. Death by a thousand cuts: granzyme pathways of programmed cell death. *Annu Rev Immunol* (2008) 26:389–420. doi: 10.1146/annurev.immunol.26.021607.090404

37. Voskoboinik I, Whisstock JC, Trapani JA. Perforin and granzymes: function, dysfunction and human pathology. *Nat Rev Immunol* (2015) 15:388–400. doi: 10.1038/nri3839

38. Ostergaard HL, Kane KP, Mescher MF, Clark WR. Cytotoxic T lymphocyte mediated lysis without release of serine esterase. *Nature* (1987) 330:71–2. doi: 10.1038/330071a0

39. Trenn G, Takayama H, Sitkovsky MV. Exocytosis of cytolytic granules may not be required for target cell lysis by cytotoxic T-lymphocytes. *Nature* (1987) 330:72–4. doi: 10.1038/330072a0

40. Young JD, Clark WR, Liu CC, Cohn ZA. A calcium- and perforin-independent pathway of killing mediated by murine cytolytic lymphocytes. *J Exp Med* (1987) 166:1894–9. doi: 10.1084/jem.166.6.1894

41. Golstein P. Cytolytic T-cell melodrama. *Nature* (1987) 327:12. doi: 10.1038/327012a0

42. Conzelmann A, Corthesy P, Cianfriglia M, Silva A, Nabholz M. Hybrids between rat lymphoma and mouse T cells with inducible cytolytic activity. *Nature* (1982) 298:170–2. doi: 10.1038/298170a0

43. Rouvier E, Luciani MF, Golstein P. Fas involvement in Ca(2+)-independent T cell-mediated cytotoxicity. *J Exp Med* (1993) 177:195–200. doi: 10.1084/jem.177.1.195

44. Watanabe-Fukunaga R, Brannan CI, Copeland NG, Jenkins NA, Nagata S. Lymphoproliferation disorder in mice explained by defects in fas antigen that mediates apoptosis. *Nature* (1992) 356:314–7. doi: 10.1038/356314a0

45. Suda T, Takahashi T, Golstein P, Nagata S. Molecular cloning and expression of the fas ligand, a novel member of the tumor necrosis factor family. *Cell* (1993) 75:1169–78. doi: 10.1016/0092-8674(93)90326-L

46. Boldin MP, Varfolomeev EE, Pancer Z, Mett IL, Camonis JH, Wallach D. A novel protein that interacts with the death domain of Fas/APO1 contains a sequence motif related to the death domain. *J Biol Chem* (1995) 270:7795–8. doi: 10.1074/jbc.270.14.7795

47. Muzio M, Chinnaiyan AM, Kischkel FC, O'Rourke K, Shevchenko A, Ni J, et al. FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death–inducing signaling complex. *Cell* (1996) 85:817–27. doi: 10.1016/S0092-8674(00)81266-0

48. Iborra S, Izquierdo HM, Martinez-Lopez M, Blanco-Menendez N, Reis e Sousa C, Sancho D. The DC receptor DNGR-1 mediates cross-priming of CTLs during vaccinia virus infection in mice. *J Clin Invest* (2012) 122:1628–43. doi: 10.1172/JCI60660

49. Iborra S, Martinez-Lopez M, Khouili SC, Enamorado M, Cueto FJ, Conde-Garrosa R, et al. Optimal generation of tissue-resident but not circulating memory T cells during viral infection requires crosspriming by DNGR-1(+) dendritic cells. *Immunity* (2016) 45:847–60. doi: 10.1016/j.immuni.2016.08.019

50. Kim TS, Gorski SA, Hahn S, Murphy KM, Braciale TJ. Distinct dendritic cell subsets dictate the fate decision between effector and memory CD8(+) T cell differentiation by a CD24-dependent mechanism. *Immunity* (2014) 40:400–13. doi: 10.1016/j.immuni.2014.02.004

51. Martinez-Lopez M, Iborra S, Conde-Garrosa R, Sancho D. Batf3-dependent CD103+ dendritic cells are major producers of IL-12 that drive local Th1 immunity against leishmania major infection in mice. *Eur J Immunol* (2015) 45:119–29. doi: 10.1002/eji.201444651

52. Mashayekhi M, Sandau MM, Dunay IR, Frickel EM, Khan A, Goldszmid RS, et al. CD8alpha(+) dendritic cells are the critical source of interleukin-12 that controls acute infection by toxoplasma gondii tachyzoites. *Immunity* (2011) 35:249–59. doi: 10.1016/j.immuni.2011.08.008

53. Muzaki AR, Tetlak P, Sheng J, Loh SC, Setiagani YA, Poidinger M, et al. Intestinal CD103(+)CD11b(-) dendritic cells restrain colitis *via* IFN-gamma-induced anti-inflammatory response in epithelial cells. *Mucosal Immunol* (2016) 9:336–51. doi: 10.1038/mi.2015.64

54. Sosinowski T, White JT, Cross EW, Haluszczak C, Marrack P, Gapin L, et al. CD8alpha+ dendritic cell trans presentation of IL-15 to naive CD8+ T cells produces antigen-inexperienced T cells in the periphery with memory phenotype and function. *J Immunol* (2013) 190:1936–47. doi: 10.4049/jimmunol.1203149

55. Yu CI, Becker C, Wang Y, Marches F, Helft J, Leboeuf M, et al. Human CD1c+ dendritic cells drive the differentiation of CD103+ CD8+ mucosal effector T cells *via* the cytokine TGF-beta. *Immunity* (2013) 38:818–30. doi: 10.1016/j.immuni.2013.03.004

56. Enamorado M, Khouili SC, Iborra S, Sancho D. Genealogy, dendritic cell priming, and differentiation of tissue-resident memory CD8(+) T cells. *Front Immunol* (2018) 9:1751. doi: 10.3389/fimmu.2018.01751

57. Schenkel JM, Fraser KA, Beura LK, Pauken KE, Vezys V, Masopust D. T Cell memory. resident memory CD8 T cells trigger protective innate and adaptive immune responses. *Science* (2014) 346:98–101. doi: 10.1126/science.1254536

58. Schenkel JM, Fraser KA, Vezys V, Masopust D. Sensing and alarm function of resident memory CD8(+) T cells. *Nat Immunol* (2013) 14:509–13. doi: 10.1038/ni.2568

59. Schenkel JM, Masopust D. Identification of a resident T-cell memory core transcriptional signature. *Immunol Cell Biol* (2014) 92:8–9. doi: 10.1038/icb.2013.67

60. Soudja SM, Chandrabos C, Yakob E, Veenstra M, Palliser D, Lauvau G. Memory-t-cell-derived interferon-gamma instructs potent innate cell activation for protective immunity. *Immunity* (2014) 40:974–88. doi: 10.1016/j.immuni.2014.05.005

61. Ariotti S, Hogenbirk MA, Dijkgraaf FE, Visser LL, Hoekstra ME, Song JY, et al. T Cell memory. skin-resident memory CD8(+) T cells trigger a state of tissue-wide pathogen alert. *Science* (2014) 346:101–5. doi: 10.1126/science.1254803

62. Nakamura Y, Watchmaker P, Urban J, Sheridan B, Giermasz A, Nishimura F, et al. Helper function of memory CD8+ T cells: heterologous CD8+ T cells support the induction of therapeutic cancer immunity. *Cancer Res* (2007) 67:10012–8. doi: 10.1158/0008-5472.CAN-07-1735

63. Le Gros G, Erard F. Non-cytotoxic, IL-4, IL-5, IL-10 producing CD8+ T cells: their activation and effector functions. *Curr Opin Immunol* (1994) 6:453–7. doi: 10.1016/0952-7915(94)90127-9

64. Crotty S. A brief history of T cell help to b cells. *Nat Rev Immunol* (2015) 15:185–9. doi: 10.1038/nri3803

65. Deenick EK, Ma CS. The regulation and role of T follicular helper cells in immunity. *Immunology* (2011) 134:361–7. doi: 10.1111/j.1365-2567.2011.03487.x

66. Ueno H. T Follicular helper cells in human autoimmunity. *Curr Opin Immunol* (2016) 43:24–31. doi: 10.1016/j.coi.2016.08.003

67. Kurata I, Matsumoto I, Sumida T. T Follicular helper cell subsets: a potential key player in autoimmunity. *Immunol Med* (2021) 44:1–9. doi: 10.1080/25785826.2020.1776079

68. Breitfeld D, Ohl L, Kremmer E, Ellwart J, Sallusto F, Lipp M, et al. Follicular b helper T cells express CXC chemokine receptor 5, localize to b cell follicles, and support immunoglobulin production. *J Exp Med* (2000) 192:1545–52. doi: 10.1084/jem.192.11.1545

69. Kim CH, Rott LS, Clark-Lewis I, Campbell DJ, Wu L, Butcher EC. Subspecialization of CXCR5+ T cells: B helper activity is focused in a germinal center-localized subset of CXCR5+ T cells. *J Exp Med* (2001) 193:1373–81. doi: 10.1084/jem.193.12.1373

70. Schaerli P, Willimann K, Lang AB, Lipp M, Loetscher P, Moser B. CXC chemokine receptor 5 expression defines follicular homing T cells with b cell helper function. *J Exp Med* (2000) 192:1553–62. doi: 10.1084/jem.192.11.1553

71. Ye L, Li Y, Tang H, Liu W, Chen Y, Dai T, et al. CD8+CXCR5+T cells infiltrating hepatocellular carcinomas are activated and predictive of a better prognosis. *Aging (Albany NY)* (2019) 11:8879–91. doi: 10.18632/aging.102308

72. Chen Y, Yu M, Zheng Y, Fu G, Xin G, Zhu W, et al. CXCR5(+)PD-1(+) follicular helper CD8 T cells control b cell tolerance. *Nat Commun* (2019) 10:4415. doi: 10.1038/s41467-019-12446-5

73. Quigley MF, Gonzalez VD, Granath A, Andersson J, Sandberg JK. CXCR5+ CCR7- CD8 T cells are early effector memory cells that infiltrate tonsil b cell follicles. *Eur J Immunol* (2007) 37:3352–62. doi: 10.1002/eji.200636746

74. Shen J, Luo X, Wu Q, Huang J, Xiao G, Wang L, et al. A subset of CXCR5(+)CD8 (+) T cells in the germinal centers from human tonsils and lymph nodes help b cells produce immunoglobulins. *Front Immunol* (2018) 9:2287. doi: 10.3389/fimmu.2018.02287

75. Valentine KM, Davini D, Lawrence TJ, Mullins GN, Manansala M, Al-Kuhlani M, et al. CD8 follicular T cells promote b cell antibody class switch in autoimmune disease. *J Immunol* (2018) 201:31–40. doi: 10.4049/jimmunol.1701079

76. Wang J, Li R, Cao Y, Gu Y, Fang H, Fei Y, et al. Intratumoral CXCR5(+)CD8 (+)T associates with favorable clinical outcomes and immunogenic contexture in gastric cancer. *Nat Commun* (2021) 12:3080. doi: 10.1038/s41467-021-23356-w

77. Le KS, Ame-Thomas P, Tarte K, Gondois-Rey F, Granjeaud S, Orlanducci F, et al. CXCR5 and ICOS expression identifies a CD8 T-cell subset with TFH features in Hodgkin lymphomas. *Blood Adv* (2018) 2:1889–900. doi: 10.1182/bloodadvances.2018017244

78. Xiao L, Jia L, Bai L, He L, Yang B, Wu C, et al. Phenotypic and functional characteristics of IL-21-expressing CD8(+) T cells in human nasal polyps. *Sci Rep* (2016) 6:30362. doi: 10.1038/srep30362

79. Adolfo RLR, Frias B, Sharma AA, Mi T, Abdelsamed H, zebley C, et al. HIV-Specific CD8 T cells from elite controllers have an epigenetic imprint that preserves effector functions. *bioRxiv* (2021). doi: 10.1101/2021.09.28.459512

80. Adams P, Iserentant G, Servais JY, Vandekerckhove L, Vanham G, Seguin-Devaux C, et al. Cytotoxic CD8+ T cells expressing CXCR5 are detectable in HIV-1 elite controllers after prolonged *In vitro* peptide stimulation. *Front Immunol* (2020) 11:622343. doi: 10.3389/fimmu.2020.622343

81. Johnston RJ, Choi YS, Diamond JA, Yang JA, Crotty S. STAT5 is a potent negative regulator of TFH cell differentiation. *J Exp Med* (2012) 209:243–50. doi: 10.1084/jem.20111174

82. Nurieva RI, Podd A, Chen Y, Alekseev AM, Yu M, Qi X, et al. STAT5 protein negatively regulates T follicular helper (Tfh) cell generation and function. *J Biol Chem* (2012) 287:11234–9. doi: 10.1074/jbc.M111.324046

83. Loyal L, Warth S, Jurchott K, Molder F, Nikolaou C, Babel N, et al. SLAMF7 and IL-6R define distinct cytotoxic versus helper memory CD8(+) T cells. *Nat Commun* (2020) 11:6357. doi: 10.1038/s41467-020-19002-6

84. Walker CM, Moody DJ, Stites DP, Levy JA. CD8+ lymphocytes can control HIV infection *in vitro* by suppressing virus replication. *Science* (1986) 234:1563–6. doi: 10.1126/science.2431484

85. Chang TL, Mosoian A, Pine R, Klotman ME, Moore JP. A soluble factor(s) secreted from CD8(+) T lymphocytes inhibits human immunodeficiency virus type 1 replication through STAT1 activation. *J Virol* (2002) 76:569–81. doi: 10.1128/JVI.76.2.569-581.2002

86. Castro BA, Homsy J, Lennette E, Murthy KK, Eichberg JW, Levy JA. HIV-1 expression in chimpanzees can be activated by CD8+ cell depletion or CMV infection. *Clin Immunol Immunopathol* (1992) 65:227–33. doi: 10.1016/0090-1229(92)90151-D

87. Morvan MG, Teque FC, Locher CP, Levy JA. The CD8(+) T cell noncytotoxic antiviral responses. *Microbiol Mol Biol Rev 85* (2021) 85(2):e00155-20. doi: 10.1128/MMBR.00155-20

88. Levy JA, Hoffman AD, Kramer SM, Landis JA, Shimabukuro JM, Oshiro LS. Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS. *Science* (1984) 225:840–2. doi: 10.1126/science.6206563

89. Jin X, Bauer DE, Tuttleton SE, Lewin S, Gettie A, Blanchard J, et al. Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques. *J Exp Med* (1999) 189:991–8. doi: 10.1084/jem.189.6.991

90. Thimme R, Wieland S, Steiger C, Ghrayeb J, Reimann KA, Purcell RH, et al. CD8(+) T cells mediate viral clearance and disease pathogenesis during acute hepatitis b virus infection. *J Virol* (2003) 77:68–76. doi: 10.1128/JVI.77.1.68-76.2003

91. Guidotti LG, Ando K, Hobbs MV, Ishikawa T, Runkel L, Schreiber RD, et al. Cytotoxic T lymphocytes inhibit hepatitis b virus gene expression by a noncytolytic mechanism in transgenic mice. *Proc Natl Acad Sci U S A* (1994) 91:3764–8. doi: 10.1073/pnas.91.9.3764

92. Xia Y, Protzer U. Control of hepatitis b virus by cytokines. *Viruses* (2017) 9:18. doi: 10.3390/v9010018

93. Lucifora J, Xia Y, Reisinger F, Zhang K, Stadler D, Cheng X, et al. Specific and nonhepatotoxic degradation of nuclear hepatitis b virus cccDNA. *Science* (2014) 343:1221–8. doi: 10.1126/science.1243462

94. Xia Y, Stadler D, Lucifora J, Reisinger F, Webb D, Hosel M, et al. Interferon-gamma and tumor necrosis factor-alpha produced by T cells reduce the HBV persistence form, cccDNA, without cytolysis. *Gastroenterology* (2016) 150:194–205. doi: 10.1053/j.gastro.2015.09.026

95. Robek MD, Wieland SF, Chisari FV. Inhibition of hepatitis b virus replication by interferon requires proteasome activity. *J Virol* (2002) 76:3570–4. doi: 10.1128/JVI.76.7.3570-3574.2002

96. Robek MD, Boyd BS, Wieland SF, Chisari FV. Signal transduction pathways that inhibit hepatitis b virus replication. *Proc Natl Acad Sci U S A* (2004) 101:1743–7. doi: 10.1073/pnas.0308340100

97. Heise T, Guidotti LG, Chisari FV. Characterization of nuclear RNases that cleave hepatitis b virus RNA near the la protein binding site. *J Virol* (2001) 75:6874–83. doi: 10.1128/JVI.75.15.6874-6883.2001

98. Heise T, Guidotti LG, Chisari FV. La autoantigen specifically recognizes a predicted stem-loop in hepatitis b virus RNA. *J Virol* (1999) 73:5767–76. doi: 10.1128/JVI.73.7.5767-5776.1999

99. Pasetto A, Frelin L, Aleman S, Holmstrom F, Brass A, Ahlen G, et al. TCR-redirected human T cells inhibit hepatitis c virus replication: hepatotoxic potential is linked to antigen specificity and functional avidity. *J Immunol* (2012) 189:4510–9. doi: 10.4049/jimmunol.1201613

100. Jo J, Aichele U, Kersting N, Klein R, Aichele P, Bisse E, et al. Analysis of CD8+ T-cell-mediated inhibition of hepatitis c virus replication using a novel immunological model. *Gastroenterology* (2009) 136:1391–401. doi: 10.1053/j.gastro.2008.12.034

101. Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol* (2004) 75:163–89. doi: 10.1189/jlb.0603252

102. Gerada C, Steain M, Campbell TM, McSharry B, Slobedman B, Abendroth A. Granzyme b cleaves multiple herpes simplex virus 1 and varicella-zoster virus (VZV) gene products, and VZV ORF4 inhibits natural killer cell cytotoxicity. *J Virol* (2019) 93:e01140-19. doi: 10.1128/JVI.01140-19

103. Knickelbein JE, Khanna KM, Yee MB, Baty CJ, Kinchington PR, Hendricks RL. Noncytotoxic lytic granule-mediated CD8+ T cell inhibition of HSV-1 reactivation from neuronal latency. *Science* (2008) 322:268–71. doi: 10.1126/science.1164164

104. Guidotti LG, Chisari FV. Noncytolytic control of viral infections by the innate and adaptive immune response. *Annu Rev Immunol* (2001) 19:65–91. doi: 10.1146/annurev.immunol.19.1.65

105. Harty JT, Tvinnereim AR, White DW. CD8+ T cell effector mechanisms in resistance to infection. *Annu Rev Immunol* (2000) 18:275–308. doi: 10.1146/annurev.immunol.18.1.275

106. Lakkis FG, Sayegh MH. Memory T cells: a hurdle to immunologic tolerance. *J Am Soc Nephrol* (2003) 14:2402–10. doi: 10.1097/01.ASN.0000085020.78117.70

107. Abou-Daya KI, Tieu R, Zhao D, Rammal R, Sacirbegovic F, Williams AL, et al. Resident memory T cells form during persistent antigen exposure leading to

allograft rejection. *Sci Immunol* (2021) 6:eabc8122. doi: 10.1126/sciimmunol.abc8122

108. Krupnick AS, Lin X, Li W, Higashikubo R, Zinselmeyer BH, Hartzler H, et al. Central memory CD8+ T lymphocytes mediate lung allograft acceptance. *J Clin Invest* (2014) 124:1130–43. doi: 10.1172/JCI71359

109. Zhang J, Xiao Z, Qu C, Cui W, Wang X, Du J. CD8 T cells are involved in skeletal muscle regeneration through facilitating MCP-1 secretion and Gr1(high) macrophage infiltration. *J Immunol* (2014) 193:5149–60. doi: 10.4049/jimmunol.1303486

110. Curato C, Slavic S, Dong J, Skorska A, Altarche-Xifro W, Miteva K, et al. Identification of noncytotoxic and IL-10-producing CD8+AT2R+ T cell population in response to ischemic heart injury. *J Immunol* (2010) 185:6286–93. doi: 10.4049/jimmunol.0903681

111. Kino T, Khan M, Mohsin S. The regulatory role of T cell responses in cardiac remodeling following myocardial infarction. *Int J Mol Sci* (2020) 21 (14):5013. doi: 10.3390/ijms21145013

112. Kohno S, Ueji T, Abe T, Nakao R, Hirasaka K, Oarada M, et al. Rantes secreted from macrophages disturbs skeletal muscle regeneration after cardiotoxin injection in cbl-b-deficient mice. *Muscle Nerve* (2011) 43:223–9. doi: 10.1002/mus.21829

113. Pannerec A, Marazzi G, Sassoon D. Stem cells in the hood: the skeletal muscle niche. *Trends Mol Med* (2012) 18:599–606. doi: 10.1016/j.molmed.2012.07.004

114. Watchmaker PB, Urban JA, Berk E, Nakamura Y, Mailliard RB, Watkins SC, et al. Memory CD8+ T cells protect dendritic cells from CTL killing. *J Immunol* (2008) 180:3857–65. doi: 10.4049/jimmunol.180.6.3857

115. Sallusto F, Kremmer E, Palermo B, Hoy A, Ponath P, Qin S, et al. Switch in chemokine receptor expression upon TCR stimulation reveals novel homing potential for recently activated T cells. *Eur J Immunol* (1999) 29:2037–45. doi: 10.1002/(SICI)1521-4141(199906)29:06<2037::AID-IMMU2037>3.0.CO;2-V

116. Sallusto F, Lenig D, Mackay CR, Lanzavecchia A. Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. *J Exp Med* (1998) 187:875–83. doi: 10.1084/jem.187.6.875

117. Brinkman CC, Sheasley-O'Neill SL, Ferguson AR, Engelhard VH. Activated CD8 T cells redistribute to antigen-free lymph nodes and exhibit effector and memory characteristics. *J Immunol* (2008) 181:1814–24. doi: 10.4049/jimmunol.181.3.1814

118. Lawrence CW, Braciale TJ. Activation, differentiation, and migration of naive virus-specific CD8+ T cells during pulmonary influenza virus infection. *J Immunol* (2004) 173:1209–18. doi: 10.4049/jimmunol.173.2.1209

119. Marco KK, Ataide A, de Casas PC, Ugur M, Eickhoff S, Zou M, et al. Lymphatic migration of unconventional T cells promotes site-specific immunity in distinct lymph nodes. *Immunity* (2022) 55:1–16. doi: 10.1016/j.immuni.2022.07.019

120. Jameson SC, Masopust D. Understanding subset diversity in T cell memory. *Immunity* (2018) 48:214–26. doi: 10.1016/j.immuni.2018.02.010

121. Gowans JL. The recirculation of lymphocytes from blood to lymph in the rat. *J Physiol* (1959) 146:54–69. doi: 10.1113/jphysiol.1959.sp006177

122. Smith ME, Ford WL. The recirculating lymphocyte pool of the rat: a systematic description of the migratory behaviour of recirculating lymphocytes. *Immunology* (1983) 49:83–94.

123. Sasaki K, Moussawy MA, Abou-Daya KI, Macedo C, Hosni-Ahmed A, Liu S, et al. Activated-memory T cells influence naive T cell fate: a noncytotoxic function of human CD8 T cells. *Commun Biol* (2022) 5:634. doi: 10.1038/s42003-022-03596-2

124. Walker CM, Moody DJ, Stites DP, Levy JA. CD8+ T lymphocyte control of HIV replication in cultured CD4+ cells varies among infected individuals. *Cell Immunol* (1989) 119:470–5. doi: 10.1016/0008-8749(89)90259-1

125. Wiviott LD, Walker CM, Levy JA. CD8+ lymphocytes suppress HIV production by autologous CD4+ cells without eliminating the infected cells from culture. *Cell Immunol* (1990) 128:628–34. doi: 10.1016/0008-8749(90)90054-U

126. Tomaras GD, Greenberg ML. CD8+ T cell mediated noncytolytic inhibition of human immunodeficiency virus type I. *Front Biosci* (2001) 6:D575–98. doi: 10.2741/tomaras

127. Mackewicz CE, Blackbourn DJ, Levy JA. CD8+ T cells suppress human immunodeficiency virus replication by inhibiting viral transcription. *Proc Natl Acad Sci U S A* (1995) 92:2308–12. doi: 10.1073/pnas.92.6.2308

128. Mackewicz CE, Ortega HW, Levy JA. CD8+ cell anti-HIV activity correlates with the clinical state of the infected individual. *J Clin Invest* (1991) 87:1462–6. doi: 10.1172/JCI115153

129. Blackbourn DJ, Mackewicz CE, Barker E, Hunt TK, Herndier B, Haase AT, et al. Suppression of HIV replication by lymphoid tissue CD8+ cells correlates with the clinical state of HIV-infected individuals. *Proc Natl Acad Sci U S AU* (1996) 93:13125–30. doi: 10.1073/pnas.93.23.13125

130. Cartwright EK, Spicer L, Smith SA, Lee D, Fast R, Paganini S, et al. CD8(+) lymphocytes are required for maintaining viral suppression in SIV-infected macaques treated with short-term antiretroviral therapy. *Immunity* (2016) 45:656–68. doi: 10.1016/j.immuni.2016.08.018

131. Newman JH, Chesson CB, Herzog NL, Bommareddy PK, Aspromonte SM, Pepe R, et al. Intratumoral injection of the seasonal flu shot converts immunologically cold tumors to hot and serves as an immunotherapy for cancer. *Proc Natl Acad Sci U S A* (2020) 117:1119–28. doi: 10.1073/pnas.1904022116

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Tumor infiltrating CD8/CD103/TIM-3-expressing lymphocytes in epithelial ovarian cancer co-express CXCL13 and associate with improved survival

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Reactivation of tumor infiltrating T lymphocytes (TILs) with immune checkpoint inhibitors or co-stimulators has proven to be an effective anti-cancer strategy for a broad range of malignancies. However, epithelial ovarian cancer (EOC) remains largely refractory to current T cell-targeting immunotherapeutics. Therefore, identification of novel immune checkpoint targets and biomarkers with prognostic value for EOC is warranted. Combining multicolor immunofluorescent staining's with single cell RNA-sequencing analysis, we here identified a TIM-3/CXCL13-positive tissue-resident memory (CD8/CD103-positive) T cell (Trm) population in EOC. Analysis of a cohort of ~175 patients with high-grade serous EOC revealed TIM-3-positive Trm were significantly associated with improved patient survival. As CXCL13-positive CD8-positive T cells have been strongly linked to patient response to anti-PD1 immune checkpoint blockade, combinatorial TIM-3 and PD-1 blockade therapy may be of interest for the (re)activation of anti-cancer immunity in EOC.

KEYWORDS

tumor infiltrating lymphocytes, epithelial ovarian cancer, TIM-3, CXCL13, RNA-seq

Introduction

For epithelial ovarian cancer (EOC) the infiltration of lymphocytes, particularly T cells, in tumor tissue is associated with a better clinical outcome (1–3), suggesting T cell immunotherapy may be of use. However, although re-activation of immune cells with immune checkpoint inhibitors (ICI) or co-stimulators is effective for a wide range of

malignancies (as reviewed in (4–6)), responses in EOC are disappointing. Most notably, objective responses in EOC do not exceed 7% with PD-1 checkpoint therapy (7–9). Accordingly, releasing the PD-1/PDL-1 immune checkpoint brake with ICI´s is not sufficient for re-establishing anti-tumor immunity in EOC. For this reason, identification of novel immune checkpoint targets on TILs as well as biomarkers with prognostic value in EOC remains essential.

Intra-epithelial localization of CD8-positive TILs is associated with improved patient survival in EOC, whereas intratumoral yet stromally located TILs do not associate with survival (10–14). More specifically, survival is associated with a subset of TILs that can be identified by CD103 expression, an αE integrin subunit regarded as a tissue-resident memory T cell marker. This TIL population additionally expresses immunotherapeutic targets, such as PD-1 and CD27, and can possibly be re-activated by a combination of PD-1 checkpoint inhibition and CD27 co-stimulators (12). This subpopulation is defined as highly activated, tumor-specific, and tissue-resident memory T cells that can also express the T cell immunoglobulin and mucin domain 3 (TIM-3) checkpoint receptor (15). In line with this, CD8-positive tumor-reactive T cells in different solid tumors co-express PD-1, LAG-3, CTLA-4 and TIM-3 (16, 17).

Initially, TIM-3 expression was described on IFN-γ producing CD4-positive helper T cells and cytotoxic CD8-positive T cells and reported to regulate macrophage activation (18). On TIL populations, TIM-3 expression is associated with T cell exhaustion, tumor progression, and poor clinical outcome in certain cancers (19–22). Reversely, for other cancers, it can associate with benefit. For instance, TIM-3 expression in TILs of triple-negative breast cancer (TNBC) patients associated with longer recurrence-free and overall survival (23). However, in a meta-analysis study of 3,072 cases from 21 published studies from a range of solid cancers TIM-3 expression on TILs did not associate with overall survival. In contrast, TIM-3 expression on malignant cells did significantly associate with poor overall survival (24). Thus, solely evaluating TIM-3 expression levels within a TIL population is likely not sufficient and a more detailed delineation of subsets of TILs that express TIM-3 is warranted. Particularly, whether such a more relevant TIL population associates with patient survival and may thus be of therapeutic interest.

Interestingly, expression of the chemokine CXCL13 has also been identified in highly exhausted TIM-3-expressing TILs, a cell population that was predictive for both response to ICIs (PD-1 blockade) and survival in non-small cell lung cancer patients and muscle-invasive bladder cancer (MIBC) (25, 26). CXCL13 expression itself associated with prognosis, immune infiltration, and T cell exhaustion in ovarian cancer (27). CXCL13 expression was TGFβ-dependent and mediated B cell recruitment and formation of tertiary lymphoid structures (TLSs) in human tumors (28). Notably, although not directly evaluated in the current study, the presence of TLSs in several human tumors has been linked to improved prognosis and outcome upon immunotherapy (29–31). Further, tumor infiltrated CD8-positive T cells in tumors without TLSs lacked prognostic benefit or even associated with increased risk of disease progression (32, 33).

Here, a cohort of EOC core samples was evaluated for the presence of tumor infiltrating CD8/CD103/TIM-3 triple-positive T cells and subsequently correlated with patient survival. Interestingly, increased tumor infiltration of CD8/CD103/TIM-3 triple-positive cells associated with improved patient survival in EOC, suggesting that CD8/CD103/TIM-3 triple-positive TILs can serve as a prognostic marker for EOC. In line with this finding, a single-cell tumor immune transcriptomic dataset revealed co-expression of TIM-3, CXCL13 and CD103 within the terminally exhausted CD8-positive T cell fraction (pre-defined by using canonical markers and curated gene signatures (34)). Expression of CXCL13 could predominantly be attributed to the CD8/CD103/TIM-3 triple-positive fraction compared to single- and double-positive counterparts in primary EOC samples. Thus, TIM-3 expression on CD8/CD103-double positive TILs may be used as surrogate marker for prognostically favorable CXCL13-positive CD8-positive TILs and may have prognostic value.

Materials and methods

Patient selection

Patient selection and construction of the tissue micro-array (TMA) were described previously (35). Briefly, a recoded database was created containing information on clinico-pathological characteristics and follow-up of patients diagnosed with advanced stage HGSOC at the University Medical Center Groningen (Groningen, The Netherlands) and Isala hospital Zwolle (Zwolle, The Netherlands) between January 2008 and January 2017. In total 176 EOC patients were included from participating centers (see Table 1). Patients were staged according to international Federation of Gynecology and Obstetrics (FIGO) criteria 2014 based on World Health Organization (WHO) guidelines. Histological subtype was confirmed by experienced gynecologic pathologists based on morphology, and when available P53 immunohistochemistry staining. The presence of tumor tissue was confirmed on H&E slides and representative locations with tumor tissue were selected for the TMA. OS was calculated from the date of initial treatment (either primary surgery or first cycle of neo-adjuvant chemotherapy) and was last updated in July 2020.

Immunohistochemical staining

For immunohistochemistry (IHC), tissue microarray (TMA) sections were constructed as described previously (36). Formalin-fixed, paraffin-embedded (FFPE) TMA slides were

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TABLE 1 Patient characteristics cohort N=176.

		N	%
FIGO stage			
	II	9	5
	III	133	76
	IV	34	19
	Unknown	0	0
BRCA status			
	Mutant	16	9
	Wildtype	50	28
	Unknown	110	63
Primary treatment			
	Primary surgery	83	47
	Neoajuvant chemotherapy	93	53
Surgery outcome			
	Macroscopic tumor	87	49
	No macroscopic tumor	87	49
	Unknown	2	1

FIGO, Fédération Internationale de Gynécologie et d'obstétrique.

dewaxed in xylene and later rehydrated by using degraded concentrations of ethanol. Antigen retrieval was initiated (10 mM citrate buffer, pH6) and endogenous peroxidase activity was blocked (30% H_2O_2 solution). Slides were stained with rabbit anti-human CD103 mAb (anti-αEβ7-integrin, Abcam, Cambridge, UK, 1:200) before incubation overnight at 4 °C. The next day, slides were incubated with Envision-HRP anti-rabbit and later amplified with fluorophore cyanine 5 according to manufacturer's instructions (TSA Cyanine 5 (Cy5) detection Kit, Perkin Elmer, 1:50). Next, the slides were stained with mouse anti-human CD8 (DAKO, Heverlee, Belgium, clone C8/144B, 1:50) before incubation overnight 4 °C. On the third day, slides were incubated with Envision-HRP anti mouse and amplified using the Fluorescein detection kit (Perkin Elmer, 753001KT, 1:50) according to the manufacturer's instructions. Afterwards, the slides were stained with rabbit anti-TIM-3 mAb before incubation overnight at 4°C. The next day, the slides were incubated with Envision-HRP anti-rabbit and amplified by using TSA Cyanine 3 (Cy3) (Perkin Elmer, 753001KT, 1:50). The sections were embedded in prolong diamond anti-fade mounting medium with DAPI (Life Technologies).

Image acquisition and analysis

Sections were scanned using a TissueFAXS imaging system (TissueGnostics, Vienna, Austria). Processed channels were merged using ImageJ. Within each core, single-positive CD8 cells, double-positive CD103/CD8 cells and triple-positive CD8/CD103/TIM-3 cells were counted, and the percentage of tumor/stromal surface was estimated. The slides were counted manually by 2 individuals who were blinded for the clinicopathological data. Afterwards, scores of the 2 individual counters were compared and differences in counts of over 10% were reanalyzed until consensus was reached.

Ethics

The study was approved by the local ethics review board under Register number 201700448.

Single cell mRNA sequencing data analysis

A single-cell tumor immune atlas based on over 500,000 cells from 217 patients and 13 cancer types (described in (34)) was utilized to evaluate gene expression in the tumor immune microenvironment (TME). The dataset was downloaded in the form of a RDS file containing the Seurat object. The data was uploaded into Seurat V4 in R language version 4.0.3. Within this cell atlas, immune cell fractions were pre-separated into 25 different clusters using canonical markers and curated gene signatures (B cells, proliferative B cells, plasma B cells, naive T cells, regulatory T cells, T helper cells, Th 17 cells, proliferative T cells, recently activated CD4-positive T cells, naive-memory CD4-positive T cells, transitional memory CD4-positive T cells, pre-exhausted CD8-positive T cells, cytotoxic CD8-positive T cells, effector memory CD8-positive T cells, terminally exhausted CD8-positive T cells, NK cells, secreted phosphoprotein 1 (SPP1) tumor-associated macrophages (TAMs), M2 TAMs, pro-inflammatory TAMs, proliferative monocytes and macrophages, monocytes, conventional dendritic cells (cDC), plasmacytoid dendritic cells (pDC), myeloid DC (mDC) and mast cells). Most cells were negative for TIM-3 and therefore cells with non-zero TIM-3 expression were considered TIM-3-positive. Differential expression was calculated by using the FindMarkers function from Seurat with MAST as the method of choice (37).

TIL flow cytometric analysis

Tumor infiltrating lymphocyte (TIL) extraction was performed on ovarian cancer tissue obtained during surgery collected in the University Medical Center Groningen, The Netherlands. This study was carried out in the Netherlands in accordance with International Ethical and Professional Guidelines (the Declaration of Helsinki and the International Conference on Harmonization Guidelines for Good Clinical Practice. The use of anonymous rest material is regulated under the code for good clinical practice in the Netherlands

and had been processed anonymously (38). Patients had given consent to use surgical material for research purposes. Primary patient TILs used for analysis of the TIL phenotype were isolated from fresh tumor samples obtained during cytoreductive surgery. Thawed TILs were resuspended in FACS tubes in a final volume of 200 µl and stimulated with Cell Stimulation Cocktail (Thermo Fisher) for 12-16h at 37°C. Golgiplug (BD Biosciences) was added the last 4h of the culture. CD3, CD8, CD103, TIM-3 and CXCL13 expression was determined by mAbs specific for the corresponding human molecules conjugated with BV785 (CD3, Biolegend), BV421 (CD8, BD Biosciences), Fluorescein Isothiocyanate (CD103, BD Biosciences), Pe-CF594 (TIM-3, BD Biosciences) and APC (CXCL13, Invitrogen). Fix and Perm solutions A and B from Nordic MuBio (Susteren, the Netherlands) were used for analysis of intracellular molecules. Acquisition was done on a CytoFLEX flow cytometer (Beckman Coulter), and analysis was performed using FlowJo V10.5.3.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics for Windows, version 23 (IBM Corp., Armonk, N.Y., USA) and R (version 3.6.2). Immune cell densities were log2 transformed for analysis. Clustering of cases was done by hierarchical clustering using Ward's minimum variance method in R using package heatmap. Correlations between immune clusters and clinical and histopathological variables were analyzed using Multiple regression analysis in SPSS. Analysis of OS as a function of immune cell density was performed in R using package Survminer. Survival curves were plotted using the Kaplan–Meier method. A p-value of <0.05 was used as cutoff for significance. Patients were divided into high or low/no infiltration clusters, determined based on the optimal cut-off.

Results

Patterns of T cell infiltrates in EOC patients

Immunofluorescent analysis of EOC patient tissue identified CD8-positive, CD103-positive, TIM-3-positive cells (Figure 1A) and all combinations thereof (Figure 1B). TIM-3-positive cells represented a relatively small subpopulation, with median cell counts of 63, 16 and 2 for CD8 single-positive (left), CD8/CD103 double-positive (middle) and CD8/CD103/TIM-3 triple-positive (right) populations, respectively (Figure 1C). Hierarchical clustering revealed that patient samples that displayed high infiltration levels of triple-positive cells were also characterized by infiltrates of double-positive cells (CD8/CD103 vs CD8/CD103/TIM-3 bars in Figure 2). Upon multiple regression analysis no significant association of the FIGO-stage, BRCA-status, primary treatment strategy or surgery outcome with any of the clusters was established (Figure 2). Interestingly, although statistical significance was not reached, an association between BRCA-status (purple bar) and the triple-positive CD8/CD103/TIM-3 cluster was identified. Further, multiple regression analysis of histopathological markers PAX8, WT1, CK7, P16 and P53 determined during diagnostic workup revealed no association with any of the immune clusters.

TIL TIM-3 expression associates with improved survival in EOC

Infiltration of CD8-positive T cells did not significantly associate with survival in the dichotomized patient cohort (Figure 3A, p = 0.12). In line with previous data, CD8/CD103-positive T cell infiltration did associate with a significant improvement in survival (Figure 3B, p = 0. 003). Importantly, although the total numbers of CD8/CD103/TIM-3 triple-positive infiltrated cells were much lower than that of single- and double-positive populations (see Figure 1B), the presence of triple-positive T cells also associated with a significantly better survival (Figure 3C, p = 0. 0028).

As the CD8/CD103 double-positive counts also include the CD8/CD103/TIM-3 triple-positive counts, an analysis was performed in which the triple-positive counts were removed from the double-positive counts, yielding a double – triple cluster (CD8/CD103-positive cells without (co-)expressing TIM-3) (Figure 3D). Interestingly, even though the double – triple population still significantly associates with (p = 0.021), the survival difference between the high and the low fraction was greatly reduced compared to the original, triple-positive high vs low cluster (Figure 3B vs 3D). Of note, a CD8 single-positive population without cells (co-)expressing CD103 and TIM-3 was even associated with reduced survival (p = 0.0012, Figures 3A vs 3E). Together, a clear survival benefit was detected in patients with high CD8/CD103/TIM-3 triple-positive tumor infiltration. Further, the higher survival probability observed for the single-positive high and double-positive high clusters can mainly be attributed to triple-positive cell also present within this population.

Tumor-infiltrating terminally exhausted CD8-positive T cells have tumor-reactive signatures and co-express CXCL13 and TIM-3

To understand the observed differential survival, we analyzed differentially expressed genes (DEGs) within the pre-defined (34) terminally exhausted CD8-positive TIL cluster versus all the other tumor infiltrating immune cell clusters.

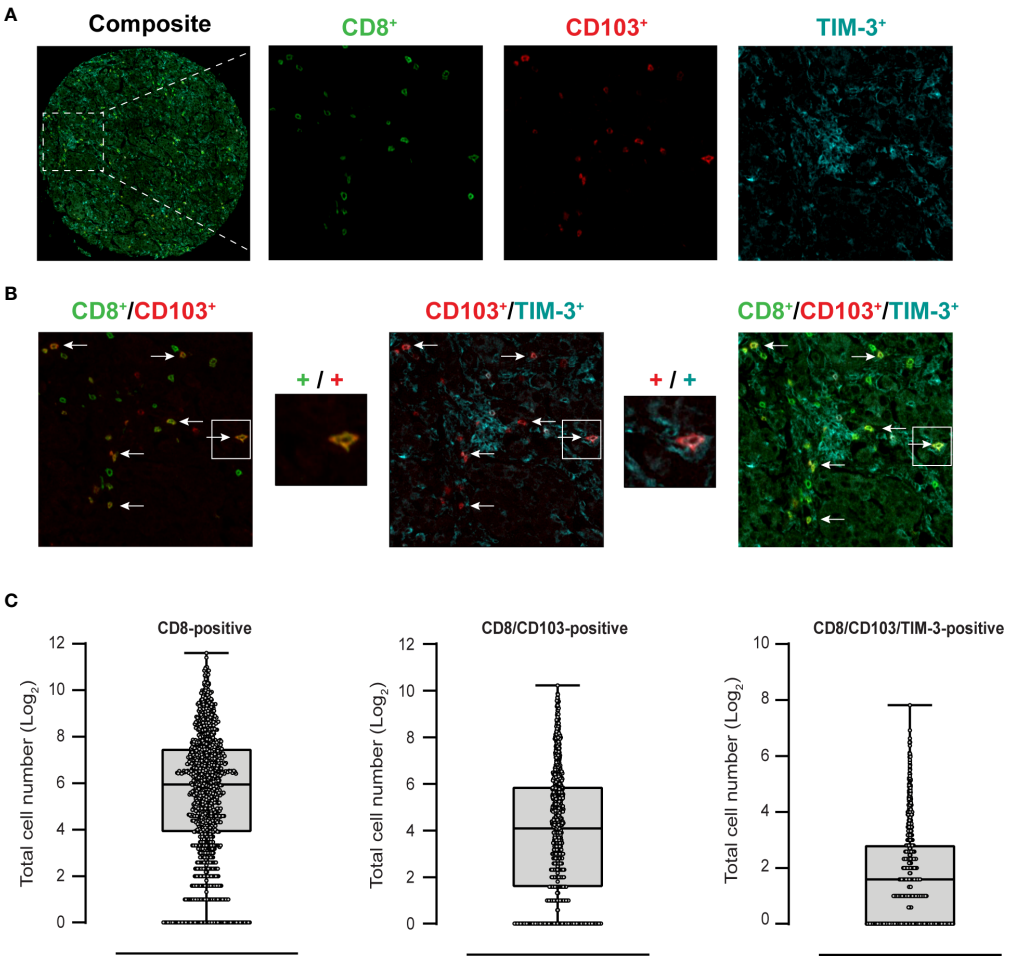

FIGURE 1
Tumor infiltration of immune cell subsets CD8-positive, CD8/CD103-positive and CD8/CD103/TIM-3-positive in EOC core samples. Exemplary processed IHC slide stained with antibodies targeting CD8 (green), CD103 (red) and TIM-3 (cyan) and corresponding secondary antibodies **(A)**. TMA sections were scanned using a TissueFAXS imaging system. Processed channels were merged using ImageJ. Fluorescent overlay analysis revealing CD8/CD103 double-positive, CD103/TIM-3 double positive and CD8/CD103/TIM-3 triple-positive cells **(B)**. Single-, double- and triple-positive cell populations were counted using ImageJ. Total raw cell counts of the single-, double- and triple-positive cell populations are Log2 transformed and displayed in **(C)**.

This analysis revealed co-expression of several genes associated with exhaustion, such as Lymphocyte Activating 3 (LAG3), TIM-3, T Cell Immunoreceptor With Ig And ITIM Domains (TIGIT), Programmed cell death protein 1 (PDCD1), CD39 (encoded by the ENTPD1 gene) and Cytotoxic T Lymphocyte Associated Protein 4 (CTLA4) (Figure 4A, left). Genes associated with cytotoxicity, such as Granzyme B (GZMB), Natural Killer Cell Granule Protein 7 (NKG7), Interferon Gamma (IFNG), Granzyme A (GZMA), Granulysin (GNLY), TNF Receptor Superfamily Member 9 (TNFRSF9/4-1BB) and CD27 were likewise upregulated (Figure 4A, left).

In line with expectation, upregulated expression of tissue-resident memory T cell marker CD103 (encoded by the ITGAE gene) was detected, as well as upregulation of the B cell recruiting chemokine CXCL13 (Figure 4A, left). CXCL13/CD103/CD8 triple-positive TILs have previously been identified with B cell recruitment, TLS formation, neo-antigen burden and cytolytic gene signatures in human tumors and TIM-3 expression has been identified on CD39/CD103 double-positive tumor-reactive CD8-positive T cells (17, 25). Therefore, a possible TIM-3 and CXCL13 co-expression pattern within the tumor infiltrating immune-repertoire was further evaluated by us.

Both TIM-3 and CXCL13 expression was found across a range of cell types, with relatively high expression of both molecules found within the terminally exhausted CD8-positive T cell fraction (Figure 4B, see arrow's). As expected, high CXCL13 expression was also found in T follicular helper cells (39). When comparing DEGs

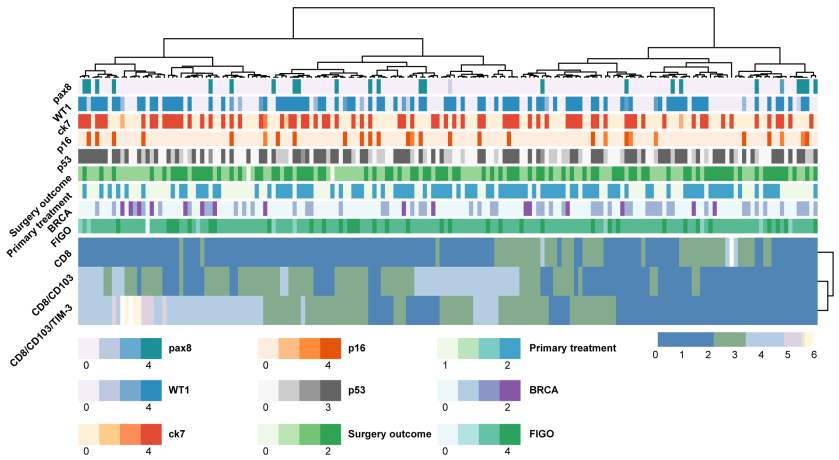

FIGURE 2
Patterns of infiltration of CD8/CD103/TIM-3-expressing immune cell subsets. Heatmap displaying infiltration of CD8/CD103/TIM-3-expressing immune cell subsets in EOC core samples. Hierarchical cluster analysis of all samples displayed three clusters based on immune cell populations: CD8-positive, CD8/CD103-positive and CD8/CD103/TIM-3-positive. Clinical characteristics are displayed for each sample, including FIGO-stage, BRCA-status, primary treatment strategy (primary debulking surgery (PDS) versus neoadjuvant chemotherapy (NACT)) and the presence of macroscopic disease after surgery (complete versus incomplete). Histopathological markers determined during diagnostic workup including p53, PAX8, WT1 and CK7 are further displayed. Clustering of cases was done by hierarchical clustering using Ward's minimum variance method in R using package pheatmap. Correlations between immune clusters and clinical and histopathological variables were analyzed using multiple regression analysis in SPSS.

within the CXCL13-positive and negative fractions from the CD8-positve TIL subset, again exhausted and cytotoxic signatures were found (e.g., upregulation of LAG3, PDCD1, CTLA4, TNFRSF18 (GITR), IFNG, TNFRSF4 (OX40) and TNFRSF9 (4-1BB)) (Figure 4C). Interestingly, in this analysis upregulated expression of TIM-3 was also detected (Figure 4C, third bar from left), suggesting a possible co-expression profile with CXCL13. Confirmatory flowcytometric evaluation of CXCL13 expression on isolated EOC TILs revealed that CXCL13 was predominantly found within the CD8/CD103/TIM-3 triple-positive fraction compared to it's single- and double-positive counterparts (Figure 4D, red bars vs all others). A representative gating strategy is displayed in Supplemental Figure 1.

Discussion

In the present study, we demonstrated that a small population of CD8/CD103/TIM-3 triple-positive TILs was present in the tumor micro-environment of EOC patients. This triple-positive population associated with improved survival in EOC. Additionally, by evaluating gene signatures in terminally exhausted CD8-positive TILs from various cancer types, an effector/exhaustive/tumor-reactive profile with a co-expression pattern of CD103, TIM-3 and CXCL13 was found.

The prognostic value of TIM-3 expression on TILs is a subject of debate, as for some cancers high expression of TIM-3 within the TIL population has been associated with poor prognosis, whereas in others it was found to have a positive impact on prognosis (reviewed in (40)). For example, even though PD-1/TIM-3 double-positive CD8-positive TILs in ovarian cancer displayed enhanced potential for cytokine production and proliferation compared to other CD8-positive TIL subsets, patients highly expressing PD-1 and TIM-3 in TILs had reduced progression free survival compared to patients with low PD-1 and TIM-3 TIL expression. However, no significant difference for overall survival was observed (21). Likewise, in oropharyngeal squamous cell carcinoma (OPSCC) TIM-3 expression in TILs was associated with a higher number of CD8-positive TILs, whereas no significant impact on overall survival was observed (41). In gastrointestinal stromal tumors (GIST) on the other hand, TIM-3 expression levels on TILs were an independent predictor of patients' overall survival and disease-free survival (42). Interestingly, although PD-1 and TIM-3 expressing TILs in diffuse large B-cell lymphoma (DLBCL) displayed an exhausted phenotype, their actual total numbers were expanded, and they expressed high levels of cytotoxic molecules (43). Promisingly, their proliferative potential and cytokine release could subsequently be restored by PD-1 or TIM-3 blockade. Solely evaluating TIM-3 expression levels on total TIL population is therefore not sufficient to define the subset of TILs that associate with survival. For this reason, the identification of a more relevant TIL population, like reported here, in terms of association with patient survival is of interest.

In the current report, no significant association of the clinical characteristics FIGO-stage, BRCA-status, primary treatment strategy or surgery outcome with any of the evaluated immune cell clusters was established, although an association between

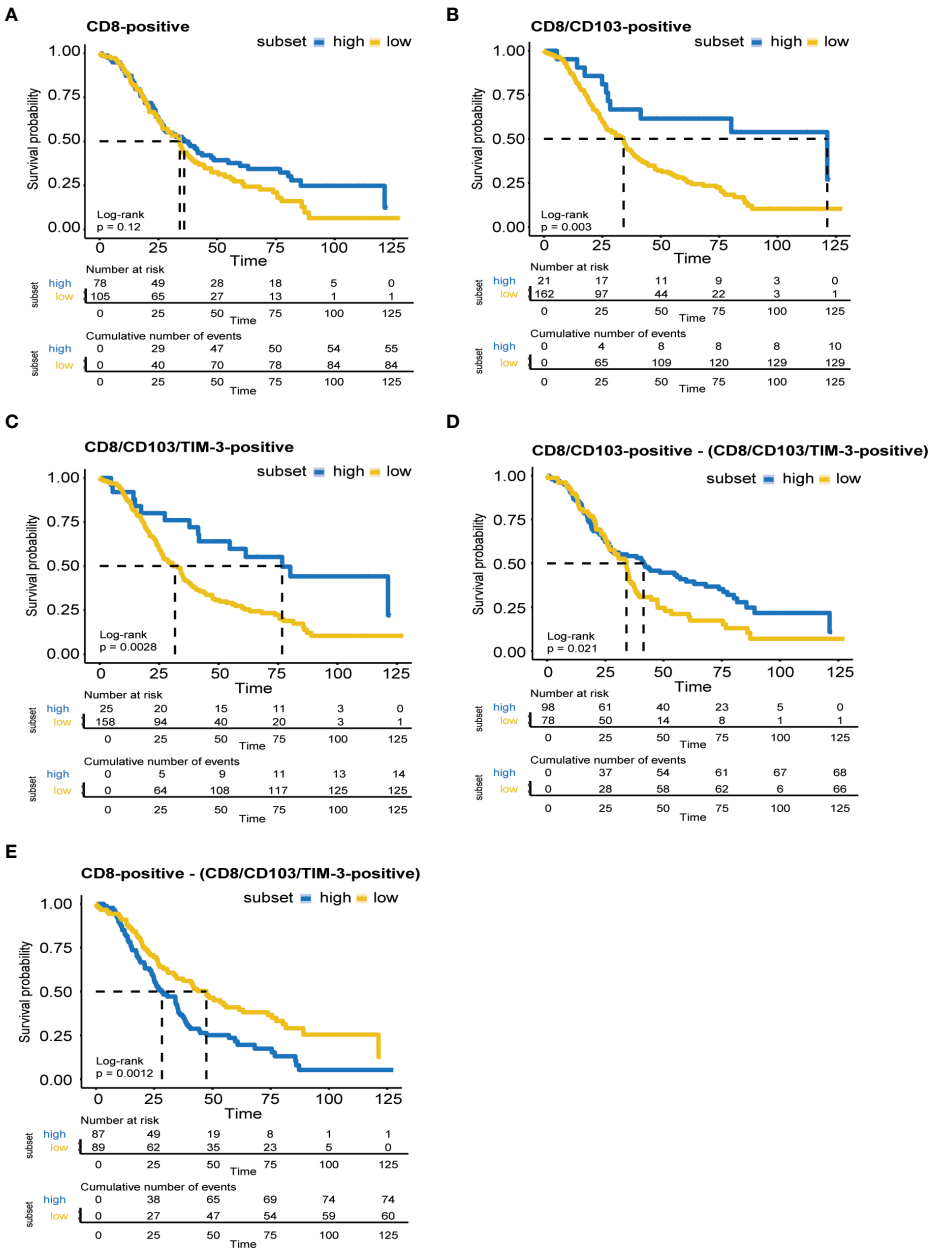

FIGURE 3
TIL TIM-3 expression associates with improved survival in EOC. Analyses of OS in months as a function of immune cell density based on immune cell populations CD8-positive **(A)**, CD8/CD103-positive **(B)**, CD8/CD103/TIM-3-positive **(C)**, CD8/CD103-positive - (CD8/CD103/TIM-3-positive) **(D)** and CD8-positive - (CD8/CD103/TIM-3-positive) **(E)** performed by Cox proportional hazard models in R using packages RMS and survival, plotted using package ggPlot2. Proportionality of hazards was confirmed by scaled Schoenfeld residuals. Optimal cutoff analysis was determined in R using package Survminer. Survival curves were plotted in R using Survminer by using the Kaplan–Meier method. A p-value of <0.05 was used as cutoff for significance.

BRCA-status and the CD8/CD103/TIM-3 triple-positive cluster was observed. A BRCA 1/2-status has previously been linked to immunogenicity and survival and might also be predictive for response to immune checkpoint inhibitors (44–46). Further, the survival benefit observed in the current report for EOC patients can mainly be attributed to the presence of CD8/CD103/TIM-3 triple-positive TILs, with limited to no impact of the CD8/CD103 double-positive or the CD8 single-positive cell population on survival. In accordance, in patients with clear cell renal cell carcinoma (ccRCC) it was shown that although extensive CD8-positive T cell infiltrate levels were observed, due to the absence of TLSs and expression of immune checkpoints there was an

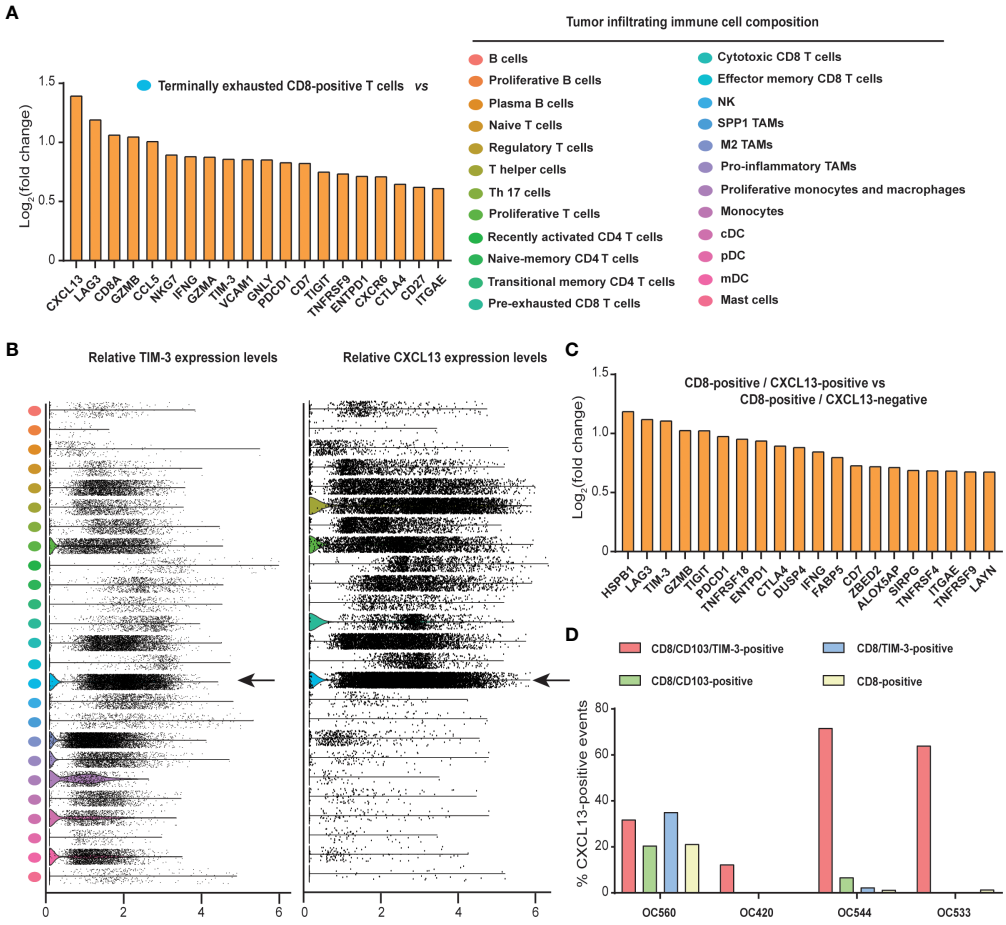

FIGURE 4
Tumor infiltrating terminally exhausted CD8-positive T cells have tumor-reactive signatures and co-express CXCL13 and TIM-3. Single-cell tumor immune atlas RNA sequencing data-set based on over 500,000 cells from 217 patients and 13 cancer types to evaluate gene expression in the tumor immune microenvironment. Immune cell fractions were pre-separated into 25 different clusters using canonical markers and curated gene signatures (**A**, right). Evaluation of DEGs within the terminally exhausted CD8-positive TIL cluster versus all the other tumor infiltrating immune cell clusters (**A**, left). Relative TIM-3 and CXCL13 expression found across the different immune cell fractions **(B)**. DEGs found within the CD8-positive CXCL13-positive vs CD8-positive CXCL13-negative analysis **(C)**. **(D)** % CXCL13 events found in CD8/CD103/TIM-3-positive (red bars), CD8/CD103-positive (green bars), CD8/TIM-3-positive (blue bars) and CD8-positive (yellow bars) populations from 4 different primary ovarian cancer patient TIL samples evaluated by flow cytometry.

increased risk of disease progression (33). As in the current manuscript no evidence is provided that CXCL13 produced by CD8/CD103/TIM-3-triple positive cells leads to TLS formation, evaluation of additional immune checkpoints and assessing the presence of TLSs in the same EOC cohort as in the present study may further help implementing of our observations.

CXCL13 is a key molecular determinant of the formation of prognostically favorable TLSs and is considered to be a surrogate marker for tumor TLS (26). Multiple studies have linked the expression of CXCL13 to patient prognosis and its potential as response biomarker to immunotherapy (26, 31, 39, 47). CXCL13 plays an important role in shaping the anti-tumor microenvironment by facilitating immune cell recruitment, their activation and regulating the adaptive immune response (48). Interestingly, in our scRNAseq analysis, CXCL13 expression was found within the terminally exhausted CD8-positive T cell fraction next to that of TIM-3 and CD103. Expression of CXCL13 was subsequently also confirmed on the CD8/CD103/TIM-3 triple-positive fraction in primary EOC samples. Within the CD8 subsets, CD8/CD103/TIM-3-positive cells predominantly express CXCL13 and their infiltration is associated with improved patient survival in EOC. However, this finding may be limited to EOC as other studies show that CD8/CXCL13-positive cells are also associated with poor clinical outcomes and display an immunoevasive contexture in the TME of ccRCC and gastric cancer (49, 50).

Multiple DEGs, collectively reflecting an exhaustive phenotype with a tumor-reactive potential, were furthermore found in the scRNAseq analysis when comparing tumor exhaustive CD8-positive TILs to the complete tumor infiltrating immune cell repertoire. For example, upregulated expression of LAG3, TIM-3, TIGIT, PDCD1, CTLA4 confirmed a transcriptome associated with exhaustion (51, 52). Further, upregulated expression of CD39 (ENTPD1) was detected, a marker of persistent TCR stimulation on exhaustive T cells (17, 53), co-expression of which with tissue-resident memory T cell marker CD103 (ITGAE) has been identified on tumor-reactive CD8-positive T cells in human solid cancers (17). The anti-tumor CD103-positive CD8-positive T cell subset has furthermore been associated with chemokine CXCL13 expression (28), and is in line with the co-expression with TIM-3 in our analysis.

Targeting inhibitory receptors like TIM-3 to reverse T cell exhaustion is of potential therapeutic interest for a variety of cancers (54–56). In this respect, antagonistic antibodies targeting TIM-3 on tumor-specific exhausted T cells alone or in combination with PD-1 or PD-L1 targeting antibodies are under clinical evaluation (57–59). As monotherapy however, none to limited anti-tumor activity has been reported so far (57, 58). A bi-specific antibody targeting both TIM-3 and PD-L1 has also been clinically evaluated, but it's further development was terminated due to unexpected immunogenicity upon targeting of both the TIM-3 and PD-L1 arms (60). Indeed, the tumor-specific role of T cell expressed TIM-3 as well as potential tumor cell-expressed TIM-3 will need to be clarified in order to rationally design TIM-3 targeted immunotherapy.

In conclusion, we identified a small set of CD8/CD103/TIM-3-expressing tumor infiltrated T cells in EOC patients associated with improved EOC patient survival. Therefore, CD8/CD103/TIM-3 triple-positive TILs may be a prognostic marker for EOC and represents a target population of interest for reactivation by immunotherapeutics. Further, DEG analysis revealed upregulated expression of co-stimulatory, cytotoxic, and exhaustive genes, and notably that of CXCL13, CD103 and TIM-3 within the terminally exhausted CD8-positive T cell fraction. Due to the observed co-expression pattern of TIM-3 and CXCL13, TIM-3 expression on CD8/CD103-double positive TILs may be used as surrogate marker for prognostically favorable CXCL13-positive CD8-positive TILs and may have prognostic value itself.

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.

Ethics statement

The study was approved by the local UMCG ethics review board under Register number 201700448. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conceptualization: MV, VB, GH, HN, MB and EB. Data curation: MV, VB, JA, AP and MB. Formal analysis: MV, VB, JA, AP and MB. Investigation: MV, VB, MB and EB. Supervision: EB. Writing – original draft: MV and VB. Writing – review & editing: VM, MB and EB. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1031746/full#supplementary-material

SUPPLEMENTARY FIGURE 1
A representative gating strategy for flowcytometric evaluation of CXCL13 expression on isolated EOC TILs within the CD8/CD103/TIM-3 triple-positive fraction compared to it's single- and double-positive counterparts. Cells were gated, followed by the selection of the viable CD3/CD8-positive subset. Here the CD8/CD103/TIM-3 subsets were evaluated for CXCL13 expression. An "all abs -CXCL13 antibody" was taken along as control.

References

1. Zhang L, Conejo-Garcia JR, Katsaros D, Gimotty PA, Massobrio M, Regnani G, et al. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. *N Engl J Med* (2003) 348(3):203–13. doi: 10.1056/NEJMoa020177

2. Sato E, Olson SH, Ahn J, Bundy B, Nishikawa H, Qian F, et al. *Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. Proc Natl Acad Sci U.S.A.* (2005) 102(51):18538–43. doi: 10.1073/pnas.0509182102

3. Li J, Wang J, Chen R, Bai Y, Lu X. The prognostic value of tumor-infiltrating T lymphocytes in ovarian cancer. *Oncotarget* (2017) 8(9):15621–31. doi: 10.18632/oncotarget.14919

4. Wei SC, Duffy CR, Allison JP. Fundamental mechanisms of immune checkpoint blockade therapy. *Cancer Discovery* (2018) 8(9):1069–86. doi: 10.1158/2159-8290.CD-18-0367

5. Leach DR, Krummel MF, Allison JP. Enhancement of antitumor immunity by CTLA-4 blockade. *Science* (1996) 271(5256):1734–6. doi: 10.1126/science.271.5256.1734

6. Rotte A. Combination of CTLA-4 and PD-1 blockers for treatment of cancer. *Journal of Experimental & Clinical Cancer Research* (2019) 38(1):1–12. J.J.o.E. and C.C. Research. doi: 10.1186/s13046-019-1259-z

7. Brahmer JR, Tykodi SS, Chow LQ, Hwu WJ, Topalian SL, Hwu P, et al. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *N Engl J Med* (2012) 366(26):2455–65. doi: 10.1056/NEJMoa1200694

8. du Bois A, Kristensen G, Ray-Coquard I, Reuss A, Pignata S, Colombo N, et al. Standard first-line chemotherapy with or without nintedanib for advanced ovarian cancer (AGO-OVAR 12): a randomised, double-blind, placebo-controlled phase 3 trial. *Lancet Oncol* (2016) 17(1):78–89. doi: 10.1016/S1470-2045(15)00366-6

9. Jayson GC, Kohn EC, Kitchener HC, Ledermann JA. Ovarian cancer. *Lancet* (9951) 2014:1376–88:384. doi: 10.1016/S0140-6736(13)62146-7

10. Webb JR, Milne K, Watson P, DeLeeuw RJ, Nelson B. Tumor-infiltrating lymphocytes expressing the tissue resident memory marker CD103 are associated with increased survival in high-grade serous ovarian cancer. *Clin Cancer Res* (2014) 20(2):434–44. doi: 10.1158/1078-0432.CCR-13-1877

11. Schenkel JM, Masopust DJI. *Tissue-resident memory T cells. Immunity* (2014) 41(6):886–97. doi: 10.1016/j.immuni.2014.12.007

12. Komdeur FL, Wouters MC, Workel HH, Tijans AM, Terwindt AL, Brunekreeft KL, et al. CD103+ intraepithelial T cells in high-grade serous ovarian cancer are phenotypically diverse TCRalphabeta+ CD8alphabeta+ T cells that can be targeted for cancer immunotherapy. *Oncotarget* (2016) 7(46):75130–44. doi: 10.18632/oncotarget.12077

13. Webb JR, Milne K, Nelson BH. PD-1 and CD103 are widely coexpressed on prognostically favorable intraepithelial CD8 T cells in human ovarian cancer. *Cancer Immunol Res* (2015) 3(8):926–35. doi: 10.1158/2326-6066.CIR-14-0239

14. Komdeur FL, Prins TM, van de Wall S, Plat A, Wisman GBA, Hollema H, et al. CD103+ tumor-infiltrating lymphocytes are tumor-reactive intraepithelial CD8+ T cells associated with prognostic benefit and therapy response in cervical cancer. *Oncoimmunology* (2017) 6(9):e1338230. doi: 10.1080/2162402X.2017.1338230

15. Djenidi F, Adam J, Goubar A, Durgeau A, Meurice G, de Montpréville V, et al. CD8+CD103+ tumor-infiltrating lymphocytes are tumor-specific tissue-resident memory T cells and a prognostic factor for survival in lung cancer patients. *J Immunol* (2015) 194(7):3475–86. doi: 10.4049/jimmunol.1402711

16. Gros A, Robbins PF, Yao X, Li YF, Turcotte S, Tran E, et al. PD-1 identifies the patient-specific CD8(+) tumor-reactive repertoire infiltrating human tumors. *J Clin Invest* (2014) 124(5):2246–59. doi: 10.1172/JCI73639

17. Duhen T, Duhen R, Montler R, Moses J, Moudgil T, de Miranda NF, et al. Co-Expression of CD39 and CD103 identifies tumor-reactive CD8 T cells in human solid tumors. *Nat Commun* (2018) 9(1):2724. doi: 10.1038/s41467-018-05072-0

18. Monney L, Sabatos CA, Gaglia JL, Ryu A, Waldner H, Chernova T, et al. Th1-specific cell surface protein Tim-3 regulates macrophage activation and severity of an autoimmune disease. *Nature* (2002) 415(6871):536–41. doi: 10.1038/415536a

19. Fourcade J, Sun Z, Benallaoua M, Guillaume P, Luescher IF, Sander C, et al. Upregulation of Tim-3 and PD-1 expression is associated with tumor antigen-specific CD8+ T cell dysfunction in melanoma patients. *J Exp Med* (2010) 207 (10):2175–86. doi: 10.1084/jem.20100637

20. Granier C, Dariane C, Combe P, Verkarre V, Urien S, Badoual C, et al. Tim-3 expression on tumor-infiltrating PD-1(+)CD8(+) T cells correlates with poor clinical outcome in renal cell carcinoma. *Cancer Res* (2017) 77(5):1075–82. doi: 10.1158/0008-5472.CAN-16-0274

21. Sawada M, Goto K, Morimoto-Okazawa A, Haruna M, Yamamoto K, Yamamoto Y, et al. PD-1+ Tim3+ tumor-infiltrating CD8 T cells sustain the potential for IFN-gamma production, but lose cytotoxic activity in ovarian cancer. *Int Immunol* (2020) 32(6):397–405. doi: 10.1093/intimm/dxaa010

22. Fucikova J, Rakova J, Hensler M, Kasikova L, Belicova L, Hladikova K, et al. TIM-3 dictates functional orientation of the immune infiltrate in ovarian cancer. *Clin Cancer Res* (2019) 25(15):4820–31. doi: 10.1158/1078-0432.CCR-18-4175

23. Do Byun K, Hwang HJ, Park KJ, Kim MC, Cho SH, Ju MH, et al. T-Cell immunoglobulin mucin 3 expression on tumor infiltrating lymphocytes as a positive prognosticator in triple-negative breast cancer. *J Breast Cancer* (2018) 21 (4):406–14. doi: 10.4048/jbc.2018.21.e61

24. Qin S, Dong B, Yi M, Chu Q, Wu K. Prognostic values of TIM-3 expression in patients with solid tumors: A meta-analysis and database evaluation. *Front Oncol* (2020) 10:1288. doi: 10.3389/fonc.2020.01288

25. Thommen DS, Koelzer VH, Herzig P, Roller A, Trefny M, Dimeloe S, et al. A transcriptionally and functionally distinct PD-1(+) CD8(+) T cell pool with predictive potential in non-small-cell lung cancer treated with PD-1 blockade. *Nat Med* (2018) 24(7):994–1004. doi: 10.1038/s41591-018-0057-z

26. Groeneveld CS, Fontugne J, Cabel L, Bernard-Pierrot I, Radvanyi F, Allory Y, et al. Tertiary lymphoid structures marker CXCL13 is associated with better survival for patients with advanced-stage bladder cancer treated with immunotherapy. *Eur J Cancer* (2021) 148:181–9. doi: 10.1016/j.ejca.2021.01.036

27. Duan H, Lv Y, Liao P, Wang Y, Zheng Z, Qu H. *CXCL13 correlates with prognosis, immune infiltration, and T cell exhaustion in ovarian cancer*. (2021) doi: 10.21203/rs.3.rs-1026024/v1

28. Workel HH, Lubbers JM, Arnold R, Prins TM, van der Vlies P, de Lange K, et al. A transcriptionally distinct CXCL13(+)CD103(+)CD8(+) T-cell population is associated with b-cell recruitment and neoantigen load in human cancer. *Cancer Immunol Res* (2019) 7(5):784–96. doi: 10.1158/2326-6066.CIR-18-0517

29. Dieu-Nosjean MC, Giraldo NA, Kaplon H, Germain C, Fridman WH, Sautès-Fridman C. Tertiary lymphoid structures, drivers of the anti-tumor responses in human cancers. *Immunol Rev* (2016) 271(1):260–75. doi: 10.1111/imr.12405

30. Cabrita R, Lauss M, Sanna A, Donia M, Skaarup Larsen M, Mitra S, et al. Tertiary lymphoid structures improve immunotherapy and survival in melanoma. *Nature* (2020) 577(7791):561–5. doi: 10.1038/s41586-019-1914-8

31. Yang M, Lu J, Zhang G, Wang Y, He M, Xu Q, et al. CXCL13 shapes immunoactive tumor microenvironment and enhances the efficacy of PD-1 checkpoint blockade in high-grade serous ovarian cancer. *J Immunother Cancer* (2021) 9(1):e001136. doi: 10.1136/jitc-2020-001136

32. Kroeger DR, Milne K, Nelson BH. Tumor-infiltrating plasma cells are associated with tertiary lymphoid structures, cytolytic T-cell responses, and superior prognosis in ovarian cancer. *Clin Cancer Res* (2016) 22(12):3005–15. doi: 10.1158/1078-0432.CCR-15-2762

33. Giraldo NA, Becht E, Skliris G, Verkarre V, Vano Y, Mejean A, et al. Orchestration and prognostic significance of immune checkpoints in the microenvironment of primary and metastatic renal cell cancer. *Clin Cancer Res* (2015) 21(13):3031–40. doi: 10.1158/1078-0432.CCR-14-2926

34. Nieto P, Elosua-Bayes M, Trincado JL, Marchese D, Massoni-Badosa R, Salvany M, et al. A single-cell tumor immune atlas for precision oncology. *Genome research* (2020) 31(10):1913–26. doi: 10.1101/2020.10.26.354829

35. Wouters MC, Komdeur FL, Workel HH, Klip HG, Plat A, Kooi NM, et al. Treatment regimen, surgical outcome, and T-cell differentiation influence prognostic benefit of tumor-infiltrating lymphocytes in high-grade serous ovarian cancer. *Clin Cancer Res* (2016) 22(3):714–24. doi: 10.1158/1078-0432.CCR-15-1617

36. De Jong RA, Leffers N, Boezen HM, Ten Hoor KA, van der Zee AGJ, Hollema H, et al. Presence of tumor-infiltrating lymphocytes is an independent prognostic factor in type I and II endometrial cancer. *Gynecol Oncol* (2009) 114 (1):105–10. doi: 10.1016/j.ygyno.2009.03.022

37. Finak G, McDavid A, Yajima M, Deng J, Gersuk V, Shalek AK, et al. MAST: a flexible statistical framework for assessing transcriptional changes and characterizing heterogeneity in single-cell RNA sequencing data. *Genome Biol* (2015) 16:278. doi: 10.1186/s13059-015-0844-5

38. *Human tissue and medical research: Code of conduct for responsible use* (2011). Available at: https://www.federa.org/sites/default/files/images/print_version_code_of_conduct_english.pdf.

39. Gu-Trantien C, Migliori E, Buisseret L, de Wind A, Brohée S, Garaud S, et al. CXCL13-producing TFH cells link immune suppression and adaptive memory in

human breast cancer. *JCI Insight* (2017) 2(11):e91487. doi: 10.1172/jci.insight.91487

40. Kandel S, Adhikary P, Li G, Cheng K. The TIM3/Gal9 signaling pathway: An emerging target for cancer immunotherapy. *Cancer Lett* (2021) 510:67–78. doi: 10.1016/j.canlet.2021.04.011

41. Wuerdemann N, Pütz K, Eckel H, Jain R, Wittekindt C, Huebbers CU, et al. LAG-3, TIM-3 and VISTA expression on tumor-infiltrating lymphocytes in oropharyngeal squamous cell carcinoma-potential biomarkers for targeted therapy concepts. *Int J Mol Sci* (2020) 22(1):379. doi: 10.3390/ijms22010379

42. Zhuang C, Ni B, Zhang ZZ, Zhao WY, Tu L, Ma XL, et al. Low distribution of TIM-3(+) cytotoxic tumor-infiltrating lymphocytes predicts poor outcomes in gastrointestinal stromal tumors. *J Immunol Res* (2021) 2021:6647292. doi: 10.1155/2021/6647292

43. Roussel M, Le KS, Granier C, Llamas Gutierrez F, Foucher E, Le Gallou S, et al. Functional characterization of PD1+TIM3+ tumor-infiltrating T cells in DLBCL and effects of PD1 or TIM3 blockade. *Blood Adv* (2021) 5(7):1816–29. doi: 10.1182/bloodadvances.2020003080

44. Strickland KC, Howitt BE, Shukla SA, Rodig S, Ritterhouse LL, Liu JF, et al. Association and prognostic significance of BRCA1/2-mutation status with neoantigen load, number of tumor-infiltrating lymphocytes and expression of PD-1/PD-L1 in high grade serous ovarian cancer. *Oncotarget* (2016) 7(12):13587–98. doi: 10.18632/oncotarget.7277

45. Wen WX, Leong CO. Association of BRCA1- and BRCA2-deficiency with mutation burden, expression of PD-L1/PD-1, immune infiltrates, and T cell-inflamed signature in breast cancer. *PLos One* (2019) 14(4):e0215381. doi: 10.1371/journal.pone.0215381

46. Zhou Z, Li M. Evaluation of BRCA1 and BRCA2 as indicators of response to immune checkpoint inhibitors. *JAMA Netw Open* (2021) 4(5):e217728. doi: 10.1001/jamanetworkopen.2021.7728

47. Ukita M, Hamanishi J, Yoshitomi H, Yamanoi K, Takamatsu S, Ueda A, et al. Tertiary lymphoid structures induced by CXCL13-producing CD4+ T cells increase tumor infiltrating CD8+ T cells and b cells in ovarian cancer. *bioRxiv* (2021). doi: 10.1101/2021.12.01.470493

48. Hsieh CH, Jian CZ, Lin LI, Low GS, Ou PY, Hsu C, et al. Potential role of CXCL13/CXCR5 signaling in immune checkpoint inhibitor treatment in cancer. *Cancers (Basel)* (2022) 14(2):294. doi: 10.3390/cancers14020294

49. Dai S, Zeng H, Liu Z, Jin K, Jiang W, Wang Z, et al. Intratumoral CXCL13(+)CD8(+)T cell infiltration determines poor clinical outcomes and immunoevasive contexture in patients with clear cell renal cell carcinoma. *J Immunother Cancer* (2021) 9(2):e001823. doi: 10.1136/jitc-2020-001823

50. Jin K, Cao Y, Gu Y, Fang H, Fei Y, Wang J, et al. Poor clinical outcomes and immunoevasive contexture in CXCL13+CD8+ T cells enriched gastric cancer patients. *Oncoimmunology* (2021) 10(1):1915560. doi: 10.1080/2162402X.2021.1915560

51. Egelston CA, Avalos C, Tu TY, Simons DL, Jimenez G, Jung JY, et al. Human breast tumor-infiltrating CD8(+) T cells retain polyfunctionality despite PD-1 expression. *Nat Commun* (2018) 9(1):4297. doi: 10.1038/s41467-018-06653-9

52. Wherry EJ, Kurachi M. Molecular and cellular insights into T cell exhaustion. *Nat Rev Immunol* (2015) 15(8):486–99. doi: 10.1038/nri3862

53. Gupta PK, Godec J, Wolski D, Adland E, Yates K, Pauken KE, et al. CD39 expression identifies terminally exhausted CD8+ T cells. *PLos Pathog* (2015) 11(10):e1005177. doi: 10.1371/journal.ppat.1005177

54. Sakuishi K, Apetoh L, Sullivan JM, Blazar BR, Kuchroo VK, Anderson AC. Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity. *J Exp Med* (2010) 207(10):2187–94. doi: 10.1084/jem.20100643

55. Ngiow SF, Von Scheidt B, Akiba H, Yagita H, Teng MW, Smyth MJ. Anti-TIM3 antibody promotes T cell IFN-γ–mediated antitumor immunity and suppresses established tumors. *Cancer research* (2011) 71(10):3540–51. doi: 10.1158/0008-5472.CAN-11-0096

56. Guo Z, Cheng D, Xia Z, Luan M, Wu L, Wang G, et al. Combined TIM-3 blockade and CD137 activation affords the long-term protection in a murine model of ovarian cancer. *J Transl Med* (2013) 11:215. doi: 10.1186/1479-5876-11-215

57. Curigliano G, Gelderblom H, Mach N, Doi T, Tai D, Forde PM, et al. Phase I/Ib clinical trial of sabatolimab, an anti-TIM-3 antibody, alone and in combination with spartalizumab, an anti-PD-1 antibody, in advanced solid tumors. *Clinical Cancer Research* (2021) 27(13):3620–9. doi: 10.1158/1078-0432.CCR-20-4746

58. Harding JJ, Moreno V, Bang YJ, Hong MH, Patnaik A, Trigo J, et al. Blocking TIM-3 in treatment-refractory advanced solid tumors: a phase ia/b study of LY3321367 with or without an anti-PD-L1 antibody. *Clinical Cancer Research* (2021) 27(8):2168–78. doi: 10.1158/1078-0432.CCR-20-4405

59. Desai J, Meniawy T, Beagle B, Li Z, Mu S, Wu J, et al. Bgb-A425, an investigational anti-TIM-3 monoclonal antibody, in combination with tislelizumab, an anti-PD-1 monoclonal antibody, in patients with advanced solid tumors: A phase I/II trial in progress. *Journal of Clinical Oncology* (2020) 38(15_suppl):TPS3146–TPS3146. doi: 10.1200/JCO.2020.38.15_suppl.TPS3146

60. Hellmann MD, Bivi N, Calderon B, Shimizu T, Delafontaine B, Liu ZT, et al. Safety and immunogenicity of LY3415244, a bispecific antibody against TIM-3 and PD-L1, in patients with advanced solid tumors. *Clinical Cancer Research* (2021) 27(10):2773–81. doi: 10.1158/1078-0432.CCR-20-3716

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Next generations of CAR-T cells - new therapeutic opportunities in hematology?

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In recent years, the introduction of chimeric antigen receptor (CAR) T-cell therapies into clinics has been a breakthrough in treating relapsed or refractory malignancies in hematology and oncology. To date, Food and Drug Administration (FDA) has approved six CAR-T therapies for specific non-Hodgkin lymphomas, B-cell acute lymphoblastic leukemia, and multiple myeloma. All registered treatments and most clinical trials are based on so-called 2nd generation CARs, which consist of an extracellular antigen-binding region, one costimulatory domain, and a CD3z signaling domain. Unfortunately, despite remarkable overall treatment outcomes, a relatively high percentage of patients do not benefit from CAR-T therapy (overall response rate varies between 50 and 100%, with following relapse rates as high as 66% due to limited durability of the response). Moreover, it is associated with adverse effects such as cytokine release syndrome and neurotoxicity. Advances in immunology and molecular engineering have facilitated the construction of the next generation of CAR-T cells equipped with various molecular mechanisms. These include additional costimulatory domains (3rd generation), safety switches, immune-checkpoint modulation, cytokine expression, or knockout of therapy-interfering molecules, to name just a few. Implementation of next-generation CAR T-cells may allow overcoming current limitations of CAR-T therapies, decreasing unwanted side effects, and targeting other hematological malignancies. Accordingly, some clinical trials are currently evaluating the safety and efficacy of novel CAR-T therapies. This review describes the CAR-T cell constructs concerning the clinical application, summarizes completed and ongoing clinical trials of next-generation CAR-T therapies, and presents future perspectives.

KEYWORDS

CAR-T cells, acute lymphoblastic leukemia, immunotherapy, lymphocyte, cytokine release syndrome, CRS, allogeneic, CRISPR

Introduction

The emergence of chimeric antigen receptor T (CAR-T) cell therapies has changed our view on treating malignancies in the field of hematology. The idea of harnessing the immune system in combat against cancer turned out to be the right way and showed outstanding treatment results. That was particularly true in the case of B cell acute lymphoblastic leukemia (B-ALL) (1) and diffuse large B-cell lymphoma (DLBCL) (2). But these hopeful outcomes were visible only in some patients, and additionally, many of them experienced severe side effects such as cytokine release syndrome (CRS) (3), neurotoxicity (4) or even death. In addition, despite initial response to the therapy, many patients eventually experienced disease relapse because of genetic mutations, short CAR-T cells persistence, immunogenicity against CAR-T cells, antigen escape, CAR-T cells exhaustion, or lineage switching (5). Consequently, the need to develop new CAR-T cells with better efficacy and safety profile emerged.

The first generation of CAR-T cells mimicked the natural cellular response by having the extracellular domain accountable for antigen recognition and was joined with the singular intracellular domain (6) (Figure 1A). The main disadvantage of that construct was the relatively short time of persistence of these cells in the patient, which is one of the known factors contributing to the response to the therapy (7). Therefore, the second generation of CAR-T cells emerged, which had an additional intracellular motif – the signaling domain of costimulatory receptors such as 4-1BB/CD137 (8) or CD28 (9) (Figure 1B). That caused the extended existence of CAR-T cells in the patient and better treatment results (10). Currently, all FDA-approved CAR-T therapies are based on this type of construct (Table 1). To improve the outcomes even further, researchers devised the idea of the third generation of CAR-T cells, which had a second costimulatory signaling domain (19) (Figure 1C). Most common, both CD28 and 4-1BB were used to enhance the effects of therapy (20). But still, the fact is that not all patients respond to that therapy (21). Therefore, the newest concepts – the fourth, fifth and other generations of CAR-T cells have emerged in recent years (22) (Figure 1D). For instance, these cells can produce IL-12 for remodeling the tumor microenvironment to break the resistance of the malignant cells (23). That construct is known as *T cells redirected for universal cytokine killing* (TRUCKS). Several other types of CAR-T cells being now under investigation are universal CAR [having no endogenous T cell receptor (TCR) or major histocompatibility complex (MHC)] (24), self-driving CAR (carrying a chemokine receptor on its surface which connects to the chemokines released by tumor cells) (25), armored CAR (resist immunosuppressive microenvironment created by malignant cells) (26), self-destruct CAR (due to administration of external signals their activity can be stopped) (27), and conditional CAR (due to administration of external signals their activity can be initiated) (28). The details regarding these constructs are going to be discussed further.

In this review, we aim to present the reader with the new constructs of CAR-T cells and show the currently recruiting clinical trials. Moreover, we summarize the results of completed clinical trials with CAR-T cells in the field of hematology and describe the perspectives for the future.

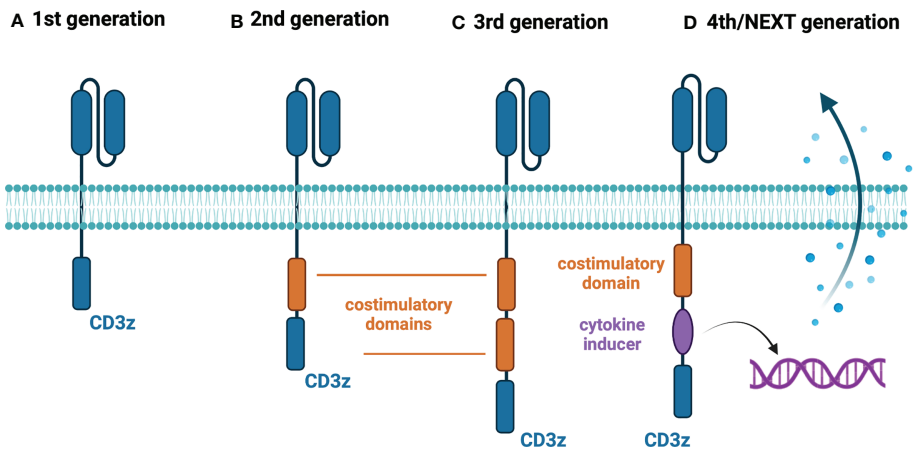

FIGURE 1
Generations of CAR-T cells. **(A)** First-generation CAR-T cells – equipped with an extracellular antigen-recognizing domain combined with intracellular CD3z accounting for signal transduction. **(B)** Second-generation CAR-T cells - equipped with an extracellular antigen-recognizing domain combined with two intracellular domains: CD3z and an additional costimulatory domain (e.g., CD28 or 4-1BB). **(C)** Third-generation CAR-T cells - equipped with an extracellular antigen-recognizing domain combined with three intracellular domains: CD3z and two additional costimulatory domains. **(D)** Fourth/Next-generation CAR-T cells – a diversified group of CAR-T constructs embracing armored CAR-T cells, cytokine-expressing CAR-T cells (illustrated above), switchable CAR-T cells, and universal CAR-T cells. Created with BioRender.com.

TABLE 1 FDA-approved CAR-T therapies – second-generation CAR-T cells.

No.	CAR-T name	Target antigen	Disease	Complete response (CR) rate	Overall response rate (ORR)	Grade 3 or higher CRS	Grade 3 or higher ICANS	Reference
1.	Axi-cel	CD19	r/r DLBCL/ FL/PMBCL	54%	82%	13%	28%	(11)
2.	Tisa-cel	CD19	r/r DLBCL	40%	52%	22%	12%	(12)
			r/r ALL in adults <25y	81%	81%	77%	40%	(13)
3.	Liso-cel	CD19	r/r DLBCL/ FL/PMBCL	53%	73%	42%	30%	(14)
4.	Brexu-cel	CD19	r/r MCL	59%	81%	15%	31%	(15)
			r/r ALL	56%	71%	24%	25%	(16)
5.	Ide-cel	BCMA	r/r MM	39%	76%	6%	3%	(17)
6.	Cilta-cel	BCMA	r/r MM	67%	97%	4%	9%	(18)

ALL, Acute Lymphoblastic Leukemia; CD, Cluster of Differentiation; CRS, Cytokine Release Syndrome; DLBCL, Diffuse Large B-cell Lymphoma; ICANS, Immune Effector Cell-Associated Neurotoxicity Syndrome; FL, follicular lymphoma; MCL, Mantle Cell Lymphoma; MM, Multiple Myeloma; PMBCL, Primary Mediastinal Large B-cell Lymphoma.

The third generation of CAR-T cells

As was said previously, the aim of developing the third generation of CAR-T cells was to enhance their efficiency. To do that, the researchers constructed a cell with two complementary costimulatory domains, most frequently combining CD28 and 4-1BB. The reason was the notion that costimulatory domains have different features, that could complement each other. That is particularly the point when it comes to the disease with a low burden as the signal for activation and persistence given by tumor cells will be limited, and the additional costimulation may be benefitable. For instance, CD28 may lead to quicker expansion of T cells and more rapid elimination of tumor cells, whereas 4-1BB is associated with longer persistence of CAR-T cells in the host (10). In that chapter, the results of trials with third - generation CAR-T cells in hematology will be summarized and still recruiting ones will be presented.

Initially, the main question about the third generation of CAR-T cells was to check whether it possesses better features than the second generation. Ramos et al. presented an *in vivo* study of third - generation *vs* second - generation CD19-specific CAR-T cells in B cell non-Hodgkin's lymphomas. They used two different constructs – one with only CD28 as a costimulating domain and the second one with two costimulating domains (CD28 and 4-1BB). That study showed better expansion (up to 40-fold) and longer persistence of 3-rd generation CAR-T cells compared with 2-nd generation. Interestingly, the difference was the most striking in the five patients with lower disease burden, which may be particularly useful in heavily pretreated patients. That study is still recruiting its patients and will end in February 2034 and have 64 participants not only with B cell lymphomas but also with ALL and chronic lymphocytic leukemia (CLL) (NCT01853631) (29).

In another study by Enblad et al., which was a phase I/IIa trial with CD-19 – targeting CAR-T cells of the third generation in patients with B-cell lymphomas and leukemias they showed similar results (NCT02132624). Six of 15 patients achieved initial complete response, and the procedure was relatively safe, with only four patients requiring hospitalization due to adverse events (30).

In the work of Schubert et al., the results confirming the previously reported outcomes were presented (NCT03676504). They reported eight patients that were infused with CD-19 – targeting CAR-T cells [2 with adult ALL, 2 CLL, 1 mantle cell lymphoma (MCL), 2 DLBCL, 1 transformed follicular lymphoma (FL)], and the clinical responses were seen in 6 of them (2 of them had CAR-T infusion just before the publication). The authors showed that the clinical responses were possible even with small numbers of CAR-T cells infused (10^6 cells/m^2 or $5x10^6$ cells/m^2), and the persistence of CAR-T cells improved (the cells were detectable three months following administration). Moreover, they migrated to CSF, which could be of great importance in the case of CNS involvement (31).

However, there are also clinical trials using third-generation CAR-T cells that are targeting other molecules than CD-19. One of them, third-generation CD-22 – targeting CAR-T cells, are now studied by Wuhan Union Hospital group (NCT04007978). In that phase 1 study, the patients with B cell lymphoma and ALL are being recruited. The study is estimated to be completed on December 30, 2022. The same group created a phase 1 study with a third-generation CAR-T cells targeting CD123 in patients with relapsed/refractory acute myeloid leukemia (AML) (NCT04014881). This molecule is a transmembrane subunit of the IL-3 receptor, expressed on AML blasts. The estimated study completion date was July 1, 2022, but the results have not been published yet. In the ENABLE phase 1 study, researchers use a third-generation CD19-targeting CAR-T cells to treat patients with r/r non-Hodgkin lymphomas to identify a safe dose (NCT04049513). The estimated study completion date is for August 2026 (32).

Altogether, although third-generation CAR-T cells show better expansion and longer persistence compared with 2-nd generation (29), some clinical assessments do not reveal such an advantage (30). Furthermore, despite encouraging overall outcomes, initial trial results do not show major improvements in response rates over the conventional CAR-T therapies (Table 1). However, currently available data are obtained from small and heterogenic samples, therefore, are insufficient to draw conclusions. Nevertheless, 3-rd generation CAR-T therapies still possess several drawbacks of the previous generation, for instance, manufacturing challenges or unsatisfactory efficacy, rationalizing investigating next-generation CAR-T cells.

The next generations of CAR-T cells

Advances in molecular engineering provided new options for managing CAR-T therapy-associated issues that have become unavoidable after its introduction into clinical practice (33). The researchers have developed various next-generation CAR-T constructs that incorporate exquisite mechanisms to overcome the constraints of currently available second-generation CAR-T therapies, namely excessive toxicity and limited efficacy (33). As the preclinical studies have shown promising outcomes, both *in vitro* and *in vivo*, many clinical trials evaluating the safety and efficacy of next-generation CAR-T cells have been commenced. As of August 2022, 85 such investigations have been registered at ClinicalTrials.gov, summarized in Supplementary Table 1. Currently, there are four distinctive approaches tested in clinics. Two aim to increase efficacy by modulating immune checkpoint pathways (9 trials) or induction of cytokine secretion (6 trials). Another investigated approach is implementing a safety-switch mechanism (40 trials) which enables the control of treatment-related adverse events, for instance, cytokine release syndrome (CRS), by disabling CAR-T cells with exogenous agents. Finally, 30 trials are evaluating genetically edited CAR-T cells suitable for allogeneic use or designated to treat T-cell malignancies. In the following sections, we describe the abovementioned approaches regarding clinical trials and confront the available results with the conventional CAR T therapies.

In addition, it is essential to emphasize that there are other approaches to utilizing CAR constructs that are beyond the scope of this review as they are based on the second generation of CAR constructs or other cell types. For instance, bispecific 2-nd generation CAR-T cells targeting two surface antigens showed promising results in preclinical and clinical studies (34, 35). The main aim of the bispecific approach is to reduce relapse rates caused by antigen loss (36). Research in CAR-NK cells is another promising field of study. Although CAR-NK cells have some advantages over conventional CAR-T cells (e.g., increased safety profile due to MHC independence and different spectrum of secreted cytokines; allogeneic use), their clinical application is limited by reduced expansion and persistence *in vivo* as well as manufacture difficulties (37). Nevertheless, all the benefits of CAR-NK cells can be achieved by engineering in next-generation CAR-T cells.

Armored CAR-T cells – immune checkpoint modulation

Immune checkpoint modulation in CAR-T therapy aims to circumvent the inhibitory stimulation in the tumor microenvironment. I hematology, all the clinical trials evaluating the feasibility of this approach rely explicitly on disrupting the programmed cell death protein 1 (PD-1) pathway. Interestingly, despite the relatively small number of trials (n=8), individual investigations present distinctive methods of dysregulating PD-1 signaling (Figure 2).

In NCT03258047, the researchers used an innovative approach in which the extracellular PD-1 was fused to the intracellular CD28 activating domain (38) (Figure 2A). Consequently, the binding of the programmed death-ligand 1 (PD-L1) to PD-1 was transformed into activating signal, thus, more potent anti-tumor efficacy had been expected (38). The study involved 17 participants suffering from B-cell non-Hodgkin lymphomas (13 – DLBCL, 2 – transformed FL, 2 – MCL) (38). The complete remission (CR) and objective response rate (ORR) achieved in this trial were 41,2% and 58,8%, respectively (38). For the DLBCL patients alone, CR was achieved by 5 of 13 patients (38,4%), whereas ORR was 54% (38). Compared to the approved second-generation CAR-T therapies for relapsed/refractory (r/r) DLBCL (CR 52%, ORR 72%) (2), these numbers show no initial advantage of next-generation CAR-T over conventional CAR-T; however, the comparison is highly biased due to small enrollment in the discussed clinical trial. The same immune checkpoint modulation approach is being Investigated in the NCT03932955 clinical trial with no results available.

Another explored method of disrupting PD-1 signaling is programming CAR-T cells to secrete PD-1 Fc fusion protein (Figure 2B). In this setting, PD-1 Fc fusion protein binds to PD-L1 and prevents its suppressive effects on T-cells. Currently, two studies are evaluating the safety and efficacy of the presented approach in r/r multiple myeloma (NCT04162119) and r/r B-cell lymphomas (NCT04163302). Unfortunately, no clinical data have been published yet.

In clinical trial NCT04836507, the investigators have presented initial results from r/r large B-cell lymphoma (LBCL) patients treated with anbalcabtagene autoleucel (Anbal-cel). This novel CAR-T cell product features knockdown of both PD-1 and T-cell immunoreceptor with Ig and ITIM (immunoreceptor tyrosine-based inhibitory motif) domains (TIGIT) (39). Anbal-cel demonstrated impressive

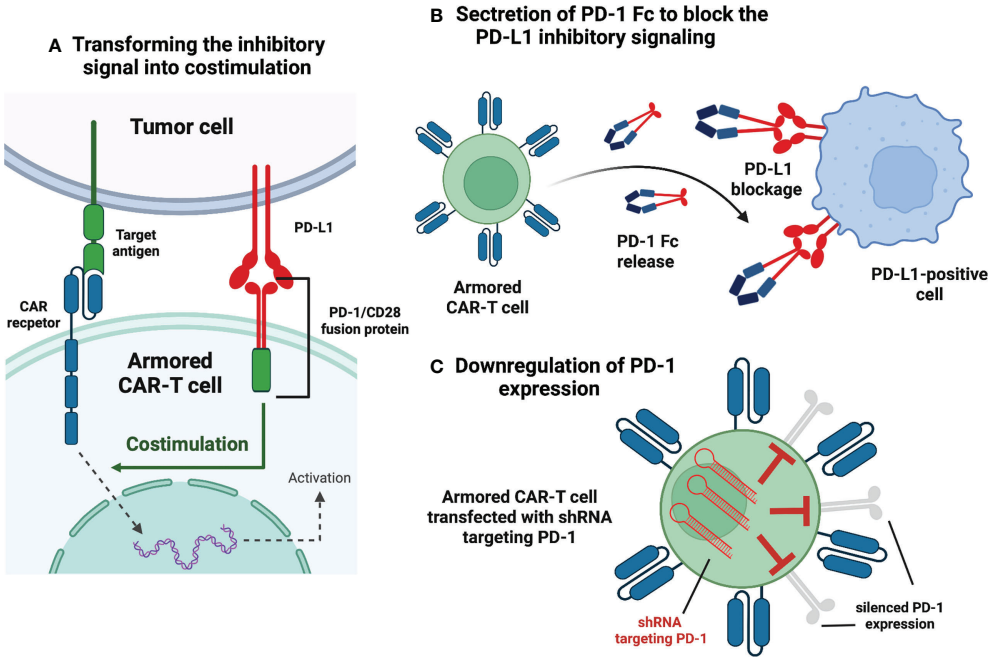

FIGURE 2
Armored CAR-T cells – immune checkpoint modulation. **(A)** Transforming the inhibitory signal into stimulation. This construct embodies the extracellular PD-1 domain fused to the intracellular CD28. Interaction between PD-1 and PD-L1 (expressed in the tumor microenvironment) is transformed into activating signal and leads to the enhanced CAR-T response. **(B)** Secretion of PD-1 Fc to block PD-L1 inhibitory signaling. The CAR-T cell is programmed to express and secrete a protein that combines the PD-1 domain and fragment crystallizable region (Fc) of an antibody. The secreted protein blocks PD-L1 molecules of malignant cells and makes them susceptible to innate immune cells. **(C)** Downregulation of PD-1 expression. The CAR-T cells are transfected with short hairpin RNA (shRNA) and subsequently, PD-1 expression is silenced *via* RNA interference. Created with BioRender.com.

outcomes with a CR of 78% (39), and further investigations are planned (39). In addition, knockdown of PD-1 is also being evaluated in NCT03208556 clinical trial (Figure 2C). Other approaches targeting PD-1 signaling include Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-mediated knockdown of PD-1 gene (NCT04213469) and incorporation of cytosolic-activated PD-1 (NCT03540303). Both trials investigate the safety and efficacy of next-generation CAR-T cells in r/r B-cell non-Hodgkin lymphomas (NHLs).

Except for the PD-1 pathway modulation, another armored CAR-T cell construct is being evaluated in the NCT04037566 clinical trial. CAR-T cells with decreased expression of Hematopoietic Progenitor Kinase 1 (HPK1) – a negative intracellular immune checkpoint (40), showed promising preliminary results (41). In all of the enrolled patients, 72,7% of them achieved CR or CRi, comparable with FDA-approved anti-CD19 therapies (41).

In conclusion, armored CAR-T cells appear to be a promising therapeutic approach in the treatment CD19 positive malignancies. In the discussed trials, complete response rates vary from 41,2% to impressive 78% (38, 39). Nevertheless, due to the small number of patients enrolled in the studies, large-scale investigations should be conducted to evaluate the feasibility of these constructs.

TRUCKs – cytokine-expressing CAR-T cells

T cells redirected for universal cytokine-mediated killing (TRUCKs) are next-generation CAR-T cells engineered to express certain cytokines to augment CAR-T cells' anti-tumor efficacy, improve their persistence, and alter characteristics of the tumor microenvironment (33) (Figure 3). Currently, six clinical trials are evaluating the rationale of TRUCKs in the treatment of hematological malignancies.

Interleukin-7 (IL-7) and Chemokine (C-C Motif) Ligand 19 (CCL19)-expressing CAR-T cells are being evaluated in four clinical trials (NCT04381741, NCT03929107, NCT04833504, NCT03778346). IL-7 fosters the proliferation and survival of T-cells, whereas CCL19 acts as a chemoattractant for both T-cells and dendritic cells (DCs) (42). Their incorporation into the CAR-T construct intends to mimic the cytokine environment in lymphoid organs (33). Investigators have presented the preliminary results of NCT04381741 trial in which patients

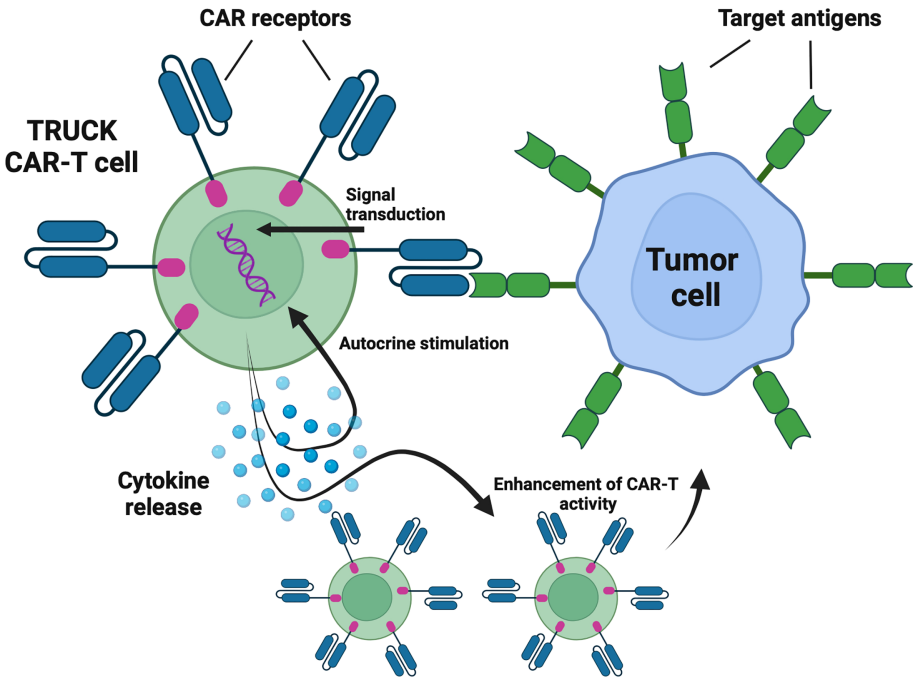

FIGURE 3
TRUCKs – cytokine-expressing CAR-T cells. TRUCKs are next-generation CAR-T cells engineered to express certain cytokines to augment CAR-T cells' antitumor efficacy, improve their persistence, and alte tumor microenvironment characteristics. Following antigen recognition, in addition to cytotoxic activity, the engineered cells release selected cytokines. Depending on the type, cytokines may promote the proliferation and survival of CAR-T cells and act as chemoattractants and enhancers of antitumor activity. Created with BioRender.com.

suffering from r/r DLBCL received IL-7/CCL19-expressing CAR-T cells with CR rate of 4/7 and ORR 5/7 (43). In addition, the same approach has been explored by NCT03778346 clinical targeting r/r multiple myeloma (MM), in which two enrolled patients achieved a CR (100%) (44). Furthermore, NCT04833504 and NCT03929107 are investigating this type of TRUCK in r/r B-cell lymphomas, however no results have been reported yet.

Another cytokine-secreting CAR-T is being evaluated in NCT04684563 clinical trial. This study aims to determine the maximum dose of interleukin-18 (IL-18) co-expressing CAR-T cells for patients with NHL and CLL. The incorporation of IL-18 into the CAR-T construct is supported by its role in the enhancement of CAR-T proliferation and anti-tumor activity (45). Interestingly, IL-18 is also associated with tumor progression (46), therefore long-term results concerning the safety of IL-18-expressing CAR-T cells in clinics are highly awaited.

NCT03602157 clinical trial represents a different approach to utilizing cytokine signaling in CAR-T cells. The investigators constructed an anti-CD30 CAR-T cell designated to treat r/r Hodgkin lymphoma and cutaneous T-cell lymphoma (CTCL) that incorporates C-C chemokine receptor type 4 (CCR4) (47). This receptor binds to Chemokine (C-C Motif) Ligand 17 (CCL17) secreted by Hodgkin lymphoma cells which in turn improves CAR-T cell trafficking into tumor site (47). The preliminary results of the trial in the Hodgkin lymphoma cohort are auspicious, 6 enrolled patients achieved CR (75%), whereas ORR was achieved by all patients (100%) (47). Unfortunately, no one achieved remission in the CTCL group of 2 persons (47).

To summarize, TRUCK CAR-T cells may serve as compelling therapeutic agents in certain diseases, with complete response rates as high as 75% in Hodgkin lymphoma and 100% in multiple myeloma (44, 47). On the other hand, results in CTCL and DLBCL are not so optimistic (43, 47). Nevertheless, similarly to armored CAR-T cells, due to the small number of patients enrolled in the studies, large-scale investigations should be conducted to evaluate the feasibility of these constructs.

Switchable CAR-T cells – control of the toxicities

The construction of switchable CAR-T cells has been prompted by treatment-associated toxicities that accompany conventional CAR-T therapies, with CRS and neurotoxicity

occurring most frequently (33). New technologies enabled researchers to incorporate safety switches that deplete CAR-T cells by inducing apoptosis, complement-dependent cytotoxicity (CDC) or antibody-dependent cellular cytotoxicity (ADCC) upon administration of an exogenous agent (48) (summarized in Figure 4). Switchable CAR-T cells are engineered by additional transduction of genes that encode easily targetable surface antigens or inducible intracellular effectors (48). As soon as the incorporated gene is expressed, the cells become susceptible to specific pharmaceuticals and can be depleted if necessary (48).

Incorporating the truncated epidermal growth factor receptor (EGFRt), a surface antigen, into CAR-T cells is a common safety-switch approach in registered clinical trials. EGFRt is targeted by monoclonal antibody cetuximab, which enables the removal of the T-cells *via* CDC or ADCC (33) (Figure 4A). As of August 2022, 20 clinical trials are investigating EGFRt-based switchable CAR-T cells in multiple hematological malignancies: 14 in CD19-positive leukemias and lymphomas, 1 in CD22 positive malignancies, 1 in CD19- and CD22-positive leukemias, 2 in CD123-positive acute myeloid leukemia (AML) and 4 in MM. Unfortunately, there is no available data concerning the launch of the EGFRt safety-switch mechanism in the human organism. The lack of such reports presumably results from the absence of life-threatening adverse events during the investigations. As the grade 3 or 4 toxicities were very rare and reversible, there could have been no need to eliminate CAR-T cells, however, no detailed explanation is provided by the research teams. Importantly, this fact implies the urgent need for evaluating the safety of triggering the therapy-controlling mechanism, and any information regarding this issue is highly awaited. In CD19-positive malignancies, clinical trials revealed significantly variable preliminary results. Correspondingly, the NCT03085173 study reported an overall CR rate of 57%, with DLBCL patients achieving a CR of 88% compared to 22% achieved by CLL patients (49). In the trials providing data from B-cell NHLs patients, complete responses were also variable matching 42% in NCT02706405 (50), 45% in NCT02153580 (51), and 75% in NCT01815749 (52). Among eight pediatric patients suffering from CD19-positive NHL enrolled in the NCT02028455 trial, CR was achieved by two patients (25%), however the response was not sustained in either patient (53). In NCT01865617, subsequently to CAR-T cells infusion, CR was achieved in 22%

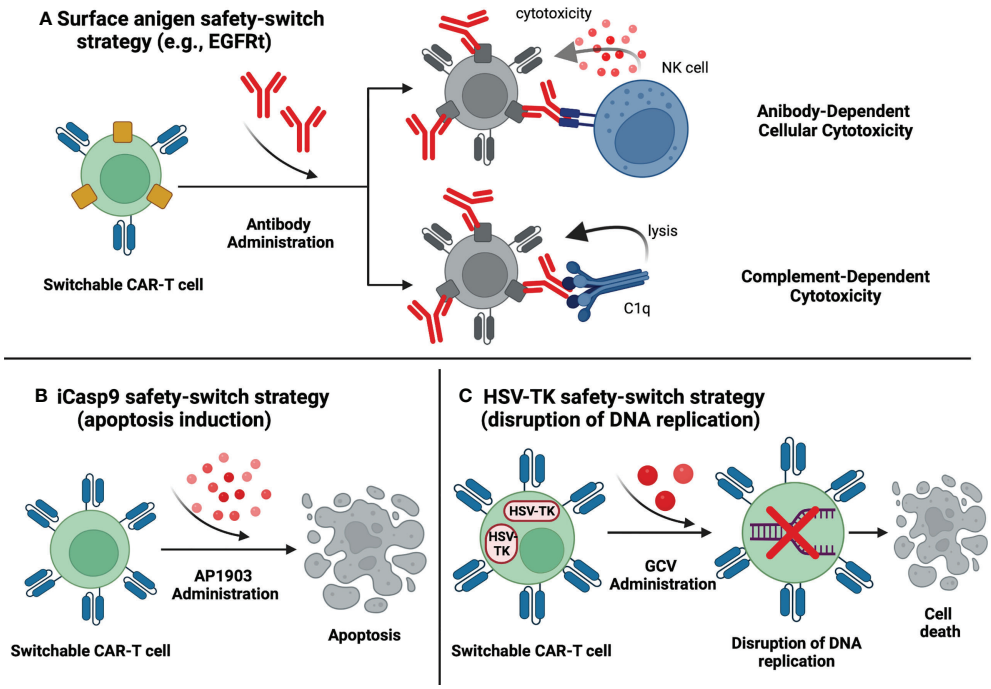

FIGURE 4
Switchable CAR-T cells – safety-switch strategies. **(A)** Surface antigen safety-switch strategy. The CAR-T cell is programmed to express a surface protein that can be targeted by a specific antibody. The binding of the antibody enables the removal of the CAR-T cells *via* antibody-dependent cytotoxicity or complement-dependent cytotoxicity. **(B)** Induction of apoptosis. Administration of AP1903 elicits dimerization of inducible caspase 9 (iCasp9) which in turn leads to activation of proapoptotic molecules and subsequent apoptosis of the iCasp9-transduced CAR-T cell. **(C)** HSV-TK safety-switch strategy. The HSV-TK gene codes an enzyme that transforms an inactive prodrug (GCV) into a competitive inhibitor of DNA polymerase. As a result, DNA replication is disrupted, and CAR-T cell undergoes apoptosis. Created with BioRender.com.

of CLL, 19% of NHL, and 21% of ALL patients (54). Impressively, in the NCT02146924 clinical trial, all patients had CR or CR with incomplete count recovery (CRi) (55). Other promising results have been obtained in the NCT03330691 clinical trial evaluating the safety and feasibility of CD19 and CD22 specific CAR-T cells in treating pediatric CD19- and CD22-positive leukemia. 84.6% of the enrolled patients achieved a CR, of which 95% were MRD negative (56). This study is especially noteworthy as CAR-T cells targeting CD19 antigen were equipped with a trastuzumab-susceptible truncated HER2 (HER2t) safety switch (56). Furthermore, two clinical trials (NCT03114670 and NCT02159495) investigate EGFRt-based switchable CAR-T cells in AML. Moreover, NCT02159495 includes treatment assessment in blastic plasmacytoid dendritic cell neoplasm (BPDCN) and does have preliminary results (57). Of the 7 AML patients, two achieved CR (29%) and one obtained morphologic leukemic-free state (MLFS), whereas, in (BPDCN) group, one patient achieved CR (50%) (57). In multiple myeloma, four clinical trials are exploring the feasibility and safety of the discussed switchable CAR-T cells. NCT03338972 study reported an ORR of 100%, but the investigators did not mention the number of CR (58). In another study, NCT03070327, ORR was achieved by 64% of patients (59). NCT03093168 revealed an ORR of 86% and CR of 29% (60). B-cell Maturation Antigen (BCMA) is the only target of CAR-T cells in the abovementioned MM-associated trials. Finally, NCT03093168 is evaluating signaling lymphocytic activation molecule F7 (SLAMF7)-aimed CAR-T in MM, however the preliminary outcomes are unknown. Results from other clinical trials concerning EGFRt-based switchable CAR-T cells included in Supplementary Table 1 have not been published yet.

The inclusion of the RQR8 suicide gene is an analogous approach to controlling CAR-T cells after therapeutic administration. RQR8 gene encodes a cell surface protein combining epitopes derived from CD20 and CD34 antigens (61). This strategy enables CAR-T cell depletion *via* CDC or ADCC following the administration of monoclonal antibody rituximab (61) (Figure 4A). Currently, NCT03590574 clinical trial is investigating RQR8-based switchable CAR-T cells in Peripheral T cell lymphomas (PTCL). Initial results show an ORR of 67%, with 56% of patients achieving complete metabolic responses (CMR) (62). Another trial, NCT03287804, has been terminated due to unsatisfactory preliminary efficacy in the treatment of multiple myeloma.

The incorporation of inducible caspase 9 (iCasp9) represents a distinctive approach to CAR-T ablation (Figure 4B). Upon administration of AP1903 (rimiducid), biologically inert small molecule, specially modified caspase 9 undergoes dimerization and triggers the apoptotic pathway (33). Currently, 17 clinical trials are evaluating the clinical application of iCasp9-based switchable CAR-T cells, however only 4 of them provide initial data. NCT03016377 clinical trial has reported a case of an ALL patient who had experienced neurotoxicity after the CAR-T infusion (63). Following the AP1903 administration, the symptoms fully resolved, and the only adverse event was grade 2 bilirubin elevation that lasted for three days (63). Interestingly, the investigators observed a clinically significant antileukemic response despite the elimination of >90% CAR-T cells (63). In the NCT02274584 trial, merely a case report of a Hodgkin lymphoma patient has been published, showing temporary partial remission (64). Additionally, partial results from NCT03050190 have been combined with information from NCT03173417 and NCT 02813837 (65), therefore, we are unable to elucidate data exclusively from NCT03050190. NCT03125577 trial reported data from 4 patients, all of whom had CRs following the CAR-T infusion (66). Noteworthy is a clinical trial (ChiCTR-OOC-16007779) registered only in the Chinese Clinical Trial Registry. This trial investigates iCasp9 switchable CAR-T therapy for patients with B-cell non-Hodgkin lymphomas. The overall CR rate was 43% (ORR 67%), with DLBCL patients achieving a CR of 33% compared to 56% in the non-DLBCL group (67). Unfortunately, except for NCT03016377, trials do not report the launch of the iCasp9 safety switch. The safety concerns may be partially answered by a study concerning graft-versus-host-disease (GvHD), in which the activation of the iCasp9 safety switch resulted in the resolution of GvHD symptoms and rapid elimination of 90% of transgenic T-cells with no subsequent adverse events (68). The remaining clinical trials regarding the iCasp9 safety switch have been summarized in Supplementary Table 1.

Another suicide gene that could be utilized as a safety switch in CAR-T therapies is herpes simplex virus thymidine kinase (HSV-TK) Mut 2 gene, the product of which is targeted by the prodrug ganciclovir (GCV). HSV-TK converts GCV into GCV-triphosphate, a competitive inhibitor of deoxyguanosine incorporation into DNA that causes cell death by disrupting the replication process (69) (Figure 4C). HSV-TK-based switchable CAR-T cells were evaluated in the terminated NCT04097301 trial. Eventually, only two patients were enrolled (both with MM) and showed no response to the treatment (70). The safety switch had not been activated due to the lack of T-cell-related toxicities (70).

In summary, reports from switchable CAR-T investigations show highly variable results. In CD19 positive malignancies treated with CAR-T cells incorporating surface targets (e.g., EGFRt, HER2, RQR8), complete response rates ranged from 19% to 84,6% (54, 56). In inducible caspase 9 (iCasp9) trials, CR rates ranged from 33% to 100% (66, 67). However, the safety-switch strategy aims to increase safety not efficacy by default. Therefore, more attention should be drawn to the results of the safety switch launch. Unfortunately, only Foster et al. reported the use of this mechanism, showing safety and full resolution of the symptoms (63). As adverse events are of major concern in CAR-T therapies, further reports are highly demanded.

Universal CAR-T cells and fratricide-resistant CAR-T cells

Nowadays, conventional CAR-T products are manufactured from autologous T-cells derived from a patient qualified for the therapy (33). This method implies several limitations, including long manufacturing time, difficulties in mobilizing the appropriate quantity of T-cells, and reduced T-cell quality in heavily treated patients (33). However, the achievements of molecular engineering enabled the generation of allogeneic CAR-T cells that could circumvent the abovementioned hurdles. To construct a universal CAR-T, major histocompatibility complex (MHC) and T-cell receptor (TCR) molecules need to be removed from donor-derived cells (33). It can be easily achieved thanks to the application of genome-editing tools such as CRISPR/Cas9 or Transcription activator-like (TAL) effector nuclease (TALEN), which facilitate gene knockout (33) (Figure 5). The initial safety and feasibility of advanced CRISPR/Cas9 technology in T-cell engineering have been demonstrated in the first-in-human pilot trial NCT03399448 (71). Notably, the study proved that multiplex CRISPR-Cas9 editing of the human genome is possible at the clinical scale (71). Correspondingly, the discussed technologies can also be implemented to generate fratricide-resistant (self-killing-resistant) CAR-T cells in which T-cell-specific antigens are removed (72). This approach allows CAR-T cells to target various T-cell malignancies (72). Currently, 30 trials are evaluating genetically edited allogeneic or fratricide-resistant CAR-T cells (all also being allogeneic except cells in NCT04767308). Therefore, all trials discussed below are examining allogeneic CAR-T cells. Crucially, due to the relatively high number of clinical trials concerning allogeneic CAR-T cells, we have decided not to include studies that do not specify the introduced modifications and mechanisms of gene editing as we could not guarantee the relevance of such information to this review.

To date, CRISPR/Cas9 engineered CAR-T cells have been investigated in 15 trials, whereas TALEN has been applied to 12 trials. Three studies used different approaches to gene editing. The Lancet has already published results from NCT02808442 and NCT02746952 studies examining TALEN-edited CAR-T cells in B-cell ALL patients. They have shown the CR or CRi of 67% with overall survival (OS) of 55% (73). The most common adverse event was CRS and was observed in 91% of patients (14% of them had grade 3 or 4 CRS) (73). Other adverse events included neurotoxicity (18%), acute skin GvHD (10%), and

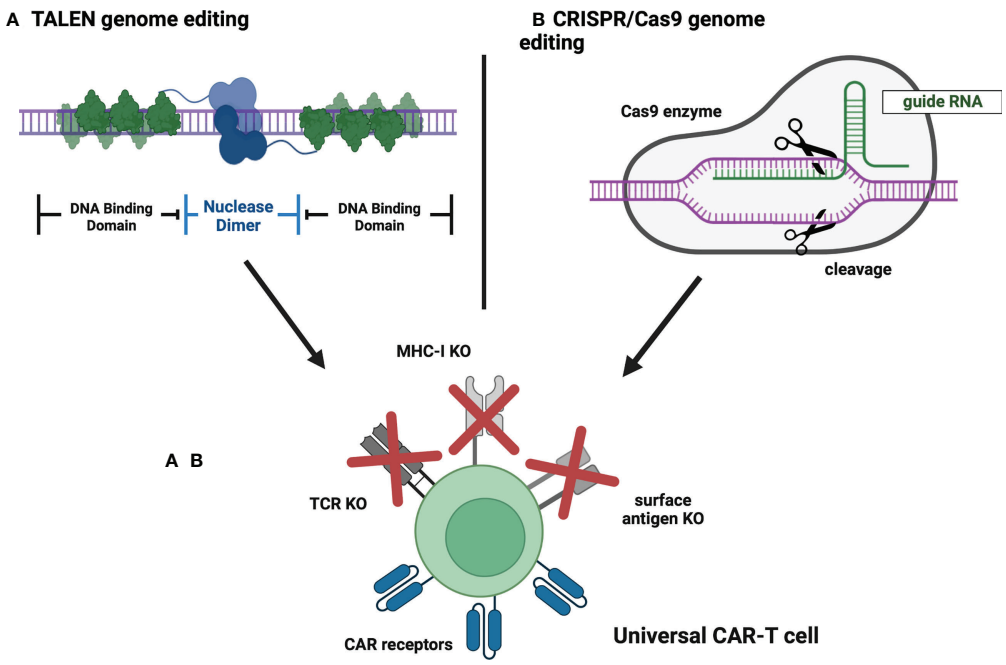

FIGURE 5
Universal CAR-T cells – genome editing strategies. **(A)** TALEN genome editing. TALEN is a genome-editing system in which a targeted DNA sequence is recognized by a pair of individually designed DNA-binding domains. Then, a pair of nuclease domains cause a double-stranded DNA break and subsequent knockout of the targeted gene. **(B)** CRISPR/Cas9 genome editing. CRISPR/Cas9 is a genome-editing system in which a targeted DNA sequence is recognized by guide RNA associated with Cas9 endonuclease that cleaves DNA strand, thus causing the knockout of a selected gene. Created with BioRender.com.

grade 4 prolonged cytopenia (32%) (74). Two treatment-related deaths were reported (73). Overall, the study showed promising efficacy accompanied by severe adverse events. In another study of TALEN-edited CAR-T cells in ALL (NCT04150497), 4 of 5 patients experienced multiple treatment-related toxicities, whereas ORR was 60% (75). Contrarily, initial data from the NCT04416984 clinical trial targeting DLBCL showed less severe adverse events, however only 25% of patients responded to the treatment (76). In addition, early data obtained in the NCT03939026 trial suggest a manageable safety profile of universal CAR-T therapy in r/r LBCL and follicular lymphoma (FL) patients, with an ORR of 78% (77).. The safety and efficacy of CRISPR/Cas9 engineered CAR-T cells in r/r B-cell acute lymphoblastic leukemia have been evaluated in NCT04227015. Five of 6 patients achieved CR or CRi (83%), with CRS occurring in all patients (including one grade 3 CRS) (78). The NCT04093596 study of universal CAR-T cells in r/r multiple myeloma showed an ORR of 33% with manageable toxicities (79).

As of August 2022, 5 trials evaluating the administration of fratricide-resistant CAR-T cells have been registered at ClinicalTrials.gov. In the NCT04502446 investigation, CAR-T cells targeting the CD70 antigen were engineered to eliminate the expression of TCR, MHC-I as well as CD70 to abolish fratricide and increase efficacy (80). 47% of patients achieved ORR, whereas CR was 20% (80). No severe adverse events were observed (80). Furthermore, the NCT04264078 trial provided data concerning universal fratricide-resistant CAR-T cell used against r/r T-cell ALL (81). CRISPR/Cas9 platform was used to disrupt TCR and CD7 genes to prevent GvHD and fratricide (81). 80% of patients (4/5) obtained CR, however the treatment was associated with severe CRS in all subjects (four patients had grade 3 CRS, one patient had grade 4 CRS) (81). The high efficacy of the treatment has been confirmed by the results from subsequently enrolled patients, with a CR of 83% (5/6), whereas safety findings were consistent with the previous observations (82).

Future perspectives

Along with the accumulation of research data and widespread use of molecular engineering, questions about the future of next-generation CAR-T cells in clinics are unavoidable. Even though it is hard to foresee whether all next-generation CAR-T approaches will become a standard of care in the future, we would like to propose improvements to currently explored strategies that could contribute to even better treatment results. In addition, we discuss the most promising approaches that could be implemented in the upcoming years.

Principally, individual augmentation strategies of the next-generation CAR-T cells aim to circumvent specific limitations of conventional CAR-T therapies (33). For instance, the incorporation of additional costimulatory domains, induction of cytokine secretion, and immune checkpoint modulation are intended to improve efficacy of eliminating malignant cells. Available reports indicate that 3rd-generation CAR-T cells equipped with additional costimulatory domains may not bring the expected benefits (30, 83). On the contrary, immune checkpoint modulation in CAR-T constructs is associated with CR rates as high as 78% and constitutes a promising method in the treatment of PD-L1 malignancies (38, 39). TRUCK CAR-T cells are designed to utilize cytokines as chemoattractants or enhancers of T-cell proliferation and survival (33). The reports show the highly variable efficacy of this therapeutic approach (CR rates ranging from 0% to impressive 100%) (43, 44, 47). Unfortunately, the number of enrolled patients in the studies regarding the abovementioned strategies is insufficient to provide an unbiased answer on whether they are likely to become a new standard of care or not. The results of ongoing trials will show whether the enhancers increase efficacy and will answer safety inquiries. Metanalyses will be necessary. If the treatment complications accompanying next-generation T-cells appeared significantly worse than conventional therapies, incorporating a safety switch would be in high demand. To date, only one study reported the use of safety switch, however with excellent outcome (63). Currently, ongoing trials regarding switchable CAR-T cells should provide adequate information about the feasibility and perspectives of this controlling strategy. Altogether, we suggest that combining the efficacy enhancers with safety switches in one CAR-T product is a reasonable strategy to increase the safety and efficacy of CAR-T therapies.

Nevertheless, the implementation of additional genes into the cellular genome brings other hazards. For instance, multiple gene insertions associated with gene editing may increase the risk of disrupting genes responsible for cell metabolism or replication, resulting in cell depletion or transformation into malignant clones (33). Additionally, induction of cytokine expression could potentially lead to toxicities associated with the pleiotropic character of these compounds.

The last paragraph is devoted exclusively to universal CAR-T cells that, in our view, have the potential to revolutionize the scene of CAR-T therapies in combination with previously described next-generation strategies. “Off-the-shelf” allogeneic CAR-T cells engineered with molecular tools like CRISPR/Cas9 demonstrate several advantages over conventional CAR-T cell therapies, even with comparable efficacy. Moreover, potential toxicities resulting from the allogeneic nature of these cells could be circumvented by incorporating a safety switch mechanism. Above all, in the case of conventional autologous CAR-T cells, a patient undergoes time-consuming procedures of manufacturing the personalized treatment. On the contrary, universal CAR-T cells can be prepared in advance so that the infusion can occur almost immediately with less time for a

disease to progress. Moreover, universal CAR-T cells allow CAR-T therapy for patients who cannot provide appropriate-quality T-cells or the quantity of their T-cells is insufficient. This approach is also more convenient for the patient and the healthcare provider as there is no need for hospitalization to perform leukapheresis. In addition, allogeneic universal CAR-T cells could be redistributed and stored in multiple locations throughout the country, thereby eliminating transport-related exclusion in healthcare. Finally, widespread application of these CAR-T therapeutics would undoubtedly lead to decreased treatment costs, a barrier that currently inhibits the clinical application of CAR-T therapies. However, despite optimistic perspectives, universal CAR-T cells have shortcomings. For instance, the knockout of MHC-related genes makes them vulnerable to natural killer cell-mediated cytotoxicity. Therefore, the results of ongoing clinical trials are highly awaited and will hopefully remove the emerging doubts.

In conclusion, we believe that the most successful next-generation CAR-T cells will be universal allogeneic CAR-T cells (manufactured with CRISPR/Cas9 technology) characterized by immune checkpoint resistance and expressing cytokines that traffic T-cells into the tumor sites. Furthermore, such a construct will incorporate a safety-switch mechanism for managing potential toxicities. As these mechanisms have been individually proven efficient in preclinical studies (33) and early clinical results discussed in this review are promising, such a combination could circumvent the current limitations of CAR-T therapies and contribute to the improvement of treatment outcomes worldwide.

Author contributions

All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1034707/full#supplementary-material

References

1. Pehlivan KC, Duncan BB, Lee DW. CAR-T cell therapy for acute lymphoblastic leukemia: Transforming the treatment of relapsed and refractory disease. *Curr Hematol Malig Rep* (2018) 13(5):396–406. doi: 10.1007/s11899-018-0470-x

2. Sermer D, Batlevi C, Palomba ML, Shah G, Lin RJ, Perales MA, et al. Outcomes in patients with DLBCL treated with commercial CAR T cells compared with alternate therapies. *Blood Adv* (2020) 4(19):4669–78. doi: 10.1182/bloodadvances.2020002118

3. Chen H, Wang F, Zhang P, Zhang Y, Chen Y, Fan X, et al. Management of cytokine release syndrome related to CAR-T cell therapy. *Front Med* (2019) 13 (5):610–7. doi: 10.1007/s11684-019-0714-8

4. Hunter BD, Jacobson CA. CAR T-cell associated neurotoxicity: Mechanisms, clinicopathologic correlates, and future directions. *J Natl Cancer Inst* (2019) 111 (7):646–54. doi: 10.1093/jnci/djz017

5. Wang L. Clinical determinants of relapse following CAR-T therapy for hematologic malignancies: Coupling active strategies to overcome therapeutic limitations. *Curr Res Transl Med* (2022) 70(1):103320. doi: 10.1016/j.retram.2021.103320

6. Gross G, Waks T, Eshhar Z. Expression of immunoglobulin-t-cell receptor chimeric molecules as functional receptors with antibody-type specificity. *Proc Natl Acad Sci U.S.A.* (1989) 86(24):10024–8. doi: 10.1073/pnas.86.24.10024

7. Kershaw MH, Westwood JA, Parker LL, Wang G, Eshhar Z, Mavroukakis SA, et al. A phase I study on adoptive immunotherapy using gene-modified T cells for ovarian cancer. *Clin Cancer Res* (2006) 12(20 Pt 1):6106–15. doi: 10.1158/1078-0432.CCR-06-1183

8. Imai C, Mihara K, Andreansky M, Nicholson IC, Pui CH, Geiger TL, et al. Chimeric receptors with 4-1BB signaling capacity provoke potent cytotoxicity against acute lymphoblastic leukemia. *Leukemia* (2004) 18(4):676–84. doi: 10.1038/sj.leu.2403302

9. Krause A, Guo HF, Latouche JB, Tan C, Cheung NK, Sadelain M. Antigen-dependent CD28 signaling selectively enhances survival and proliferation in genetically modified activated human primary T lymphocytes. *J Exp Med* (1998) 188(4):619–26. doi: 10.1084/jem.188.4.619

10. van der Stegen SJ, Hamieh M, Sadelain M. The pharmacology of second-generation chimeric antigen receptors. *Nat Rev Drug Discovery* (2015) 14(7):499–509. doi: 10.1038/nrd4597

11. Neelapu SS, Locke FL, Bartlett NL, Lekakis LJ, Miklos DB, Jacobson CA, et al. Axicabtagene ciloleucel CAR T-cell therapy in refractory Large b-cell lymphoma. *N Engl J Med* (2017) 377(26):2531–44. doi: 10.1056/NEJMoa1707447

12. Schuster SJ, Bishop MR, Tam CS, Waller EK, Borchmann P, McGuirk JP, et al. Tisagenlecleucel in adult relapsed or refractory diffuse Large b-cell lymphoma. *N Engl J Med* (2019) 380(1):45–56. doi: 10.1056/NEJMoa1804980

13. Maude SL, Laetsch TW, Buechner J, Rives S, Boyer M, Bittencourt H, et al. Tisagenlecleucel in children and young adults with b-cell lymphoblastic leukemia. *N Engl J Med* (2018) 378(5):439–48. doi: 10.1056/NEJMoa1709866

14. Abramson JS, Palomba ML, Gordon LI, Lunning MA, Wang M, Arnason J, et al. Lisocabtagene maraleucel for patients with relapsed or refractory large b-cell lymphomas (TRANSCEND NHL 001): a multicentre seamless design study. *Lancet* (2020) 396(10254):839–52. doi: 10.1016/S0140-6736(20)31366-0

15. Wang M, Munoz J, Goy A, Locke FL, Jacobson CA, Hill BT, et al. KTE-X19 CAR T-cell therapy in relapsed or refractory mantle-cell lymphoma. *N Engl J Med* (2020) 382(14):1331–42. doi: 10.1056/NEJMoa1914347

16. Shah BD, Ghobadi A, Oluwole OO, Logan AC, Boissel N, Cassaday RD, et al. KTE-X19 for relapsed or refractory adult b-cell acute lymphoblastic leukaemia: phase 2 results of the single-arm, open-label, multicentre ZUMA-3 study. *Lancet* (2021) 398(10299):491–502. doi: 10.1016/S0140-6736(21)01222-8

17. Lin Y, Raje NS, Berdeja JG, Siegel DS, Jagannath S, Madduri D, et al. Idecabtagene vicleucel (ide-cel, bb2121), a BCMA-directed CAR T cell therapy, in patients with relapsed and refractory multiple myeloma: Updated results from phase 1 CRB-401 study. *Blood* (2020) 136:26–7. doi: 10.1182/blood-2020-134324

18. Berdeja JG, Madduri D, Usmani SZ, Jakubowiak A, Agha M, Cohen AD, et al. Ciltacabtagene autoleucel, a b-cell maturation antigen-directed chimeric antigen receptor T-cell therapy in patients with relapsed or refractory multiple myeloma (CARTITUDE-1): a phase 1b/2 open-label study. *Lancet* (2021) 398 (10297):314–24. doi: 10.1016/S0140-6736(21)00933-8

19. Carpenito C, Milone MC, Hassan R, Simonet JC, Lakhal M, Suhoski MM, et al. Control of large, established tumor xenografts with genetically retargeted human T cells containing CD28 and CD137 domains. *Proc Natl Acad Sci U.S.A.* (2009) 106(9):3360–5. doi: 10.1073/pnas.0813101106

20. Zhong XS, Matsushita M, Plotkin J, Riviere I, Sadelain M. Chimeric antigen receptors combining 4-1BB and CD28 signaling domains augment PI3kinase/AKT/Bcl-XL activation and CD8+ T cell-mediated tumor eradication. *Mol Ther* (2010) 18(2):413–20. doi: 10.1038/mt.2009.210

21. Cheng J, Zhao L, Zhang Y, Qin Y, Guan Y, Zhang T, et al. Understanding the mechanisms of resistance to CAR T-cell therapy in malignancies. *Front Oncol* (2019) 9:1237. doi: 10.3389/fonc.2019.01237

22. Tokarew N, Ogonek J, Endres S, von Bergwelt-Baildon M, Kobold S. Teaching an old dog new tricks: Next-generation CAR T cells. *Br J Cancer* (2019) 120(1):26–37. doi: 10.1038/s41416-018-0325-1

23. Chmielewski M, Abken H. CAR T cells transform to trucks: chimeric antigen receptor-redirected T cells engineered to deliver inducible IL-12 modulate the tumour stroma to combat cancer. *Cancer Immunol Immunother* (2012) 61(8):1269–77. doi: 10.1007/s00262-012-1202-z

24. Zhao J, Lin Q, Song Y, Liu D. Universal CARs, universal T cells, and universal CAR T cells. *J Hematol Oncol* (2018) 11(1):132. doi: 10.1186/s13045-018-0677-2

25. Liu G, Rui W, Zheng H, Huang D, Yu F, Zhang Y, et al. CXCR2-modified CAR-T cells have enhanced trafficking ability that improves treatment of hepatocellular carcinoma. *Eur J Immunol* (2020) 50(5):712–24. doi: 10.1002/eji.201948457

26. Yeku OO, Purdon TJ, Koneru M, Spriggs D, Brentjens RJ. Armored CAR T cells enhance antitumor efficacy and overcome the tumor microenvironment. *Sci Rep* (2017) 7(1):10541. doi: 10.1038/s41598-017-10940-8

27. Straathof KC, Pule MA, Yotnda P, Dotti G, Vanin EF, Brenner MK, et al. An inducible caspase 9 safety switch for T-cell therapy. *Blood* (2005) 105(11):4247–54. doi: 10.1182/blood-2004-11-4564

28. Park S, Pascua E, Lindquist KC, Kimberlin C, Deng X, Mak YSL, et al. Direct control of CAR T cells through small molecule-regulated antibodies. *Nat Commun* (2021) 12(1):710. doi: 10.1038/s41467-020-20671-6

29. Ramos CA, Rouce R, Robertson CS, Reyna A, Narala N, Vyas G, et al. In vivo fate and activity of second- versus third-generation CD19-specific CAR-T cells in b cell non-hodgkin's lymphomas. *Mol Ther* (2018) 26(12):2727–37. doi: 10.1016/j.ymthe.2018.09.009

30. Enblad G, Karlsson H, Gammelgård G, Wenthe J, Lövgren T, Amini RM, et al. A phase I/IIa trial using CD19-targeted third-generation CAR T cells for lymphoma and leukemia. *Clin Cancer Res* (2018) 24(24):6185–94. doi: 10.1158/1078-0432.CCR-18-0426

31. Schubert M-L, Schmitt A, Neuber B, Hückelhoven-Krauss A, Kunz A, Wang L, et al. Third-generation CAR T cells targeting CD19 are associated with an excellent safety profile and might improve persistence of CAR T cells in treated patients. *Blood* (2019) 134:51. doi: 10.1182/blood-2019-125423

32. George P, Dasyam N, Giunti G, Mester B, Bauer E, Andrews B, et al. Third-generation anti-CD19 chimeric antigen receptor T-cells incorporating a TLR2 domain for relapsed or refractory b-cell lymphoma: a phase I clinical trial protocol (ENABLE). *BMJ Open* (2020) 10(2):e034629. doi: 10.1136/bmjopen-2019-034629

33. Rafiq S, Hackett CS, Brentjens RJ. Engineering strategies to overcome the current roadblocks in CAR T cell therapy. *Nat Rev Clin Oncol* (2020) 17(3):147–67. doi: 10.1038/s41571-019-0297-y

34. Spiegel JY, Patel S, Muffly L, Hossain NM, Oak J, Baird JH, et al. CAR T cells with dual targeting of CD19 and CD22 in adult patients with recurrent or refractory b cell malignancies: a phase 1 trial. *Nat Med* (2021) 27(8):1419–31. doi: 10.1038/s41591-021-01436-0

35. Cordoba S, Onuoha S, Thomas S, Pignataro DS, Hough R, Ghorashian S, et al. CAR t cells with dual targeting of CD19 and CD22 in pediatric and young adult patients with relapsed or refractory b cell acute lymphoblastic leukemia: a phase 1 trial. *Nat Med* (2021) 27(10):1797–805. doi: 10.1038/s41591-021-01497-1

36. Zhao J, Song Y, Liu D. Clinical trials of dual-target CAR T cells, donor-derived CAR T cells, and universal CAR T cells for acute lymphoid leukemia. *J Hematol Oncol* (2019) 12(1):17. doi: 10.1186/s13045-019-0705-x

37. Lu H, Zhao X, Li Z, Hu Y, Wang H. From CAR-T cells to CAR-NK cells: A developing immunotherapy method for hematological malignancies. *Front Oncol* (2021) 11:720501. doi: 10.3389/fonc.2021.720501

38. Liu H, Lei W, Zhang C, Yang C, Wei J, Guo Q, et al. CD19-specific CAR T cells that express a PD-1/CD28 chimeric switch-receptor are effective in patients with PD-L1-positive b-cell lymphoma. *Clin Cancer Res* (2021) 27(2):473–84. doi: 10.1158/1078-0432.CCR-20-1457

39. Kim WS, Kim SJ, Yoon S, Kim JR. Phase 1/2 study of anbalcabtagene autoleucel, novel anti-CD19 CAR-T cell therapy with dual silencing of PD-1 and TIGIT in relapsed or refractory large b-cell lymphoma. *J Clin Oncol* (2022) 40 (16_suppl):7522–2. doi: 10.1200/JCO.2022.40.16_suppl.7522

40. You D, Hillerman S, Locke G, Chaudhry C, Stromko C, Murtaza A, et al. Enhanced antitumor immunity by a novel small molecule HPK1 inhibitor. *J Immunother Cancer* (2021) 9(1):1–12. doi: 10.1136/jitc-2020-001402

41. Zhang N, Si J, Li G, Wang Y, Long F, Wang T, et al. Decreasing HPK1 expression in CD19 CAR-T cells: A novel strategy to overcome challenges of cell therapy for adult (r/r) b-ALL. *J Clin Oncol* (2022) 40(16_suppl):7041–1. doi: 10.1200/JCO.2022.40.16_suppl.7041

42. Adachi K, Kano Y, Nagai T, Okuyama N, Sakoda Y, Tamada K. IL-7 and CCL19 expression in CAR-T cells improves immune cell infiltration and CAR-T cell survival in the tumor. *Nat Biotechnol* (2018) 36(4):346–51. doi: 10.1038/nbt.4086

43. Qian W, Zhao A, Liu H, Lei W, Liang Y, Yuan X. Safety and efficacy of CD19 CAR-T cells Co-expressing IL-7 and CCL19 in combination with anti-PD-1 antibody for Refractory/Relapsed DLBCL: Preliminary data from the phase Ib trial (NCT04381741). *Blood* (2021) 138(Supplement 1):3843–3. doi: 10.1182/blood-2021-144523

44. Duan D, Wang K, Wei C, Feng D, Liu Y, He Q, et al. The BCMA-targeted fourth-generation CAR-T cells secreting IL-7 and CCL19 for therapy of Refractory/Recurrent multiple myeloma. *Front Immunol* (2021) 12:609421. doi: 10.3389/fimmu.2021.609421

45. Hu B, Ren J, Luo Y, Keith B, Young RM, Scholler J, et al. Augmentation of antitumor immunity by human and mouse CAR T cells secreting IL-18. *Cell Rep* (2017) 20(13):3025–33. doi: 10.1016/j.celrep.2017.09.002

46. Vidal-Vanaclocha F, Mendoza L, Telleria N, Salado C, Valcárcel M, Gallot N, et al. Clinical and experimental approaches to the pathophysiology of interleukin-18 in cancer progression. *Cancer Metastasis Rev* (2006) 25(3):417–34. doi: 10.1007/s10555-006-9013-3

47. Grover NS, Ivanova A, Moore DT, Cheng CJA, Babinec C, West J, et al. CD30-directed CAR-T cells Co-expressing CCR4 in Relapsed/Refractory Hodgkin lymphoma and CD30+ cutaneous T cell lymphoma. *Blood* (2021) 138(Supplement 1):742–2. doi: 10.1182/blood-2021-148102

48. Moghanloo E, Mollanoori H, Talebi M, Pashangzadeh S, Faraji F, Hadjilooei F, et al. Remote controlling of CAR-T cells and toxicity management: Molecular switches and next generation CARs. *Transl Oncol* (2021) 14(6):101070. doi: 10.1016/j.tranon.2021.101070

49. Park JH, Palomba ML, Batlevi CL, Riviere I, Wang X, Senechal B, et al. A phase I first-in-Human clinical trial of CD19-targeted 19-28z/4-1BBL "Armored" CAR T cells in patients with relapsed or refractory NHL and CLL including richter's transformation. *Blood* (2018) 132(Supplement 1):224–4. doi: 10.1158/1078-0432.CCR-21-3867

50. Hirayama AV, Gauthier J, Hay KA, Sheih A, Cherian S, Chen X, et al. Efficacy and toxicity of JCAR014 in combination with durvalumab for the treatment of patients with Relapsed/Refractory aggressive b-cell non-Hodgkin lymphoma. *Blood* (2018) 132(Supplement 1):1680–0. doi: 10.1182/blood-2018-99-116745

51. Siddiqi T, Wang X, Blanchard MS, Wagner J, Vyas V, Clark MC, et al. Results from a phase 1 study to evaluate a memory-enriched CD19-specific CAR T cell platform in adult patients with CD19+ b-cell non-Hodgkin lymphoma (B-NHL). *Blood* (2021) 138(Supplement 1):2836–6. doi: 10.1182/blood-2021-147082

52. Wang X, Popplewell LL, Wagner JR, Naranjo A, Blanchard MS, Mott MR, et al. Phase 1 studies of central memory-derived CD19 CAR T-cell therapy following autologous HSCT in patients with b-cell NHL. *Blood* (2016) 127 (24):2980–90. doi: 10.1182/blood-2015-12-686725

53. Rivers J, Annesley C, Summers C, Finney O, Pulsipher MA, Wayne AS, et al. Early response data for pediatric patients with non-Hodgkin lymphoma treated with CD19 chimeric antigen receptor (CAR) T-cells. *Blood* (2018) 132(Supplement 1):2957–7. doi: 10.1182/blood-2018-99-113744

54. Gauthier J, Hirayama AV, Hay KA, Li D, Sheih A, Wu V, et al. Factors associated with duration of response after CD19-specific CAR-T cell therapy for refractory/relapsed b-cell non-Hodgkin lymphoma. *J Clin Oncol* (2018) 36 (15_suppl):7567–7. doi: 10.1200/JCO.2018.36.15_suppl.7567

55. Khaled SK, Blanchard S, Wang X, Wagner J, Naranjo A, Simpson J, et al. Adult patients with ALL treated with CD62L+ T Naïve/Memory-enriched T cells expressing a CD19-CAR mediate potent antitumor activity with a low toxicity profile. *Blood* (2018) 132(Supplement 1):4016–6. doi: 10.1182/blood-2018-99-119883

56. Gardner RA, Annesley C, Wilson A, Summers C, Prabha Narayanaswamy Wu V, et al. Efficacy of SCRI-CAR19x22 T cell product in b-ALL and persistence of anti-CD22 activity. *J Clin Oncol* (2020) 38(15_suppl):3035–5. doi: 10.1200/JCO.2020.38.15_suppl.3035

57. Budde LE, Song J, Real MD, Kim Y, Toribio C, Wood B, et al. Abstract PR14: CD123CAR displays clinical activity in relapsed/refractory (r/r) acute myeloid leukemia (AML) and blastic plasmacytoid dendritic cell neoplasm (BPDCN): Safety and efficacy results from a phase 1 study. *Cancer Immunol Res* (2020) 8 (4_Supplement):PR14–4. doi: 10.1158/2326-6074.TUMIMM18-PR14

58. Green DJ, Pont M, Sather BD, Cowan AJ, Turtle CJ, Till BG, et al. Fully human bcma targeted chimeric antigen receptor T cells administered in a defined composition demonstrate potency at low doses in advanced stage high risk multiple myeloma. *Blood* (2018) 132(Supplement 1):1011–1. doi: 10.1182/blood-2018-99-117729

59. Mailankody S, Ghosh A, Staehr M, Purdon TJ, Roshal M, Halton E, et al. Clinical responses and pharmacokinetics of MCARH171, a human-derived bcma targeted CAR T cell therapy in Relapsed/Refractory multiple myeloma: Final results of a phase I clinical trial. *Blood* (2018) 132(Supplement 1):959–9. doi: 10.1182/blood-2018-99-119717

60. Liu Y, Chen Z, Wei R, Shi L, He F, Shi Z, et al. Remission observed from a phase 1 clinical study of CAR-T therapy with safety switch targeting BCMA for patients with relapsed/refractory multiple myeloma. *J Clin Oncol* (2018) 36 (15_suppl):8020–0. doi: 10.1200/JCO.2018.36.15_suppl.8020

61. Philip B, Kokalaki E, Mekkaoui L, Thomas S, Straathof K, Flutter B, et al. A highly compact epitope-based marker/suicide gene for easier and safer T-cell therapy. *Blood* (2014) 124(8):1277–87. doi: 10.1182/blood-2014-01-545020

62. Cwynarski K, Tholouli E, Iacoboni G, Menne T, Irvine D, Wood L, et al. S261: Safety and preliminary efficacy findings of AUTO4, a TRBC1-targetting car, in relapsed/refractory TRBC1 positive selected T cell non-hodgkin lymphoma. *HemaSphere* (2022) 6:162–3. doi: 10.1097/01.HS9.0000843936.81776.ac

63. Foster MC, Savoldo B, Lau W, Rubinos C, Grover N, Armistead P, et al. Utility of a safety switch to abrogate CD19.CAR t-cell–associated neurotoxicity. *Blood* (2021) 137(23):3306–9. doi: 10.1182/blood.2021010784

64. Ying Z-T, Chang L-J, Kuo H-H, Liu Y-C, Song Y-Q, Wang X-P, et al. 415. first-In-Patient proof of safety and efficacy of a ^{4th}h generation chimeric antigen receptor-modified T cells for the treatment of relapsed or refractory CD30 positive lymphomas. *Mol Ther* (2015) 23:S164. doi: 10.1016/S1525-0016(16)34024-2

65. Chen YH, Zhang X, Cheng YF, Chen H, Mo XD, Yan CH, et al. Long-term follow-up of CD19 chimeric antigen receptor T-cell therapy for relapsed/refractory acute lymphoblastic leukemia after allogeneic hematopoietic stem cell transplantation. *Cytotherapy* (2020) 22(12):755–61. doi: 10.1016/j.jcyt.2020.08.002

66. Jiao C, Zvonkov E, Lai X, Zhang R, Liu Y, Qin Y, et al. 4SCAR2.0: a multi-CAR-T therapy regimen for the treatment of relapsed/refractory b cell lymphomas. *Blood Cancer J* (2021) 11(3):59. doi: 10.1038/s41408-021-00455-x

67. Zhou X, Tu S, Wang C, Huang R, Deng L, Song C, et al. Phase I trial of fourth-generation anti-CD19 chimeric antigen receptor T cells against relapsed or refractory b cell non-Hodgkin lymphomas. *Front Immunol* (2020) 11:564099. doi: 10.3389/fimmu.2020.564099

68. Di Stasi A, Tey SK, Dotti G, Fujita Y, Kennedy-Nasser A, Martinez C, et al. Inducible apoptosis as a safety switch for adoptive cell therapy. *N Engl J Med* (2011) 365(18):1673–83. doi: 10.1056/NEJMoa1106152

69. Bonifant CL, Jackson HJ, Brentjens RJ, Curran KJ. Toxicity and management in CAR T-cell therapy. *Mol Ther Oncolytics* (2016) 3:16011. doi: 10.1038/mto.2016.11

70. S.p.A, A.G.C.B and C. Horizon - European. *Study of CAR T-cell therapy in acute myeloid leukemia and multiple myeloma* (2021). Available at: https://ClinicalTrials.gov/show/NCT04097301.

71. Stadtmauer EA, Fraietta JA, Davis MM, Cohen AD, Weber KL, Lancaster E, et al. CRISPR-engineered T cells in patients with refractory cancer. *Science* (2020) 367(6481):976. doi: 10.1126/science.aba7365

72. Cooper ML, Choi J, Staser K, Ritchey JK, Devenport JM, Eckardt K, et al. An "off-the-shelf" fratricide-resistant CAR-T for the treatment of T cell hematologic malignancies. *Leukemia* (2018) 32(9):1970–83. doi: 10.1038/s41375-018-0065-5

73. Benjamin R, Graham C, Yallop D, Jozwik A, Mirci-Danicar OC, Lucchini G, et al. Genome-edited, donor-derived allogeneic anti-CD19 chimeric antigen receptor T cells in paediatric and adult b-cell acute lymphoblastic leukaemia: results of two phase 1 studies. *Lancet* (2020) 396(10266):1885–94. doi: 10.1016/S0140-6736(20)32334-5

74. Ward GA, McGraw KL, Abbas-Aghababazadeh F, Meyer BS, McLemore AF, Vincelette ND, et al. Oxidized mitochondrial DNA released after inflammasome activation is a disease biomarker for myelodysplastic syndromes. *Blood Adv* (2021) 5(8):2216–28. doi: 10.1182/bloodadvances.2020003475

75. Jain N, Roboz GJ, Konopleva M, Liu H, Jabbour E, Poirot C, et al. Preliminary results of balli-01: A phase I study of UCART22 (allogeneic engineered T-cells expressing anti-CD22 chimeric antigen receptor) in adult patients with relapsed or refractory (R/R) CD22+ b-cell acute lymphoblastic leukemia (B-ALL). *Blood* (2020) 136(Supplement 1):7–8. doi: 10.1182/blood-2020-138594

76. Locke FL, Malik S, Tees MT, Neelapu SS, Popplewell L, Abramson JS, et al. First-in-human data of ALLO-501A, an allogeneic chimeric antigen receptor (CAR) T-cell therapy and ALLO-647 in relapsed/refractory large b-cell lymphoma (R/R LBCL): ALPHA2 study. *J Clin Oncol* (2021) 39(15_suppl):2529–9. doi: 10.1200/JCO.2021.39.15_suppl.2529

77. Neelapu SS, Munoz J, Locke FL, Miklos DB, Brown R, McDevitt JT, et al. First-in-human data of ALLO-501 and ALLO-647 in relapsed/refractory large b-cell or follicular lymphoma (R/R LBCL/FL): ALPHA study. *J Clin Oncol* (2020) 38 (15_suppl):8002–2. doi: 10.1200/JCO.2020.38.15_suppl.8002

78. Hu Y, Zhou Y, Zhang M, Ge W, Li Y. The safety and efficacy of a CRISPR/Cas9-engineered universal CAR-T cell product (CTA101) in patients with Relapsed/Refractory b-cell acute lymphoblastic leukemia. *Blood* (2020) 136 (Supplement 1):52–2. doi: 10.1182/blood-2020-142262

79. Mailankody S, et al. Universal: An allogeneic first-in-Human study of the anti-bcma ALLO-715 and the anti-CD52 ALLO-647 in Relapsed/Refractory multiple myeloma. *Blood* (2020) 136(Supplement 1):24–5. doi: 10.1182/blood-2020-140641

80. Iyer SP, et al. S262: The COBALT-LYM study of CTX130: A phase 1 dose escalation study of CD70-targeted allogeneic CRISPR-CAS9–engineered CAR T cells in patients with relapsed/refractory (R/R) T-cell malignancies. *HemaSphere* (2022) 6:163–4. doi: 10.1097/01.HS9.0000843940.96598.e2

81. Wang X, et al. Abstract CT052: Clinical safety and efficacy study of TruUCAR^{TM} GC027: The first-in-human, universal CAR-T therapy for adult relapsed/refractory T-cell acute lymphoblastic leukemia (r/r T-ALL). *Cancer Res* (2020) 80(16_Supplement):CT052–2. doi: 10.1158/1538-7445.AM2020-CT052

82. Li S, et al. Abstract LB147: Updates on clinical safety and efficacy result of GC027, the first-in-human, "Off-the-Shelf" CD7 CAR-T stand-alone therapy for adult patients with relapsed/refractory T-cell lymphoblastic leukemia (r/r T-ALL). *Cancer Res* (2021) 81(13_Supplement):LB147–7. doi: 10.1158/1538-7445.AM2021-LB147

83. Sterner RC, Sterner RM. CAR-T cell therapy: current limitations and potential strategies. *Blood Cancer J* (2021) 11(4):69. doi: 10.1038/s41408-021-00459-7

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Sleeping beauty generated CD19 CAR T-Cell therapy for advanced B-Cell hematological malignancies

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Chimeric antigen receptor (CAR) T-cell therapy has emerged recently as a standard of care treatment for patients with relapsed or refractory acute lymphoblastic leukemia (ALL) and several subtypes of B-cell non-Hodgkin lymphoma (NHL). However, its use remains limited to highly specialized centers, given the complexity of its administration and its associated toxicities. We previously reported our experience in using a novel Sleeping Beauty (SB) CD19-specific CAR T-cell therapy in the peri-transplant setting, where it exhibited an excellent safety profile with encouraging survival outcomes. We have since modified the SB CD19 CAR construct to improve its efficacy and shorten its manufacturing time. We report here the phase 1 clinical trial safety results. Fourteen heavily treated patients with relapsed/refractory ALL and NHL were infused. Overall, no serious adverse events were directly attributed to the study treatment. Three patients developed grades 1-2 cytokine release syndrome and none of the study patients experienced neurotoxicity. All dose levels were well tolerated and no dose-limiting toxicities were reported. For efficacy, 3 of 8 (38%) patients with ALL achieved CR/CRi (complete remission with incomplete count recovery) and 1 (13%)

patient had sustained molecular disease positivity. Of the 4 patients with DLBCL, 2 (50%) achieved CR. The SB-based CAR constructs allow manufacturing of targeted CAR T-cell therapies that are safe, cost-effective and with encouraging antitumor activity.

KEYWORDS

sleeping beauty, non-viral gene transfer, CD19, CAR, T cells, lymphoid malignancy, acute lymphoblastic leukemia, non-hodgkin lymphoma

Introduction

Despite the advances made over the past decade and the introduction of several novel therapeutics, there remains an unmet need to further improve the outcomes of patients with advanced hematologic malignancies. CD19-targeted chimeric antigen receptor (CAR) T-cell therapy has emerged recently as one of the new standard treatments for patients with relapsed or refractory acute lymphoblastic leukemia (ALL) and several subtypes of B-cell non-Hodgkin lymphoma (NHL) (1–4). However, durable responses are noted in less than 50% of these patients (5), and the widespread use of this promising therapy is hampered by the known unique and potentially serious toxicities, particularly cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS) (6). Hence, there is a need for CAR constructs with a better safety profile that at a minimum maintain this practice-changing therapy's efficacy, if not improve it.

We have previously reported our experience in using a novel Sleeping Beauty (SB) (7) CD19-specific CAR T-cell therapy in two phase 1 clinical trials (NCT00968760 and NCT01497184) (8, 9). T-cells were genetically modified using the SB transposon/transposase system to produce a second-generation CAR construct (10), with co-signaling through CD3 and CD28 (11). Through incorporating SB CAR T-cells into the hematopoietic stem cell transplantation (HCT) setting (infusing cells 2 days after stem cell infusion), we showed an excellent safety profile, long-term persistence of the genetically modified T-cells (median of 4.5 years), and potentially improved outcomes in patients with advanced B-cell lymphoid malignancies (8, 12). Twenty-six patients were treated in these phase 1 studies, with no unexpected acute or delayed toxicities noted. Based on the promising phase 1 findings in patients with low tumor burden at time of cell infusion, we made modifications to the CAR stalk to reduce binding to Fc receptors, and modifications to the manufacturing process to shorten the production time in efforts to improve efficacy in patients with bulky disease, and improve ease of administration, respectively (13). Herein, we report the final safety and efficacy results of this clinical trial (NCT02807883; IND# 16474).

Materials and methods

Study design

This was a prospective, open-label, single-arm, single center, phase 1 clinical trial evaluating 5 dose escalation/de-escalation levels (DL -1: $\leq 1 \times 10^5$/kg; DL +1 > 1×10^5/kg but $\leq 1 \times 10^6$/kg; DL +2 > 1×10^6/kg but $\leq 1 \times 10^7$/kg; DL +3 > 1×10^7/kg but $\leq 1 \times 10^8$/kg; DL +4 > 1×10^8/kg but $\leq 1 \times 10^9$/kg). The phase 1 clinical trial design we employed in this study was as previously described by Ji et al. (14). Dose-limiting toxicity (DLT) was defined as a non-reversible grade 3 or any grade 4-5 non-hematological organ toxicities and/or allergic/autoimmune reactions related to the study cell infusion. Adverse events were graded using the Common Terminology Criteria for Adverse Events version 4 (CTCAE V 4.0). Responses were assessed and defined per disease category, as previously described (15).

Ethics approval and patient consent

The study was conducted after the protocol was reviewed and approved by MD Anderson Cancer Center's Institutional Review Board (IRB). Patients provided informed consent prior to enrollment in the clinical study in accordance with the Declaration of Helsinki. This phase 1 clinical trial was registered at ClinicalTrials.gov (NCT02807883).

Patient eligibility

Patients with relapsed/refractory $CD19^+$ B-cell lymphoid malignancies, ages 1 through 80 years, were eligible. B-cell

lymphoid malignances included acute lymphoblastic leukemia (ALL), diffuse large B-cell lymphoma (DLBCL), chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL), follicular lymphoma, marginal zone lymphoma, and mantle cell lymphoma, with confirmed positive CD19 by flow cytometry on the malignant cells. At study entry, patients were required to have adequate organ function, Karnofsky performance status (KPS) >60%, and with no evidence of active hepatitis B or C infection. Patients with a history of HIV infection were excluded. Those with prior allogeneic HCT were allowed after at least 3 months following transplant. Patients had measurable disease at study entry and had failed standard frontline therapy. Bridging chemotherapy to control disease while waiting for CAR T-cell production was allowed and at the discretion of the treating physician. Notably, patients must have had measurable disease and adequate organ function at time of starting lymphodepletion prior to CAR T infusion.

DNA constructs

This study used a second generation CD19-specific CAR (10, 16, 17) consisting of an anti-CD19scfv held on the cell surface by a CD8α stalk and with signaling through CD3ζ and CD28 co-stimulatory endodomains (CD19RCD8CD28) (13). The CAR was expressed in a SB transposon and SB transposase was encoded by a pCMV-SB11 plasmid (18).

Cell lines

All cell lines were cultured in complete media (RPMI 1640, 10% heat inactivated fetal bovine serum (HyClone), and 1% Glutamax-100 (Gibco)) at 5% CO_2 and 37°C. Daudiβ_2m, NALM-6, EL-4 and EL-4 modified to express CD19 (CD19$^+$ EL-4) were maintained, as previously described (18). K562 clone #1 AaPC was developed as previously described (19) and expressed CD19, CD32, CD64, CD86, CD137L and membrane bound IL-15. A working cell bank of clone #1 was used to propagate AaPC in WAVE Bioreactors, γ-irradiated, and cryopreserved for future use, as described previously (8). Thawed irradiated AaPC were utilized for generation of CAR T-cells. Cell lines were negative for mycoplasma and endotoxin. The identities of cell lines were established by STR DNA fingerprinting performed by the Characterized Cell Line Core at MD Anderson Cancer Center.

Generation of CAR T-cells

Peripheral blood mononuclear cells (PBMC) were obtained *via* patient derived leukapheresis products. PBMC were isolated using the Biosafe Sepax II platform, as described previously (18). In short, the Sepax II is a closed system centrifugation instrument that utilizes an automated Ficoll gradient protocol to separate PBMCs from non-target cell types. After Ficoll gradient isolation, PBMCs were washed twice with PBS/EDTA supplemented with human serum albumin and the washed cells were transferred into a 200 mL blood banking bag. The PBMCs were then cryopreserved for future manufacturing purposes. Genetic modification to generate CAR T-cells was performed, as described earlier (8). PBMC were thawed and rested in complete media for two hours at 37°C, 5% CO_2. The rested cells were resuspended at a concentration of $2x10^7$/100μL of a mixture containing 15μg transposon DNA plasmid coding for CD19RCD8CD28 transposon, 5μg transposase DNA plasmid (pCMV-SB11) coding for SB11 transposase, and Human T-cell kit reagent (cat# VPA-1002, Lonza). The mixture was transferred to a cuvette, electroporated using program U-14 of the Nucleofector II device (Amaxa, Lonza) and transferred to complete media for a two-hour rest at 37°C, 5% CO_2. A half media change was performed and the electroporated cells were then incubated overnight at 37°C, 5% CO_2. The next day, cells were harvested, counted, and phenotyped by flow cytometry. Cells were then co-cultured with 100Gy irradiated K562 clone #1 AaPCs at a 1:1 ratio (AaPC: CAR$^+$ T-cell) along with IL-21 (PeproTech, 30ng/mL). Media changes and cytokine additions were performed every 2-3 days. IL-2 (Aldesleukin, Novartis, 50U/mL) was incorporated into the media changes starting at day 7 to avoid early outgrowth of natural killer (NK) cells. T-cell cultures were evaluated for CAR$^+$ expression and re-stimulated every 7 days with 100Gy irradiated clone #1 AaPCs with the addition of IL-21 and IL-2. T-cells were expanded in culture to reach appropriate patient dose levels and cryopreserved thereafter.

Lymphodepletion and CAR T-cell infusion

Lymphodepletion was recommended for all study patients, unless there were remarkable cytopenias from prior therapies. Lymphodepletion consisted of fludarabine 30 mg/m^2 and cyclophosphamide 500 mg/m^2 for 3 consecutive days, followed by CAR T-cell infusion at least 48 hours after completion of lymphodepletion. Reduced intensity lymphodepletion (fludarabine 25 mg/m^2 and cyclophosphamide 250 mg/m^2) was allowed at the discretion of the treating physician. Prior to CAR T-cell infusion, patients should have been off steroids for at least 72 hours (unless on physiological dose replacements), with no active infection, and with resolution of any non-hematologic toxicity from lymphodepletion to < grade 3. The day of CAR T-cell infusion was designated as Day 0. The CAR T-cell dose was defined by the dose group per the phase 1 dose escalation/de-escalation schedule, as described in the study design section.

Safety and evaluations

Disease assessments with peripheral blood studies, bone marrow examinations, and PET when clinically relevant were done prior to study entry and at 30 days following CAR T infusion to assess for response. The CTCAE V 4.0 was used to grade toxicities.

Response definitions and outcome measures

The primary objectives were to determine the safety profile and maximum tolerated dose (MTD) of SB CAR T-cells. Secondary objectives included assessment of disease response and to determine persistence of CAR T-cells. CR was defined as having ≤ 5% malignant blasts in the bone marrow, recovery of normal blood counts with absolute neutrophil count ≥ 0.5 x 10^9/L and platelet count > 20 x 10^9/L, normal karyotype, and absence of extramedullary disease. MRD was assessed using multiparameter flow cytometry with a threshold of > 0.01%. CR for lymphoma was defined by CT and/or PET, as per Cheson criteria (15).

Flow cytometry

Immunophenotyping by flow cytometric analysis was performed by staining T-cell suspensions with a live/dead stain followed by surface antibody staining for anti-CD3, CD4, CD8, CD45, CD56, CD11c, CD19, CD14, CD16, CD20, CD32, CD45RO, CD27, CD95, CD45RA, CD28 CD62L, CD197, TCRαβ, TCRγδ and CAR (Supplementary Methods). All experiments were performed using a BD Fortessa or BD FACS Calibur. Data was analyzed using FlowJo software.

Chromium release assay

Specific lysis of $CD19^+$ targets by CAR^+ T-cells was determined using a standard 4 hour chromium release assay (18).

Gene integration (CAR copy number)

Assessment of integrated CAR copy number of SB-modified T-cells was determined by droplet digital PCR (ddPCR), a sensitive method of detecting and quantifying infrequent target DNA molecules (20), as described previously (8). 50ng of genomic DNA was multiplexed using primer/probe sets for the CAR and a housekeeping gene (EIF2C1) (Supplementary Methods). PCR droplets were generated and analyzed using a QX-100 Digital Droplet PCR System (Bio-Rad).

Serum cytokines

Blood samples collected post CAR T-cell infusion were processed to isolate serum and cryopreserved in aliquots at -80° C for analysis. For evaluation of cytokines in the serum of patients post infusion, serum samples were thawed and processed using a Bio-Plex Pro Human Cytokine 27-plex Assay (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Briefly, serum samples were diluted (1:4) in complete media (RPMI containing 10% FBS with Glutamax-1), incubated with capture beads, and read in a Bio-Plex 200 system (Bio-Rad).

Statistical analysis

Progression-free survival (PFS) time was computed from date of cell infusion to date of progression or death, whichever came first. Patients who were alive at their last follow-up date who had not progressed were censored. Overall survival (OS) time was computed from date of cell infusion to date of death. Patients who were alive at their last follow-up date were censored. The Kaplan-Meier method was used to estimate PFS and OS.

Results

Generation of CAR T-cells for clinical trial

Genetically modified T-cells were co-cultured with γ-irradiated AaPCs (clone #1) in the presence of exogenous cytokines (IL-2, IL-21) for a median of 22 days (mean ± SD, 23.4 ± 5.19) and cryopreserved for infusion (Figure 1A). Twenty-six patients were enrolled on the study from June 2016 through April 2019. CAR product was successfully generated for 23 patients, while product manufacture failure occurred for 3 patients (Supplementary Table S1). The gene-modified T-cells had an average expansion of 4681-fold for CAR^+ T-cells, median 97.9% $CD3^+$ (mean ± SD, 91.4% ± 13.26%), median 80.5% CAR^+ (mean ± SD, 70.1% ± 29.7%) and were predominantly $CD8^+$ (mean ± SD, 70.2% ± 18.7%; mean CD4/CD8, 0.19) (Figures 1B, C, Supplementary Table S1). They were able to effectively lyse at a effector:target ratio of 5:1 various $CD19^+$ B-cell lines, Daudi (Burkitt's Lymphoma) co-expressing β_2-microglobulin (21) (Daudiβ_2m) to reduce lysis by LAC or NK cells (mean ± SD, 44.6% ± 18.2%) and NALM-6 (pre-B ALL; mean ± SD, 38.4% ± 15.1%) in a cytotoxicity assay. An increase of 4.8-fold (5:1, E:T) in killing of $CD19^+$ EL-4 cells as compared to unmodified $CD19^{neg}$ EL-4 (mouse T-cell lymphoma) targets demonstrated specificity for CD19 by CAR T cells. (Supplementary Figure S1, Supplementary Table S2). Expanded T cells were cryopreserved, passed release testing to generate a certificate of analysis (22), and were thawed on the day of infusion after the recipient met eligibility.

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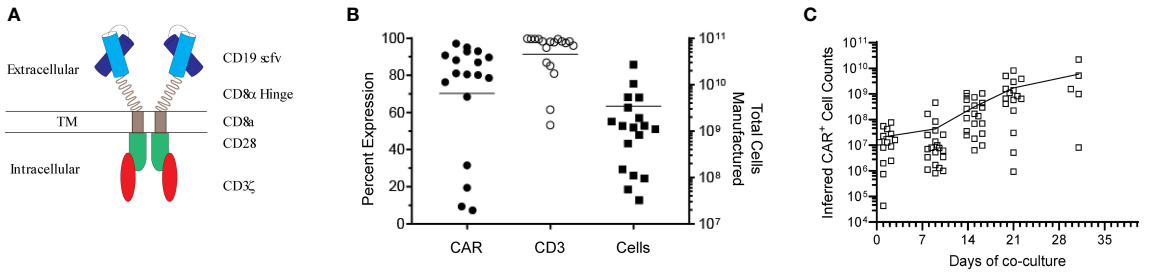

FIGURE 1
Design and characterization of the CD19 CAR. **(A)** Schematic of the CD19-specific CAR, which was held on the cell surface *via* a CD8α hinge stalk and signaled through CD28 and CD3ζ endodomains. **(B)** Characterization of the infusion product. Patient derived CAR$^+$ T-cells were generated by co-culture of genetically modified T-cells with K562 AaPCs. Cells were phenotyped and enumerated every 7 days. The total cells generated and percent CAR and CD3 expression of the cell products at time of cryopreservation is shown. **(C)** Expansion kinetics of all the manufactured patient derived CAR$^+$ T-cells over time. Each symbol represents an individual patient.

Patient characteristics and safety

Fourteen patients with a median age of 40 years (range, 16-73 years) received CAR T-cell therapy and were included in the final safety and efficacy analysis (Table 1). Nine patients did not have cells infused due to the following reasons: rapid disease progression with clinical deterioration and death (n=4), no disease at time of CAR availability (n=3, 2 of which remain in remission and 1 patient relapsed with CNS involvement and died from disease progression), allogeneic HCT (n=1), and loss of insurance (n=1).

Of the 14 patients who received thawed SB CD19-specific CAR T-cells (viability, mean ± SD, 98.8% ± 2.13%, Supplementary Table S1), 8 patients had B-ALL, 4 patients

TABLE 1 Study patient characteristics and treatment outcomes in detail for the infused patients, N=14.

Acc #	Age	Gender	Diagnosis	Prior lines of therapy	Prior trans-plant	Cohort	CRS	ICANS	Response	Progressed	Status at last follow-up
2	68	M	CLL	3	No	1	No	No	No response	Yes	Died, secondary cancer
4	40	M	ALL	6	Yes	1	No	No	CRi	Yes	Died, active disease
6	36	F	DLBCL	4	Yes	-1	No	No	No response	Yes	Died, active disease
8	40	F	ALL	4	Yes	1	No	No	CR	Yes	Died, active disease
9	29	F	ALL	4	No	2	Yes	No	No response	Yes	Died, active disease
13	46	M	ALL	5	Yes	2	Yes	No	MRD negative	Yes	Died, active disease
14	72	M	DLBCL	5	Yes	2	Yes	No	CR	No	Alive, in remission
16	16	M	ALL	3	Yes	1	No	No	No response	Yes	Died, active disease
20	47	F	DLBCL*	3	No	3	No	No	CR	Yes	Alive, in remission
21	31	M	ALL	4	Yes	3	No	No	No response	Yes	Died, active disease
22	73	F	DLBCL	2	No	-1	No	No	Progression	Yes	Died, active disease
23	57	F	CLL	3	Yes	3	No	No	No response	Yes	Alive, active disease
25	34	M	ALL	4	Yes	1	No	No	No response	Yes	Died, active disease
26	39	M	ALL	2	Yes	1	No	No	MRD positive	No	Alive, in remission

ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; CR, complete remission; CRi, CR with incomplete platelet and/or neutrophil recovery; CRS, cytokine release syndrome; ICANS, immune effector cell associated neurotoxicity syndrome; DLBCL, diffuse large B-cell lymphoma; F, female; M, male; MRD, minimal residual disease.
*Transformed from marginal zone lymphoma.

had DLBCL and 2 patients had CLL. Patients were heavily pretreated, with a median of 4 (range, 2-6) prior lines of therapy. Additionally, 10 patients had prior allogeneic HCT, of whom 3 received two transplants (one patient received an autologous and then an allogeneic transplant, and two patients received two prior allogeneic transplants). Two patients, numbers 2 and 16 in Table 1, received reduced intensity lymphodepletion due to significant cytopenia at time of study treatment.

Overall, no serious adverse events were directly attributed to the study treatment. Only 1 patient had a grade 2 infusion reaction, which resolved with supportive treatment. No unexpected acute or delayed toxicities were observed. Three patients developed grades 1-2 CRS and none of the study patients had ICANS. Three events of grade 3 non-hematological adverse events occurred; one each for infection, elevated alanine aminotransferase, and elevated aspartate aminotransferase. All dose levels were well tolerated with no DLTs reported.

Response and survival

Of the 14 patients assessed for efficacy, 5 (36%) patients achieved objective responses, 2 (14%) had stable disease (both had CLL), and 1 patient had sustained minimal residual disease (MRD) positivity (ALL patient). Table 1 presents the disease and treatment characteristics of the 14 individual patients in the study and their respective outcomes. Of the 8 patients with ALL, 3 (38%) patients achieved CR/CRi (complete remission with incomplete count recovery) at 1 month, 1 (13%) patient had sustained molecular measurable residual disease (MRD) positivity, and 4 (50%) patients had no response. Of the responding 3 patients in remission, all progressed during the study period (Figure 2). Of the 4 patients with DLBCL, 2 (50%) achieved complete remission, 1 patient had progressive disease at 1 month after CAR T-cell therapy, and 1 patient had rapid leptomeningeal central nervous system progression and transitioned to hospice before day 30 disease assessments. The 2 responding patients had durable remissions; 1 remains in remission at 3 years after cell infusion and the second patient progressed at 18 months after therapy (Figure 2). The 2 CLL patients did not respond to CAR T-cell therapy (Figure 2).

The median (range) follow-up for the 14 study patients was 14.8 months (0.9-62.0 months). The 1-year PFS rate for all study patients was 21%; 13% for the ALL patients and 50% for the DLBCL patients. The respective 1-year OS rates for all study patients, ALL, and DLBCL were 57%, 50%, and 50%, respectively.

Persistence of CAR T-cells in patients

Peripheral blood was collected serially over time from the patients, and the presence of genetically modified CAR$^+$ T-cells was investigated using both ddPCR and flow cytometry. CAR T-

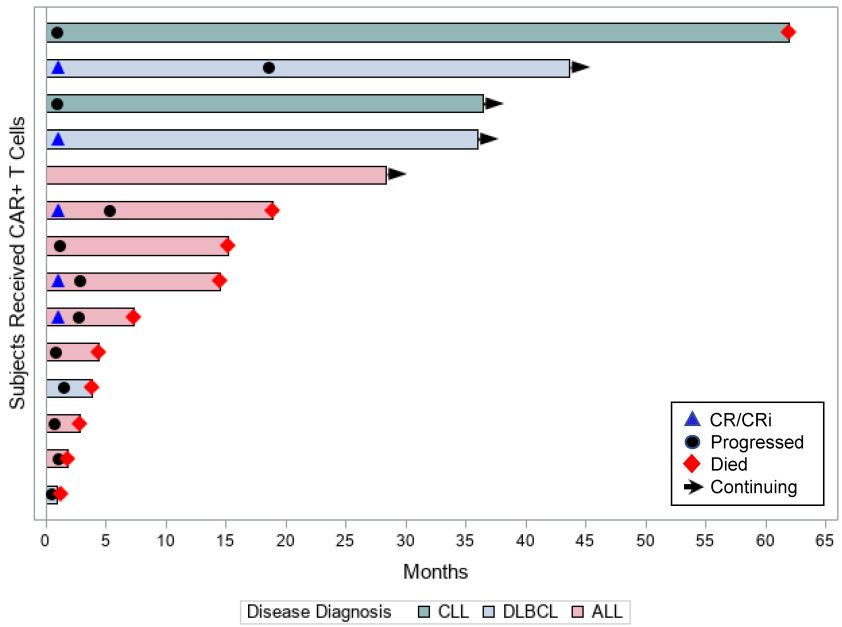

FIGURE 2
Patient responses. Swimmer's plot displays each individual patient's time in the study (in months), their disease diagnosis, and outcomes.

cells could be detected by flow cytometry up to 30 days post infusion, after which the level of detection was at background level. Using the more sensitive ddPCR method, CAR T-cells could be detected up to an average of 203 days post infusion. Data for select patients are shown in Figure 3, and data for all patients is shown in Supplementary Figure S2. We did not observe any correlation between persistence and response.

Serum cytokines

Persistence of CAR$^+$ T-cells depends on signaling through the CAR moiety and through cytokine receptors. We observed no difference in the levels of cytokines signaling through the common cytokine receptor γ (gamma) chain before and after infusion of the T-cells. We noted low levels of IL-2 and IL-15, normal levels of IL-4 and IL-7, and elevated levels of IL-9. Of the cytokines implicated in cytokine release syndrome, IFN-γ and CXCL10 were altered 1 week ($p<0.05$) and 2 weeks ($p<0.05$) post infusion, respectively (Supplementary Figure S3).

Discussion

We report on the long-term findings of non-viral, SB generated, autologous CD19 directed CAR T-cells in patients with advanced lymphoid malignancies. We note our ability to consistently manufacture up to dose level three (10^7 CAR T-cells/kg). Furthermore, similar to our previous trial (8), the CAR T infusion was very well tolerated, with minimal rates of CRS and no noted ICANS. The intent of this trial was to investigate whether modifications to the CAR T product would increase efficacy. Our previous clinical trials infused T-cells expressing a 2nd generation CAR (designated CD19RCD28) with an IgG$_4$-Fc stalk that activated T-cells *via* chimeric CD28 and CD3ζ (8). Modifications to the CAR stalk to reduce binding of Fc receptor

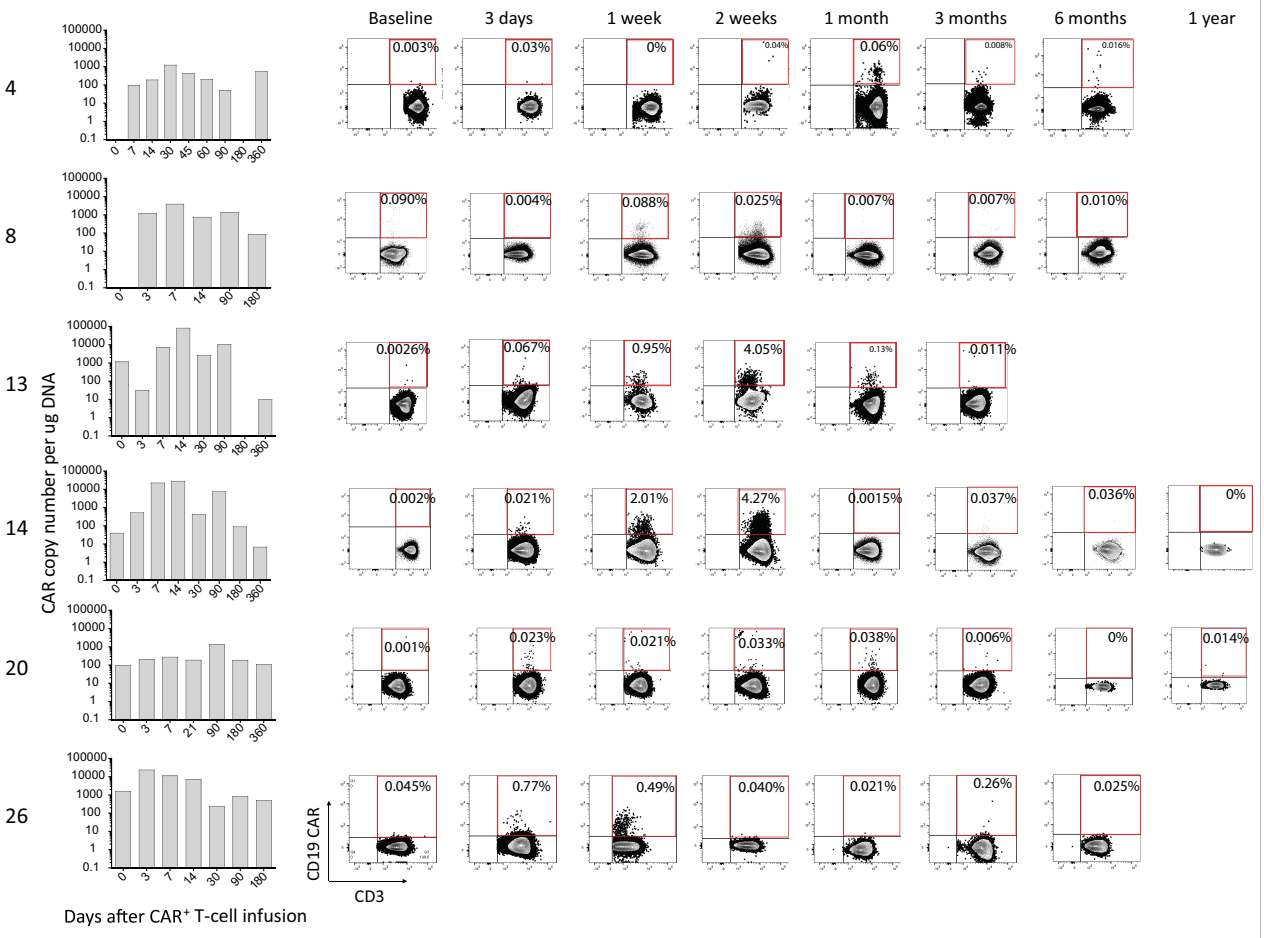

FIGURE 3
Persistence of CAR$^+$ T-cells in selected patients. The presence of CAR T-cells was evaluated by ddPCR (bar graphs) and flow cytometry (dot-plots) before (baseline) and at various timepoints after infusion of the gene-modified T-cells.

(s) and antigen recognition has shown to further improve the persistence of the genetically modified T-cells (23–25). Of the various stalks tested, including the IgG_4-Fc mutant EQ (L235E and N297Q), CD8α hinge, and 12aa IgG_1 hinge, the CAR with a CD8α-derived hinge (CD19RCD8CD28) showed reduced binding to Fcγ receptors (FcγR) and superior efficacy and persistence in NSG xenograft MRD leukemic models (13). Moreover, repeated stimulation cycles can erode the therapeutic potential of *ex vivo* propagated T-cells (26, 27). Therefore, we shortened the length of time in tissue culture to sustain the outgrowth of CAR^+ T-cells that preserves a "memory" T-cell phenotype and genotype. Reducing the number of recursive stimulation cycles on aAPCs from 4x to 2x showed an improved memory phenotype (CCR7/CD45RA) (28) of the CAR T-cells, which led to improved efficacy and survival in mouse models (13).

With these modifications, we noted robust *in vivo* expansion of the CAR T product and responses in some patients. We treated a heterogeneous patient population, which precludes our ability to study any predictors for response. We noted long-term persistence in some patients but there did not appear to be any correlation between *in vivo* CAR T persistence and response.

Further modifications to the CAR T construct, as well as exploring other tumor associated antigens (TAA) and other substrates for CAR T production, are under investigation in efforts to improve the efficacy of immunotherapies using the SB platform. Magnani et al. reported on a phase I/II trial using donor-derived CD19 CAR T-cells generated with the SB transposon and differentiated into cytokine-induced killer (CIK) cells for patients with B-ALL who relapsed after allogeneic HCT (29). The product was successfully made for all 13 treated patients and was generated from a peripheral blood collection from the donor. It consisted of mainly $CD3^+$ lymphocytes, with 43% CAR expression. Patients received a single dose of the CAR T product. Six of the 7 patients treated at the highest dose level had a CR or CRi, including 5 with an MRD negative response. Robust expansion was achieved in the majority of the patients. CAR T-cells were measurable by transgene copy PCR for up to 10 months. Toxicities reported included 2 patients with grade I and 1 patient with grade II CRS at the highest dose in the absence of graft-versus-host disease (GVHD), neurotoxicity, or DLT (29).

Our study highlights the success of genetic engineering of T cells based on SB nonviral gene transfer system combined with *ex vivo* expansion on AaPCs to generate CD19 CAR T cells from patients with a variety of lymphoid malignancies. By utilizing an improved CAR design and shorter *ex vivo* expansion protocol, we observed persistence of T cells by both flow cytometry and PCR in 42% of the patients, an improvement from our previous trials, with active disease. Furthermore, SB-modified CAR T cells were well tolerated and no severe CRS or ICANS were observed. Further studies to improve persistence and efficacy are warranted, and we are adapting cytokine (IL-15) co-stimulation to support T cell *in vivo* persistence and maintenance of an immature differentiation state (30).

Therefore, SB platform allows for more cost efficient and nimble construction of CAR T products. CAR DNA constructs can be easily and rapidly produced at much lower cost (10) compared to clinical grade lentivirus or retrovirus (10, 31). Outsourcing and the need for specialized handling along with limited GMP facilities for generation of viruses and long wait times due to unprecedented demand, all make the use of recombinant viruses tedious and unattractive and hence the need for alternative non-viral transduced CAR constructs. Additionally, for early proof-of-concept trials, the reduced pricing for plasmid DNA allows for speed in translating preclinical data into clinical trials. This approach may be particularly useful in the setting of immunotherapy for patients with solid tumors, where identifying optimal TAAs is critical and under active investigation. Further studies are needed to improve efficacy of these promising therapies.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Institutional Review Board of the University of Texas MD Anderson Cancer Center. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

HS, SS, LC, and PK conceptualized the study. DM completed the statistical analysis. HS, SS, JM, CD, MG, MA, SO, HH, EG, DM, DP, AO, PA, JI, IK, CH, KR, RC, ES, LC, and PK enrolled patients, monitored clinical responses, completed laboratory studies, and/or analyzed data. All authors contributed to the article and approved the submitted version.

Funding

Clinical trial was supported by Alaunos Therapeutics.

Conflict of interest

The technology was advanced through research conducted at MD Anderson by LC. In January 2015, the technology was

licensed by The University of Texas MD Anderson Cancer Center for commercial application to Alaunos Therapeutics formerly Ziopharm Oncology, Inc., and Precigen formerly Intrexon Corporation, in exchange for equity interests in each of these companies. LC and some co-authors received equity because of the licensing of this technology. From 2015 to 2021 LC was Chief Executive Officer at ZIOPHARM. The information being reported in this publication is research in which The University of Texas MD Anderson Cancer Center has an institutional financial conflict of interest. Because The University of Texas MD Anderson Cancer Center is committed to the protection of human subjects and the effective management of its financial conflicts of interest in relation to its research activities, The University of Texas MD Anderson Cancer Center has implemented an Institutional Conflict of Interest Management and Monitoring Plan to manage and monitor the conflict of interest with respect to The University of Texas MD Anderson Cancer Center's conduct of this research. EG and LC were formerly employed by Alaunos Therapeutics and have equity ownership in the company. KR and The University of Texas MD Anderson Cancer Center have an institutional financial conflict of interest with Takeda Pharmaceutical and Affimed GmbH. KR participates on the Scientific Advisory Board for GemoAb, AvengeBio, Virogin Biotech, GSK, Bayer, Navan Technologies, and Caribou Biosciences. ES participates on Scientific Advisory Boards for Adaptimmune, Axio, Navan, Fibroblasts and Fibrobiologics, and the NY Blood Center; has licensing or patents with Takeda and Affimed; and honorarium from Bayer Healthcare Pharmaceuticals. PK has served on advisory boards for Kite and Pfizer; received research support from Amgen and Alaunos; and has been a consultant for Jazz.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be constructed 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.2022.1032397/full#supplementary-material

References

1. Schuster SJ, Bishop MR, Tam CS, Waller EK, Borchmann P, McGuirk JP, et al. Tisagenlecleucel in adult relapsed or refractory diffuse Large b-cell lymphoma. *N Engl J Med* (2019) 380(1):45–56. doi: 10.1056/NEJMoa1804980

2. Locke FL, Ghobadi A, Jacobson CA, Miklos DB, Lekakis LJ, Oluwole OO, et al. Long-term safety and activity of axicabtagene ciloleucel in refractory large b-cell lymphoma (ZUMA-1): a single-arm, multicentre, phase 1-2 trial. *Lancet Oncol* (2019) 20(1):31–42. doi: 10.1016/S1470-2045(18)30864-7

3. Maude SL, Laetsch TW, Buechner J, Rives S, Boyer M, Bittencourt H, et al. Tisagenlecleucel in children and young adults with b-cell lymphoblastic leukemia. *N Engl J Med* (2018) 378(5):439–48. doi: 10.1056/NEJMoa1709866

4. Shah BD, Ghobadi A, Oluwole OO, Logan AC, Boissel N, Cassaday RD, et al. KTE-X19 for relapsed or refractory adult b-cell acute lymphoblastic leukaemia: phase 2 results of the single-arm, open-label, multicentre ZUMA-3 study. *Lancet* (2021) 398(10299):491–502. doi: 10.1016/S0140-6736(21)01222-8

5. Greenbaum U, Mahadeo KM, Kebriaei P, Shpall EJ, Saini NY. Chimeric antigen receptor T-cells in b-acute lymphoblastic leukemia: State of the art and future directions. *Front Oncol* (2020) 10:1594. doi: 10.3389/fonc.2020.01594

6. Sheth VS, Gauthier J. Taming the beast: CRS and ICANS after CAR T-cell therapy for ALL. *Bone Marrow Transplant* (2021) 56(3):552–66. doi: 10.1038/s41409-020-01134-4

7. Ivics Z, Hackett PB, Plasterk RH, Izsvak Z. Molecular reconstruction of sleeping beauty, a Tc1-like transposon from fish, and its transposition in human cells. *Cell* (1997) 91(4):501–10. doi: 10.1016/S0092-8674(00)80436-5

8. Kebriaei P, Singh H, Huls MH, Figliola MJ, Bassett R, Olivares S, et al. Phase I trials using sleeping beauty to generate CD19-specific CAR T cells. *J Clin Invest* (2016) 126(9):3363–76. doi: 10.1172/JCI86721

9. Kebriaei P, Huls H, Jena B, Munsell M, Jackson R, Lee DA, et al. Infusing CD19-directed T cells to augment disease control in patients undergoing autologous hematopoietic stem-cell transplantation for advanced b-lymphoid malignancies. *Hum Gene Ther* (2012) 23(5):444–50. doi: 10.1089/hum.2011.167

10. Singh H, Manuri PR, Olivares S, Dara N, Dawson MJ, Huls H, et al. Redirecting specificity of T-cell populations for CD19 using the sleeping beauty system. *Cancer Res* (2008) 68(8):2961–71. doi: 10.1158/0008-5472.CAN-07-5600

11. Kowolik CM, Topp MS, Gonzalez S, Pfeiffer T, Olivares S, Gonzalez N, et al. CD28 costimulation provided through a CD19-specific chimeric antigen receptor enhances in vivo persistence and antitumor efficacy of adoptively transferred T cells. *Cancer Res* (2006) 66(22):10995–1004. doi: 10.1158/0008-5472.CAN-06-0160

12. Srour SA, Singh H, McCarty J, de Groot E, Huls H, Rondon G, et al. Long-term outcomes of sleeping beauty-generated CD19-specific CAR T-cell therapy for relapsed-refractory b-cell lymphomas. *Blood* (2020) 135(11):862–5. doi: 10.1182/blood.2019002920

13. Singh H, Huls MH, Figliola MJ, Zhang L, Mi TJ, Maiti S, et al. Next-generation non-viral gene transfer to redirect T-cell specificity. *Mol Ther* (2016) 24:S110–S1. doi: 10.1016/S1525-0016(16)33087-8

14. Ji Y, Liu P, Li Y, Bekele BN. A modified toxicity probability interval method for dose-finding trials. *Clin Trials* (2010) 7(6):653–63. doi: 10.1177/1740774510382799

15. Cheson BD, Fisher RI, Barrington SF, Cavalli F, Schwartz LH, Zucca E. Recommendations for initial evaluation, staging, and response assessment of Hodgkin and non-Hodgkin lymphoma: the lugano classification. *J Clin Oncol* (2014) 32(27):3059–68. doi: 10.1200/JCO.2013.54.8800

16. Singh H, Figliola MJ, Dawson MJ, Huls H, Olivares S, Switzer K. CD19-specific T cells with IL-21 signaling can improve adoptive immunotherapy of b-lineage malignancies. *Cancer Res* (2011) 71(10):3516–27. doi: 10.1158/0008-5472.CAN-10-3843

17. Davies JK, Singh H, Huls H, Yuk D, Lee DA, Kebriaei P, et al. Combining CD19 redirection and alloanergization to generate tumor-specific human T cells for allogeneic cell therapy of b-cell malignancies. *Cancer Res* (2010) 70(10):3915–24. doi: 10.1158/0008-5472.CAN-09-3845

18. Singh H, Figliola MJ, Dawson MJ, Olivares S, Zhang L, Yang G, et al. Manufacture of clinical-grade CD19-specific T cells stably expressing chimeric

antigen receptor using sleeping beauty system and artificial antigen presenting cells. *PloS One* (2013) 8(5):e64138. doi: 10.1371/journal.pone.0064138

19. Deniger DC, Yu J, Huls MH, Figliola MJ, Mi T, Maiti SN, et al. Sleeping beauty transposition of chimeric antigen receptors targeting receptor tyrosine kinase-like orphan receptor-1 (ROR1) into diverse memory T-cell populations. *PloS One* (2015) 10(6):e0128151. doi: 10.1371/journal.pone.0128151

20. Maiti SN, Huls H, Singh H, Dawson M, Figliola M, Olivares S, et al. Sleeping beauty system to redirect T-cell specificity for human applications. *J Immunother* (2013) 36(2):112–23. doi: 10.1097/CJI.0b013e3182811ce9

21. Rabinovich BA, Ketchem RR, Wolfson M, Goldstein L, Skelly M, Cosman D. A role for the MHC class I-like mill molecules in nutrient metabolism and wound healing. *Immunol Cell Biol* (2008) 86(6):489–96. doi: 10.1038/icb.2008.41

22. Singh H, Huls H, Kebriaei P, Cooper LJ. A new approach to gene therapy using sleeping beauty to genetically modify clinical-grade T cells to target CD19. *Immunol Rev* (2014) 257(1):181–90. doi: 10.1111/imr.12137

23. Jonnalagadda M, Mardiros A, Urak R, Wang X, Hoffman LJ, Bernanke A, et al. Chimeric antigen receptors with mutated IgG4 fc spacer avoid fc receptor binding and improve T cell persistence and antitumor efficacy. *Mol Ther* (2015) 23 (4):757–68. doi: 10.1038/mt.2014.208

24. Hudecek M, Lupo-Stanghellini MT, Kosasih PL, Sommermeyer D, Jensen MC, Rader C, et al. Receptor affinity and extracellular domain modifications affect tumor recognition by ROR1-specific chimeric antigen receptor T cells. *Clin Cancer Res* (2013) 19(12):3153–64. doi: 10.1158/1078-0432.CCR-13-0330

25. Hudecek M, Sommermeyer D, Kosasih PL, Silva-Benedict A, Liu L, Rader C, et al. The nonsignaling extracellular spacer domain of chimeric antigen receptors is decisive for in vivo antitumor activity. *Cancer Immunol Res* (2015) 3(2):125–35. doi: 10.1158/2326-6066.CIR-14-0127

26. Gattinoni L, Klebanoff CA, Restifo NP. Paths to stemness: building the ultimate antitumour T cell. *Nat Rev Cancer* (2012) 12(10):671–84. doi: 10.1038/nrc3322

27. Bucks CM, Norton JA, Boesteanu AC, Mueller YM, Katsikis PD. Chronic antigen stimulation alone is sufficient to drive CD8+ T cell exhaustion. *J Immunol* (2009) 182(11):6697–708. doi: 10.4049/jimmunol.0800997

28. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* (1999) 401(6754):708–12. doi: 10.1038/44385

29. Magnani CF, Gaipa G, Lussana F, Belotti D, Gritti G, Napolitano S, et al. Sleeping beauty-engineered CAR T cells achieve antileukemic activity without severe toxicities. *J Clin Invest* (2020) 130(11):6021–33. doi: 10.1172/JCI138473

30. Hurton LV, Singh H, Najjar AM, Switzer KC, Mi T, Maiti S, et al. Tethered IL-15 augments antitumor activity and promotes a stem-cell memory subset in tumor-specific T cells. *Proc Natl Acad Sci U.S.A.* (2016) 113(48):E7788–e97. doi: 10.1073/pnas.1610544113

31. Yip A, Webster RM. The market for chimeric antigen receptor T cell therapies. *Nat Rev Drug Discovery* (2018) 17(3):161–2. doi: 10.1038/nrd.2017.266

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Spatial architecture of regulatory T-cells correlates with disease progression in patients with nasopharyngeal cancer

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Purpose: This study aims to investigate the prognostic value of composition and spatial architecture of tumor-infiltrating lymphocytes (TILs) as well as PDL1 expression on TILs subpopulations in nasopharyngeal carcinoma (NPC).

Methods: A total of 121 patients with NPC were included and divided into two groups: favorable (n = 68) and unfavorable (n = 53). The archived tumor tissues of the included patients were retrieved, and a tissue microarray was constructed. The density and spatial distribution of TILs infiltration were analyzed using the multiplex fluorescent immunohistochemistry staining for CD3, CD4, CD8, Foxp3, cytokeratin (CK), PDL1, and 4′,6-diamidino-2-phenylindole (DAPI). The infiltration density of TILs subpopulations and PDL1 expression were compared between the two groups. The Gcross function was calculated to quantify the relative proximity of any two types of cells. The Cox proportional hazards regression model was used to identify factors associated with overall survival (OS) and disease-free survival (DFS).

Results: The densities of regulatory T-cells (Tregs), effector T-cells (Teffs), PDL1 + Tregs, and PDL1+ Teffs were significantly higher in patients with unfavorable outcomes. PDL1 expression on tumor cells (TCs) or overall TILs was not associated with survival. Multivariate analysis revealed that higher PDL1+ Tregs infiltration density was independently associated with inferior OS and DFS, whereas Tregs infiltration density was only a prognostic marker for DFS. Spatial analysis revealed that unfavorable group had significantly stronger Tregs and PDL1+ Tregs engagement in the proximity of TCs and cytotoxic T lymphocyte (CTLs). Gcross analysis further revealed that Tregs and PDL1+

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Tregs were more likely to colocalize with CTLs. Moreover, increased G_{TC} : $_{Treg}$ (Tregs engagement surrounding TCs) and G_{CTL} : $_{PDL1+\ Treg}$ were identified as independent factors correlated with poor outcomes.

Conclusion: TILs have a diverse infiltrating pattern and spatial distribution in NPC. Increased infiltration of Tregs, particularly PDL1+ Tregs, as well as their proximity to TCs and CTLs, correlates with unfavorable outcomes, implying the significance of intercellular immune regulation in mediating disease progression.

KEYWORDS

tumor infiltrating lymphocytes (TILs), programmed cell death ligand 1 (PDL1), nasopharyngeal carcinoma (NPC), cell spatial distribution, regulatory T cells (Tregs), cytotoxic T lymphocytes (CTLs), immune supression, proximity

Background

Nasopharyngeal carcinoma (NPC) is characterized by its close association with Epstein–Barr virus (EBV) infection, poor differentiation, and sensitivity to radiotherapy and chemotherapy (1, 2). Intensity-modulated radiotherapy (IMRT) and advanced chemotherapeutic regimens have provided excellent overall loco-regional management for NPC (3–5). However, distant metastasis and local recurrence continue to occur in approximately 30%–40% of patients, and the response rate to immune checkpoint inhibitors is only 20%–30% (6–11). Therefore, it is crucial to identify additional robust prognostic markers of NPC and guide treatment beyond the well-known staging system and EBV DNA load (12).

The tumor microenvironment (TME) is an intricately organized landscape occupied by infiltrating immune cells, epithelial cells, vascular and lymphatic vessels, cytokines, and chemokines (13). The tumor immune microenvironment (TIME) is critical in the development and progression of many solid tumors (14–19). TIME analysis reveals the diverse composition and functional states of immune cells (20). Tumor-infiltrating lymphocytes (TILs), being the most important component of TIME, play a vital role in mediating antitumor immunity in the TME. Previous studies have demonstrated that TILs have a prognostic impact on a variety of solid cancers (15–19). Nevertheless, tumor cells (TCs) can evade immune surveillance in a variety of ways, including upregulating immune checkpoint receptor ligands such as programmed death-ligand 1 (PDL1) (21). Furthermore, regulatory T-cells (Tregs) and other suppressive signals can enhance tumor progression by attenuating antitumor immunity (13–15, 20).

A few studies have been conducted over the last several decades to investigate the immunological landscape of NPC using hematoxylin and eosin (H&E) staining, immunohistochemical (IHC) staining, and flow cytometry. Recent studies have demonstrated that the immunological components such as CD8 + T-cell infiltration and PD1/PDL1 expression may have prognostic value, but the results are still controversial (22–24). Asides from TILs composition, a few recent studies have shown that the spatial architecture of the TIME may also play an essential role in mediating cancer progression (25, 26). Thus, investigating the TIME composition and spatial architecture of NPC samples may provide additional critical insights into the complex and heterogeneous immunological landscape associated with disease progression.

The present study provides a comprehensive analysis of the composition and abundance of TILs, as well as PDL1 expression in the TME using the multiplex fluorescent immunohistochemistry (mfIHC) approach, aiming to evaluate the prognostic role of TILs in NPC. Furthermore, the spatial architecture of TCs and TILs is studied using multispectral imaging analysis. This allows researchers to assess the role of TILs' intercellular proximity and distribution pattern in mediating disease progression, revealing a potential treatment-responsive biomarker for immune-modulatory therapy.

Abbreviations: NPC, nasopharyngeal carcinoma; PDL1, programmed cell death-ligand 1; PD1, programmed cell death protein 1; TILs, tumor infiltrating lymphocytes; OS, overall survival; DFS, disease free survival; CTLs, cytotoxic T lymphocytes; Teffs, effector T cells; Tregs, regulatory T cells; IC, immune cell; TC, tumor cell; IQR, interquartile range; HR, hazard ratio; CI, confidence interval; KPS, Karnofsky performance status.

Materials

Study population

In this study, patients with NPC who were staged as I–IVA according to the 8th American Joint Committee on Cancer

(AJCC) TNM staging system, had no concomitant immune system disease, received IMRT at our institution between March 2010 and July 2014, and had sufficient tumor sample collection prior to any anticancer treatment were included. Eligible patients were then divided into two groups with comparable clinicopathological characteristics but distinct posttreatment outcomes. For attaining a balance between the two groups, clinicopathological data such as age, sex, smoking history, histological classification, AJCC 8th TNM stage, Karnofsky performance status, lactate dehydrogenase level, hemoglobin level, platelet count, and treatment modality were considered. The 5-year disease progression rate was the main prognostic index in this study. Finally, 121 patients were included, with 68 in the favorable group surviving at least 5 years without disease progression (Group 1) and 53 in the unfavorable group having disease progression within 5 years (Group 2).

Samples for mfIHC stains

All fresh tumor samples were preserved at our institution at −80°C liquid nitrogen with signed written informed consent. Formalin-fixed, paraffin-embedded (FFPE) blocks were prepared using a standard method. All H&E-stained slides were reassessed independently by two pathologists, and they were blinded to clinical data. After reviewing H&E-stained slides, one 1.5 mm diameter tumor tissue core from representative sections of FFPE blocks was used to construct the tissue microarray (TMA) (Shanghai Outdo Biotech Co., Ltd).

Seven-color immunohistochemical multiplex

The Opal 7-color manual IHC kit 50 slides (Akoya, NEL811001KT) was used according to the manufacturer's protocol. Briefly, TMA block sections were deparaffinized in an automatic dyeing machine (Leica ST5020, Leica) and subjected to antigen retrieval by microwave treatment in Citrate buffer (pH=6.0). Sections were then incubated in 3% hydrogen peroxide in methanol for 30 min at room temperature and subsequently with a blocking solution containing 0.3% bovine serum albumin in 0.05% Tween solution for 30 min. Then, the sections were incubated with primary antibody for 60 min at room temperature and its corresponding HRP-conjugated secondary antibody for 10 min, followed by opal fluorophores for 10 min. The staining sequence of primary antibodies and corresponding fluorescence channels was anti-CD4, CD3, PDL1, CD8, Foxp3 and CK, with the corresponding opal fluorophores 520, 690, 570, 620, 540 and 650, respectively. After staining the above markers in turn, sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Life Tech) and mounted with VECTASHIELD fluorescence mounting medium (Vector Labs, Burlingame, CA).

TILs phenotyping

To study the infiltration composition of T-cell subpopulations in NPC and their potential correlation with posttreatment progression, the following markers were used: cytokeratin (CK), CD3, CD4, CD8, Foxp3, and 4′,6-diamidino-2-phenylindole (DAPI,nuclear stain). CK was used to identify epithelial cancer cells in NPC tumor samples. The detailed information on biomarkers and antibodies used is presented in Supplementary Table 1. T-cells were identified using the CD3 marker. Furthermore, T-cell subpopulations were identified according to the standard staining protocol as cytotoxic T lymphocytes (CTLs, CD3+CD8+), CD4+ effector T-cells (Teffs, CD3+CD4+Foxp3-), Tregs (CD3+CD4+Foxp3+), and other T-cells (CD3+CD4-CD8-Foxp3-) (27). Finally, all other cells that were not recruited in our phenotyping categories, such as normal nasopharyngeal epithelial cells, blood vessels, nerves, macrophages, and other nuclear cells, were grouped into one category and labeled as "others."

Multispectral imaging

Multiplex fluorescent-stained TMA slides were scanned using the image analysis software StrataQuest (TissueGnostics-StrataQuest 7.7.1.165 version) for multicellular contextual tissue analysis in both bright field and fluorescence images. The total counts of various cell phenotypes derived from all available cores were analyzed. Additionally, the core density was calculated by dividing the total number of cells by the area of each core (cells/mm^2). The spectral signature for each fluorophore was determined using single-antigen staining and captured using a multispectral fluorescent microscope, which records an image every 10 nm over the full-emission spectrum. This enabled the simultaneous capture of seven different fluorophores into a single composite image, which could then be unmixed and separated into six unique images representing each fluorophore and the nuclear stain DAPI, as well as the precise x and y spatial coordinates of each identified cell.

PDL1 evaluation

PDL1 immunostaining was observed in the membrane and/or cytoplasm of the TCs and lymphocytes. At the cellular level, PDL1 expression was measured as the percentage of tumor or immune cells with positive staining (range: 0%–100%). At the patient level, specific phenotypes of cells with a PDL1 staining

score of at least 5% were considered positive PDL1 expression (22). Since the evaluation and cutoff values for TILs are not standardized, the immune cell count in our study was based on a predetermined threshold of fluorescence intensity, which was identified by the mean value of fluorescence intensity of stained cells manually counted at a magnification (200×) in 10 random views. The median density of immune cells was chosen to divide the patient cohort into high and low expression groups (28). All staining was assessed by two independent pathologists who were blinded to the clinicopathologic data.

Spatial distribution

The spatial distribution of TILs surrounding TCs was first analyzed in various tissue compartments (inner tumor vs. stroma). Next, the stroma area within 200 μm of the tumor edges was divided into 10 intervals, and the infiltration density of each type of TILs within each interval was quantitatively estimated. Furthermore, the Gcross function (G*ij*(r)) was calculated to estimate the distribution probability of finding at least one specified point "*j*" within a given radius "*r*" (μm) of any specified point "*i*," allowing quantification of the relative proximity of any two cell types (29). Therefore, the Gcross function value becomes a quantitative index of TILs infiltration when the "*i*" is applied as a TC and "*j*" as TILs, with greater $G_{TC:TIL}$ values indicating a higher TILs infiltration density near TCs. Additionally, the area under the curve (AUC) of the Gcross curve was calculated to represent the accumulated infiltration level of cell type "*j*" within a given distance from cell type "*i*." Accordingly, larger AUCs indicate higher immune cell interaction around TCs. Typical Gcross function curves indicating high (left), intermediate (middle), and low (right) levels of infiltration are illustrated in Supplementary Figure 1.

Statistical analysis

The continuous variables, such as percentage, density, and Gcross value, were presented as median and interquartile range (IQR) and compared between two groups using the Mann–Whitney U-test. The categorical data were compared using the Chi-squared test. Disease-free survival (DFS) was defined as the time between the first date of diagnosis and disease progression or death, whereas overall survival (OS) was defined as the time between the first day of treatment and death from any cause or the last follow-up. The survival index was estimated using the Kaplan–Meier method, and the significance of the difference was assessed using the log-rank test. The Cox proportional hazards regression model was used to identify factors related to survival variables and to calculate the hazard ratio and corresponding confidence interval. To further evaluate the prognostic significance of TME, the densities and spatial architectures of TIL phenotypes were respectively assessed in multivariable Cox regression models that initially included age, sex, smoking history, histological type, N stage, T stage, and TNM stage. All statistical tests were two-sided, and a *p*-value of 0.05 or less was considered statistically significant. All statistical analyses were conducted by using the GraphPad Prism 8.0 software (GraphPad Software Inc.), the Statistical Package for the Social Sciences (SPSS) 22.0 software (IBM Inc.), and the R 3.6.3 software (R Foundation for Statistical Computing).

Results

Patients ' clinicopathological characteristics

In the final analysis, 121 patients with NPC were included and classified into two groups: favorable (Group 1, n = 68) and unfavorable (Group 2, n = 53). The overall population had a median follow-up time of 78.0 months (IQR: 57.5–93.2 months). The 5-year OS and DFS rates in the favorable and unfavorable groups were 100% vs. 45.3% ($p < 0.001$) and 100% vs. 0% ($p < 0.001$), respectively. Table 1 demonstrates the clinicopathological characteristics of all studied patients. The median age in the favorable and unfavorable groups was 48 and 47 years, respectively, with males accounting for the majority of patients in both groups. Nearly 90% of patients had stage III to IVA diseases and received concurrent chemoradiotherapy. The pathological classification of all patients was nonkeratinizing undifferentiated subtype. Furthermore, the clinicopathological characteristics were comparable between the two groups.

TILs subpopulation heterogeneity and disease progression

TILs subpopulations and PDL1 expression on TCs and TILs were assessed for each core using mfIHC staining. Figure 1 shows a representative immunofluorescence image. When the TILs compositions of the two groups were compared, patients in Group 2 had a significantly higher proportion of Tregs than those in Group 1 (0.8% vs. 0.3%, respectively, $p = 0.023$), whereas there was no significant difference in the proportions of TCs, total TILs, Teffs, and CTLs (Figures 2A–D).

Aside from cell proportion, cell density (calculated by dividing cell counts by area) can reflect cell distribution to some extent. The median densities of the total TILs, CTLs, Teffs, and Tregs were 2260.1 (IQR: 1721.1–3213.1), 582.1 (IQR: 242.7–1225.1), 140.1 (IQR: 69.8–318.6), and 58.2 (IQR: 19.0–142.0) cells/mm^2, respectively. There were no significant differences in total TILs or CTLs density between the two groups (Figures 2E, F), but the median Tregs density (103.6 vs. 34.0 cells/mm^2, $p = 0.002$) and median Teffs density (184.4 vs.

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TABLE 1 General characteristics of patients in two groups.

Characteristics	Group 1 (n=68)	Group 2 (n=53)	*P* value
	n (%)	n (%)	
Age (years)			0.964
Median(range)	48 (18-76)	47 (23-74)	
Sex			0.667
Male	53 (77.9)	43 (81.1)	
Female	15 (22.1)	10 (18.9)	
Smoking history			0.845
No	29 (43.3)	22 (41.5)	
Yes	38 (56.7)	31 (58.5)	
Histological type			0.900
WHO II	29 (42.6)	22 (41.5)	
WHO III	39 (57.4)	31 (58.5)	
T stage			0.900
T1	11 (16.2)	10 (18.9)	
T2	14 (20.6)	9 (17.0)	
T3	22 (32.4)	20 (37.7)	
T4	21 (30.9)	14 (26.4)	
N stage			0.082
N0	7 (10.3)	2 (3.8)	
N1	18 (26.5)	9 (17.0)	
N2	21 (30.9)	28 (52.8)	
N3	22 (32.4)	14 (26.4)	
Overall stage			0.653
I	2 (2.9)	1 (1.9)	
II	6 (8.8)	2 (3.8)	
III	23 (33.8)	22 (41.5)	
IVA	37 (54.4)	28 (52.8)	
KPS			1.000
<80	3 (4.4)	2 (3.8)	
≥80	65 (95.6)	51 (96.2)	
LDH(U/L)			0.085
<245	67 (98.5)	48 (90.6)	
≥245	1 (1.5)	5 (9.4)	
HB (g/L)			0.327
<130	9 (13.2)	11 (20.8)	
≥130	59 (86.8)	42 (79.2)	
PLT ($\times 10^9$/L)			0.432
<300	59 (88.1)	44 (83.0)	
≥300	8 (11.9)	9 (17.0)	
Treatment pattern			0.825
CCRT	44 (64.7)	33 (62.2)	
IC+CCRT	10 (14.7)	10 (18.9)	
RT/IC+RT/CCRT +AC	14 (20.6)	10 (18.9)	

WHO II, non-keratinizing differentiated carcinoma; WHO III, non-keratinizing undifferentiated carcinoma; KPS, karnofsky performance status; LDH, lactate dehydrogenase; HB, haemoglobin; PLT, platelet; CCRT, concurrent chemoradiotherapy; IC, induction chemotherapy; RT, radiation therapy; AC, adjuvant chemotherapy.

113.3 cells/mm^2, $p = 0.023$) were significantly higher in patients with an unfavorable outcome than in those with a favorable outcome, respectively (Figures 2G, H). The detailed densities of each subtype are presented in Supplementary Table 2. When the median value was used as a cutoff, no significant associations were found between the infiltration densities of any of the TILs subpopulations and clinicopathological characteristics (Supplementary Table 3), implying that TILs have a prognostic impact that is independent of clinicopathological features.

Furthermore, PDL1 expression was assessed in various cell subtypes. The median percentages of PDL1 positive TCs (PDL1+ TCs) and PDL1 positive TILs (PDL1+ TILs) in the favorable and unfavorable groups were 20.3% vs. 18.6% ($p = 0.775$) and 71.1% vs. 60.0% ($p = 0.166$), respectively. In the overall population, the median densities of PDL1+ TCs and PDL1+ TILs were 3113.8 (IQR: 1043.4–5461.6) and 1037.4 (IQR: 711.5–1709.1) cells/mm^2, respectively. In terms of PDL1 expression on TILs subpopulations, densities of PDL1+ Tregs (56.1 vs. 17.7 cells/mm^2, $p = 0.001$) and PDL1+ Teffs (112.7 vs. 58.0 cells/mm^2, $p = 0.011$) were significantly higher in patients with unfavorable outcomes than in those with favorable ones, respectively, with no significant differences in densities of PDL1+ TCs, PDL1+ TILs, or PDL1+ CTLs between the two groups (Figures 2I–L).

When the median value was used as a cutoff, no significant association was found between the infiltration densities of PDL1 + TCs, PDL1+ TILs, or any PDL1+ TILs subpopulations and the clinicopathological characteristics (Supplementary Table 4), implying that the higher infiltration density of PDL1+ Tregs and PDL1+ Teffs in the unfavorable group was independent of other prognostic factors for NPC.

Tregs spatial distribution and disease progression

The spatial distribution pattern of TILs subpopulations was analyzed to further investigate the impact of Tregs and PDL1+ Tregs on disease progression. Despite the similarity in the total TILs infiltration between the inner and stroma areas in the overall population, the stroma area had significantly higher infiltrations of CTLs ($p = 0.001$), Teffs ($p < 0.001$), Tregs ($p = 0.04$), and PDL1+ Tregs ($p < 0.001$) (Figure 3A). There was no significant difference between the two groups in terms of total TILs, CTLs, Teffs, Tregs, or PDL1+ Tregs engagement within the inner tumor area (Figure 3B). Patients in the unfavorable group had greater infiltration of Tregs ($p = 0.008$) and PDL1+ Tregs ($p = 0.015$) within the stroma area (Figure 3C). Detailed infiltration densities of TIL subpopulations in the inner and stromal areas are presented in Supplementary Table 5. The Gcross function was used as a more precise descriptive

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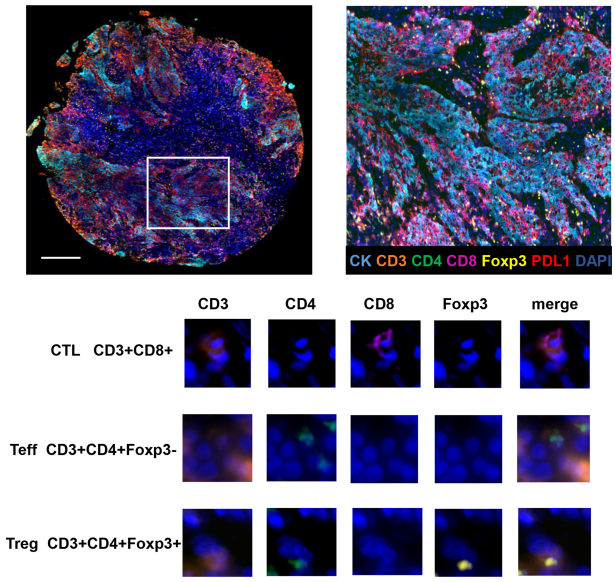

FIGURE 1
Opal seven-color multiplex analysis of NPC tumor tissue identifies specific TILs subtypes. Representative image of multiplex fluorescence staining and the enlarged subsection are displayed on the top panel. In the lower panel, images for unmixed single marker of CD3, CD4, CD8 and Foxp3 are presented in the left four columns. The right column demonstrates merged fluorescence image of various combination of four markers, resulting in the identification of typical TILs phenotypes such as CTLs, Teffs and Tregs.

method to better investigate spatial intercellular interactions. Figure 3D depicts a schematic model for various scenarios of infiltration with the same number of immune cells located within a 20 μm radius of the TC. Despite the same infiltration density, the Gcross function can better reflect the distinct engagement level of immune cells. Within a 100 μm radius, the AUCs of the Gcross functions reflecting various cellular interactions were compared between the two groups, with patients with poor outcomes having significantly higher Gcross AUCs of the TC : Tregs (Figure 3E). Figures 3F–H show Gcross function values at specific radii of 20, 30, and 50 μm. Similarly, patients with poor outcomes had significantly higher $G_{TC:\ Treg}$ at radii of 30 and 50 μm, as well as higher $G_{TC:\ PDL1+\ Treg}$ at the radius of 50 μm. Detailed Gcross function values for each radius for the two groups are presented in Supplementary Table 6. The spatial distributions of Tregs and PDL1+ Tregs surrounding CTLs were also investigated, in addition to intercellular distances between TILs and TCs. The Gcross AUCs of CTL : Treg and CTL : PDL1+ Treg were significantly higher in the unfavorable group, implying that Tregs and PDL1+ Tregs are strongly engaged in the proximity of CTLs. Consistent with the AUC analysis, both $G_{CTL:\ Treg}$ and $G_{CTL:\ PDL1+\ Treg}$ at the specific radius of 20, 30, and 50 μm were significantly higher in patients with disease progression, indicating the potential role of intercellular interaction between Tregs, PDL1+ Tregs, and CTLs in mediating tumor progression. It should be noted that both Tregs and PDL1+ Tregs had much higher infiltration probabilities near CTLs than TCs (Supplementary Table 7).

Univariate and multivariate analyses for OS and DFS

In the overall study population, Table 2 presents univariate and multivariate analyses of density and Gcross function score of TILs subpopulations for OS and DFS. Patients with higher densities of Tregs, PDL1+ Teffs, and PDL1+ Tregs had significantly lower DFS in univariate analysis. Patients with more abundant PDL1+ Tregs infiltration had a lower OS, with a p-value approaching statistical significance. Further multivariate analysis revealed that higher infiltrations of Tregs, Teffs, PDL1+ Teffs, and PDL1+ Tregs were significantly associated with lower DFS, whereas only abundant PDL1+ Tregs infiltration may be associated with lower OS trending toward significance.

Furthermore, the impact of Tregs spatial architecture on disease progression was investigated. Univariate analysis of the Gcross function revealed that the TC : Treg, TC : PDL1+ Treg, CTL : Treg, and CTL : PDL1+ Treg colocalizations were all associated with worse DFS to varying degrees. Further

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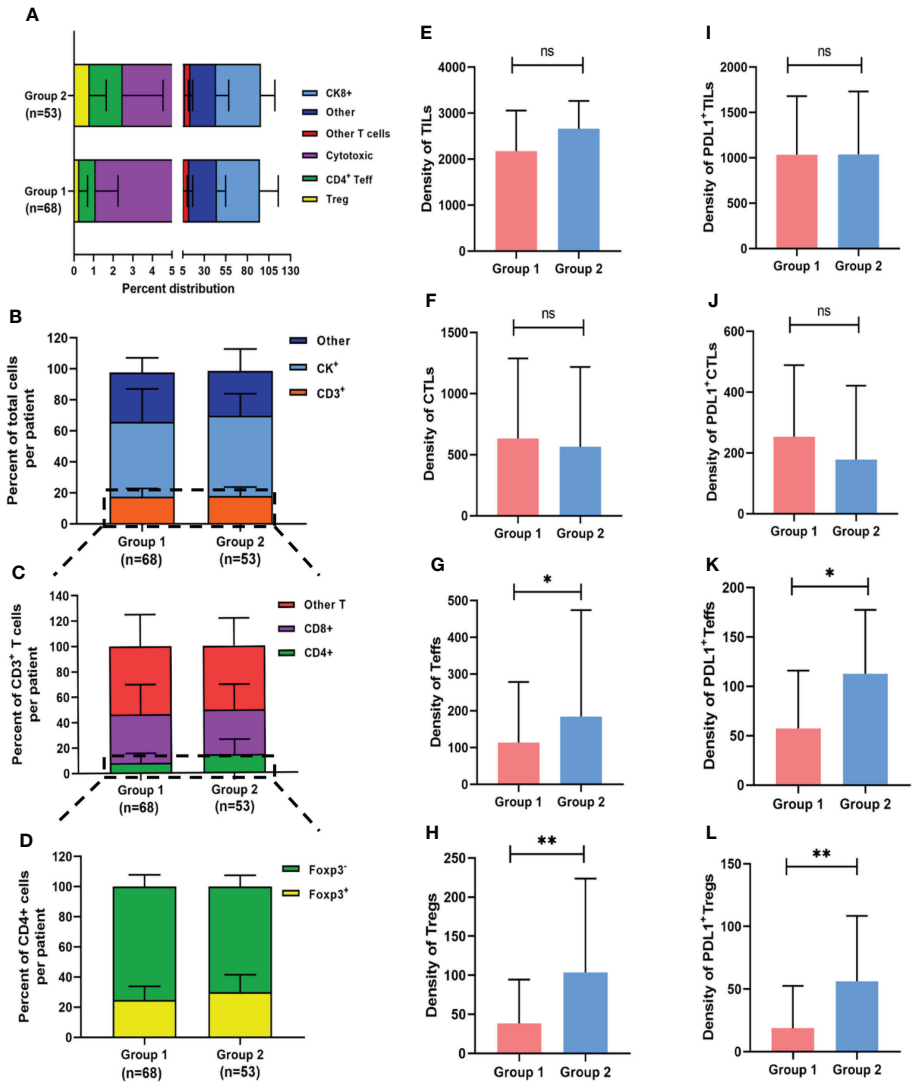

FIGURE 2
Composition of heterogeneous infiltrating immune cell subpopulations in Group 1 and Group 2. **(A)** Relative distribution of all analyzed cell phenotypes in NPC sample tissues. **(B-D)** Relative distribution analysis of different T cell subtypes between two groups, firstly by separating the total cell number into other, CK+ cells or CD3+ T cells (including all T cell subtypes) **(B)**; then focusing on CD3+ T cells and dividing them into CD4+ T (CD3+CD4+), CD8+ (CD3+CD8+) and other (CD3+CD4-CD8-) cells **(C)**; and finally focusing on CD3+CD4+ T cells and dividing them into Foxp3+ and Foxp3- T cells **(D)**. **(E-H)** Pairwise comparisons of the density of TIL subpopulations between the two groups for TILs **(E)**, CTLs **(F)**, Teffs **(G)** and Tregs **(H)**. **(I-L)** Pairwise comparisons of PDL1 positive TIL subpopulations between the two groups. *$p < 0.05$, **$p < 0.01$, ns, not significant.

multivariate analysis confirmed that higher G_{TC} : $_{Treg}$, G_{TC} : $_{PDL1+ Treg}$, G_{CTL} : $_{Treg}$, and G_{CTL} : $_{PDL1+ Treg}$ all had independently negative effects on DFS. Table 2 and Figures 4A, B present detailed data on the univariable and multivariable analyses for DFS.

Although TC : Treg, TC : PDL1+ Treg, CT : Treg, and CTL : PDL1+ Treg colocalization within a certain radius had a significant correlation with OS, multivariable analysis revealed that only G_{TC} : $_{Treg}$ and G_{CTL} : $_{PDL1+ Treg}$ were independently correlated with lower OS. Table 2 and Figures 4C, D present detailed data on univariate and multivariate Cox regression analyses for OS.

To better investigate the prognostic role of the aforementioned elements, Figure 5 depicts the Kaplan–Meier survival curves for OS and DFS between subgroups with high vs. low density of Tregs, PDL1+ Tregs, and the high vs. low Gcross functions of TC : Treg and CTL : PDL1+ Treg. The survival curves for OS and DFS between subgroups with high and low infiltration, as well as other TIL colocalization, are shown in Supplementary Figure 2.

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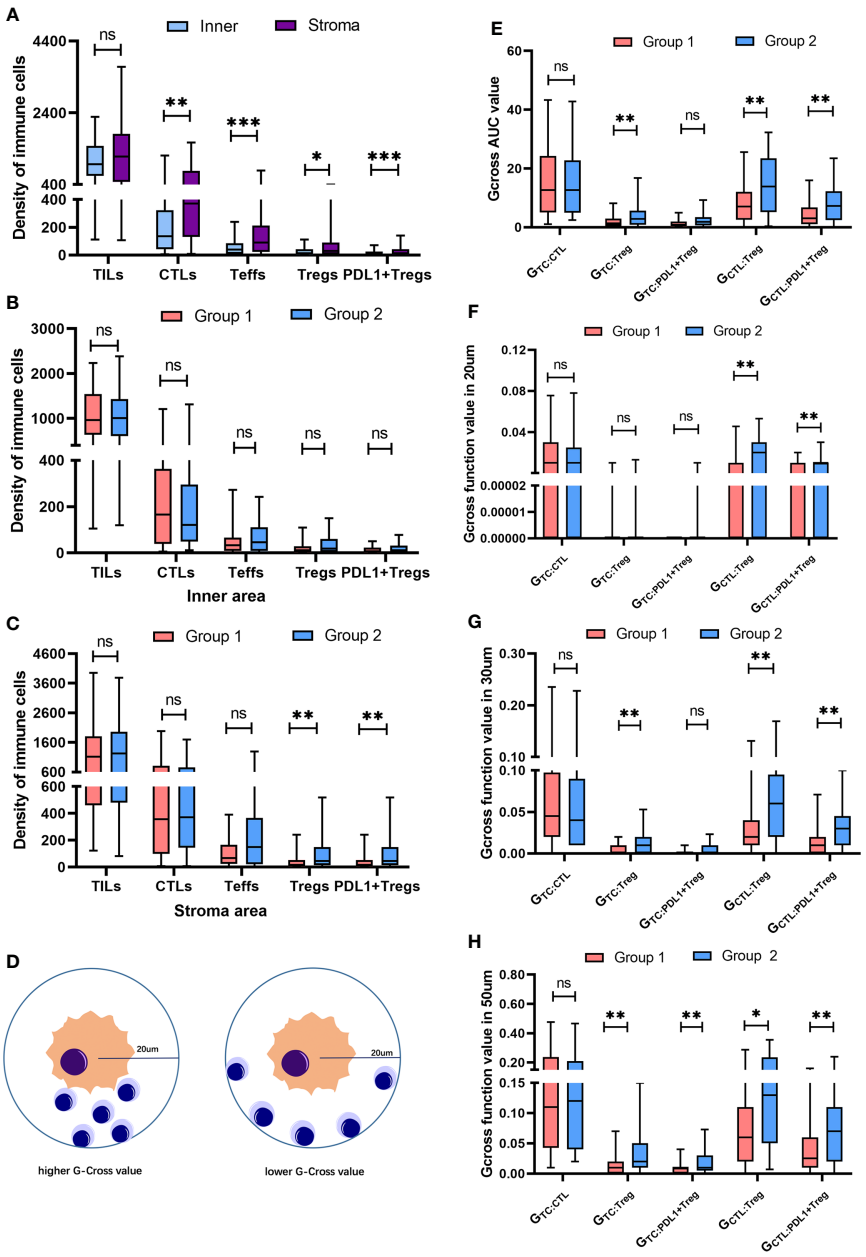

FIGURE 3
Spatial distribution of heterogeneous infiltrating immune cell subpopulations. **(A)** Pairwise comparison of the infiltration differences for TILs, CTLs, Teffs, Tregs and PDL1+ Tregs in the whole population between inner and stroma area. **(B)** Pairwise comparison of the infiltration differences for TILs, CTLs, Teffs, Tregs and PDL1+ Tregs within inner area between two groups. **(C)** Pairwise comparison of the infiltration differences for TILs, CTLs, Teffs, Tregs and PDL1+ Tregs in stroma area between two groups. **(D)** Schematic model for different scenarios of infiltration with the same number of immune cells locating within a 20 um radius of the tumor cell, reflecting distinct engagement level of immune cells. **(E)** Pairwise comparisons of the Gcross-AUC values for $G_{TC:CTL}$, $G_{TC:Treg}$, $G_{TC:Teff}$, $G_{TC:PDL1+Treg}$, $G_{CTL:Treg}$ and $G_{CTL:PDL1+Treg}$ between two groups. **(F-H)** Pairwise comparisons of the G-cross values at 20 um **(F)**, 30 um **(G)** and 50 um **(H)** radii for $G_{TC:CTL}$, $G_{TC:Treg}$, $G_{TC:Teff}$, $G_{TC:PDL1+Treg}$, $G_{CTL:Treg}$ and $G_{CTL:PDL1+Treg}$ between two groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns, not significant.

Discussion

In this study, we comprehensively analyzed the composition and spatial distribution of TILs and PDL1 expression in NPC using mfIHC and multispectral imaging analysis. Tregs and PDL1+ Tregs compositionally higher density and spatial closeness to TCs were significantly associated with worse outcomes. Furthermore, increased Tregs engagement, particularly PDL1+ Tregs surrounding CTLs, was highly associated with poor outcomes. Overall, our findings

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TABLE 2 Univariate and multivariate analysis for OS and DFS according to densities and Gcross functions of TILs subpopulations.

Variables	Median IQR (cells/mm^2)	Disease-free survival		Overall survival	
		Univariate HR (95% CI)	Multivariate HR (95% CI)[a]	Univariate HR (95% CI)	Multivariate HR (95% CI)[a]
Density					
TILs	2260.1 (1721.2-3213.1)				
High vs Low		1.13 (0.66-1.94)	1.27 (0.73-2.22)	1.12 (0.56-2.22)	1.19 (0.58-2.42)
p value		0.647	0.402	0.747	0.636
CTLs	582.1 (242.7-1225.1)				
High vs Low		0.84 (0.49-1.44)	0.75 (0.42-1.32)	0.77 (0.38-1.53)	0.69 (0.34-1.42)
p value		0.539	0.317	0.45	0.316
Teffs	140.1 (69.8-318.6)				
High vs Low		1.64 (0.94-2.84)	1.83 (1.03-3.23)	1.47 (0.73-2.95)	1.38 (0.67-2.82)
p value		0.078	0.039	0.279	0.382
Tregs	58.2 (19.0-142.0)				
High vs Low		1.79 (1.03-3.12)	1.94 (1.09-3.42)	1.71 (0.85-3.46)	1.71 (-.83-3.51)
p value		0.040	0.023	0.136	0.146
PDL1+TILs	1037.4 (711.5-1709.1)				
High vs Low		0.90 (0.53-1.55)	0.934 (0.54-1.62)	1.01 (0.54-2.13)	1.23 (0.61-2.46)
p value		0.701	0.807	0.836	0.563
PDL1+TCs	3113.8 (1043.4-5461.6)				
High vs Low		0.78 (0.46-1.34)	0.76 (0.42-1.35)	1.39 (0.70-2.77)	0.72 (0.35-1.47)
p value		0.371	0.351	0.342	0.369
PDL1+CTLs	223.7 (76.0-466.6)				
High vs Low		0.74 (0.43-1.27)	0.68 (0.38-1.20)	0.54 (0.26-1.09)	0.55 (0.26-1.13)
p value		0.274	0.180	0.085	0.105
PDL1+Teffs	69.7 (33.5-148.3)				
High vs Low		1.87 (1.07-3.27)	2.07 (1.18-3.65)	1.27 (0.64-2.52)	1.40 (0.69-2.83)
p value		0.027	0.012	0.497	0.348
PDL1+Tregs	32.2 (11.5-63.7)				
High vs Low		2.34 (1.32-4.14)	2.54 (1.42-4.56)	2.00 (0.98-4.08)	2.01 (0.98-4.15)
p value		0.003	0.002	0.055	0.058
Gcross function value[b]					
$G_{TC:Treg}$	1.80 (0.55-4.35)				
High vs Low		2.35 (1.33-4.15)	2.43 (1.34-4.42)	2.27 (1.10-4.69)	2.20 (1.04-4.63)
p value		0.003	0.004	0.026	0.039
$G_{TC:PDL1+Treg}$	1.07 (0.40-2.61)				
High vs Low		1.61(0.93-2.79)	1.68 (0.96-2.95)	1.62 (0.81-3.26)	1.57 (0.77-3.18)
p value		0.090	0.072	0.176	0.215
$G_{CTL:Treg}$	9.19 (3.49-17.12)				
High vs Low		2.01 (1.14-3.52)	2.16 (1.21-3.87)	1.96 (0.96-4.00)	1.78 (0.86-3.68)
p value		0.015	0.009	0.066	0.121
$G_{CTL:PDL1+Treg}$	4.21 (1.38-9.41)				
High vs Low		2.08 (1.18-3.65)	2.15 (1.21-3.80)	2.03 (0.99-4.15)	2.08 (1.00-4.29)
p value		0.011	0.009	0.053	0.049
20 um radius					
$G_{TC:Treg}$	0.0008 (0.0002-0.0031)				
High vs Low		1.67 (0.96-2.89)	1.75 (0.98-3.12)	1.42 (0.71-2.83)	1.44 (0.70-2.95)
p value		0.069	0.060	0.32	0.326

(Continued)

TABLE 2 Continued

Variables	Median IQR (cells/mm^2)	Disease-free survival		Overall survival	
		Univariate HR (95% CI)	Multivariate HR (95% CI)[a]	Univariate HR (95% CI)	Multivariate HR (95% CI)[a]
$G_{TC:PDL1+Treg}$	0.0006 (0.0000-0.0017)				
High vs Low		1.21 (0.71-2.07)	1.23 (0.70-2.15)	1.09 (0.55-2.15)	1.04 (0.51-2.10)
p value		0.493	0.467	0.808	0.918
$G_{CTL:Treg}$	0.0089 (0.0014-0.0212)				
High vs Low		2.29 (1.29-4.04)	2.39 (1.33-4.29)	2.31 (1.11-4.80)	2.06 (0.99-4.30)
p value		0.004	0.003	0.025	0.053
$G_{CTL:PDL1+Treg}$	0.0034 (0.000-0.0104)				
High vs Low		2.01 (1.15-3.53)	2.14 (1.21-3.80)	1.70 (0.84-3.44)	1.79 (0.88-3.64)
p value		0.011	0.009	0.139	0.110
30 um radius					
$G_{TC:Treg}$	0.0037 (0.001-0.0111)				
High vs Low		2.17 (1.24-3.81)	2.25 (1.24-4.09)	1.98 (0.98-4.04)	2.01 (0.97-4.19)
p value		0.007	0.008	0.058	0.062
$G_{TC:PDL1+Treg}$	0.0026 (0.0006-0.0067)				
High vs Low		1.64 (0.94-2.84)	1.72 (0.97-3.04)	1.63 (0.81-3.28)	1.62 (0.79-3.22)
p value		0.080	0.065	0.170	0.183
$G_{CTL:Treg}$	0.0326 (0.0088-0.0670)				
High vs Low		2.26 (1.28-3.40)	2.37 (1.31-4.30)	2.29 (1.20-4.75)	1.91 (0.91-4.01)
p value		0.005	0.004	0.027	0.087
$G_{CTL:PDL1+Treg}$	0.0161 (0.0028-0.0319)				
High vs Low		2.20 (1.25-3.87)	2.43 (1.37-4.30)	2.14 (1.04-4.37)	2.20 (1.07-4.54)
p value		0.006	0.002	0.038	0.032
50 um radius					
$G_{TC:Treg}$	0.0127 (0.0036-0.0342)				
High vs Low		2.42 (1.37-4.28)	2.38 (1.32-4.30)	2.33 (1.13-4.80)	2.19 (1.05-4.58)
p value		0.002	0.004	0.022	0.037
$G_{TC:PDL1+Treg}$	0.0071 (0.0027-0.0201)				
High vs Low		1.99 (1.12-3.49)	2.00(1.12-3.56)	2.19 (1.06-4.51)	2.07 (0.99-4.32)
p value		0.017	0.019	0.034	0.054
$G_{CTL:Treg}$	0.0842 (0.0265-0.1597)				
High vs Low		1.78 (1.02-3.10)	1.91 (1.07-3.41)	1.70 (0.84-3.44)	1.56 (0.76-3.21)
p value		0.042	0.028	0.139	0.230
$G_{CTL:PDL1+Treg}$	0.0384 (0.0110-0.0855)				
High vs Low		2.34 (1.32-4.14)	2.45 (1.38-4.38)	2.37 (1.14-4.91)	2.35 (1.12-4.92)
p value		0.003	0.003	0.021	0.023

[a],Cox proportional hazards regression model adjusted for age, sex, N stage, T stage, TNM stage, smoking history, histological type.
[b],Gcross function values measured as the probability of finding at least one Treg/PDL1+Tregs within a given radius from a tumor cell or CTLs.
CI, confidence interval; HR, hazard ratio; IQR, interquartile range.
TILs, tumor infiltrating lymphocytes; CTLs, cytotoxic T lymphocytes; Teffs, positive effector T cells; Tregs, regulatory T cells; PDL1+ TILs, PDL1 positive tumor infiltrating lymphocytes; PDL1 +TCs, PDL1 positive tumor cells; PDL1+CTLs, PDL1 positive cytotoxic T lymphocytes; PDL1+Teffs, PDL1 positive effector T cells; PDL1+Tregs, PDL1 positive regulatory T cells.

demonstrate that a suppressive immune microenvironment had a propelling effect on NPC progression regardless of potential clinical confounders, with crucial implications for prognosis prediction and immune-modulatory therapy.

TILs are well known for their vital role in mediating antitumor immune responses. Cellular factors including myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages(TAMs), CD8+ T cells and regulatory T cells (Tregs) in TME also may impact the prognosis of sloid tumors (30). Therefore, a thorough understanding of the diversity and complexity of TME emerges as a crucial approach to identifying more valid biomarkers for failure prediction and therapeutic targets. Previous studies on the prognostic significance of TILs in various tumors, including NPC, have yielded conflicting results.

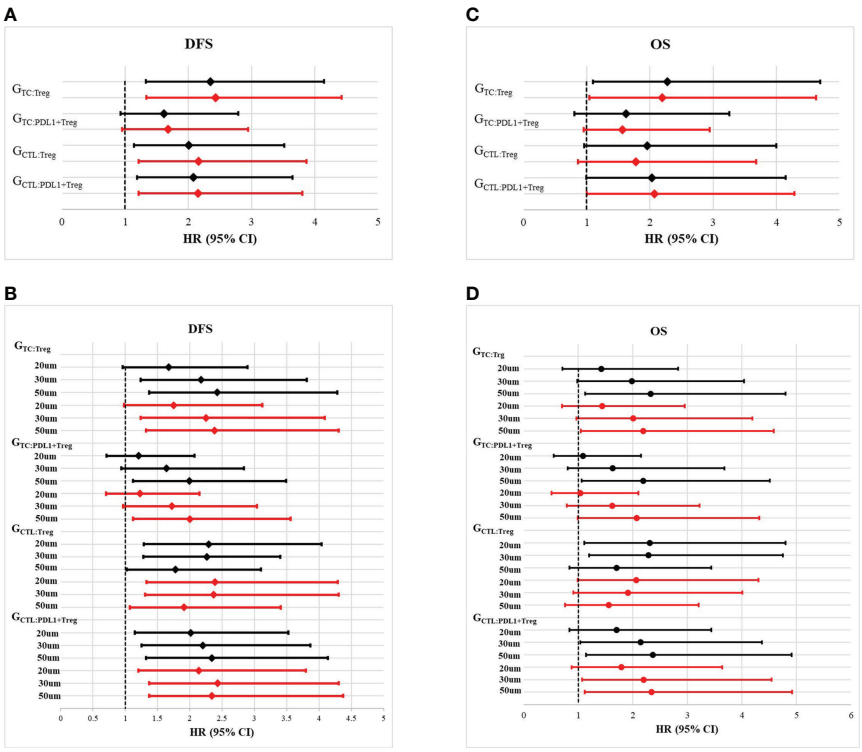

FIGURE 4
Forest plots of multivariate analysis and univariate analysis of Gcross function score in the whole cohort. **(A)** Hazard ratio of univariate (black solid line) and multivariate (red solid line) analysis of G_{TC} : $_{Treg}$, G_{TC} : $_{PDL1+Treg}$, G_{CTL} : $_{Treg}$ and G_{CTL} : $_{PDL1+Treg}$ for disease free survival (DFS). **(B)** Hazard ratio of univariate (black solid line) and multivariate (red solid line) analysis of G_{TC} : $_{Treg}$, G_{TC} : $_{PDL1+Treg}$, G_{CTL} : $_{Treg}$ and G_{CTL} : $_{PDL1+Treg}$ within certain raidus (20um, 30um and 50um) for disease free survival (DFS). **(C)** Hazard ratio of univariate (black solid line) and multivariate (red solid line) analysis of G_{TC} : $_{Treg}$, G_{TC} : $_{PDL1+Treg}$, G_{CTL} : $_{Treg}$ and G_{CTL} : $_{PDL1+Treg}$ for overall survival (OS). **(D)** Hazard ratio of univariate (black solid line) and multivariate (red solid line) analysis of G_{TC} : $_{Treg}$, G_{TC} : $_{PDL1+Treg}$, G_{CTL} : $_{Treg}$ and G_{CTL} : $_{PDL1+Treg}$ within certain raidus (20um, 30um and 50um) for overall survival (OS).

Wang et al. (19) and Almangush et al. (23) used H&E-stained slides to assess the prognostic value of TILs in endemic and nonendemic areas of NPC, respectively. Both studies found that overall TILs were significantly associated with survival, whereas TILs subtypes were not further evaluated. Ooft et al. (24), Al-Rajhi et al. (31), and Zhu et al. (32) found that increasing intratumoral CD3+ TILs infiltration was associated with superior OS and DFS without further investigation of subphenotypes. Ono et al. investigated TILs subpopulations and found that higher CTLs density was a significant factor in favorable prognosis (33). However, in our study, neither the abundance nor the density of TILs or CTLs was found to be associated with clinical outcomes. In agreement with our finding, Larbcharoensub et al. found that CTLs abundance was not associated with a significant difference in clinical survival (34). These inconsistencies suggest the presence of significant heterogeneity in TME and the need for further investigation of TME's impact on the antitumor immune response.

Tregs play crucial roles in suppressing antitumor immunity in TME by expressing ligands for inhibitory checkpoint receptors and secreting suppressive cytokines, promoting the occurrence and development of tumors (35, 36). Therefore, it is not surprising that Tregs are often associated with a poor prognosis in cancer. Although the high density of Foxp3 positive TILs was consistently associated with poor survival in patients with operable tongue cancer (37), breast cancer (38), hepatocellular cancer (39), ovarian cancer (40), and esophageal cancer (41), it was also reported to be associated with favorable outcomes in patients with head and neck squamous cell cancer (42), colorectal cancer (43), and SCLC (44). Such controversial findings have also been reported in patients with NPC. Ooft et al. found that a high Foxp3 count was an independent predictor of better OS (45). In our study, patients with a higher infiltration of Tregs had a significantly inferior OS and DFS, which was consistent with Lu's study findings (46). Lab work conducted by Huo et al. demonstrated that EBV-EBNA1 enhanced the chemotactic migration of Treg cells through the TGFβ1-SMAD3-PI3K-AKT-c-JUN-miR-200a-CXCL12-CXCR4 axis in NPC microenvironment, thereby promoting NPC immune escape (47). Alternatively, Tregs can secrete immunosuppressive cytokines including TGF-β, IL-10, and IL-35, and subsequently suppress

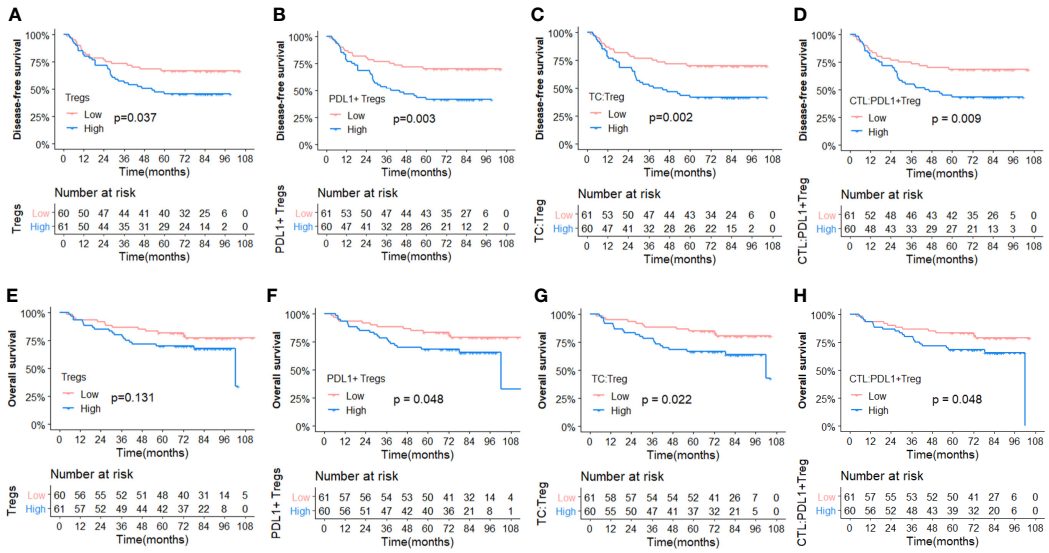

FIGURE 5
Comparisons of Kaplan-Meier survival curves between high and low infiltration density of TILs and Gcross function scores. (Upper panel) Disease free survival curves for Tregs **(A)**, PDL1+ Tregs **(B)**, $G_{TC:Treg}$ **(C)** and $G_{CTL:PDL1+Treg}$ **(D)**. (Lower panel) Overall survival curves for Tregs **(E)**, PDL1+ Tregs **(F)**, $G_{TC:Treg}$ **(G)** and $G_{CTL:PDL1+Treg}$ **(H)**.

cytotoxic effect of CD8 positive CTL and effector T cells (Teff) (48, 49). Therefore, overcoming the suppressive signal of Tregs may be critical to restoring exhausted CTLs function and enhancing patient responsiveness to immune-modulatory therapy.

The PD1/PDL1 axis is a well-known immune checkpoint that attenuates T-cells' antitumor immune response and mediates immunological escape (50). Despite the fact that a considerable number of studies assessed the prognostic value of PDL1 expression in NPC, the results were inconsistent among studies (23, 24, 32, 33, 51–55). Zhang et al. and Li et al. reported that high PDL1 expression on TCs was significantly associated with poor DFS or OS (55) (56). However, Zhu et al. found that positive PDL1 expression on TCs is a favorable prognostic factor in patients with NPC (32). Conversely, Liu et al. found that high PDL1 expression on TILs and TCs was highly associated with decreased local recurrence in patients with NPC after radiotherapy (54). Similarly, Ono et al. demonstrated that patients with higher PDL1 expression on TILs had longer progression-free survival and OS (33). However, another two previous studies found no association between PDL1 expression on TILs and survival outcomes (34, 51). Likewise, neither PDL1+ TCs nor PDL1+ TILs densities were found to be associated with survival outcomes in patients with NPC in our present study.

Aside from PDL1 expression on TCs and overall TILs, our study used the mfIHC method to conduct a more extensive and meticulous investigation into the prognostic significance of PDL1 expression on TILs subphenotypes. The combination of mfIHC, high-quality image acquisition, and multispectral imaging analysis, as advanced technology, allows for simultaneous multimarker labeling as well as cellular proximity analysis in a single core of tissue, providing a novel insight into TME research. One notable finding in the present study was that patients with more abundant PDL1+ Treg infiltration had the worst survival. A similar scenario has been reported in other solid tumors. DiDomenico et al. found that PDL1 was important in the expansion and maintenance of Tregs immunosuppression activity in glioma (57). Furthermore, Wu et al. found that the frequency of PD-L1^{hi} Tregs was positively correlated with PD-1-positive CD8 in the tumor stroma of non-small cell lung cancer (58). Additionally, Wu et al. found that PD-1^{hi} CD8 with PD-L1^{hi} Tregs group had the lowest proportion of tumor necrosis factor-alpha- and interferon-gamma-producing CTLs while achieving the best response to PD-1 blockade immunotherapy. Based on the present research, it is plausible to speculate that PDL1 inhibitors may aid in the recovery of CTL tumor-killing capacity by attenuating PDL1+ Treg suppression, thereby introducing another appealing mechanism of PD1/PDL1 axis blockade. De et al. reported that tumor infiltrating Tregs can express surface specific molecules such as PD-L1 and PD-L2 in order to bind their receptors on the surface of CD8+ T cells, inhibiting CD8+ T-cell activation, which also supported our outcomes and hypothesis (36).

In addition to the composition of the TILs subpopulation, our study revealed the intercellular spatial association in TME. Although a few studies have researched the TME either by using mIF (59) or by applying spatial analysis (60) in NPC, mIF based TME composition and spatial structure have not been comprehensively investigated. As far as we know, our study

was the first to investigate both the compositional abundance and the spatial distribution of TILs in NPC. In the present study, Gcross analysis was adopted to quantify the intercellular proximity between any two types of cells. Herein, radii of 20, 30, 50, and 100 μm were selected as distances of interest for this study since distances between 20 and 110 μm have been previously suggested to represent physiological distances for direct intercellular crosstalk (25, 61). According to Gcross analysis, significant engagement of Tregs surrounding TCs was independently associated with poor outcomes. These findings are consistent with recent findings in lung cancer (15) and esophageal cancer (17), highlighting the significance of the close proximity of Tregs to TCs in prompting progression.

Another significant finding of the present study is that closer and denser infiltration of PDL1+ Tregs surrounding CTLs was independently associated with a worse outcome. Furthermore, we found that Tregs and PDL1+ Tregs had a substantially higher probability of infiltrating near CTLs than TCs. These findings support our hypothesis that PDL1+ Tregs interact with CTLs *via* the PD1/PDL1 axis and subsequently mediate CTL dysfunction in antitumor activity, resulting in enhanced immune suppression. This has implications for future clinical investigations and mechanisms of prognosis prediction, as well as immunotherapy for patients with NPC.

Our study also has other strengths. To the best of our knowledge, this is the first study to investigate both the compositional abundance and the spatial distribution of TILs in NPC. Second, our study population included two groups of patients with well-matched characteristics but distinct DFS, which reduced the confounding effect of traditional prognostic factors and aided the identification of valid differential immunomarkers. Third, rather than semiquantitative measurements, the mfIHC technology allows for the codetection of multiple markers at a single cell level, demonstrating the high quality of cell phenotyping and accurate cell densities.

Nevertheless, there are several limitations to our study. First, patients in our study received treatment in the early 2010s, when EBV DNA was not well known as a prognostic factor. Therefore, the EBV DNA data in our database were incomplete and thus were not considered in the present study. Second, we only investigated classical TILs subpopulations along with PDL1 expression, whereas other markers of TILs functional state were not covered in this study. Future studies incorporating alternative lymphocyte markers could offer a more comprehensive landscape of TME. Third, although we established the prognostic role of PDL1+ Tregs infiltration in NPC, other immune-suppressive cell populations, such as MDSCs and M2 macrophages, may also play vital roles in immune suppression. Future studies should delve into more abundant cell subpopulations to provide a more precise cell–cell interaction network. Finally, TMA cannot represent the whole slide, just as the whole slide cannot represent the whole tumor. The heterogeneity always exists within the tumor, especially in those with large tumor burden. However, many studies have shown good concordance rate between TMA and whole slide (62).

To conclude, our study comprehensively demonstrates the infiltrating profile and spatial distribution characteristics of TILs in NPC. Increased Tregs infiltration, particularly PDL1+ Tregs, as well as their proximity to TCs and CTLs, correlates with unfavorable outcomes, highlighting the essential role of dynamic intercellular interactions between heterogeneous T-cell subtypes in disease progression. This study offers new insights into the immunological landscape of NPC, adding evidence of the prognostic value of TILs and the potential mechanism of PDL1/PD1 axis blockade in the era of immune-modulatory therapy.

Data availability statement

The data used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics statement

The study was approved by the Institutional Review Board (IRB) of Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College (IRB approval no. NCC 2462). All patients provided informed consent for the collection of tissue samples.

Author contributions

FZ and GS: data curation, formal analysis, methodology, visualization, writing-original draft preparation. XC, YZ, RW, JZ, XH, KW, and YQ: investigation, data curation. SS, QL, YL, and XS: data curation, methodology. JL and Y-XL: investigation, supervision. BL and JW: software, visualization. JY and JW: conceptualization, project administration, writing - review and editing, and funding acquisition. All authors contributed to the article and approved the submitted version.

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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.2022.1015283/full#supplementary-material

References

1. Chien YC, Chen JY, Liu MY, Yang HI, Hsu MM, Chen CJ, et al. Serologic markers of Epstein-Barr virus infection and nasopharyngeal carcinoma in Taiwanese men. *New Engl J Med* (2001) 345(26):1877–82. doi: 10.1056/NEJMoa011610

2. Zhang B, Mo Z, Du W, Wang Y, Liu L, Wei Y. Intensity-modulated radiation therapy versus 2D-RT or 3D-CRT for the treatment of nasopharyngeal carcinoma: A systematic review and meta-analysis. *Oral Oncol* (2015) 51(11):1041–6. doi: 10.1016/j.oraloncology.2015.08.005

3. Chen L, Zhang Y, Lai SZ, Li WF, Hu WH, Sun R, et al. 10-year results of therapeutic ratio by intensity-modulated radiotherapy versus two-dimensional radiotherapy in patients with nasopharyngeal carcinoma. *Oncol* (2019) 24(1): e38–45. doi: 10.1634/theoncologist.2017-0577

4. Au KH, Ngan RKC, Ng AWY, Poon DMC, Ng WT, Yuen KT, et al. Treatment outcomes of nasopharyngeal carcinoma in modern era after intensity modulated radiotherapy (IMRT) in Hong Kong: A report of 3328 patients (HKNPCSG 1301 study). *Oral Oncol* (2018) 77:16–21. doi: 10.1016/j.oraloncology.2017.12.004

5. Tang LQ, Lu TY, Li Y, Guo SY, Zhong QY, Zou MS, et al. Patterns of failure and survival trends of 720 patients with stage I nasopharyngeal carcinoma diagnosed from 1990-2012: A Large-scale retrospective cohort study. *J Cancer* (2018) 9(7):1308–17. doi: 10.7150/jca.21009

6. Wang J, Huang X, Sun S, Wang K, Qu Y, Chen X, et al. Stage-dependent conditional survival and failure hazard of non-metastatic nasopharyngeal carcinoma after intensity-modulated radiation therapy: Clinical implications for treatment strategies and surveillance. *Cancer Med* (2021) 10(11):3613–21. doi: 10.1002/cam4.3917

7. Ma BBY, Lim WT, Goh BC, Hui EP, Lo KW, Pettinger A, et al. Antitumor activity of nivolumab in recurrent and metastatic nasopharyngeal carcinoma: An international, multicenter study of the Mayo clinic phase 2 consortium (NCI-9742). *J Clin Oncol Off J Am Soc Clin Oncol* (2018) 36(14):1412–8. doi: 10.1200/JCO.2017.77.0388

8. Hsu C, Lee SH, Ejadi S, Even C, Cohen RB, Le Tourneau C, et al. Safety and antitumor activity of pembrolizumab in patients with programmed death-ligand 1-positive nasopharyngeal carcinoma: Results of the KEYNOTE-028 study. *J Clin Oncol Off J Am Soc Clin Oncol* (2017) 35(36):4050–6. doi: 10.1200/JCO.2017.73.3675

9. Wang FH, Wei XL, Feng J, Li Q, Xu N, Hu XC, et al. Efficacy, safety, and correlative biomarkers of toripalimab in previously treated recurrent or metastatic nasopharyngeal carcinoma: A phase II clinical trial (POLARIS-02). *J Clin Oncol Off J Am Soc Clin Oncol* (2021), 39(7):704–712. doi: 10.1200/JCO.20.02712

10. Yang Y, Zhou T, Chen X, Li J, Pan J, He X, et al. Efficacy, safety, and biomarker analysis of camrelizumab in previously treated recurrent or metastatic nasopharyngeal carcinoma (CAPTAIN study). *J Immunother Cancer* (2021) 9(12): e003790. doi: 10.1136/jitc-2021-003790

11. Lee AW, Ma BB, Ng WT, Chan AT. Management of nasopharyngeal carcinoma: Current practice and future perspective. *J Clin Oncol Off J Am Soc Clin Oncol* (2015) 33(29):3356–64. doi: 10.1200/JCO.2015.60.9347

12. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *New Engl J Med* (2012) 366(26):2443–54. doi: 10.1056/NEJMoa1200690

13. Fridman WH, Pagès F, Sautès-Fridman C, Galon J. The immune contexture in human tumours: Impact on clinical outcome. *Nat Rev Cancer* (2012) 12(4):298–306. doi: 10.1038/nrc3245

14. Khoury T, Nagrale V, Opyrchal M, Peng X, Wang D, Yao S. Prognostic significance of stromal versus intratumoral infiltrating lymphocytes in different subtypes of breast cancer treated with cytotoxic neoadjuvant chemotherapy. *Appl Immunohistochem Mol Morphol AIMM* (2018) 26(8):523–32. doi: 10.1097/PAI.0000000000000466

15. Barua S, Fang P, Sharma A, Fujimoto J, Wistuba I, Rao AUK, et al. Spatial interaction of tumor cells and regulatory T cells correlates with survival in non-small cell lung cancer. *Lung Cancer* (2018) 117:73–9. doi: 10.1016/j.lungcan.2018.01.022

16. Corredor G, Wang X, Zhou Y, Lu C, Fu P, Syrigos K, et al. Spatial architecture and arrangement of tumor-infiltrating lymphocytes for predicting likelihood of recurrence in early-stage non-small cell lung cancer. *Clin Cancer Res an Off J Am Assoc Cancer Res* (2019) 25(5):1526–34. doi: 10.1158/1078-0432.CCR-18-2013

17. Pan C, Wang Y, Liu Q, Hu Y, Fu J, Xie X, et al. Phenotypic profiling and prognostic significance of immune infiltrates in esophageal squamous cell carcinoma. *Oncoimmunology* (2021) 10(1):1883890. doi: 10.1080/2162402X.2021.1883890

18. Toss MS, Abidi A, Lesche D, Joseph C, Mahale S, Saunders H, et al. The prognostic significance of immune microenvironment in breast ductal carcinoma in situ. *Br J Cancer* (2020) 122(10):1496–506. doi: 10.1038/s41416-020-0797-7

19. Wang YQ, Chen YP, Zhang Y, Jiang W, Liu N, Yun JP, et al. Prognostic significance of tumor-infiltrating lymphocytes in nondisseminated nasopharyngeal carcinoma: A large-scale cohort study. *Int J Cancer* (2018) 142(12):2558–66. doi: 10.1002/ijc.31279

20. Zhao J, Guo C, Xiong F, Yu J, Ge J, Wang H, et al. Single cell RNA-seq reveals the landscape of tumor and infiltrating immune cells in nasopharyngeal carcinoma. *Cancer Lett* (2020) 477:131–43. doi: 10.1016/j.canlet.2020.02.010

21. Kim JM, Chen DS. Immune escape to PD-L1/PD-1 blockade: seven steps to success (or failure). *Ann Oncol Off J Eur Soc Med Oncol* (2016) 27(8):1492–504. doi: 10.1093/annonc/mdw217

22. Herbst RS, Soria JC, Kowanetz M, Fine GD, Hamid O, Gordon MS, et al. Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. *Nature* (2014) 515(7528):563–7. doi: 10.1038/nature14011

23. Almangush A, Ruuskanen M, Hagström J, Hirvikoski P, Tommola S, Kosma VM, et al. Tumor-infiltrating lymphocytes associate with outcome in nonendemic nasopharyngeal carcinoma: a multicenter study. *Hum Pathol* (2018) 81:211–9. doi: 10.1016/j.humpath.2018.07.009

24. Ooft ML, van Ipenburg JA, Braunius WW, Zuur CI, Koljenović S, Willems SM. Prognostic role of tumor infiltrating lymphocytes in EBV positive and EBV negative nasopharyngeal carcinoma. *Oral Oncol* (2017) 71:16–25. doi: 10.1016/j.oraloncology.2017.05.015

25. Väyrynen SA, Zhang J, Yuan C, Väyrynen JP, Dias Costa A, Williams H, et al. Composition, spatial characteristics, and prognostic significance of myeloid cell infiltration in pancreatic cancer. *Clin Cancer Res an Off J Am Assoc Cancer Res* (2021) 27(4):1069–81. doi: 10.1158/1078-0432.CCR-20-3141

26. Lazarus J, Maj T, Smith JJ, Perusina Lanfranca M, Rao A, D'Angelica MI, et al. Spatial and phenotypic immune profiling of metastatic colon cancer. *JCI Insight* (2018) 3(22): e121932. doi: 10.1172/jci.insight.121932

27. Carstens JL, Correa de Sampaio P, Yang D, Barua S, Wang H, Rao A, et al. Spatial computation of intratumoral T cells correlates with survival of patients with pancreatic cancer. *Nat Commun* (2017) 8:15095. doi: 10.1038/ncomms15095

28. Hu C, Tian S, Lin L, Zhang J, Ding H. Prognostic and clinicopathological significance of PD-L1 and tumor infiltrating lymphocytes in hypopharyngeal squamous cell carcinoma. *Oral Oncol* (2020) 102:104560. doi: 10.1016/j.oraloncology.2019.104560

29. Baddeley A, Rubak E, Turner R. *Spatial point patterns: Methodoloty and applications with r*. London: Chapman and Hall/CRC Press (2015).

30. Rad HS, Shiravand Y, Radfar P, Ladwa R, Perry C, Han X, et al. Understanding the tumor microenvironment in head and neck squamous cell carcinoma. *Clin Trans Immunol* (2022) 11(6):e1397. doi: 10.1002/cti2.1397

31. Al-Rajhi N, Soudy H, Ahmed SA, Elhassan T, Mohammed SF, Khoja HA, et al. CD3+T-lymphocyte infiltration is an independent prognostic factor for advanced nasopharyngeal carcinoma. *BMC Cancer* (2020) 20(1):240. doi: 10.1186/s12885-020-06757-w

32. Zhu Q, Cai MY, Chen CL, Hu H, Lin HX, Li M, et al. Tumor cells PD-L1 expression as a favorable prognosis factor in nasopharyngeal carcinoma patients with pre-existing intratumor-infiltrating lymphocytes. *Oncoimmunology* (2017) 6 (5):e1312240. doi: 10.1080/2162402X.2017.1312240

33. Ono T, Azuma K, Kawahara A, Sasada T, Matsuo N, Kakuma T, et al. Prognostic stratification of patients with nasopharyngeal carcinoma based on tumor immune microenvironment. *Head Neck* (2018) 40(9):2007–19. doi: 10.1002/hed.25189

34. Larbcharoensub N, Mahaprom K, Jiarpinitnun C, Trachu N, Tubthong N, Pattaranutaporn P, et al. Characterization of PD-L1 and PD-1 expression and CD8+ tumor-infiltrating lymphocyte in Epstein-Barr virus-associated nasopharyngeal carcinoma. *Am J Clin Oncol* (2018) 41(12):1204–10. doi: 10.1097/COC.0000000000000449

35. Li C, Jiang P, Wei S, Xu X, Wang J. Regulatory T cells in tumor microenvironment: New mechanisms, potential therapeutic strategies and future prospects. *Mol Cancer* (2020) 19(1):116. doi: 10.1158/1557-3125.HIPPO19-B11

36. De Simone M, Arrigoni A, Rossetti G, Gruarin P, Ranzani V, Politano C, et al. Transcriptional landscape of human tissue lymphocytes unveils uniqueness of tumor-infiltrating T regulatory cells. *Immunity* (2016) 45(5):1135–47. doi: 10.1016/j.immuni.2016.10.021

37. Chen WY, Wu CT, Wang CW, Lan KH, Liang HK, Huang BS, et al. Prognostic significance of tumor-infiltrating lymphocytes in patients with operable tongue cancer. *Radiat Oncol (London England)* (2018) 13(1):157. doi: 10.1186/s13014-018-1099-6

38. Merlo A, Casalini P, Carcangiu ML, Malventano C, Triulzi T, Mènard S, et al. FOXP3 expression and overall survival in breast cancer. *J Clin Oncol Off J Am Soc Clin Oncol* (2009) 27(11):1746–52. doi: 10.1200/JCO.2008.17.9036

39. Gao Q, Qiu SJ, Fan J, Zhou J, Wang XY, Xiao YS, et al. Intratumoral balance of regulatory and cytotoxic T cells is associated with prognosis of hepatocellular carcinoma after resection. *J Clin Oncol Off J Am Soc Clin Oncol* (2007) 25(18):2586–93. doi: 10.1200/JCO.2006.09.4565

40. Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* (2004) 10(9):942–9. doi: 10.1038/nm1093

41. Wang G, Liu G, Liu Y, Li X, Su Z. FOXP3 expression in esophageal cancer cells is associated with poor prognosis in esophageal cancer. *Hepato-gastroenterology* (2012) 59(119):2186–91. doi: 10.5754/hge11961

42. Bron L, Jandus C, Andrejevic-Blant S, Speiser DE, Monnier P, Romero P, et al. Prognostic value of arginase-II expression and regulatory T-cell infiltration in head and neck squamous cell carcinoma. *Int J Cancer* (2013) 132(3):E85–93. doi: 10.1002/ijc.27728

43. Salama P, Phillips M, Grieu F, Morris M, Zeps N, Joseph D, et al. Tumor-infiltrating FOXP3+ T regulatory cells show strong prognostic significance in colorectal cancer. *J Clin Oncol Off J Am Soc Clin Oncol* (2009) 27(2):186–92. doi: 10.1200/JCO.2008.18.7229

44. Bonanno L, Pavan A, Dieci MV, Di Liso E, Schiavon M, Comacchio G, et al. The role of immune microenvironment in small-cell lung cancer: Distribution of PD-L1 expression and prognostic role of FOXP3-positive tumour infiltrating lymphocytes. *Eur J Cancer (Oxford Engl 1990)* (2018) 101:191–200. doi: 10.1016/j.ejca.2018.06.023

45. Ooft ML, van Ipenburg JA, Sanders ME, Kranendonk M, Hofland I, de Bree R, et al. Prognostic role of tumour-associated macrophages and regulatory T cells in EBV-positive and EBV-negative nasopharyngeal carcinoma. *J Clin Pathol* (2018) 71(3):267–74. doi: 10.1136/jclinpath-2017-204664

46. Lu J, Chen XM, Huang HR, Zhao FP, Wang F, Liu X, et al. Detailed analysis of inflammatory cell infiltration and the prognostic impact on nasopharyngeal carcinoma. *Head Neck* (2018) 40(6):1245–53. doi: 10.1002/hed.25104

47. Huo S, Luo Y, Deng R, Liu X, Wang J, Wang L, et al. EBV-EBNA1 constructs an immunosuppressive microenvironment for nasopharyngeal carcinoma by promoting the chemoattraction of treg cells. *J Immunother Cancer* (2020) 8(2): e001588. doi: 10.1136/jitc-2020-001588.

48. Toomer KH, Malek TR. Cytokine signaling in the development and homeostasis of regulatory T cells. *Cold Spring Harbor Perspect Biol* (2018) 10(3): a028597. doi: 10.1101/cshperspect.a028597.

49. Wei X, Zhang J, Gu Q, Huang M, Zhang W, Guo J, et al. Reciprocal expression of IL-35 and IL-10 defines two distinct effector treg subsets that are required for maintenance of immune tolerance. *Cell Rep* (2017) 21(7):1853–69. doi: 10.1016/j.celrep.2017.10.090

50. Chen S, Crabill GA, Pritchard TS, McMiller TL, Wei P, Pardoll DM, et al. Mechanisms regulating PD-L1 expression on tumor and immune cells. *J Immunother Cancer* (2019) 7(1):305. doi: 10.1186/s40425-019-0770-2

51. Chan OS, Kowanetz M, Ng WT, Koeppen H, Chan LK, Yeung RM, et al. Characterization of PD-L1 expression and immune cell infiltration in nasopharyngeal cancer. *Oral Oncol* (2017) 67:52–60. doi: 10.1016/j.oraloncology.2017.02.002

52. Fang W, Zhang J, Hong S, Zhan J, Chen N, Qin T, et al. EBV-driven LMP1 and IFN-gamma up-regulate PD-L1 in nasopharyngeal carcinoma: Implications for oncotargeted therapy. *Oncotarget* (2014) 5(23):12189–202. doi: 10.18632/oncotarget.2608

53. Zhou Y, Shi D, Miao J, Wu H, Chen J, Zhou X, et al. PD-L1 predicts poor prognosis for nasopharyngeal carcinoma irrespective of PD-1 and EBV-DNA load. *Sci Rep* (2017) 7:43627. doi: 10.1038/srep43627

54. Liu YJ, Tsang NM, Hsueh C, Yeh CJ, Ueng SH, Wang TH, et al. Low PD-L1 expression strongly correlates with local recurrence in Epstein-Barr virus-positive nasopharyngeal carcinoma after radiation-based therapy. *Cancers* (2018) 10(10): 374. doi: 10.3390/cancers10100374

55. Zhang J, Fang W, Qin T, Yang Y, Hong S, Liang W, et al. Co-Expression of PD-1 and PD-L1 predicts poor outcome in nasopharyngeal carcinoma. *Med Oncol* (2015) 32(3):86. doi: 10.1007/s12032-015-0501-6

56. Li YF, Ding JW, Liao LM, Zhang ZL, Liao SS, Wu Y, et al. Expression of programmed death ligand-1 predicts poor outcome in nasopharyngeal carcinoma. *Mol Clin Oncol* (2017) 7(3):378–82. doi: 10.3892/mco.2017.1318

57. DiDomenico J, Lamano JB, Oyon D, Li Y, Veliceasa D, Kaur G, et al. The immune checkpoint protein PD-L1 induces and maintains regulatory T cells in glioblastoma. *Oncoimmunology* (2018) 7(7):e1448329. doi: 10.1080/2162402X.2018.1448329

58. Wu SP, Liao RQ, Tu HY, Wang WJ, Dong ZY, Huang SM, et al. Stromal PD-L1-Positive regulatory T cells and PD-1-Positive CD8-positive T cells define the response of different subsets of non-small cell lung cancer to PD-1/PD-L1 blockade immunotherapy. *J Thorac Oncol Off Publ Int Assoc Study Lung Cancer* (2018) 13 (4):521–32. doi: 10.1016/j.jtho.2017.11.132

59. Liu SL, Bian LJ, Liu ZX, Chen QY, Sun XS, Sun R, et al. Development and validation of the immune signature to predict distant metastasis in patients with nasopharyngeal carcinoma. *J Immunother Cancer* (2020) 8(1):e000205. doi: 10.1136/jitc-2019-000205.

60. Wang YQ, Liu X, Xu C, Jiang W, Xu SY, Zhang Y, et al. Spatial heterogeneity of immune infiltration predicts the prognosis of nasopharyngeal carcinoma patients. *Oncoimmunology* (2021) 10(1):1976439. doi: 10.1080/2162402X.2021.1976439

61. Feichtenbeiner A, Haas M, Büttner M, Grabenbauer GG, Fietkau R, Distel LV. Critical role of spatial interaction between $CD8^+$ and $Foxp3^+$ cells in human gastric cancer: The distance matters. *Cancer Immunol Immunother CII* (2014) 63 (2):111–9.doi: 10.1007/s00262-013-1491-x

62. Ciesielska U, Piotrowska A, Kobierzycki C, Pastuszewski W, Podhorska-Okolow M, Dziegiel P, et al. Comparison of TMA technique and routine whole slide analysis in evaluation of proliferative markers expression in laryngeal squamous cell cancer. *In Vivo (Athens Greece)* (2020) 34(6):3263–70. doi: 10.21873/invivo.12163

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Progress in the clinical application of immune checkpoint inhibitors in small cell lung cancer

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Small cell lung cancer (SCLC) is a refractory cancer with poor prognosis due to its aggressive malignancy and high rates of metastasis, recurrence and drug resistance. These characteristics have also greatly impeded the identification of new treatment methods and drugs. The traditional model of SCLC treatment that has been reliant on platinum combined with etoposide for decades has been superseded by the emergence of immune checkpoint inhibitors (ICIs), which have shown significant therapeutic effects and broad application prospects as a monotherapy. This has led to the evaluation of ICIs with different mechanisms of action and their use in combination with radiotherapy or a variety of molecular targeted drugs to achieve synergy, complementary advantages, and reduce adverse reactions. Here, we review the progress in the use of ICIs as a monotherapy or in combination therapy for SCLC and consider the current limitations of these approaches as well as prospects for future developments.

KEYWORDS

small cell lung cancer, immune checkpoint inhibitors, combined immunotherapy, clinical trials, CTLA- 4, PD1,PD-L1

1 Introduction

Small cell lung cancer (SCLC), which accounts for approximately 15% of all types of lung cancer, is a neuroendocrine tumor with rapid growth, early metastasis and poor prognosis (1). Platinum and etoposide (EP)-based systemic chemotherapy has long been considered the first-line treatment for extensive-stage SCLC. Chemotherapy is effective in early-stage SCLC, but the vast majority of patients will rapidly relapse and die within a few months (2).

Immunotherapy has become an important strategy for the treatment of tumors. Cancer immunotherapies include tumor vaccines, cytokines, chimeric antigen receptor T cell immunotherapy (CAR-T), and immune checkpoint inhibitors (ICIs), which have become a focus of research in recent years (3). ICIs eliminate tumors by inhibiting the immune escape of tumor cells and enhancing the immune response of T cells (4). ICIs have been

positively correlated with tumor mutation burden (5). SCLC is a smoking-related disease characterized by a high tumor mutation burden, indicating that SCLC may be highly sensitive to ICI-based immunotherapy (2). The ICIs used to treat SCLC include inhibitors of programmed cell death 1 (PD-1), programmed cell death ligand (PD-L1) and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) (6). Numerous clinical studies are ongoing to further explore the role of ICIs as adjuvant or neoadjuvant therapy for lung cancer patients.

The field of SCLC research was widely considered to be a "forbidden zone" until the emergence of ICIs offered the potential for more efficient and less toxic modes of immunotherapy both alone and in combination with radiotherapy or a variety of molecular targeted drugs. Here, we review the clinical trials of ICIs as monotherapy and in combination therapy for SCLC (Table 1), and discuss the progress in this field as well as the limitations and prospects for future developments that will pave the way for improved outcomes for patients with SCLC.

2 CTLA-4 inhibitors

CTLA-4 is a negative regulator of T cell activation. As the first ICIs for SCLC (32), CTLA-4 inhibitors include ipilimumab and tremelimumab. Ipilimumab is a human monoclonal IgG1 antibody against CTLA-4, which blocks the immunosuppressive interaction between CTLA-4 and its ligands on cells (CD80/CD86) to promote the activation and proliferation of T cells, and enhance anti-tumor immune function (33). Tremelimumab is a fully human monoclonal IgG2 antibody that is still in preclinical testing.

2.1 Ipilimumab combined with chemotherapy

The CA184-041 study (7) was a randomized control trial of 130 treatment-naive patients with extensive-stage small-cell lung cancer (ES-SCLC) who were randomly allocated to a staged treatment group (paclitaxel and carboplatin combined with ipilimumab), a concurrent chemotherapy group (paclitaxel and carboplatin combined with ipilimumab) and a control group (paclitaxel and carboplatin). While the objective response rate (ORR) and immune-related progression-free survival (irPFS) were increased in the phase-therapy ipilimumab group, there were no significant improvements in the concurrent chemotherapy group. Compared with the control group, the staged ipilimumab regimen improved irPFS (HR = 0.64; $P = 0.03$), but not in the concurrent ipilimumab regimen (HR = 0.75; $P = 0.11$). Median irPFS was 5.3 months for control, 6.4 months for staged ipilimumab, and 5.7 months for concurrent ipilimumab regimens. However, treatment-related grade III/IV immune adverse events (AEs) were more common in the ipilimumab arm. Interpretation of the results of this study is limited by its small sample size and the availability of only preclinical data on ipilimumab plus chemotherapy.

The efficacy and safety of ipilimumab or placebo combined with platinum and etoposide in the treatment of newly diagnosed ES-SCLC patients have been evaluated in a phase III clinical study (CA184-156) (8). Among 1,132 patients randomly assigned to receive ipilimumab or placebo, the median OS was 11.0 months and 10.9 months (HR = 0.94; 95% CI: 0.81–1.09; $P = 0.3775$), respectively, and PFS was 4.6 months and 4.4 months (HR = 0.85; 95%CI: 0.75–0.97, $P = 0.016$), respectively. These results showed that there was no significant improvement in the primary endpoint of OS compared with chemotherapy alone, so the difference in the secondary endpoint PFS could not be considered statistically significant. Diarrhea, rash, and colitis were more common with chemotherapy plus ipilimumab, while other treatment-related AEs were of similar frequency and severity in the two groups. Treatment-related discontinuation was higher with ipilimumab (18% vs. 2% with placebo). Five treatment-related deaths occurred in the ipilimumab group and two in the placebo group. The toxicity of ipilimumab combined with carboplatin and etoposide (ICE) in the treatment of ES-SCLC was also found in the study reported by Edurne et al. (34). It is not clear why ipilimumab was not more effective than etoposide+ platinum-based chemotherapy, although one possible explanation is that ipilimumab does not effectively stimulate peripheral T cell activation, and thus activated T cells that could effectively enhance antitumor immune responses are not present in the tumor microenvironment.

3 PD-1 and PD-L1 inhibitors

PD-1 and PD-L1 play an important role in regulating T cell function to maintain protective immunity and immune balance, homeostasis and tolerance. The combination of PD-1 and PD-L1 has an immunosuppressive effect, transmits negative signals, inhibits T cell proliferation, cytokine production and cytolytic function, and maintains the balance of the immune system (35). Currently, pembrolizumab and nivolumab are PD-1 inhibitors that are widely studied in the field of SCLC, and PD-L1 inhibitors include atezolizumab, durvalumab and avelumab (36).

3.1 Pembrolizumab

Pembrolizumab is a highly selective humanized monoclonal antibody that binds to the PD-1 receptor and directly blocks the interaction between PD-1 and its ligand, thereby enhancing the function of tumor-directed T cells and mediating tumor destruction (37).

3.1.1 Pembrolizumab in monotherapy

The KEYNOTE-028 study (9) included 24 patients with SCLC who failed to respond to standard chemotherapy and had PD-L1 expression confirmed by immunohistochemistry. The results of this study showed that the ORR was 33%, the median OS was 7.7 months, the PFS was 1.9 months, and the 1-year survival rate was 37.7%. This study confirmed that pembrolizumab monotherapy showed promising anti-tumor activity and was well-tolerated in the treatment of PD-L1-positive, previously treated SCLC. However, all

TABLE 1 Completed and ongoing clinical trials of immunotherapy for SCLC.

Study ID	Trial identifier	Phase	Patients	Treatment methods	Estimated primary completion date	Status	Reference
CA184-041	NCT00527735	II	Untreated SCLC	Ipilimumab+paclitaxel/carboplatin vs. paclitaxel/carboplatin	December 2011	Completed	(7)
CA184-156	NCT01450761	III	ES-SCLC	Ipilimumab+etoposide/platinum vs. etoposide/platinum	May 17, 2017	Completed	(8)
KEYNOTE-028	NCT02054806	IB	PD-L1-ES-SCLC	Pembrolizumab	April 30, 2021	Completed	(9)
KEYNOTE-158	NCT02628067	II	Advanced SCLC	Pembrolizumab	June 18, 2026	Ongoing	(10)
KEYNOTE-604	NCT03066778	III	ES-SCLC	Pembrolizumab+EP vs. placebo+EP	September 21, 2021	Completed	(11)
REACTION	NCT02580994	II	Untreated ES-SCLC	Etoposide and cis/carboplatin ± pembrolizumab	December 2023	Ongoing	(12)
–	NCT02402920	I	SCLC	Pembrolizumab and concurrent chemoradiotherapy or radiation therapy	July 31, 2023	Ongoing	(13)
KEYLYNK-013	NCT04624204	III	ES-SCLC	Pembrolizumab+concurrent chemoradiation therapy followed by pembrolizumab ± olaparib vs. concurrent chemoradiation therapy	October 28, 2027	Ongoing	(14)
CheckMate 032	NCT01928394	I/II	Recurrent SCLC	Nivolumab vs. nivolumab+ipilimumab	April 30, 2023	Ongoing	(15)
CheckMate331	NCT02481830	III	Relapsed SCLC	Nivolumab vs. chemotherapy	August 22, 2022	Completed	(16)
CheckMate 451	NCT02538666	III	ES-SCLC	Nivolumab vs. nivolumab+ipilimumab vs. placebo	November 11, 2021	Completed	(17)
IMpower133	NCT02763579	I/III	Untreated ES-SCLC	Carboplatin+etoposide ± atezolizumab	July 8, 2022	Completed	(18, 19)
SKYSCRAPER-02	NCT04256421	III	Untreated ES-SCLC	Atezolizumab+carboplatin+etoposide ± tiragolumab	March 21, 2024	Ongoing	(20)
CASPIAN	NCT03043872	III	Untreated ES-SCLC	Durvalumab ± tremelimumab in combination with Platinum-based chemotherapy	December 30, 2022	Completed	(21)
BALTIC	NCT02937818	II	Platinum Refractory ES-SCLC	Durvalumab+tremelimumab followed by durvalumab monotherapy	December 29, 2023	Ongoing	(22)
–	NCT02701400	–	Relapsed SCLC	Tremelimumab+durvalumab combination ± radiation	August 7, 2020	Completed	(23)
PAVE	NCT03568097	II	Advanced SCLC	Avelumab combined with chemotherapy	April 2023	Ongoing	(24)
QUILT-3.055	NCT03228667	IIb	Previously received treatment with PD-1/PD-L1 ICIs	Avelumab	December 2023	Ongoing	(25)
JAVELIN Medley	NCT02554812	Ib/II	SCLC	Avelumab+utomilumab	February 28, 2023	Ongoing	(26)
–	NCT05429866	II	SCLC	Immune checkpoint inhibitor(s) (ICI) alone or in combination with chemotherapy or targeted therapy	December 1, 2024	Ongoing	(27)
CAPSTONE-1	NCT03711305	III	Untreated ES-SCLC	Carboplatin+etoposide with or without Adebrelimab	December 2023	Ongoing	(28)
–	NCT03041311	II	Untreated ES-SCLC	Carboplatin, Etoposide, and Atezolizumab With or Without Trilaciclib	October 29, 2020	Completed	(29)

(Continued)

TABLE 1 Continued

Study ID	Trial identifier	Phase	Patients	Treatment methods	Estimated primary completion date	Status	Reference
–	NCT02514447	Ib/IIa	ES-SCLC Receiving Topotecan Chemotherapy Previously	Trilaciclib and topotecan or placebo and topotecan	October 4, 2021	Completed	(30)
–	NCT02499770	Ib/IIa	Untreated ES-SCLC	Trilaciclib/placebo + carboplatin/etoposide	February 22, 2019	Completed	(31)

patients experienced treatment-related AEs, the most common of which were fatigue (n = 7) and cough (n = 6). The incidence of immune-related toxicities was 12.5% (3/24), including immune thyroiditis, infusion site reactions, cytokine release syndrome, and colitis. Toxicity was consistent with that observed previously for pembrolizumab therapy in other solid tumors. Subsequently, the KEYNOTE-158 study (10) was conducted to better identify biomarkers that would more accurately identify SCLC patients who might respond to pembrolizumab. In patients with relapsed or metastatic SCLC who received pembrolizumab monotherapy (regardless of PD-L1 expression), the ORR was 18.7%, median OS was 8.7 months, and median PFS was 2.0 months. In both studies, pembrolizumab had a favorable safety profile, which was consistent with the safety profile of this monotherapy in other tumor types. Chung et al. (38) conducted a pooled analysis of these two studies, and the median OS and PFS (7.7 and 2.0 months, respectively) were similar to those observed in the subgroup populations of the two studies. In this pooled analysis, pembrolizumab showed promising antitumor activity and durable clinical benefit, supporting the use of pembrolizumab monotherapy in third-line or later treatment for patients with SCLC. Pembrolizumab was recently approved by the U.S. Food and Drug Administration for patients with previously treated metastatic SCLC who had disease progression during, or after platinum-based chemotherapy on the basis of the KEYNOTE-028 and KEYNOTE-158 studies (39).

3.1.2 Pembrolizumab combined with chemotherapy

Studies have shown that ICIs combined with chemotherapy drugs can activate immune cells. ICIs can maintain the activation state of T cells after stimulating specific anti-tumor immune cells with high frequency and low dose chemotherapy. Therefore, ICIs combined with chemotherapy can produce a synergistic effect and enhance the anti-tumor immune response; this raises the possibility of eliminating drug-resistant tumor cells, which is not possible with any of the current treatment modalities (40). The randomized, double-blind, phase III KEYNOTE-604 study (11) compared pembrolizumab/placebo plus etoposide and platinum (EP) in previously untreated patients with ES-SCLC. A total of 453 participants were randomized to receive pembrolizumab plus EP or placebo plus EP. The estimated 12-month PFS was 13.6% with pembrolizumab plus EP and 3.1% with placebo plus EP. The incidence of AEs from any cause was 76.7% and 74.9% for grade 3-4 and 6.3% and 5.4% for grade 5 in the pembrolizumab + EP and placebo + EP groups, respectively. The results showed that adding pembrolizumab to standard first-line EP significantly improved PFS in patients with ES-SCLC (HR = 0.75; 95%CI = 0.61–0.91; P = 0.0023), and no unexpected toxicities were observed. Many ongoing studies, such as the REACTION study (NCT02580994), are also evaluating pembrolizumab in combination with standard chemotherapy regimens for the first-line treatment of SCLC (12). Overall, these data support the benefit of pembrolizumab in SCLC, adding to a growing body of evidence supporting the value of immune checkpoint inhibitors (ICIs) in this historically difficult-to-treat cancer.

3.1.3 Pembrolizumab combined with radiation therapy

In preclinical models, ionizing radiation induces PD-L1 expression in tumor and stromal cells, along with an increase in myeloid-derived suppressor cells (41, 42). In addition, tumor-associated antigens released after radiation-induced cell death may be highly immunogenic, thereby enhancing the anti-tumor efficacy of systemic immunotherapy agents, even at distant tumor sites (43–45). Anti-PD-L1 inhibitors combined with radiotherapy have shown synergistic effects in xenograft models of pancreatic, colon, and breast cancer (43–45). Therefore, the combination of the two can enhance the local and systemic anti-tumor immune response and improve the success rate of treatment (46).

A phase I trial (NCT02402920) (13) evaluated the safety of pembrolizumab combined with thoracic radiation therapy (TRT) after induction chemotherapy in patients with ES-SCLS. The results showed that pembrolizumab combined with TRT was well-tolerated, and the incidence of serious AEs was low. However, studies with a longer follow-up time and larger sample size are still needed to improve outcomes compared with immunotherapy or TRT alone. However, in European subclinical trials (47), the OS of this combination treatment group and the TRT alone treatment group were 8.4 months and 8 months, respectively, and the PFS were 6.1 months and 4 months, respectively, showing the advantage of the combination therapy. The phase I trial to evaluate the safety and efficacy of this combination regimen laid a solid foundation for future prospective studies.

Ongoing studies include comparisons of pembrolizumab plus concurrent chemoradiotherapy followed by pembrolizumab with or without olaparib are ongoing in patients with newly diagnosed LS-

SCLC (KEYLYNK-013, NCT04624204) (14) in addition to phase II studies of pembrolizumab and lenvatinib plus chemotherapy in the treatment of ES-SCLC. We expect these studies to provide more evidence that will guide the use of pembrolizumab in the treatment of SCLC.

3.2 Nivolumab

Nivolumab is the first fully human IgG4 antibody approved by the FDA and the first to be studied clinically in non-small cell lung cancer. In 2018, nivolumab was approved for second-line treatment of SCLC, marking a great leap forward in the treatment of SCLC and indicating that immunotherapy is gradually changing the overall treatment layout of this disease (48).

In 2020, the CheckMate 032 study (15), which provided the latest body of data, demonstrated that nivolumab monotherapy and nivolumab+ ipilimumab showed anti-tumor activity with durable efficacy and manageable safety in previously treated SCLC patients. Ready et al. (49) further reported the efficacy of nivolumab monotherapy as a third-line or late-stage treatment for relapsed SCLC. The ORR was 11.9% (95% CI: 6.5–19.5), and the 12-month and 18-month overall survival rates were 28.3% and 20.0%, respectively, with an incidence of grade 3–4 AEs of 11.9%. These results demonstrate that nivolumab has durable efficacy and is well-tolerated as third-line or late-stage treatment for relapsed SCLC. However, in the CheckMate 331 study (16), 569 patients with SCLC who relapsed after first-line chemotherapy were randomized to receive nivolumab or chemotherapy (topotecan or amrubicin). The results showed that nivolumab was not effective in improving the survival of patients with relapsed SCLC compared with chemotherapy (median OS: 7.5 months vs. 8.4 months; HR = 0.86; 95% CI = 0.72–1.04; P = 0.11), and no new safety signals were observed. In addition, the CheckMate 451 study (17) compared the efficacy of nivolumab monotherapy versus nivolumab combined with ipilimumab in patients with ES-SCLC. The results showed that for patients who did not progress on first-line chemotherapy, the combination group (HR = 0.92; 95% CI: 0.75–1.12) and single-agent group (HR = 0.84; 95% CI: 0.69–1.02) did not significantly improve OS compared with placebo and with the same safety profile. Although the trial results indicated that maintenance nivolumab monotherapy or combination therapy was not effective for patients with ES-SCLC, patients in the combination group showed a trend toward benefit. At present, several relevant clinical trials are still ongoing and expected to yield more data on optimizing the combination regimen.

Tumor cells have multiple immune signaling pathways, and inhibition of only one may lead to compensatory upregulation of other immune checkpoint molecules. This mechanism has a significant limiting effect on ICI monotherapy; however, two different types of ICIs can be combined to regulate T cells by acting on different sites. Thus, the synergistic anti-tumor effects of the ICI combination can stimulate the production of a large number of specific T cells in the early stage, and restore the immune function of exhausted T cells in the late stage (50). In this regard, the combination of nivolumab, a PD-1/PD-L1 inhibitor, and ipilimumab, a CTLA4 inhibitor, shows encouraging promise (15).

3.3 Atezolizumab

Atezolizumab was the first PD-L1 inhibitor to be studied in SCLC. Studies have shown that atezolizumab combined with conventional chemotherapy regimens can significantly prolong OS and PFS as the first-line treatment of ES-SCLC, with comparable safety. Liu et al. (18) reported the latest OS data based on a large sample population. The IMpower133 study (18, 19) evaluated the efficacy and safety of atezolizumab combined with carboplatin plus etoposide as first-line treatment for ES-SCLC. A total of 403 patients with ES-SCLC were randomized to atezolizumab+carboplatin+etoposide or placebo+carboplatin +etoposide. The results showed that at a median follow-up of 13.9 months, the median OS was 12.3 months in the atezolizumab group and 10.3 months in the placebo group (HR = 0.70, 95%CI: 0.54–0.91; P = 0.007), and the median PFS was 5.2 months and 4.3 months, respectively (HR = 0.77; 95%CI: 0.62–0.96; P = 0.02) (1). The IMpower133 study was terminated early because the efficacy was so good that OS and PFS had already reached positive results at the time of the interim analysis. Mansfield et al. (51) evaluated AEs in the IMpower133 study and found that grade 3–4 AEs were similar in the two groups. The IMpower133 trial is the first clinical study to achieve dual positive endpoints in the first-line treatment of ES-SCLC in more than 30 years. As a result, this new regimen has been adopted as the first-line treatment for ES-SCLC, representing an important milestone in SCLC immunotherapy. Indeed, based on this study, atezolizumab combined with carboplatin/etoposide chemotherapy is now recommended as a class I regimen for the first-line treatment of ES-SCLC in the 2019 edition of the NCCN SCLC clinical guidelines. Atezolizumab has also become the first immunotherapy agent approved for the first-line treatment of SCLC.

Tiragolumab is a human monoclonal antibody that targets T cell immunoreceptor with immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domains (TIGIT), which is expressed by natural killer (NK) cells in the majority of tumors and competes with the costimulatory molecule CD226 (DNAM-1) for binding to the ligands CD155 and CD112. A number of preclinical trials have shown that anti-TIGIT antibody and anti-PD-1/PD-L1 antibody function synergistically to provide anti-tumor effects and enhance the anti-tumor responses. Consequently, this combination has become an immune checkpoint of great interest after CTLA-4 and PD-1/PD-L1. The phase III trial SKYSCRAPER-02 (20) compared tiragolumab+ atezolizumab+ carboplatin+ etoposide (CE) with placebo +atezolizumab+CE in chemotherapy-naive patients with ES-SCLC. Tiragolumab was found to provide no additional benefit when added to atezolizumab and chemotherapy. The PFS and OS observed in the control group supported the results of the IMpower133 trial and further confirmed the validity of this combination as the standard of care in the first-line treatment of

patients with ES-SCLC. The SKYSCRAPER-02 study will continue with OS analysis and biomarker analysis.

3.4 Durvalumab

Durvalumab, a selective human IgG1 monoclonal antibody directed against PD-L1, exerts antitumor activity by preventing immune escape mediated by the PD-L1 pathway (52).

3.4.1 Durvalumab combined with chemotherapy

In the CASPIAN study (21), durvalumab, a selective human IgG1 monoclonal antibody directed against PD-L1, was shown to exert anti-tumor activity by preventing immune escape mediated by the PD-L1 pathway. Similar to the IMpower133 trial, the EP regimen plus durvalumab significantly improved median OS compared with the EP regimen alone (13 months vs. 10.3 months, HR = 0.73, 95% CI: 0.59–0.91, P = 0.0047). The ORR was also improved (79.5% vs. 70.3%), although the incidence of grade 3–4 AEs and AE mortality were similar in the two groups. Based on the results of this study, the 2020 National Comprehensive Cancer Network Clinical Practice Guidelines (3rd edition) recommended durvalumab plus EP as the preferred first-line treatment for patients with ES-SCLC. The U.S. FDA subsequently approved this regimen for first-line treatment of ES-SCLC in March 2020.

3.4.2 Durvalumab combined with tremelimumab

Inhibitors of CTLA-4 and PD-1/PD-L1can restore anti-tumor immune responses, resulting in long-term benefits in a substantial proportion of patients treated. ICI combination therapy is an emerging treatment option (53). The meta-analysis by Francesco et al. suggested that the current PD-1/CTLA-4 inhibitor combination therapy has a limited effect in advanced NSCLC patients with high and/or low PD-L1, but may be an effective and tolerable option in the PD-L1-negative subgroup (54). In ES-SCLC, studies have shown that PD-1/PD-L1 inhibitors combined with chemotherapy are safer and more effective than chemotherapy alone, whereas PD-1/PD-L1 inhibitors combined with CTLA-4 inhibitors did not improve the efficacy (55).

The combination regimen of durvalumab+ tremelimumab (D +T), which acts *via* the same mechanism, has attracted widespread attention. The phase II clinical trial NCT02937818 (22) initially confirmed the good safety and reliable anti-tumor activity of this "golden partner" group. A preliminary analysis of the phase III CASPIAN study (56), in which durvalumab+ tremelimumab+ platin-etoposide was compared with platin-etoposide alone, were reported in 2021, and the analysis of the total OS of durvalumab+ platin-etoposide and platin-etoposide alone were updated after 11 months of follow-up. Patients were randomized to receive durvalumab+ tremelimumab+ platinum-etoposide (268 patients), durvalumab+ platinum-etoposide (268 patients), or platinum-etoposide (269 patients). Durvalumab+ tremelimumab+ platinum-etoposide did not significantly improve overall OS compared with platinum-etoposide treatment (HR = 0.82,95% CI: 0.68–1.00; P = 0.045), with a median total OS of 10.4 months (95% CI: 9.6–12.0) vs. 10.5 months (9.3–11.2). Compared with the platinum-etoposide group, the durvalumab+ platinum-etoposide group had a significant improvement in OS (HR = 0.75, 95% CI: 0.62–0.91; P = 0.0032), with a median OS of 12.9 months (95% CI: 11.3–14.7) vs. 10.5 months (9.3–11.2). Durvalumab+platinum-etoposide showed a sustained improvement in OS, but the addition of tremelimumab to durvalumab did not significantly improve prognosis. These results support durvalumab+platinum-etoposide as first-line treatment for ES-SCLC. This study and the IMpower133 study provide compelling evidence that PD-L1 monoclonal antibody combined with chemotherapy is a successful first-line treatment strategy for ES-SCLC.

3.4.3 Durvalumab combined with radiation therapy

A phase II study (NCT02701400) (23) of patients with relapsed SCLC who had received ≤2 lines of prior therapy were randomly assigned to two groups: (1) Group A: received durvalumab (D) tremelimumab (T), but did not receive stereotactic body radiation therapy (SBRT); (2) Group B: immune susceptibility SBRT (9 Gy × 3 F) was performed on a selected tumor site, before patients received D/T. The median PFS times of groups A and B were 2.1 months and 3.3 months (HR = 2.44, 95% CI: 0.75–7.93, P = 0.122), respectively, and the median OS times were 2.8 months and 5.7 months (HR = 1.50, 95% Cl: 0.45–4.99, P = 0.507), respectively. These studies showed that radiotherapy combined with immunotherapy improved efficacy, although there were no significant difference in the OS and PFS times between the two groups, which is worthy of further exploration in relapsed SCLC. Future studies should take full advantage of the synergy between radiation and immunotherapy in the early stages of disease, while also seeking enrichment strategies for patients who may benefit from immunotherapy.

3.5 Avelumab

A phase II study evaluating the safety and efficacy of avelumab +cisplatin or carboplatin+etoposide (NCT03568097) in 55 subjects, with the primary endpoint of 1-year PFS rate, is expected to be completed in April 2023 (24). Another phase IIb multicenter immune-combination study (NCT03228667) is ongoing to validate avelumab in SCLC patients previously treated with PD-1/PD-L1 ICIs (25). Two additional studies (NCT02554812 and NCT05429866) are also ongoing and may lead to the development of new ways to treat patients with SCLC (26, 27).

3.6 Adebrelimab

Adebrelimab is a novel humanized IgG4 monoclonal antibody directed against PD-L1. In the phase III CAPSTONE-1 study (28), 462 treatment-naive patients with ES-SCLC were randomized to receive adebrelimab+ chemotherapy (n = 230) or placebo+

chemotherapy (n = 232). The results presented at the American Association for Cancer Research annual meeting in April 2022 showed that adebrelimab significantly improved OS compared with chemotherapy, with a median OS of 15.3 and 12.8 months (HR = 0.72, P = 0.0017), respectively, and PFS of 5.8 months and 5.6 months (HR = 0.67, 95% CI 0.54–0.83), respectively. Grade 3 or higher treatment-related AEs occurred in 85.7% and 84.9% of the patients in the two groups, respectively. Hematologic toxicity most common (≥5%) AE in the two groups. At present, chemotherapy is still the main treatment for SCLC. The wide application of immunotherapy and the emergence of various adjuvant therapies is expected lead to new treatment methods that will overcome the problem of chemoresistance.

4 Summary and prospect

With the development of molecular biology, several candidate therapeutic targets for SCLC have been reported including poly ADP-ribose polymerase (PARP), enhancer of zeste homologue 2 (EZH2), and delta-like ligand 3 (DLL3). ICIs combined with PARP inhibitors and DLL3-targeted antibody conjugated drugs will become a new direction for the treatment of drug-resistant SCLC (57).

Trilaciclib is a selective, reversible cyclin-dependent kinase 4 and 6 (CDK4/6) inhibitor that reduces bone marrow hematopoietic stem cell depletion during treatment and protects the immune system (58). Based on the results of three phase II clinical trials (NCT03041311, NCT02514447, NCT02499770) (29–31), the U.S. FDA approved Trilaciclib in February 2021 before treatment with platinum-based/etoposide or topotecan-based regimens in adult patients with ES-SCLC to reduce the incidence of chemotherapy-induced myelosuppression (59).

The advent of ICIs has facilitated major breakthroughs in the first- and third-line treatment of SCLC, which is gradually changing the overall therapeutic landscape. ICI monotherapy and combination therapy are now the standard treatment options for patients with SCLC. Extensive research on the immune mechanism and tumor microenvironment has led to a gradual standardization of combined immunotherapy, As an emerging research hotspot, it is hoped that future studies will lead to diversification of strategies using ICIs in combinations that will improve their therapeutic effects in SCLC patients.

Author contributions

All authors contributed to the article and approved the submitted version.

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.

Publisher's note

References

1. van Meerbeeck JP, Fennell DA, De Ruysscher DKM. Small-cell lung cancer. *Lancet* (2011) 378(9804):1741–55. doi: 10.1016/S0140-6736(11)60165-7

2. George J, Lim JS, Jang SJ, Cun Y, Ozretic L, Kong G, et al. Comprehensive genomic profiles of small cell lung cancer. *Nature* (2015) 524(7563):47–53. doi: 10.1038/nature14664

3. Kottschade LA. The future of immunotherapy in the treatment of cancer. *Semin Oncol Nurs* (2019) 35(5):150934. doi: 10.1016/j.soncn.2019.08.013

4. Yao S, Zhu Y, Chen L. Advances in targeting cell surface signalling molecules for immune modulation, nature reviews. *Drug Discovery* (2013) 12(2):130–46. doi: 10.1038/nrd3877

5. Hellmann MD, Callahan MK, Awad MM, Calvo E, Ascierto PA, Atmaca A, et al. Tumor mutational burden and efficacy of nivolumab monotherapy and in combination with ipilimumab in small-cell lung cancer. *Cancer Cell* (2018) 33(5):853–861.e4. doi: 10.1016/j.ccell.2018.04.001

6. Bukamur H, Katz H, Alsharedi M, Alkrekshi A, Shweihat YR, Munn NJ. Immune checkpoint inhibitor-related pulmonary toxicity: Focus on nivolumab. *South Med J* (2020) 113(11):600–5. doi: 10.14423/SMJ.0000000000001166

7. Reck M, Bondarenko I, Luft A, Serwatowski P, Barlesi F, Chacko R, et al. Ipilimumab in combination with paclitaxel and carboplatin as first-line therapy in extensive-disease-small-cell lung cancer: results from a randomized, double-blind, multicenter phase 2 trial. *Ann Oncol* (2013) 24(1):75–83. doi: 10.1093/annonc/mds213

8. Reck M, Luft A, Szczesna A, Havel L, Kim SW, Akerley W, et al. Phase III randomized trial of ipilimumab plus etoposide and platinum versus placebo plus etoposide and platinum in extensive-stage small-cell lung cancer. *J Clin Oncol* (2016) 34 (31):3740–8. doi: 10.1200/JCO.2016.67.6601

9. Ott EEPA, Hiret S, Kim D-W, Morosky A, Saraf S, Piperdi B, et al. Pembrolizumab in patients with extensive-stage small-cell lung cancer: Results from the phase ib KEYNOTE-028 study. *J Clin Oncol* (2017) 35(34):3823–9. doi: 10.1200/JCO.2017.72.5069

10. Chung HC, Lopez-Martin JA, Kao SCH, Miller WH, Ros W, Gao B, et al. Phase 2 study of pembrolizumab in advanced small-cell lung cancer (SCLC): KEYNOTE-158. *J Clin Oncol* (2018) 36:8506. doi: 10.1200/JCO.2018.36.15_suppl.8506

11. Rudin CM, Awad MM, Navarro A, Gottfried M, Peters S, Csőszi T, et al. Pembrolizumab or placebo plus etoposide and platinum as first-line therapy for extensive-stage small-cell lung cancer: Randomized, double-blind. *Phase III KEYNOTE-604 Study* (2020) 38(21):2369–79. doi: 10.1200/JCO.20.00793

12. Available at: https://www.clinicaltrials.gov/ct2/show/NCT02580994?term=NCT02580994&draw=2&rank=1.

13. Welsh JW, Heymach JV, Chen D, Verma V, Cushman TR, Hess KR, et al. Phase I trial of pembrolizumab and radiation therapy after induction chemotherapy for extensive-stage small cell lung cancer. *J Thorac Oncol* (2020) 15(2):266–73. doi: 10.1016/j.jtho.2019.10.001

14. Available at: https://www.clinicaltrials.gov/ct2/show/NCT04624204?term=NCT04624204&draw=2&rank=1.

15. Antonia SJ, López-Martin JA, Bendell J, Ott PA, Taylor M, Eder JP, et al. Nivolumab alone and nivolumab plus ipilimumab in recurrent small-cell lung cancer (CheckMate 032): a multicentre, open-label, phase 1/2 trial. *Lancet Oncol* (2016) 17 (7):883–95. doi: 10.1016/S1470-2045(16)30098-5

16. Spigel DR, Vicente D, Ciuleanu TE, Gettinger S, Peters S, Horn L, et al. Second-line nivolumab in relapsed small-cell lung cancer: CheckMate 331(). *Ann Oncol* (2021) 32(5):631–41. doi: 10.1016/j.annonc.2021.01.071

17. Owonikoko TK, Park K, Govindan R, Ready N, Reck M, Peters S, et al. Nivolumab and ipilimumab as maintenance therapy in extensive-disease small-cell lung cancer: CheckMate. *J Clin Oncol* (2021) 39(12):1349–59. doi: 10.1200/JCO.20.02212

18. Liu SV. Updated overall survival and PD-L1 subgroup analysis of patients with extensive-stage small-cell lung cancer treated with atezolizumab, carboplatin, and etoposide (IMpower133). *J Clin Oncol* (2020) 39(6):619–30. doi: 10.1200/JCO.20.01055

19. Horn L, Mansfield AS, Szczesna A, Havel L, Krzakowski M, Hochmair MJ, et al. First-line atezolizumab plus chemotherapy in extensive-stage small-cell lung cancer. *N Engl J Med* (2018) 379(23):2220–9. doi: 10.1056/NEJMoa1809064

20. Rudin CM, Liu SV, Lu S, Soo SL, Hong MH, Lee JS, et al. SKYSCRAPER-02: primary results of a phase III, randomized, double-blind, placebo controlled study of atezolizumab (atezo) + carboplatin + etoposide (CE) with or without tiragolumab (tira) in patients (pts) with untreated extensive-stage small cell lung cancer (ES-SCLC). *J Clin Oncol* (2022) 40(suppl 17):LBA8507. doi: 10.1200/JCO.2022.40.17_suppl.LBA8507

21. Paz-Ares L, Dvorkin M, Chen Y, Reinmuth N, Hotta K, Trukhin D. Durvalumab plus platinum–etoposide versus platinum–etoposide in first-line treatment of extensive-stage small-cell lung cancer (CASPIAN): a randomised, controlled, open-label, phase 3 trial. *Lancet* (2019) 394(10212):1929–39. doi: 10.1016/S0140-6736(19)32222-6

22. Available at: https://clinicaltrials.gov/ct2/show/NCT02937818.

23. Pakkala S, Higgins K, Chen Z, Sica G, Steuer C, Zhang C, et al. Durvalumab and tremelimumab with or without stereotactic body radiation therapy in relapsed small cell lung cancer: a randomized phase II study. *J Immunother Cancer* (2020) 8(2): e001302. doi: 10.1136/jitc-2020-001302

24. Available at: https://www.clinicaltrials.gov/ct2/show/NCT03568097?term=NCT03568097&draw=2&rank=1.

25. Available at: https://www.clinicaltrials.gov/ct2/show/NCT03228667?term=NCT03228667&draw=2&rank=1.

26. Available at: https://www.clinicaltrials.gov/ct2/show/NCT02554812?term=NCT02554812&draw=2&rank=1.

27. Available at: https://www.clinicaltrials.gov/ct2/show/NCT05429866?term=NCT05429866&draw=2&rank=1.

28. Wang J, Zhou C, Yao W, Wang Q, Min X, Chen G, et al. Adebrelimab or placebo plus carboplatin and etoposide as first-line treatment for extensive-stage small-cell lung cancer (CAPSTONE-1): a multicentre, randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet Oncol* (2022) 23(6):739–47. doi: 10.1016/S1470-2045(22)00224-8

29. Daniel D, Kuchava V, Bondarenko I, Ivashchuk O, Reddy S, Jaal J, et al. Trilaciclib prior to chemotherapy and atezolizumab in patients with newly diagnosed extensive-stage small cell lung cancer: A multicentre, randomised, double-blind, placebo-controlled phase II trial. *Int J Cancer* (2020) 148(10):2557–70. doi: 10.1002/ijc.33453

30. Weiss JM, Csoszi T, Maglakelidze M, Hoyer RJ, Beck JT, Gomez MD, et al. Myelopreservation with the CDK4/6 inhibitor trilaciclib in patients with small-cell lung cancer receiving first-line chemotherapy: a phase ib/randomized phase II trial. *Ann Oncol* (2019) 30(10):1613–21. doi: 10.1093/annonc/mdz278

31. Hart LL, Ferrarotto R, Andric ZG, Beck JT, Subramanian J, Radosavljevic DZ, et al. Myelopreservation with trilaciclib in patients receiving topotecan for small cell lung cancer: Results from a randomized, double-blind, placebo-controlled phase II study. *Adv Ther* (2021) 38(1):350–65. doi: 10.1007/s12325-020-01538-0

32. Topalian SL. Targeting immune checkpoints in cancer therapy. *JAMA* (2017) 318(17):1647–8. doi: 10.1001/jama.2017.14155

33. Klein O, Ebert LM, Nicholaou T, Browning J, Russell SE, Zuber M, et al. Melan-a-specific cytotoxic T cells are associated with tumor regression and autoimmunity following treatment with anti-CTLA-4. *Clin Cancer Res* (2009) 15(7):2507–13. doi: 10.1158/1078-0432.CCR-08-2424

34. Arriola E, Wheater M, Galea I, Cross N, Maishman T, Hamid D, et al. Outcome and biomarker analysis from a multicenter phase 2 study of ipilimumab in combination with carboplatin and etoposide as first-line therapy for extensive-stage SCLC. *J Thorac Oncol* (2016) 11(9):1511–21. doi: 10.1016/j.jtho.2016.05.028

35. Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, et al. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med* (2002) 8(8):793–800. doi: 10.1038/nm730

36. Kim S-K, Wu X, Ragupathi G, Gathuru J, Koide F, Cheung N-K, et al. Impact of minimal tumor burden on antibody response to vaccination. *Cancer Immunology Immunotherapy* (2011) 60(5):621–7. doi: 10.1007/s00262-011-0975-9

37. Sul J, Blumenthal GM, Jiang X, He K, Keegan P, Pazdur R. FDA Approval summary: Pembrolizumab for the treatment of patients with metastatic non-small cell lung cancer whose tumors express programmed death-ligand 1. *Oncologist* (2016) 21 (5):643–50. doi: 10.1634/theoncologist.2015-0498

38. Chung HC, Piha-Paul SA, Lopez-Martin J, Schellens JHM, Kao S, Miller WHJr., et al. Pembrolizumab after two or more lines of previous therapy in patients with recurrent or metastatic SCLC: Results from the KEYNOTE-028 and KEYNOTE-158 studies. *J Thorac Oncol* (2020) 15(4):618–27. doi: 10.1016/j.jtho.2019.12.109

39. M.S.D. Corp. *KEYTRUDA® (pembrolizumab) full prescribing information*. Whitehouse Station, NJ: Merck Sharp & Dohme Corp (2019).

40. Kareva I. A combination of immune checkpoint inhibition with metronomic chemotherapy as a way of targeting therapy-resistant cancer cells, international journal of molecular sciences 18(10). *Int J Mol Sci* (2017) 18(10):2134. doi: 10.3390/ijms18102134

41. Chen D, Barsoumian HB, Yang L, Younes AI, Verma V, Hu Y, et al. SHP-2 and PD-L1 inhibition combined with radiotherapy enhances systemic antitumor effects in an anti-PD-1-Resistant model of non-small cell lung cancer. *Cancer Immunol Res* (2020) 8(7):883–94. doi: 10.1158/2326-6066.CIR-19-0744

42. Chen D BH, Yang L, Younes AI, Verma V, Hu Y, Menon H, et al. *Oncotarget* Consolidative thoracic radiotherapy for extensive stage small cell lung cancer. (2017) 8 (13):22251–261. doi: 10.18632/oncotarget.14759

43. Blanquicett C, Saif MW, Buchsbaum DJ, Eloubeidi M, Vickers SM, Chhieng DC, et al. Antitumor efficacy of capecitabine and celecoxib in irradiated and lead-shielded, contralateral human BxPC-3 pancreatic cancer xenografts: clinical implications of abscopal effects. *Clin Cancer Res* (2005) 11(24 Pt 1):8773–81. doi: 10.1158/1078-0432.CCR-05-0627

44. Deng L, Liang H, Burnette B, Beckett M, Darga T, Weichselbaum RR, et al. Irradiation and anti-PD-L1 treatment synergistically promote antitumor immunity in mice. *J Clin Invest* (2014) 124(2):687–95. doi: 10.1172/JCI67313

45. Deng L, Liang H, Burnette B, Weicheslbaum RR, Fu YX. Radiation and anti-PD-L1 antibody combinatorial therapy induces T cell-mediated depletion of myeloid-derived suppressor cells and tumor regression. *Oncoimmunology* (2014) 3:e28499. doi: 10.4161/onci.28499

46. Patel SH, Rimner A, Cohen RB. Combining immunotherapy and radiation therapy for small cell lung cancer and thymic tumors. *Transl Lung Cancer Res* (2017) 6 (2):186–95. doi: 10.21037/tlcr.2017.03.04

47. Slotman BJ, van Tinteren H, Praag JO, Knegjens JL, El Sharouni SY, Hatton M, et al. Use of thoracic radiotherapy for extensive stage small-cell lung cancer: a phase 3 randomised controlled trial. *Lancet* (2015) 385(9962):36–42. doi: 10.1016/S0140-6736(14)61085-0

48. Kalemkerian GP, Loo BW, Akerley W, Attia A, Bassetti M, Boumber Y. NCCN guidelines insights: Small cell lung cancer, version 2.2018. *J Natl Compr Canc Netw* (2018) 16(10):1171–82. doi: 10.6004/jnccn.2018.0079

49. Ready N, Farago AF, de Braud F, Atmaca A, Hellmann MD, Schneider JG, et al. Third-line nivolumab monotherapy in recurrent SCLC: CheckMate 032. *J Thorac Oncol* (2019) 14(2):237–44. doi: 10.1016/j.jtho.2018.10.003

50. Wei SC, Duffy CR, Allison JP. Fundamental mechanisms of immune checkpoint blockade therapy. *Cancer Discovery* (2018) 8(9):1069–86. doi: 10.1158/2159-8290.CD-18-0367

51. Mansfield AS, Kazarnowicz A, Karaseva N, Sanchez A, De Boer R, Andric Z, et al. Safety and patient-reported outcomes of atezolizumab, carboplatin, and etoposide in extensive-stage small-cell lung cancer (IMpower133): a randomized phase I/III trial. *Ann Oncol* (2020) 31(2):310–7. doi: 10.1016/j.annonc.2019.10.021

52. Stewart R, Morrow M, Hammond SA, Mulgrew K, Marcus D, Poon E, et al. Identification and characterization of MEDI4736, an antagonistic anti-PD-L1 monoclonal antibody. *Cancer Immunol Res* (2015) 3(9):1052–62. doi: 10.1158/2326-6066.CIR-14-0191

53. Curran MA, Montalvo W, Yagita H, Allison JP. PD-1 and CTLA-4 combination blockade expands infiltrating T cells and reduces regulatory T and myeloid cells within B16 melanoma tumors. *Proc Natl Acad Sci U.S.A.* (2010) 107(9):4275–80. doi: 10.1073/pnas.0915174107

54. Passiglia F, Galvano A, Gristina V, Barraco N, Castiglia M, Perez A, et al. Is there any place for PD-1/CTLA-4 inhibitors combination in the first-line treatment of advanced NSCLC?–a trial-level meta-analysis in PD-L1 selected subgroups. *Trans Lung Cancer Res* (2021) 10(7):3106–19. doi: 10.21037/tlcr-21-52

55. Gristina V, Galvano A, Castellana L, Insalaco L, Cusenza S, Graceffa G, et al. Is there any room for PD-1 inhibitors in combination with platinum-based chemotherapy as frontline treatment of extensive-stage small cell lung cancer? a systematic review and meta-analysis with indirect comparisons among subgroups and landmark survival analyses. *Ther Adv Med Oncol* (2021) 13:17588359211018018. doi: 10.1177/17588359211018018

56. Goldman JW, Dvorkin M, Chen Y, Reinmuth N, Hotta K, Trukhin D. Durvalumab, with or without tremelimumab, plus platinum–etoposide versus platinum–etoposide alone in first-line treatment of extensive-stage small-cell lung cancer (CASPIAN): updated results from a randomised, controlled, open-label, phase 3 trial. *Lancet Oncol* (2021) 22(1):51–65. doi: 10.1016/S1470-2045(20)30539-8

57. Saito M, Shiraishi K, Goto A, Suzuki H, Kohno T, Kono K. Development of targeted therapy and immunotherapy for treatment of small cell lung cancer. *Jpn J Clin Oncol* (2018) 48(7):603–8. doi: 10.1093/jjco/hyy068

58. Reck M, Liu SV, Mansfield AS, Mok TSK, Scherpereel A, Reinmuth N, et al. IMpower133: Updated overall survival (OS) analysis of first-line (1L) atezolizumab (atezo) + carboplatin + etoposide in extensive-stage SCLC (ES-SCLC). *Ann Oncol* (2019) 30:v710–1. doi: 10.1093/annonc/mdz264

59. G1 Therapeutics Inc. *COSELA™ (trilaciclib). prescribing information* (2021). Available at: https://www.g1therapeutics.com/cosela/pi/.

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T cell-derived exosomes in tumor immune modulation and immunotherapy

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Exosomes are nanoscale vesicles secreted by most cells and have a phospholipid bilayer structure. Exosomes contain DNA, small RNA, proteins, and other substances that can carry proteins and nucleic acids and participate in communication between cells. T cells are an indispensable part of adaptive immunity, and the functions of T cell-derived exosomes have been widely studied. In the more than three decades since the discovery of exosomes, several studies have revealed that T cell-derived exosomes play a novel role in cell-to-cell signaling, especially in the tumor immune response. In this review, we discuss the function of exosomes derived from different T cell subsets, explore applications in tumor immunotherapy, and consider the associated challenges.

KEYWORDS

T cell, exosome, tumor, cancer, immune modulation, immunotherapy

1 Introduction

Exosomes are nanoscale vesicles (30–160 nm) secreted by most cells and have a phospholipid bilayer structure (1). Exosomes contain DNA, small RNA, proteins, and other substances that can carry proteins and nucleic acids and participate in communication between cells (2). Previous studies have suggested that exosomes function as cellular garbage bags, eliminating redundant and non-functional cellular components (3). Recent studies have shown that exosomes are intercellular junctions that transport proteins, lipids, and nucleic acids to target cells, play a role in various biological processes (such as angiogenesis, antigen presentation, apoptosis, and inflammation), and can be used as diagnostic and therapeutic tools for diseases (4). It can also participate in various pathophysiological processes such as tissue repair, immune response, inflammation, and tumor growth and metastasis (5, 6).

T-lymphocytes are derived from pluripotent stem cells in the bone marrow (7). During the embryonic and primary stages of human life, pluripotent stem cells or proT cells in the bone marrow migrate to the thymus and mature into immunoactive T cells under the induction of thymus hormones (8). Intercellular communication is an essential hallmark of multicellular organisms and can be mediated through direct cell-cell contact or the transfer of secreted molecules (9). Increasing studies have shown that immune cells participate in cellular communication by secreting exosomes (10, 11). Among the immune cell-derived exosomes, T cell-derived exosomes have recently been reported to be involved in antitumor effects in cancer immunotherapy by mimicking the role of parental cells (12–15). The upregulation and downregulation of exosome production by T cells is a new method for regulating the immune response to tumors (16). Therefore, fully exploiting the characteristics of T cell-derived exosomes can effectively treat tumors. In this review, we summarize the pathogenesis and secretion of exosomes and describe the role of T cell-derived exosomes in tumor immune regulation and the application of T cell-derived exosomes in tumor immunotherapy to provide new ideas for the future treatment of cancers.

2 Biogenesis and secretion of exosomes

Exosomes are intraluminal vesicles (ILVs) formed by inward budding of the endosomal membrane during maturation of multivesicular bodies (MVBs). Subsequently, MVBs fuse with the plasma membrane to release the contained ILVs as exosomes or fuse with lysosomes or autophagosomes for degradation (17) (Figure 1). Various sorting mechanisms are involved in different steps of exosome formation (18). First, the limited membrane regions of MVBs are generally referred to as the dispersed microdomains. The formation of the cluster microdomain and the external mechanical action promote membrane budding, followed by the division of the plasma membrane into the extracellular medium or the limiting membrane of MVBs into the MVB lumen. Currently, the mechanism of exosome formation is well understood, and the subunits involved in the endosomal sorting complex required for transport (ESCRT) play an important role (19, 20). When ILVs enter the lumen of MVBs, the involvement of ESCRT-III is required to varying degrees; however, the processes of inclusion aggregation and membrane budding are not entirely dependent on ESCRT (21, 22). ESCRT-independent pathways have also been identified as alternative mechanisms and may coexist with ESCRT-dependent machinery in the formation of MVBs and sorting of internalized cargo (23, 24). Exosome production is complex and often depends on the host and the type of parent cell as well as other stimuli received by the cell. These inclusions participate in the germination, fission, and release of exosomes through progressive aggregation (25). In addition, the properties and content of exosome inclusions are specific and are often influenced by the physiological or pathological state of the maternal cell, stimuli that regulate their production and release, and molecular mechanisms that facilitate their production (2).

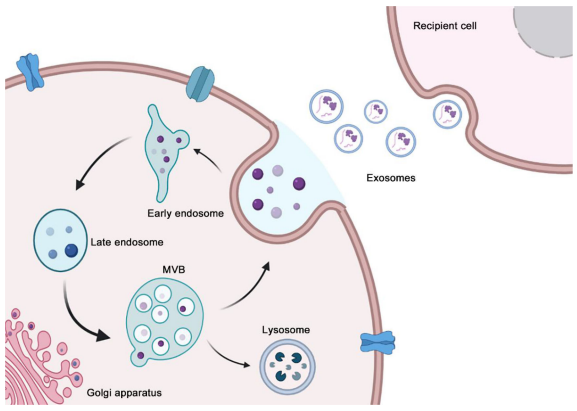

FIGURE 1
The process of exosome biogenesis and secretion. The biogenesis of exosome begins at early endosome formation through endocytosis at the plasma membrane, and then the invagination of the plasma membrane of LSEs forms ILVs that are ultimately secreted as exosomes. In the end, MVBs fuse with the plasma membrane to release exosomes. Ectosome originates from the outward budding and fission of the plasma membrane, subsequently, the nascent ectosomes are released into the extracellular space.

3 T cell-derived exosomes in tumor immune modulation

Similar to other cells, T cells produce exosomes that reflect their characteristics, such as directly killing target cells, assisting or inhibiting B cells to produce antibodies, responding to specific antigens and mitogens, and producing cytokines, thereby creating an optimal microenvironment for immune cell function in paracrine and autocrine forms (26). T cell-derived exosomes can activate other immune cells, suppress immune responses, and participate in the licensing of antigen-presenting cells (APCs) (26). In a recent study, researchers attached interleukin (IL)-2 to the transmembrane domain of platelet-derived growth factor receptor *via* a flexible linker and then incorporated the gene into lentiviruses for Jurkat T cell infection. The infected Jurkat T cells then secreted IL-2-exosomes, which showed significant changes in the expression of miR-181a-3p and miR-223-3p in IL-2-exosomes relative to untreated exosomes. miRNAs increase the activity of $CD8^+$ T cells and decrease the expression of programmed death ligand 1 (PD-L1) in melanoma, resulting in increased sensitivity to $CD8^+$ T cell-mediated cytotoxicity (27). T cells can regulate the release of distinct exosome subpopulations depending on their activation status (28). In the following sections, we discuss the role of different T cell subsets in tumor immunomodulation (Table 1).

3.1 $CD8^+$ T cell-derived exosomes

$CD8^+$ T cells are cytotoxic T lymphocytes (CTLs), a subset of white blood cells that secrete various cytokines to specifically kill target cells. It can remove virus-infected cells, tumor cells, and other

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TABLE 1 Role of T cell-derived exosomes in immune modulation.

The origin of exosomes	Mechanism of action	Content	Reference
$CD8^+$ T cells	Attenuating PD-L1-induced suppression of tumor-specific cytotoxic T cell activity	PD-1	(32)
$CD8^+$ T cells	Associated with the expression of immune checkpoint receptors on the surface of $CD8^+$ T cells	uPAR	(36)
$CD8^+$ T cells	Inhibited antitumor effect by decreasing the MHC-I in DCs and $CD8^+$ T cell activity	LFA-1	(38)
$CD8^+$ T cells	Limiting estrogen-driven development of UCEC *via* regulation of the miR-765/PLP2 axis	miR-765	(39)
$CD8^+$ T cells	Mediating depletion of mesenchymal tumor stromal cells	–	(14)
Exhausted $CD8^+$ T cells	Impairing the anticancer function of normal $CD8^+$ T cells	lncRNAs	(40)
$CD8^+$ T cells	Activating ERK and NF-κB pathways to induce melanoma metastasis	FasL	(41)
$CD4^+$ T cells	Inducing $CD8^+$ T cell-mediated antitumor responses	miR-25-3p, miR-155-5p, miR-215-5p, and miR-375	(43)
$CD4^+$ T cells	Inhibiting $CD8^+$ cytotoxic T lymphocyte responses and antitumor immunity in melanoma	LFA-1	(44)
$CD4^+$ T cells	Involving in the regulation of humoral immunity	CD40L	(45)
Tregs	As a potential non-invasive tumor and immune cell biomarkers in HNSCC	–	(54)
Tregs	Resulting the production of a tolerogenic phenotype in DCs	miR-150-5p and miR-142-3p	(55)
$CD8^+$ $CD25^+$ Tregs	Inhibiting DC-induced cytotoxic T lymphocyte responses and antitumor immunity	–	(56)
Tregs	Promoting the expression of M2 macrophage markers	–	(57)
Tregs	Inhibiting the proliferation of $CD4^+$ T cells	miR-146a-5p	(63)

antigenic substances and is an important defense line of antiviral and antitumor immunity (29). An increasing number of studies have revealed that $CD8^+$ T cell-derived exosomes mediate information exchange between immune cells and tumor cells, thereby regulating tumor development. $CD8^+$ CTLs fully activated by tumor antigens enhance the activation of low-affinity $CD8^+$ T cells by secreting exosomes, thus participating in the tumor killing process (30, 31). For instance, Qiu et al. (32). found that programmed cell death 1 (PD-1), which is widely expressed in tumor-infiltrating lymphocytes of triple-negative breast cancer (TNBC) and is significantly associated with poor prognosis of TNBC (33, 34), can be secreted by activated T cells on the surface of exosomes, interacting remotely with PD-L1 on the cell surface or exosomes, and restoring tumor surveillance by attenuating PD-L1-induced suppression of tumor-specific cytotoxic T cell activity. In another clinical study, considering the effect of urokinase-type plasminogen activator (uPAR) signaling on tumors (35), Porcelli et al. collected blood samples from 71 patients with metastatic melanoma treated with immune checkpoint inhibitors (including responders and non-responders) and analyzed $CD8^+$ T cell-derived $uPAR^+$ exosome levels. The results of this study indicated that patients with immune checkpoint inhibitor-resistant melanoma had low levels of $CD8^+$ T cell-derived $uPAR^+$ exosomes in their blood (36). These findings suggest that $CD8^+$ T cell-derived $uPAR^+$ exosomes are associated with the expression of immune checkpoint receptors on the surface of $CD8^+$ T cells, which is a direction for future research. The above studies provide a potential therapeutic strategy for modifying the exosome surface with membrane-bound inhibitory immune checkpoint receptors to attenuate the suppressive tumor immune microenvironment. Interestingly, $CD8^+$ T cell-derived exosomes can also be endocytosed by APCs, cells in the body that can ingest, process, and transfer antigen information to induce the immune response of T and B cells (37), *via* pMHC-I/TCR interactions, and inhibit antigen-specific dendritic cell (DC)-mediated indirect $CD8^+$ CTL responses (38). Specifically, exosomes derived from activated $CD8^+$ T cells inhibited antitumor effects by decreasing MHC-I in DCs and $CD8^+$ T cell activity in melanoma models (38). In addition to participating in the regulation of tumor growth by mediating information exchange between immune cells, $CD8^+$ T cell-derived exosomes directly inhibit tumor progression. For example, Zhou et al. found that $CD45RO^-CD8^+$ T cell-derived exosomes released more miR-765 than $CD45RO^+CD8^+$ T cells. These exosomes miR-765 derived from $CD45RO^-CD8^+$ T cells limit estrogen-driven development of uterine corpus endometrial cancer (UCEC) *via* regulation of the miR-765/proteolipid protein 2 (PLP2) axis (39). Additionally, $CD8^+$ T cells can inhibit tumor progression by exosome-mediated depletion of mesenchymal tumor stromal cells, in addition to

their conventional direct cytotoxicity against tumor cells (14). The above studies support the idea that $CD8^+$ T cell-derived exosomes are involved in the inhibition of tumor progression. However, $CD8^+$ T cell-derived exosomes play a double-edged sword in tumorigenesis and development.

Wang et al. found that exosomes derived from exhausted $CD8^+$ T cells can be taken up by normal $CD8^+$ T cells and impair their proliferation (Ki67) and cell activity (CD69) and the production of cytokines such as IFN-γ and IL-2, impairing the anticancer function of normal $CD8^+$ T cells, causing tumor progression (40). The research team further used microarray and functional enrichment analyses to identify 257 lncRNAs that actively participate in various processes regulating the activity of $CD8^+$ T cells, such as metabolism, gene expression, and biosynthesis processes (40). Notably, in the above content, we demonstrated that $CD8^+$ T cell-derived exosomes can activate $CD8^+$ T cells with low affinity, which is contrary to the conclusion of this study. This can be attributed to the differences in $CD8^+$ T cell subsets and activation states. In addition, $CD8^+$ T cell-derived exosomes have been reported to be involved in directly promoting tumor progression, which is inconsistent with the function of the corresponding source cells. Exosomes from activated $CD8^+$ T cells were shown to activate the ERK and NF-κB pathways in melanoma cells, leading to melanoma metastasis *in vivo* by increasing the expression of MMP9 *via* Fas signaling, suggesting a role for $CD8^+$ T cell-derived exosomes in tumor immune escape (41). Owing to the dual role of $CD8^+$ T cell-derived exosomes in tumor progression, tumor therapy strategies targeting exosomes need to consider the balancing mechanism involved.

3.2 $CD4^+$ T cell-derived exosomes

T cells can be divided into various subsets based on their immunophenotypes, mainly $CD4^+$ T helper cells and cytotoxic $CD8^+$ T cells. $CD4^+$ T cells can be further divided into Th1, Th2, Th9, Th17, Th22, follicular helper T cells, and regulatory T cells (Tregs), each of which produce specific effector cytokines under unique transcriptional regulation (42). $CD4^+$ T cells interact with other cells, such as NK cells, macrophages, and $CD8^+$ T cells, through the cytokines they produce. Shin et al. revealed that $CD4^+$ T cell-derived exosomes increased the antitumor response of $CD8^+$ T cells without affecting Tregs, thereby suppressing melanoma growth. Mechanistically, miR-25-3p, miR-155-5p, miR-215-5p, and miR-375 within $CD4^+$ T cell-derived exosomes are responsible for inducing $CD8^+$ T cell-mediated antitumor responses (43). This further supports the notion that exosomes are a novel form of $CD8^+$ T cell activation by $CD4^+$ T cells in addition to cytokines. However, the opposite was observed in another study, which suggested that exosomes released by $CD4^+$ T cells inhibited $CD8^+$ CTL responses and antitumor immunity in melanoma (44). It is worth considering whether this opposite result is caused by the heterogeneity of exosomes and whether there is a balancing mechanism.

In addition to influencing cellular immunity, $CD4^+$ T cell-derived exosomes are involved in the regulation of humoral immunity (45). In this study, mice vaccinated with the hepatitis B surface antigen (HBsAg) vaccine showed a stronger humoral immune response to $CD4^+$ T-cell-derived exosomes, indicating higher serum levels of hepatitis B surface antibody (HBsAb) (45). Additionally, $CD4^+$ T cell-derived exosomes play an important role in B cell responses *in vitro*, which significantly promotes B cell activation, proliferation, and antibody production (45). It is well known that hepatitis B virus is the main cause of hepatocellular carcinoma (46–48), and the synergistic effect of $CD4^+$ T cell-derived exosomes on HBsAb may contribute to the inhibition of hepatocellular carcinoma. Further research is required to confirm this hypothesis.

3.3 Treg-derived exosomes

Tregs are a group of lymphocytes that negatively regulate the immune response of the body and participate in tumor cells to evade immune surveillance (49). Owing to the significant immunosuppressive effects of Treg-derived exosomes, an increasing number of studies have focused on their role in tumor immune escape (50). Interestingly, Tregs have been reported to secrete more exosomes that express CD25, CTLA-4, and CD73 on the surface than other T cells. Exosomes expressing CD73 perform immunosuppressive functions by producing adenosine, which plays an important role in the anti-inflammatory response (51–53). In a recent phase I clinical trial, 18 patients with head and neck squamous cell carcinoma who received a combination of cetuximab, ipilimumab, and radiation therapy were serially monitored for Treg-derived exosomes, and Treg-derived exosomes were found to increase from the baseline levels (54), supporting the potential role of Treg-derived exosomes as non-invasive tumor and immune cell biomarkers in cancer. To promote clinical translation, researchers have further carried out relevant basic research. Tung et al. demonstrated for the first time that miRNAs, particularly miR-150-5p and miR-142-3p, are transferred from Tregs to DCs *via* Treg-derived exosomes, resulting in the production of a tolerogenic phenotype in DCs (55). Similarly, Xie et al. found that exosomes derived from natural $CD8^+$ $CD25^+$ Tregs significantly inhibited DC-induced CTL responses and antitumor immunity in a mouse B16 melanoma model (56). In addition to DCs, Treg-derived exosomes inhibit the expression of M1 macrophage markers and promote M2 macrophage markers (57). Macrophages are divided into classically activated M1 macrophages, which mainly exert anti-inflammatory and antitumor functions (58), and alternately activated M2 macrophages, which have immunosuppressive and tumor-promoting abilities (59). Therefore, induction of M2 macrophages by Treg-derived exosomes may promote tumor growth. Immunosuppression of Tregs mainly inhibits the activation and proliferation of $CD4^+$ and $CD8^+$ T cells (60). Studies have shown that exosomes derived from Tregs suppress T-cell proliferation (61, 62). In addition, Torri et al. revealed the inhibition of $CD4^+$ T cell proliferation by Treg-derived exosomes (63). However, these studies have not yet confirmed the role of

Treg-derived exosome-mediated immunosuppression of infiltrating T lymphocytes in tumor progression, which remains to be explored further.

4 T cell-derived exosomes in tumor immunotherapy

4.1 Engineered T cell-derived exosomes

Engineered exosomes mainly refer to modified exosomes with enhanced drug-loading efficiency, targeting, and resistance to body clearance after natural exosomes are treated with bioengineering techniques. Usually, the size and shape of these exosomes do not change significantly (64–67), but their membrane loaders or contents may differ significantly depending on the research purpose. Studies have shown that the clinical therapeutic effect of exosomes can be improved by changing their contents and surface substances to improve their targeting and drug-loading rate. For example, Lou et al. constructed an miR-199a-modified engineered exosome through genetic engineering and found that it could effectively transfer miR-199a to liver cancer cells. The miR-199a-modified engineered exosomes significantly increased the sensitivity of liver cancer cells to Adriamycin *in vitro*. It can also significantly promote the antitumor effect of Adriamycin in liver cancer *in vivo* (68). Another example is the loading of siRNA and oxaliplatin into bone marrow mesenchymal stem cell-derived exosomes *via* electroporation, which blocks the connection of tumor cells to macrophages, thus inhibiting the polarization of macrophages in the tumor microenvironment (69). Jung et al. generated IL-2-tethered exosomes from engineered Jurkat T cells expressing IL-2 at the plasma membrane *via* a flexible linker to induce an autocrine effect. Levels of miRNA in T cell-derived exosomes using IL-2 surface engineering were significantly altered, and differentially expressed miRNAs activated $CD8^+$ T cells, enhancing their antitumor immune effects (27). Therefore, strengthening immune activity through engineering modification of $CD4^+$ T cells and $CD8^+$ T cell-derived exosomes is a novel strategy to improve the efficacy of tumor immunotherapy.

4.2 Depleting exosomes or blocking the uptake of exosomes

Given the role of Treg-derived exosomes and some $CD8^+$ T cell-derived exosomes in tumor immune escape, depleting exosomes or blocking their uptake may be a novel cancer immunotherapy (70). The Aethlon ADAPT^{TM} system, a novel device that can remove blood components below 200 nm, including exosomes that interact with the immobilized affinity agent of the device, was successfully applied for the first time in patients with hepatitis C virus. It could be speculated that if the Aethlon ADAPT^{TM} system is used to eliminate immunosuppressive exosomes from T cells, it may improve the efficacy of antitumor immunotherapy.

4.3 Chimeric antigen receptor T cell-derived exosomes

Chimeric antigen receptor (CAR) T cells conjugate the antigen-binding part of an antibody that recognizes a tumor antigen and the intracellular part of the CD3-ζ chain or FcεRIγ into a chimeric protein *in vitro*, and patient T cells are then transfected with gene transduction to express CAR (71). Patient T cells are "reprogrammed" to generate a large number of tumor-specific CAR-T cells, which have been successfully designed and used to treat malignant blood diseases (72). However, in the process of treating malignant tumors, CAR-T therapy inevitably has side effects such as cytokine release syndrome, neurotoxicity, and organ failure (73, 74). The management of CAR-T cell toxicity remains a challenge.

CAR-T cell-derived exosomes have been reported to reduce the cytotoxicity of CAR-T therapy and cross the blood-brain barrier and blood-tumor barrier (13). CAR-T cell-derived exosomes express high levels of cytotoxic molecules (FasL, Apo2L, perforin, and grazyme A and B), making them effective vectors to provide pro-apoptotic cues to target tumor cells carrying homologous antigens (75). Several preclinical studies have confirmed that CAR-T cell-derived exosomes exert inhibitory effects on solid tumors, including TNBC and lung cancer, and are relatively safe (13, 76, 77). The mechanism of tumor apoptosis induced by CAR-T cell-derived exosomes is independent of FasL, Apo2L, perforin, and grazyme. A recent study demonstrated that CAR T cells contain RNA components of the tumor-suppressive signal-recognition particle 7SL1 (RN7SL1), a non-coding RNA that activates interferon-IFN stimulator genes (78). Notably, RN7SL1 is selectively transferred to immune cells *via* CAR-T cell-derived exosomes, restricting the development of bone marrow-derived suppressor cells and enhancing the immunostimulatory properties of DCs, thus effectively activating melanoma with endogenous $CD8^+$ T cells that reject CAR antigens (78). Additionally, anticancer drugs can be loaded into exosomes from CAR-T cells to kill target tumor cells because of their excellent potential to penetrate the extracellular matrix of solid tumors (79). The above studies have shown that activated CAR-T cells can secrete exosomes to function in solid tumors and can affect the immune microenvironment of tumors; however, the current study seems to have failed to conclude whether CAR-T cell-derived exosomes play a role in hot or cold tumors (Figure 2).

5 Conclusions

This review summarized the role of $CD8^+$ T cells, $CD4^+$ T cells, and Treg-derived exosomes in tumor immune modulation and revealed the potential application of T cell-derived exosomes in tumor immunotherapy, including engineered T cell-derived exosomes, depleting exosomes, or blocking the uptake of exosomes and CAR-T cell-derived exosomes. However, studies on T cell-derived exosomes remain in the exploratory stage. There are still many hurdles to overcome before T cell-derived exosomes can

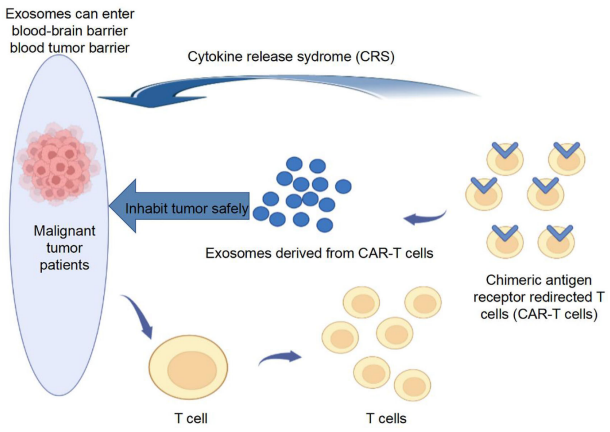

FIGURE 2
CAR-T cell-derived exosomes for tumor treatment. CAR-T cell-derived exosomes express high levels of cytotoxic molecule, making them effective vectors to provide pro-apoptotic cues to target tumor cells carrying homologous antigens.

transition from the laboratory to the clinic. First, the purification and characterization methods of exosomes vary from laboratory to laboratory, and different methods may confuse the subgroups and physicochemical properties of exosomes. Therefore, researchers need to refer to the International Society of Extracellular Vesicles and the standardization efforts for exosome isolation, purification, and use for therapeutics. The second problem is exosome production. The number of exosomes extracted from cells is small and it is often difficult to meet the requirements of drug delivery. Therefore, to continue expanding the applications of exosomes, a large-scale production mode is needed. In addition, the stability and toxicity of exosomes after modification or drug loading need to be further explored, especially as vectors for tumor nanomedical applications. These findings will facilitate clinical transformation of exosomes (80). Additionally, the best exosome therapy candidate payload is currently inconclusive and needs to be explored further in the future.

Exosomes have many advantages over other drug delivery systems, especially their high stability, low immunogenicity, ability to avoid clearance by mononuclear phagocytes, good biocompatibility, high bioactivity, and high targeting efficiency. We believe that with the joint efforts of immunologists, molecular biologists, chemists, and physicians, T cell-based exosomes will become a powerful tool in the fight against tumors in the future.

Author contributions

QZ and SW wrote the manuscript and created the figures. HW, YL and SF collected and prepared the related papers. YC and CW conceived the final approval of the version to be submitted and obtaining of the funding. All authors read and approved the final manuscript.

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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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References

1. Théry C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the international society for extracellular vesicles and update of the MISEV2014 guidelines. *J Extracellular Vesicles* (2018) 7:1535750. doi: 10.1080/20013078.2018.1535750

2. Buzas EI. The roles of extracellular vesicles in the immune system. *Nat Rev Immunol* (2023) 4:236–250. doi: 10.1038/s41577-022-00763-8

3. Buschow SI, Nolte-'t Hoen EN, van Niel G, Pols MS, ten Broeke T, Lauwen M, et al. MHC II in dendritic cells is targeted to lysosomes or T cell-induced exosomes *via* distinct multivesicular body pathways. *Traffic* (2009) 10:1528–42. doi: 10.1111/j.1600-0854.2009.00963.x

4. Luo X, Li Y, Hua Z, Xue X, Wang X, Pang M, et al. Exosomes-mediated tumor metastasis through reshaping tumor microenvironment and distant niche, J control release. (2022) 353:327–336. doi: 10.1016/j.jconrel.2022.11.050

5. Isaac R, Reis FCG, Ying W, Olefsky JM. Exosomes as mediators of intercellular crosstalk in metabolism. *Cell Metab* (2021) 33:1744–62. doi: 10.1016/j.cmet.2021.08.006

6. Mathivanan S, Fahner CJ, Reid GE, Simpson RJ. ExoCarta 2012: database of exosomal proteins, RNA and lipids. *Nucleic Acids Res* (2012) 40(2012):D1241–1244. doi: 10.1093/nar/gkr828

7. Pramanik A, Bhattacharyya S. Myeloid derived suppressor cells and innate immune system interaction in tumor microenvironment. *Life Sci* (2022) 305:120755. doi: 10.1016/j.lfs.2022.120755

8. Kyrysyuk O, Wucherpfennig KW. Designing cancer immunotherapies that engage T cells and NK cells. *Annu Rev Immunol* (2022). doi: 10.1146/annurev-immunol-101921-044122

9. Singer SJ. Intercellular communication and cell-cell adhesion. *Science* (1992) 255:1671–7. doi: 10.1126/science.1313187

10. Hazrati A, Soudi S, Malekpour K, Mahmoudi M, Rahimi A, Hashemi SM, et al. Immune cells-derived exosomes function as a double-edged sword: role in disease progression and their therapeutic applications. *biomark Res* (2022) 10:30. doi: 10.1186/s40364-022-00374-4

11. Alahdal M, Elkord E. Promising use of immune cell-derived exosomes in the treatment of SARS-CoV-2 infections. *Clin Transl Med* (2022) 12:e1026. doi: 10.1002/ctm2.1026

12. Wahlgren J, Karlson Tde L, Glader P, Telemo E, Valadi H. Activated human T cells secrete exosomes that participate in IL-2 mediated immune response signaling. *PloS One* (2012) 7:e49723. doi: 10.1371/journal.pone.0049723

13. Fu W, Lei C, Liu S, Cui Y, Wang C, Qian K, et al. CAR exosomes derived from effector CAR-T cells have potent antitumour effects and low toxicity. *Nat Commun* (2019) 10:4355. doi: 10.1038/s41467-019-12321-3

14. Seo N, Shirakura Y, Tahara Y, Momose F, Harada N, Ikeda H, et al. Activated CD8(+) T cell extracellular vesicles prevent tumour progression by targeting of lesional mesenchymal cells. *Nat Commun* (2018) 9:435. doi: 10.1038/s41467-018-02865-1

15. Hong J, Kang M, Jung M, Lee YY, Cho Y, Kim C, et al. T-Cell-Derived nanovesicles for cancer immunotherapy. *Adv Mater* (2021) 33:e2101110. doi: 10.1002/adma.202101110

16. Shao Y, Pan X, Fu R. Role and function of T cell-derived exosomes and their therapeutic value. *Mediators Inflammation* (2021) 2021:8481013. doi: 10.1155/2021/8481013

17. Huang M, Peng X, Yang L, Yang S, Li X, Tang S, et al. Non-coding RNA derived from extracellular vesicles in cancer immune escape: Biological functions and potential clinical applications. *Cancer Lett* (2021) 501:234–46. doi: 10.1016/j.canlet.2020.11.005

18. van Niel G, D'Angelo G, Raposo G. Shedding light on the cell biology of extracellular vesicles. *Nat Rev Mol Cell Biol* (2018) 19:213–28. doi: 10.1038/nrm.2017.125

19. Shehzad A, Islam SU, Shahzad R, Khan S, Lee YS. Extracellular vesicles in cancer diagnostics and therapeutics. *Pharmacol Ther* (2021) 223:107806. doi: 10.1016/j.pharmthera.2021.107806

20. Moller A, Lobb RJ. The evolving translational potential of small extracellular vesicles in cancer. *Nat Rev Cancer* (2020) 20:697–709. doi: 10.1038/s41568-020-00299-w

21. Colombo M, Moita C, van Niel G, Kowal J, Vigneron J, Benaroch P, et al. Analysis of ESCRT functions in exosome biogenesis, composition and secretion highlights the heterogeneity of extracellular vesicles. *J Cell Sci* (2013) 126:5553–65. doi: 10.1242/jcs.128868

22. Banuelos C, Betanzos A, Javier-Reyna R, Galindo A, Orozco E. Molecular interplays of the entamoeba histolytica endosomal sorting complexes required for transport during phagocytosis. *Front Cell Infect Microbiol* (2022) 12:855797. doi: 10.3389/fcimb.2022.855797

23. Babst M. MVB vesicle formation: ESCRT-dependent, ESCRT-independent and everything in between. *Curr Opin Cell Biol* (2011) 23:452–7. doi: 10.1016/j.ceb.2011.04.008

24. van Niel G, Charrin S, Simoes S, Romao M, Rochin L, Saftig P, et al. The tetraspanin CD63 regulates ESCRT-independent and -dependent endosomal sorting during melanogenesis. *Dev Cell* (2011) 21:708–21. doi: 10.1016/j.devcel.2011.08.019

25. Naito Y, Yoshioka Y, Ochiya T. Intercellular crosstalk between cancer cells and cancer-associated fibroblasts *via* extracellular vesicles. *Cancer Cell Int* (2022) 22:367. doi: 10.1186/s12935-022-02784-8

26. Arenaccio C, Chiozzini C, Columba-Cabezas S, Manfredi F, Affabris E, Baur A, et al. Exosomes from human immunodeficiency virus type 1 (HIV-1)-infected cells license quiescent CD4+ T lymphocytes to replicate HIV-1 through a nef- and ADAM17-dependent mechanism. *J Virol* (2014) 88:11529–39. doi: 10.1128/JVI.01712-14

27. Jung D, Shin S, Kang SM, Jung I, Ryu S, Noh S, et al. Reprogramming of T cell-derived small extracellular vesicles using IL2 surface engineering induces potent anti-cancer effects through miRNA delivery. *J Extracell Vesicles* (2022) 11:e12287. doi: 10.1002/jev2.12287

28. van der Vlist EJ, Arkesteijn GJ, van de Lest CH, Stoorvogel W, Nolte-'t Hoen EN, Wauben MH. CD4(+) T cell activation promotes the differential release of distinct populations of nanosized vesicles. *J Extracell Vesicles* (2012) 1. doi: 10.3402/jev.v1i0.18364

29. Notarbartolo S, Abrignani S. Human T lymphocytes at tumor sites. *Semin Immunopathol* (2022) 44:883–901. doi: 10.1007/s00281-022-00970-4

30. Wu SW, Li L, Wang Y, Xiao Z. CTL-derived exosomes enhance the activation of CTLs stimulated by low-affinity peptides. *Front Immunol* (2019) 10:1274. doi: 10.3389/fimmu.2019.01274

31. Li L, Jay SM, Wang Y, Wu SW, Xiao Z. IL-12 stimulates CTLs to secrete exosomes capable of activating bystander CD8(+) T cells. *Sci Rep* (2017) 7:13365. doi: 10.1038/s41598-017-14000-z

32. Qiu Y, Yang Y, Yang R, Liu C, Hsu JM, Jiang Z, et al. Activated T cell-derived exosomal PD-1 attenuates PD-L1-induced immune dysfunction in triple-negative breast cancer. *Oncogene* (2021) 40:4992–5001. doi: 10.1038/s41388-021-01896-1

33. Chen F, Chen N, Gao Y, Jia L, Lyu Z, Cui J. Clinical progress of PD-1/L1 inhibitors in breast cancer immunotherapy. *Front Oncol* (2021) 11:724424. doi: 10.3389/fonc.2021.724424

34. Nakhjavani M, Shigdar S. Future of PD-1/PD-L1 axis modulation for the treatment of triple-negative breast cancer. *Pharmacol Res* (2022) 175:106019. doi: 10.1016/j.phrs.2021.106019

35. Del Rosso M, Margheri F, Serrati S, Chilla A, Laurenzana A, Fibbi G. The urokinase receptor system, a key regulator at the intersection between inflammation, immunity, and coagulation. *Curr Pharm Des* (2011) 17:1924–43. doi: 10.2174/138161211796718189

36. Porcelli L, Guida M, De Summa S, Di Fonte R, De Risi I, Garofoli M, et al. uPAR (+) extracellular vesicles: a robust biomarker of resistance to checkpoint inhibitor immunotherapy in metastatic melanoma patients. *J Immunother Cancer* (2021) 9(5): e002372. doi: 10.1136/jitc-2021-002372

37. Jhunjhunwala S, Hammer C, Delamarre L. Antigen presentation in cancer: insights into tumour immunogenicity and immune evasion. *Nat Rev Cancer* (2021) 21:298–312. doi: 10.1038/s41568-021-00339-z

38. Xie Y, Zhang H, Li W, Deng Y, Munegowda MA, Chibbar R, et al. Dendritic cells recruit T cell exosomes via exosomal LFA-1 leading to inhibition of CD8+ CTL responses through downregulation of peptide/MHC class I and fas ligand-mediated cytotoxicity. *J Immunol* (2010) 185:5268–78. doi: 10.4049/jimmunol.1000386

39. Zhou WJ, Zhang J, Xie F, Wu JN, Ye JF, Wang J, et al. CD45RO(-)CD8(+) T cell-derived exosomes restrict estrogen-driven endometrial cancer development *via* the ERbeta/miR-765/PLP2/Notch axis. *Theranostics* (2021) 11:5330–45. doi: 10.7150/thno.58337

40. Wang X, Shen H, He Q, Tian W, Xia A, Lu XJ. Exosomes derived from exhausted CD8+ T cells impaired the anticancer function of normal CD8+ T cells. *J Med Genet* (2019) 56:29–31. doi: 10.1136/jmedgenet-2018-105439

41. Cai Z, Yang F, Yu L, Yu Z, Jiang L, Wang Q, et al. Activated T cell exosomes promote tumor invasion via fas signaling pathway. *J Immunol* (2012) 188:5954–61. doi: 10.4049/jimmunol.1103466

42. Zhu J, Yamane H, Paul WE. Differentiation of effector CD4 T cell populations (*). *Annu Rev Immunol* (2010) 28:445–89. doi: 10.1146/annurev-immunol-030409-101212

43. Shin S, Jung I, Jung D, Kim CS, Kang SM, Ryu S, et al. Novel antitumor therapeutic strategy using CD4+ T cell-derived extracellular vesicles. *Biomaterials* (2022) 289:121765. doi: 10.1016/j.biomaterials.2022.121765

44. Zhang H, Xie Y, Li W, Chibbar R, Xiong S, Xiang J. CD4(+) T cell-released exosomes inhibit CD8(+) cytotoxic T-lymphocyte responses and antitumor immunity. *Cell Mol Immunol* (2011) 8:23–30. doi: 10.1038/cmi.2010.59

45. Lu J, Wu J, Xie F, Tian J, Tang X, Guo H, et al. CD4(+) T cell-released extracellular vesicles potentiate the efficacy of the HBsAg vaccine by enhancing b cell responses. *Adv Sci (Weinh)* (2019) 6:1802219. doi: 10.1002/advs.201802219

46. Yang Z, Sun B, Xiang J, Wu H, Kan S, Hao M, et al. Role of epigenetic modification in interferon treatment of hepatitis b virus infection. *Front Immunol* (2022) 13:1018053. doi: 10.3389/fimmu.2022.1018053

47. Hu N, Wu Y, Tang M, Luo T, Yuan S, Li C, et al. Case report: Hepatic tuberculosis mimicking hepatocellular carcinoma in a patient with cirrhosis induced by hepatitis b virus. *Front Med (Lausanne)* (2022) 9:1005680. doi: 10.3389/fmed.2022.1005680

48. Xiang Q, Tao JS, Li JJ, Tian RB, Li XH. What is the role of Von willebrand factor in chronic hepatitis b virus infection to hepatocellular carcinoma: a review article. *Ther Adv Chronic Dis* (2022) 13:20406223221125683. doi: 10.1177/20406223221125683

49. Baek H, Ye M, Kang GH, Lee C, Lee G, Choi DB, et al. Neuroprotective effects of CD4+CD25+Foxp3+ regulatory T cells in a 3xTg-AD alzheimer's disease model. *Oncotarget* (2016) 7:69347–57. doi: 10.18632/oncotarget.12469

50. Lin C, Guo J, Jia R. Roles of regulatory T cell-derived extracellular vesicles in human diseases. *Int J Mol Sci* (2022) 23(19):11206. doi: 10.3390/ijms231911206

51. Smyth LA, Ratnasothy K, Tsang JY, Boardman D, Warley A, Lechler R, et al. CD73 expression on extracellular vesicles derived from CD4+ CD25+ Foxp3+ T cells contributes to their regulatory function. *Eur J Immunol* (2013) 43:2430–40. doi: 10.1002/eji.201242909

52. Clayton A, Al-Taei S, Webber J, Mason MD, Tabi Z. Cancer exosomes express CD39 and CD73, which suppress T cells through adenosine production. *J Immunol* (2011) 187:676–83. doi: 10.4049/jimmunol.1003884

53. Yang Y, Bucan V, Baehre H, von der Ohe J, Otte A, Hass R. Acquisition of new tumor cell properties by MSC-derived exosomes. *Int J Oncol* (2015) 47:244–52. doi: 10.3892/ijo.2015.3001

54. Theodoraki MN, Yerneni S, Gooding WE, Ohr J, Clump DA, Bauman JE, et al. Circulating exosomes measure responses to therapy in head and neck cancer patients treated with cetuximab, ipilimumab, and IMRT. *Oncoimmunology* (2019) 8:1593805. doi: 10.1080/2162402X.2019.1593805

55. Tung SL, Boardman DA, Sen M, Letizia M, Peng Q, Cianci N, et al. Regulatory T cell-derived extracellular vesicles modify dendritic cell function. *Sci Rep* (2018) 8:6065. doi: 10.1038/s41598-018-24531-8

56. Xie Y, Zhang X, Zhao T, Li W, Xiang J. Natural CD8(+)25(+) regulatory T cell-secreted exosomes capable of suppressing cytotoxic T lymphocyte-mediated immunity against B16 melanoma. *Biochem Biophys Res Commun* (2013) 438:152–5. doi: 10.1016/j.bbrc.2013.07.044

57. Hu H, Wu J, Cao C, Ma L. Exosomes derived from regulatory T cells ameliorate acute myocardial infarction by promoting macrophage M2 polarization. *IUBMB Life* (2020) 72:2409–19. doi: 10.1002/iub.2364

58. Gao J, Liang Y, Wang L. Shaping polarization of tumor-associated macrophages in cancer immunotherapy. *Front Immunol* (2022) 13:888713. doi: 10.3389/fimmu.2022.888713

59. Xiang X, Wang J, Lu D, Xu X. Targeting tumor-associated macrophages to synergize tumor immunotherapy. *Signal Transduct Target Ther* (2021) 6:75. doi: 10.1038/s41392-021-00484-9

60. Sanders JM, Jeyamogan S, Mathew JM, Leventhal JR. Foxp3+ regulatory T cell therapy for tolerance in autoimmunity and solid organ transplantation. *Front Immunol* (2022) 13:1055466. doi: 10.3389/fimmu.2022.1055466

61. Aiello S, Rocchetta F, Longaretti L, Faravelli S, Todeschini M, Cassis L, et al. Extracellular vesicles derived from T regulatory cells suppress T cell proliferation and prolong allograft survival. *Sci Rep* (2017) 7:11518. doi: 10.1038/s41598-017-08617-3

62. Yu X, Huang C, Song B, Xiao Y, Fang M, Feng J, et al. CD4+CD25+ regulatory T cells-derived exosomes prolonged kidney allograft survival in a rat model. *Cell Immunol* (2013) 285:62–8. doi: 10.1016/j.cellimm.2013.06.010

63. Torri A, Carpi D, Bulgheroni E, Crosti MC, Moro M, Gruarin P, et al. Extracellular MicroRNA signature of human helper T cell subsets in health and autoimmunity. *J Biol Chem* (2017) 292:2903–15. doi: 10.1074/jbc.M116.769893

64. Kooijmans SAA, Fliervoet LAL, van der Meel R, Fens M, Heijnen HFG, van Bergen En Henegouwen PMP, et al. PEGylated and targeted extracellular vesicles display enhanced cell specificity and circulation time. *J Control Release* (2016) 224:77–85. doi: 10.1016/j.jconrel.2016.01.009

65. Li S, Wu Y, Ding F, Yang J, Li J, Gao X, et al. Engineering macrophage-derived exosomes for targeted chemotherapy of triple-negative breast cancer. *Nanoscale* (2020) 12:10854–62. doi: 10.1039/D0NR00523A

66. Milbank E, Dragano NRV, Gonzalez-Garcia I, Garcia MR, Rivas-Limeres V, Perdomo L, et al. Small extracellular vesicle-mediated targeting of hypothalamic AMPKalpha1 corrects obesity through BAT activation. *Nat Metab* (2021) 3:1415–31. doi: 10.1038/s42255-021-00467-8

67. Fuhrmann G, Serio A, Mazo M, Nair R, Stevens MM. Active loading into extracellular vesicles significantly improves the cellular uptake and photodynamic effect of porphyrins. *J Control Release* (2015) 205:35–44. doi: 10.1016/j.jconrel.2014.11.029

68. Lou G, Chen L, Xia C, Wang W, Qi J, Li A, et al. MiR-199a-modified exosomes from adipose tissue-derived mesenchymal stem cells improve hepatocellular carcinoma chemosensitivity through mTOR pathway. *J Exp Clin Cancer Res* (2020) 39:4. doi: 10.1186/s13046-019-1512-5

69. Zhou W, Zhou Y, Chen X, Ning T, Chen H, Guo Q, et al. Pancreatic cancer-targeting exosomes for enhancing immunotherapy and reprogramming tumor microenvironment. *Biomaterials* (2021) 268:120546. doi: 10.1016/j.biomaterials.2020.120546

70. Kalluri R. The biology and function of exosomes in cancer. *J Clin Invest* (2016) 126:1208–15. doi: 10.1172/JCI81135

71. Zhou Z, Tao C, Li J, Tang JC, Chan AS, Zhou Y. Chimeric antigen receptor T cells applied to solid tumors. *Front Immunol* (2022) 13:984864. doi: 10.3389/fimmu.2022.984864

72. Al-Haideri M, Tondok SB, Safa SH, Maleki AH, Rostami S, Jalil AT, et al. CAR-T cell combination therapy: the next revolution in cancer treatment. *Cancer Cell Int* (2022) 22:365. doi: 10.1186/s12935-022-02778-6

73. June CH, O'Connor RS, Kawalekar OU, Ghassemi S, Milone MC. CAR T cell immunotherapy for human cancer. *Science* (2018) 359:1361–5. doi: 10.1126/science.aar6711

74. Chan JD, Lai J, Slaney CY, Kallies A, Beavis PA, Darcy PK. Cellular networks controlling T cell persistence in adoptive cell therapy. *Nat Rev Immunol* (2021) 21:769–84. doi: 10.1038/s41577-021-00539-6

75. Tang XJ, Sun XY, Huang KM, Zhang L, Yang ZS, Zou DD, et al. Therapeutic potential of CAR-T cell-derived exosomes: a cell-free modality for targeted cancer therapy. *Oncotarget* (2015) 6:44179–90. doi: 10.18632/oncotarget.6175

76. Yang P, Cao X, Cai H, Feng P, Chen X, Zhu Y, et al. The exosomes derived from CAR-T cell efficiently target mesothelin and reduce triple-negative breast cancer growth. *Cell Immunol* (2021) 360:104262. doi: 10.1016/j.cellimm.2020.104262

77. Aharon A, Horn G, Bar-Lev TH, Zagagi Yohay E, Waks T, Levin M, et al. Extracellular vesicles derived from chimeric antigen receptor-T cells: a potential therapy for cancer. *Hum Gene Ther* (2021) 32:1224–41. doi: 10.1089/hum.2021.192

78. Johnson LR, Lee DY, Eacret JS, Ye D, June CH, Minn AJ. The immunostimulatory RNA RN7SL1 enables CAR-T cells to enhance autonomous and endogenous immune function. *Cell* (2021) 184:4981–4995.e4914. doi: 10.1016/j.cell.2021.08.004

79. Yang T, Martin P, Fogarty B, Brown A, Schurman K, Phipps R, et al. Exosome delivered anticancer drugs across the blood-brain barrier for brain cancer therapy in danio rerio. *Pharm Res* (2015) 32:2003–14. doi: 10.1007/s11095-014-1593-y

80. Susa F, Limongi T, Dumontel B, Vighetto V, Cauda V. Engineered extracellular vesicles as a reliable tool in cancer nanomedicine. *Cancers (Basel)* (2019) 11(12):1979. doi: 10.3390/cancers11121979

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Advancements in cancer immunotherapies targeting CD20: from pioneering monoclonal antibodies to chimeric antigen receptor-modified T cells

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CD20 located predominantly on the B cells plays a crucial role in their development, differentiation, and activation, and serves as a key therapeutic target for the treatment of B-cell malignancies. The breakthrough of monoclonal antibodies directed against CD20, notably exemplified by rituximab, revolutionized the prognosis of B-cell malignancies. Rituximab, approved across various hematological malignancies, marked a paradigm shift in cancer treatment. In the current landscape, immunotherapies targeting CD20 continue to evolve rapidly. Beyond traditional mAbs, advancements include antibody-drug conjugates (ADCs), bispecific antibodies (BsAbs), and chimeric antigen receptor-modified (CAR) T cells. ADCs combine the precision of antibodies with the cytotoxic potential of drugs, presenting a promising avenue for enhanced therapeutic efficacy. BsAbs, particularly CD20xCD3 constructs, redirect cytotoxic T cells to eliminate cancer cells, thereby enhancing both precision and potency in their therapeutic action. CAR-T cells stand as a promising strategy for combatting hematological malignancies, representing one of the truly personalized therapeutic interventions. Many new therapies are currently being evaluated in clinical trials. This review serves as a comprehensive summary of CD20-targeted therapies, highlighting the progress and challenges that persist. Despite significant advancements, adverse events associated with these therapies and the development of resistance remain critical issues. Understanding and mitigating these challenges is paramount for the continued success of CD20-targeted immunotherapies.

KEYWORDS

CD20, B cell, leukemia, lymphoma, immunotherapy, monoclonal antibody, antibody-drug conjugate (ADC), CAR-T

Introduction

CD20 is a surface protein that exhibits ubiquitous expression in B cells with minimal occurrence in other tissues, rendering it an ideal target for immunotherapy against B cell-derived malignancies. CD20 expression initiates during the pre-B cell stage and persists until B cells undergo terminal differentiation into plasma cells (Figure 1). Immunotherapy directed at CD20 is extensively employed for treating mature B cell-derived malignancies, such as chronic lymphocytic leukemia (CLL) and various B cell-derived non-Hodgkin lymphomas (B-NHL), including follicular lymphoma (FL), diffuse large B-cell lymphoma (DLBCL), and mantle cell lymphoma (MCL). CD20 is also present in multiple subtypes of B cell precursor acute lymphoblastic leukemia (B-ALL), albeit its expression at diagnosis is heterogeneous and frequently low (1–3). Notably, documented upregulation of CD20 after induction treatment suggests a potential expansion of CD20-directed immunotherapy applications for B-ALL (4, 5). CD20-specific therapies offer precise B cell targeting, minimizing impact on other cell types. These therapies efficiently deplete CD20-expressing B cells without hindering the replenishment of the B-cell compartment from early B cell precursors. Hence, upon cessation of anti-CD20 treatment, the B-cell population can recover (6). Notably, the absence of CD20 on fully mature plasma cells enables patients to maintain protective humoral immunity against previously encountered pathogens during treatment (6).

CD20-targeted immunotherapy encompasses diverse modalities administered at various treatment stages. Rituximab, the pioneering anti-CD20 monoclonal antibody (mAb) introduced in 1997, stands out as a well-studied, low-toxicity immunotherapy with manageable side effects. It is a crucial component of the common therapy regimens, such as BR (bendamustine + rituximab) or FCR (fludarabine + cyclophosphamide + rituximab), which are often used as a first-line treatment in specific groups of CLL and B-NHL patients. In addition, following positive phase 3 trial results, rituximab has been recently integrated into chemotherapy for adult B-ALL patients with at least 20% CD20-positive leukemic cells (7). Beyond rituximab, the engineered anti-CD20 mAb obinutuzumab is registered and employed in combination with chemotherapy as first-line therapy for defined cases of CLL and FL. In addition to mAbs, new immunotherapies targeting CD20 have been developed and successfully introduced into the clinic for patients refractory to first-line therapy or with relapsed disease (r/r). These include bispecific antibodies (BsAbs) targeting the CD20 molecule and simultaneously recruiting cytotoxic T cells, as well as adoptive therapies using autologous T cells modified with chimeric antigen receptors (CAR-T). Three BsAbs targeting CD20 have received FDA approval, while CD20-specific CAR-T cells are presently undergoing clinical trials. Notably, CAR-T cells simultaneously targeting CD19 and CD20 aim to address CD19-negative clones, with ongoing clinical trials in advanced r/r B-cell malignancies (Table 1).

This comprehensive review explores various CD20-directed immunotherapies, including mAbs, radio-immunoconjugates, BsAbs, and CD20 CAR-T cells. The discussion encompasses both approved drugs and novel solutions undergoing investigation in preclinical and clinical trials (Figures 2, 3; Tables 1–3). Mechanisms of resistance to CD20-directed immunotherapies are presented (Figure 4), and the potential for various combinations with immunotherapies is discussed.

CD20 antigen: structure, function, and expression regulation

CD20 is a transmembrane protein whose significance as a target for immunotherapy is well recognized, although its biological role remains elusive. Encoded by the *MS4A1* gene, CD20 is part of the MS4A family, which consists of 18 proteins with similar structures. The CD20 protein spans the cell membrane with four transmembrane helices and features two extracellular loops, which are the main epitopes recognized by anti-CD20 mAbs. Notably, both the N-terminal and C-terminal ends of CD20 are situated inside the cell. The detailed structure and dimeric assembly of CD20 on the cell membrane have recently been extensively

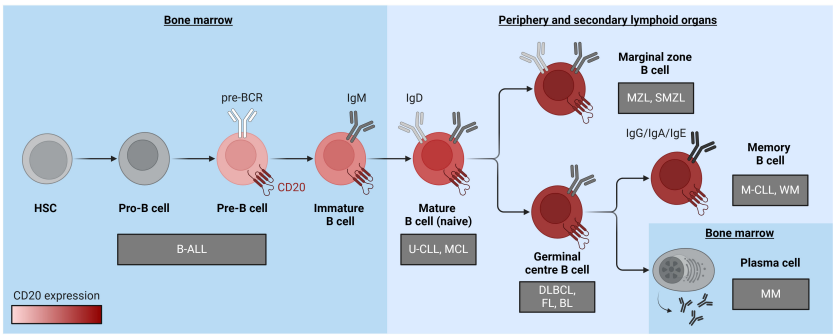

FIGURE 1
A diagram illustrating B cell differentiation and maturation, emphasizing the pronounced increase in CD20 expression levels depicted through a red color gradient. Associated malignancies are positioned near the cell of origin and represented within grey boxes. B-ALL, B cell acute lymphoblastic leukemia; BL, Burkitt lymphoma; DLBCL, diffuse large B cell lymphoma; FL, follicular lymphoma; HSC, hematopoietic stem cell; MCL, mantle cell lymphoma; M-CLL, mutated chronic lymphocytic leukemia; MM, multiple myeloma; MZL, marginal zone lymphoma; SMZL, splenic marginal zone lymphoma; U-CLL, unmutated chronic lymphocytic leukemia; WM, Waldenstrom macroglobulinaemia. The figure was created using BioRender.com.

TABLE 1 Clinically tested CD20-targeting CAR-T therapies.

Name	Effector cells	Structure	Indications	Clinical trial phase	Clinical trial identifier
CD20 CAR-T	autologous T cells	CD20 scFv with CD8a H/TM, 4-1BB, CD3ζ domains	r/r B-NHL	phase I	NCT04036019
CD19/CD20 CAR-T	autologous T cells	CD20 and CD19 scFv with CD8a H/TM, 41BB, CD3ζ domains	r/r B-cell malignancies	phase I/II	NCT03097770
CD20/CD22 CAR-T	allogeneic T cells	CD20 and CD22 scFv with CD8α H/TM, 41BB, CD3ζ domains	r/r B-NHL	phase I/II	NCT05607420
CD19/CD20/CD22 CAR-T	autologous T cells	CD19, CD20 and CD22 scFv with CD8α H/TM, 41BB CD3ζ domains	r/r B-NHL	phase I	NCT05418088

characterized (9). This structural research sheds light on the molecular architecture of CD20 and anti-CD20 mAbs biding modes, contributing to our understanding of its potential as a target for immunotherapy (9).

While no identified physiological ligand binds to CD20, it is known to form nanoclusters on the B cell membrane with proteins such as IgD or IgM-class B cell receptors (BCR), CD19, CXCR4, and CD40 (10). A recent study utilizing CRISPR/Cas9-mediated CD20 elimination from mature B cells revealed CD20's role as a gatekeeper in maintaining the resting state. The knockout of CD20 resulted in the translocation of the BCR toward the CD19 coreceptor, transient B cell activation, and internalization of various B cell-specific proteins (10). Additionally, initial research proposed that CD20 may function as a calcium channel (11), however, subsequent findings suggested that calcium flux induction is mediated by the CD20-BCR complex rather than CD20 alone (12). Therefore, the role of CD20 as a regulator of B cell activity seems to be inherently linked to interactions of CD20 with other surface proteins, primarily with the BCR complex.

The regulation of the *MS4A1* gene was attributed to several transcription factors, summarized in (13). The described positive regulators include transcription factors essential for B cell development and maturation, such as PU.1, PiP (IRF4), NFκB (14–16), as well as other factors, such as USF, TFE3.1 (16), OCT1, OCT2 (17), ELK1, ETS1 (18), SP1, CHD4 and MBD2 (19). Recently, another member of interferon regulatory factors (IRF)

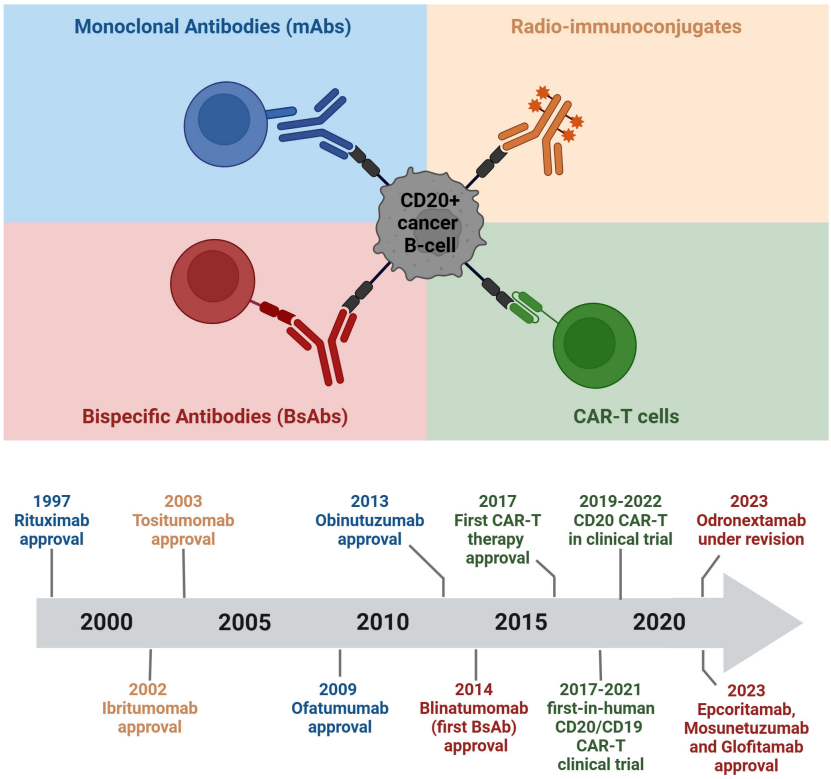

FIGURE 2
Chronology of clinical approvals and recent breakthroughs in CD20-targeted immunotherapies. The figure was created using BioRender.com.

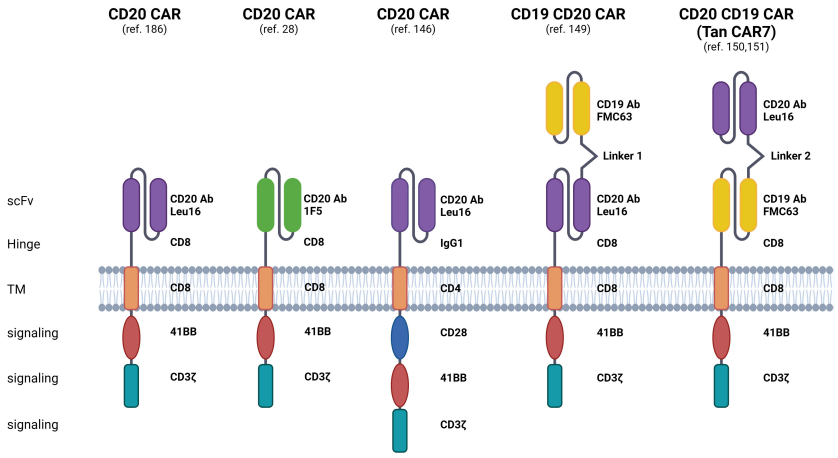

FIGURE 3
Displayed are CAR constructs, including second- and third-generation designs specifically recognizing the CD20 molecule, alongside dual-specificity CAR constructs recognizing both CD20 and CD19 molecules. Additionally, preclinical and clinical investigations explore the efficacy of bi- or tri-cistronic vectors for the expression of these CARs. Linker 1: (G4S)5, Linker 2: (EAAAK)3. The figure was created using BioRender.com.

TABLE 2 Clinically approved and tested anti-CD20 mAbs and radio-immunoconjugates.

Name	Structure	Origin	CD20 epitope characteristics	Fc domain	Approval date*	Indications	Common dosages in clinical setting	Clinical trials
Rituximab	IgG1κ	chimeric	$A_{(170)}NPS_{(173)}$ region on large extracellular loop	unmodified	1997	CLL, DLBCL, FL	375 mg/m2 per infusion	PMID: 9310469
Ofatumumab	IgG1	human	FLKMESLNFIRAHT region on large extracellular loop and A74T, I76A, Y77S residues on small extracellular loop	unmodified	2009	CLL	300-2000 mg per infusion	NCT00092274
Obinutuzumab	glycoengineered IgG1κ	humanized	residues 172–176 on large extracellular loop	reduced fucosylation of Fc region	2013	CLL, r/r FL	100-1000 mg per infusion	NCT22431570
Ublituximab	glycoengineered IgG1κ	chimeric	residues 168–171 and 158–159 on large extracellular loop	reduced fucosylation of Fc region	not approved	CLL	≤150 - 900 mg per infusion	NCT02301156
Ocaratuzumab	glycoengineered IgG1	humanized	$A_{(170)}NPS_{(173)}$ region on large extracellular loop. Increased affinity to CD20	reduced fucosylation of Fc region; protein-engineered to improve affinity to 158-F FcγRIIIa carriers	not approved	r/r FL	375 mg/m2 per infusion	NCT00354926
90Y-Ibritumomab Tiuxetan	90-yttrium labeled IgG1κ	murine	$A_{(170)}NPS_{(173)}$ region on large extracellular loop	unmodified	2002	FL, r/r NHL	14.8 MBq/kg	PMID: 12777454
131I-Tositumomab	131-iodium-linked IgG2aλ	murine	$A_{(170)}NPS_{(173)}$ region on large extracellular loop	unmodified	2003	r/r NHL (withdrawn)	75 cGy	PMID:15689582 PMID:11579112

(*) regarding approval in oncological indications.
Information about CD20 epitopes recognized by subsequent Abs is described in (8).

TABLE 3 Clinically approved and tested CD20xCD3 BsAbs.

Name	Structure	Antigen binding domain	Fc domain	Production	Approval date	Indications	Common dosages in clinical setting	Clinical trials
Epcoritamab	full-length IgG1	1 anti-CD20 Fab 1 anti-CD3 Fab	FcγR and C1q binding abolished FcRn binding maintained	controlled Fab arm exchange	2023	r/r DLBCL	0,16-48 mg s.c. in 28-day cycles with step-up dosing	NCT03625037
Mosunetuzumab	full-length IgG1	1 anti-CD20 Fab 1 anti-CD3 Fab	FcγR binding abolished FcRn binding maintained	knobes-into-holes	2023	r/r FL	1-60 mg i.v. in 21-day cycles	NCT02500407
Glofitamab	full-length IgG1	2 anti-CD20 Fab 1 anti-CD3 Fab	FcγR and C1q binding abolished FcRn binding maintained	head to tail fusion via flexible linker	2023	r/r DLBCL	2,5-30 mg i.v. in 21-day cycles with step-up dosing and obinutuzumab pretreatment	NCT03075696
Odronextamab	full-length IgG4	1 anti-CD20 Fab 1 anti-CD3 Fab	FcγRIII binding abolished FcRn binding maintained	heavy chains with different affnities and common light chains	Review	r/r DLBCL, r/r FL	0,1-320 mg i.v. in 21-day cycles with step-up dosing	NCT02290951 NCT03888105
Imvotamab	IgM	10 anti-CD20 Fabs 1 anti-CD3 scFv	unmodified	IgM platform with recombinant J-chain	Not approved	r/r DLBCL, r/r FL	15-300 mg i.v. in 21-day cycles with step-up dosing	NCT04082936
Plamotamab	IgG1	1 anti-CD20 Fab 1 anti-CD3 scFv	FcγR binding abolished FcRn binding maintained	Fab-scFv-Fc format	Not approved	r/r DLBCL, r/r FL	dose-escalation study	NCT02924402

engaged in B cell development, IRF8, was shown to promote CD20 expression in DLBCL as well as in healthy B cells (20). Negative regulators of CD20 include FOXO1 (21), CREM (19), SMAD2/3 (22), and MYC (23, 24).

Additional regulation of CD20 expression occurs on the epigenetic level. Histone deacetylase (HDAC) family members HDAC1/2, HDAC1/4, and HDAC6 as well as methyltransferase enzyme EZH2 can repress CD20 in healthy and malignant B cells (25–27). Recently, the occurrence of four 5'-UTR variants of *MS4A1* mRNA with differential translation efficacy was described (28).

Anti-CD20 mAbs and immunoconjugates

The evolution of anti-CD20 mAbs marks a progression toward enhanced compatibility and reduced immunogenicity. The first therapeutic anti-CD20 mAb, rituximab, comprises a chimeric murine-human structure, contributing to the development of immune response and infusion-related reactions due to its limited resemblance to natural human antibodies (29, 30). Enhanced human content in subsequent mAbs correlates with decreased immunogenicity and improved binding affinity to human Fc receptors. The newer generations of anti-CD20 mAbs, exhibiting humanized (obinutuzumab) and fully human (ofatumumab) designs have reduced immunogenicity (31–33). Furthermore, heightened human sequence content enhances interactions with immune effector cells and FcRn receptors on hepatic and epithelial cells, thereby prolonging IgG antibodies' half-life (34).

CD20-targeting mAbs elicit their cytotoxic function by at least four different mechanisms (35). Upon binding CD20 on target cells, they can activate complement-mediated cytotoxicity (CDC), engage immune effector cells to mediate antibody-dependent cytotoxicity (ADCC) and phagocytosis (ADCP), as well as directly induce cell death. The anti-CD20 mAbs currently employed in cancer treatment vary in their degree of activating specific mechanisms. These differences are the basis for a categorization of anti-CD20 mAbs into two types. The majority of mAbs are characterized as type I, which exhibit the ability to cluster CD20 into membrane lipid rafts, which is associated with potent induction of CDC. On the other hand, type I antibodies display a higher rate of internalization, which can limit their therapeutic efficacy (36). Type II mAbs do not stabilize CD20 in lipid rafts and are weak inducers of CDC, but they potently evoke direct cell death (37).

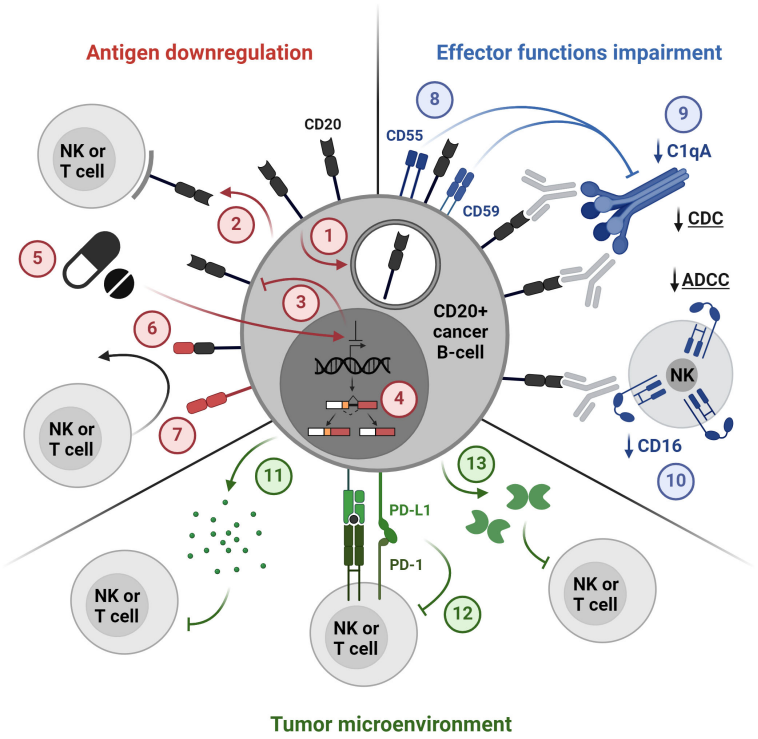

FIGURE 4
Mechanisms of resistance to CD20-directed immunotherapies. 1. Internalization, 2. Trogocytosis, 3. Loss of antigen expression, 4. Alternative splicing, 5. Drug-induced antigen downregulation, 6. Loss of an epitope, 7. Lineage switch, 8. Overexpression of complement regulatory proteins, 9. Downregulation of complement proteins, 10. CD16 downregulation, 11. Secretion of immunosuppressive cytokines, 12. Immune checkpoints, 13. Secretion of suppressive molecules, e.g. galectin 1. The figure was created using BioRender.com.

Rituximab, ofatumumab, and ublituximab are classified as the type I anti-CD20 mAbs, whereas obinutuzumab represents a type II anti-CD20 mAb.

CD20-directed mAbs are also used in the form of immunoconjugates – Abs linked with drugs (ADCs), toxins (immunotoxins or engineered toxin bodies – ETBs) or radioactive isotopes (radio-immunoconjugates). Unlike conventional antibodies, which largely depend on the immune effector cells or the complement system for their cytotoxic effects, immunoconjugates can directly induce apoptosis of cancer cells. Due to the relatively poor internalization of CD20, few ADCs and immunotoxins were developed. A single-chain variable fragment-based targeting CD20 and conjugated with Shiga-like toxin A subunit, MT-3724, presented promising preclinical and early clinical results, but its development was ceased by the manufacturer (38). Another strategy that does not require CD20 internalization for direct and targeted cell killing is the use of radio-immunoconjugates. This approach has gained significant attention in targeting lymphoma cells, which are highly radiosensitive (39). Radio-immunoconjugates utilize ionizing radiation to induce cytotoxicity of the target cell. Concurrently, they can trigger classical effector mechanisms such as CDC, ADCC, and ADCP.

In this section, we describe anti-CD20 mAbs and conjugates that were approved for clinical use in lymphoid malignancies. Additionally, other anti-CD20 agents that displayed effectiveness in clinical trials of B-cell neoplasms are listed in Table 2.

Rituximab

Rituximab is the first mAb used for cancer therapy. It is a chimeric mouse/human IgG1 anti-CD20 mAb targeting the epitope on a large extracellular loop of CD20. As a type I mAb, rituximab elicits its function mostly by CDC, ADCC, and ADCP (35). Since gaining its first approval for low grade FL in 1997 (40), rituximab has consistently demonstrated its efficacy, both as part of combination drug regimens and as a standalone agent, across various clinical trials. Rituximab is currently employed in a broad spectrum of conditions including DLBCL, Burkitt lymphoma (BL), MCL, FL, marginal zone lymphoma (MZL), hairy cell leukemia (HCL), and CLL, as comprehensively reviewed in (41). A relatively recent hematologic application of rituximab involves its use in $CD20^{+}$ adult B-ALL, serving as an adjunct to chemotherapy throughout all stages of treatment (7). The popularity and effectiveness of rituximab, as well as the expiration of its patent, has catalyzed an increase in the production of biosimilars. Following prior studies confirming its bioavailability, a new formulation of rituximab with hyaluronidase has been approved for subcutaneous use in FL, CLL, and DLBCL (42). Despite the success of rituximab, some patients experience relapses due to various resistance mechanisms, including trogocytosis, complement exhaustion, internalization of CD20 and others, described in the section *Resistance to CD20-directed immunotherapies* (43, 44). Attempts to increase the efficacy of

rituximab prompted the trials combining rituximab with other drugs that could potentiate its cytotoxicity, ideally in chemotherapy-free schemes. A phase 3 study AUGUMENT confirmed the benefit of the addition of the immunomodulatory drug lenalidomide to the rituximab in r/r FL and MZL (45). Strategies involving the addition of mTOR inhibitors to rituximab combined with classic chemotherapeutics are also under investigation for the treatment of patients with r/r DLBCL, with promising results from phase 1 and 2 trials (46, 47).

Ofatumumab

Ofatumumab (2F2) is a fully human anti-CD20 IgG1κ mAb developed by Genmab and Glaxo PLC. It binds to an epitope distinct from that of rituximab, targeting both small and large extracellular loops of CD20 (48). Preclinical tests have shown that ofatumumab induces CDC more potently than rituximab, while the ADCC efficacy is comparable to that of rituximab (49, 50). The superior CDC efficacy of ofatumumab may be in part associated with the location of its target epitope more proximally to the cell membrane than the epitope recognized by rituximab (51). Recent structural studies also revealed that ofatumumab complexes show optimal geometry for complement recruitment (52). Additionally, ofatumumab demonstrates a slower off-rate than rituximab (49), allowing prolonged binding to the target cells. The first approval of ofatumumab was granted in 2009 for refractory CLL. Despite promising preclinical results, there is limited clinical evidence to confirm its superiority over other anti-CD20 agents (53). Clinical trials comparing ofatumumab to rituximab in FL (54) and DLBCL (55) relapsed after a rituximab-containing therapy showed no superiority of ofatumumab. On the other hand, the comparison of the treatment composed of hyper-fractionated cyclophosphamide, vincristine, doxorubicin, dexamethasone with ofatumumab (HCVAD-O) to the historical cohort of B-ALL $CD20^{+}$ Ph^{-} patients treated with HCVAD with rituximab (HCVAD-R) showed improvement in event-free survival (EFS) and overall survival (OS) (56). Currently, ofatumumab is rarely used in its initial indication, being replaced by newer agents such as obinutuzumab or ibrutinib (57–59).

Obinutuzumab

Obinutuzumab (GA101) is a humanized, glycoengineered IgG1 type II mAb that targets the epitope on the large extracellular loop of CD20, which partially overlaps with the rituximab epitope. The novelty of obinutuzumab design lies predominantly in the glycoengineering modifications, which were applied to improve affinity to the FcγR receptors on effector cells (60). Specifically, obinutuzumab exhibits reduced fucosylation of oligosaccharides attached to Asp297 in its Fc region, which results in improved binding of FcγRIII (61). In preclinical tests, obinutuzumab presented a slower internalization rate after binding to CD20 and superior efficacy in ADCC than rituximab and ofatumumab (62). The ADCP efficacy was comparable between the three antibodies (63). As a type II mAb, it exhibits reduced levels of CDC (60, 63), but was suggested to have the ability to induce direct cell death (DCD) via a non-apoptotic, lysosome-mediated mechanism, in some, but not all target cell types (64, 65). While obinutuzumab has consistently demonstrated greater effectiveness than equivalent doses of rituximab in the preclinical *in vivo* models (60, 66, 67), the exact reasons behind this advantage remain incompletely understood. The underlying mechanism appears to be multifaceted and potentially attributable to the combination of several factors including greater induction of ADCC and DCD, as well as being less prone to internalization (63). Importantly, obinutuzumab demonstrated superior efficacy as a part of the chemotherapy regimen in comparison with the same chemotherapy but with rituximab in first-line treatment of CLL patients, demonstrating improved progression-free survival (PFS) and OS in a phase 3 trial (68). This resulted in the approval of obinutuzumab in combination with chlorambucil for the treatment of patients with previously untreated CLL in 2013 (69). Recently published results from the phase 3 trial have also demonstrated the benefit of obinutuzumab over rituximab when used as a part of immunochemotherapy in the first-line treatment of FL (70). On the other hand, no advantage over rituximab was observed in advanced DLBCL (9, 71, 72). It is also important to note that the overall doses of obinutuzumab in the clinical trials were higher for most patients (68, 70, 73). An ongoing trial will assess the efficacy of obinutuzumab versus rituximab in B-ALL (NCT04920968). Additionally, promising results of the phase 1 trial of the combination of obinutuzumab with the novel oral cereblon-modulating agent avadomide suggest the potential for new chemotherapy-free regimens for NHL (74). Comprehensive information about obinutuzumab and its efficacy is reviewed in (69, 73, 75).

Radio-immunoconjugates: 90-Y-Ibritumomab tiuxetan and 131I-Tositumomab

Y-90-Ibritumomab tiuxetan is a murine anti-CD20 IgG1 mAb linked with Y-90 isotope of yttrium, which emits beta radiation and decays to non-radioactive Zirconium-90. A randomized controlled trial of 90-Y ibritumomab tiuxetan in r/r low-grade, follicular, or transformed NHL showed a significant improvement in overall response rate (ORR) and complete response (CR) rates (ORR 80% vs. 56%; CR 30% vs. 16%) in comparison to the rituximab treatment (76). 90-Y ibritumomab tiuxetan was approved in 2002 for r/r NHL patients, and in 2014 the approval was expanded for the first-line consolidation in NHL (77, 78).

131I-Tositumomab is a murine anti-CD20 monoclonal IgG2 antibody linked with Iodium-131, which emits beta and gamma radiation. It was approved for use in r/r NHL in 2003. Despite the documented efficacy in FL and r/r NHL (79, 80) 131I-Tositumomab was replaced by modern agents and its sale was discontinued in 2014.

While the use of radio-immunoconjugates is linked to an increased risk of secondary malignancies and myelotoxicity, their overall toxicity profile was considered acceptable and comparable to

other therapies (39, 79, 81–84). Nonetheless, neither of the two radio-immunotherapeutic agents has been widely used in clinical practice, mainly due to economic and logistic problems, such as radiation safety concerns (85). Radio-immunotherapeutics targeting CD20 are extensively reviewed in (86).

Side effects of anti-CD20 mAbs and their management

The toxicity of anti-CD20 mAbs is relatively low, with hypersensitivity reactions, myelosuppression, and immunosuppression being the most common. Other common side effects include chest pain, arrythmia, paresthesia, nausea, diarrhea, abdominal pain, and muscle pain (87, 88). Rarely, more severe complications may occur, including tumor lysis syndrome or progressive multifocal leukoencephalopathy (PML). Common strategies for reducing hypersensitivity reaction incidence include premedication by steroids or antihistamine drugs and a slow rate of first infusion (89). For CLL patients with high lymphocyte counts (over 25 x 10^9/L), administration of *i.v.* prednisone or prednisolone is recommended before the infusion of rituximab to decrease the risk of acute infusion reactions and/or cytokine release syndrome (CRS) (88). Additionally, a recent study confirmed that obinutuzumab - as a humanized and potentially less immunogenic antibody - can be used as an alternative to rituximab after a hypersensitivity reaction (31). In the case of hypogammaglobulinemia, intravenous immunoglobulin (IVIG) replacement should be considered to reduce the risk of infections (90). Radiolabeled antibodies exhibit additional toxicities related to the emitted radiation, including the risk of myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) (86). Additionally, the use of 131I-Tositumomab can lead to hypothyroidism. This was addressed by oral administration of potassium iodide to inhibit the thyroid uptake of Iodium-131 (86).

CD20-directed BsAbs

BsAbs represent one of the most promising classes of off-the-shelf immunotherapies for the treatment of r/r B cell malignancies (91, 92). Notably, several BsAbs targeting the CD20 antigen received clinical approvals in 2023, with numerous others showing promising results in ongoing clinical trials (Figure 2, Table 3) (93). These engineered proteins, featuring dual binding sites, can simultaneously target two different antigens or two epitopes of the same antigen. This dual specificity allows BsAbs to bridge immune cells, such as T cells, with target tumor cells, promoting their interaction and subsequent cytotoxicity against the tumor cells.

Over the past few years, there has been a rapid development of this technology, resulting in various molecular BsAb formats, including IgG-like and non-IgG-like platforms (91, 92). IgG-like BsAbs mimic the structure of IgG, featuring an Fc region for effector functions, like ADCC and CDC, and provide a larger molecular weight, which increases solubility, stability, serum half-life. This allows for a wider spectrum of dosing frequency from daily to weekly or even less frequent and its administration both intravenously and subcutaneously. In contrast, non-IgG-like BsAbs lack the Fc region, typically have a smaller molecular weight, and primarily exert therapeutic effects through direct antigen binding (92). This applies also to blinatumomab, the first BsAb approved for medical use. Blinatumomab is a CD3xCD19 bispecific T-cell engager that consists only of two scFvs connected by a linker, which contributes to a relatively short half-life and the necessity for frequent dosing in prolonged infusions (94).

Among the various formats, the anti-CD20xCD3 BsAb engaging cytotoxic T cells is the most popular format. Here we focus on CD20-targeting IgG-like BsAbs as a new therapeutic option for patients with B-cell malignancies who have already undergone several lines of mAbs and CD19 CAR-T therapy.

Epcoritamab (DuoBody-CD3xCD20, GEN3013)

It is a full-length IgG1 BsAb generated by Fab-arm exchange of a humanized CD3 mAb and human CD20 mAb (95). In preclinical studies, epcoritamab has demonstrated its efficacy by eliciting robust T-cell activation and T-cell-mediated cytotoxicity against NHL cell lines *in vitro* (95). It also showed high effectiveness against primary cells derived from lymph node biopsies from newly diagnosed and r/r B-NHL patients (96). Moreover, epcoritamab-mediated cytotoxicity was observed against primary CLL cells *in vitro* and *in vivo* in patient-derived xenografts (PDX), where epcoritamab demonstrated a reduction in both blood and spleen disease burden. This effect was enhanced when used in combination with Bruton's tyrosine kinase (BTK) and BCL2 inhibitors (97).

These promising results led to the testing of epcoritamab in clinical trials. In the first-in-human trial in patients with r/r B-cell lymphoma, including DLBCL, FL, MCL, high-grade B-cell lymphoma (HGBCL), primary mediastinal large B-cell lymphoma (PMBCL), small lymphocytic lymphoma (SLL) and MZL (EPCORE™ NHL-1, NCT03625037), epcoritamab administered as a single agent subcutaneously in 68 patients exhibited notable efficacy (88% ORR and 38% CR at 48 mg) (98). In an ongoing clinical trial evaluating the safety and efficacy of epcoritamab in patients with r/r CLL and Richter's syndrome (EPCORE™ CLL-1, NCT04623541) so far epcoritamab was well tolerated (99). Several other clinical trials using this BsAb are currently underway, including testing a combination with rituximab for the first-line FL (NCT05783609). In 2023 the encouraging outcomes of clinical trials resulted in FDA and EMA approval of epcoritamab for r/r DLBCL after at least two lines of systemic therapy (100, 101).

Odronextamab (REGN1979)

This hinge-stabilized, fully human IgG4 BsAb targeting CD20 and CD3, has demonstrated both *in vitro* and *in vivo* efficacy (102, 103), leading to further evaluation of its effectiveness in clinical trials. In a phase 1, multicenter trial (ELM-1, NCT02290951) investigating the safety and tolerability of odronextamab in 145 patients with $CD20^+$ B-NHL pretreated with CD19 CAR-T therapy or refractory to the last

line of therapy, ORR among all patients reached 51% (72 of 142 patients), but among those with FL who received doses of 5 mg or higher 91% ORR (29 of 32 patients) and 72% CRR (23 of 32 patients). DLBCL patients who received doses of 80 mg or higher without previous CAR T-cell therapy reached 53% ORR and all responses were complete, and those pretreated with CAR T-cell 33% ORR and 27% CR (104). Additionally, the efficacy and safety of odronextamab were demonstrated in a case report of two patients with r/r B-NHL refractory to CAR-T therapy, who achieved complete responses that persisted for over 2 years of follow-up (105). In the light of these results, currently the phase 2 clinical trial is conducted. It assesses the anti-tumor activity and safety of odronextamab in pretreated patients with B-NHL (ELM-2, NCT03888105).

Odronextamab is not yet fully approved for marketing, but in September 2023 FDA accepted it for Priority Review for the treatment of adult patients with r/r FL and r/r DLBCL after at least two prior systemic therapies (106). Almost at the same time, EMA has accepted it for review in the same medical indications (107). Previously this drug was designated by EMA as an orphan drug for FL and DLBCL.

Mosunetuzumab (BTCT4465A)

It is a full-length, humanized IgG1 CD20xCD3 BsAb, generated using "knobs-into-holes" heterodimerization technology, which allows the combination of two heavy chains, one with the 'knob' mutation and the other with the 'hole' mutation, into one BsAb (108, 109). It was effective *in vitro* against tumor B cells obtained from PBMC of CLL patients, and *in vivo* in mice and cynomolgus monkeys, causing complete B cell depletion in peripheral blood and lymphoid tissues also in the presence of a competitive anti-CD20 mAb (108).

It has been tested in phase 1/2 clinical trial verifying it as a single agent and combined with atezolizumab (anti-PD-L1 mAb) in NHL and CLL (NCT02500407) and demonstrated notable efficacy and a manageable safety profile in patients with r/r FL (ORR 78%, CR 60%) (110). In patients with r/r DLBCL, including those previously treated with CAR-T cells, ORR was 42% and CR 23.9% (111). It is also being investigated in combination with polatuzumab vedotin (CD79b-directed ADC approved for patients with previously untreated DLBCL, NOS and HGBL with International Prognostic Index (IPI) score of at least 2) in B-NHL (NCT03671018) where it shows a favorable safety profile with highly durable responses (112). Currently, many other single-agent and combination studies of mosunetuzumab in r/r and previously untreated B-NHL are ongoing. In June 2022 mosunetuzumab obtained conditional approval from EMA (113) and in January 2023 FDA approved it for adult patients with r/r FL after two or more lines of systemic therapy (114).

Glofitamab (RO7082859)

It is CD20xCD3 heterodimeric human IgG1 BsAb with two anti-CD20 and one anti-CD3 Fabs (115). In preclinical studies, it showed higher potency than classical 1:1 IgG BsAbs, and its main treatment-related risk of CRS was mitigated by prior treatment with obinutuzumab (115). This combination of anti-CD20 therapies was evaluated in phase 1/2 clinical trial in patients with r/r B-NHL (NCT03075696), where it demonstrated durable responses, with most patients in CR (116, 117). These clinical findings were also confirmed in a group of 46 heavily pretreated patients with r/r DLBCL, who were given the drug under compassionate use and reached 7 months median OS (118). Glofitamab is also tested in combination with polatuzumab vedotin plus rituximab, cyclophosphamide, doxorubicin and prednisone (R-pola-CHP) and shows promising early results (ORR 100% and CR 76,5% among 17 patients) (119). Therefore in June 2023 FDA granted accelerated approval to glofitamab for r/r DLBCL, not otherwise specified (NOS) or large B-cell lymphoma (LBCL) arising from FL, after two or more lines of systemic therapy (120), and in July EMA approved it for conditional use for adults with r/r DLBCL after at least two previous treatments (121).

Imvotamab (IGM-2323)

This CD20xCD3 IgM BsAb is generated from 10 high-affinity CD20 binding domains and a single anti-CD3 scFv fused through the recombinant J-chain (122, 123). It exhibits a higher avidity for the CD20 binding and induces CDC against CD20-expressing cells with a greater potency than IgG BsAbs *in vitro* (122). Moreover, it exhibits vastly reduced cytokine release *in vitro* and *in vivo* (122) and seems to maintain higher effectiveness in the presence of rituximab than IgG BsAbs (124). A combination of imvotamab and loncastuximab tesirine (CD19-directed ADC approved in r/r LBCL after two or more lines of systemic therapy) demonstrated enhanced cytotoxic effect in preclinical studies (125) and is currently tested in first-in-human clinical trial in patients with r/r NHL (NCT04082936). So far imvotamab shows notable safety and tolerability profile due to repeatable IFNγ-dominant cytokine profile (123, 126).

Plamotamab (XmAb13676)

This humanized CD20xCD3 IgG1 BsAb is heterodimer with one IgG Fab arm exchanged for a scFv (127, 128). Preclinical *in vivo* data show its efficiency both in circulation and lymphoid organs (127). A phase 1 clinical trial (NCT02924402) evaluating its safety and tolerability in patients with CD20-expressing hematologic malignancies is ongoing and demonstrated so far evidence of clinical activity in heavily pretreated patients with DLBCL and FL, including earlier treatment with CAR-T therapy (129, 130).

Other BsAbs

There are several new directions in the further development of BsAbs, involving the use of antigens other than CD3. These include, among others, CD20xNKG2D antibodies, which engage the cytotoxic

activity of NK cells against leukemic cells *in vitro* (131, 132). Another novel type of BsAb tested in preclinical studies is the CD95xCD20 antibody, which induces apoptosis in malignant B cells both *in vitro* and *in vivo* (133, 134). Finally, CD20xCD28 antibodies, which were first created over 20 years ago, however, due to the high production costs using conventional methods, were not developed for a long time (135, 136).

Side effects of CD20-directed BsAbs

Although significant therapeutic successes have been observed in clinical trials, CD20xCD3 BsAbs are associated with certain side effects. The most common is CRS, primarily associated with the initial doses and confined to the first cycle of treatment (110, 137). This is related to the simultaneous binding of BsAb to CD3 of effector cells and FcγR of other immune system cells or complement factor C1q, which results in premature activation and release of cytokines, hampering the effectiveness of therapy and increasing its toxicity. Therefore, currently used BsAbs have silencing mutations in the Fc regions that prevent binding to FcγR and C1q but retain binding to FcRn, which ensures extended plasma half-life (138). Another strategies to overcome CRS are step-up dosing of BsAbs (111, 139) and premedication with anti-CD20 mAb, which depletes B-cells in both peripheral blood and secondary lymphoid organs and decreases T cells activation (115). Other common adverse events include pyrexia, fatigue, injection-site reaction, nausea, diarrhea, hypophosphatemia, hematological toxicities: neutropenia, anemia, lymphopenia, thrombocytopenia, as well as neurological adverse events: headache, insomnia, dizziness (98, 99, 104, 110–112, 117–119, 126, 129, 130, 137, 139–143). Immune effector cell-associated neurotoxicity syndrome (ICANS) is a rare complication that occurs in less than 5% of patients treated with BsAb (119, 137, 139). The frequency and severity of these side effects vary. To mitigate risks, careful patient monitoring, premedication with anti-CD20 mAb, and dose adjustments are implemented to enhance the safety profile of CD20xCD3 BsAb therapies.

CD20-directed CAR-T cells

CD20 is also under exploration as a target for CAR-T cells in preclinical and clinical trials. CARs are synthetic constructs comprising extracellular antigen recognition domains, hinge and transmembrane regions, and intracellular signaling domains responsible for their activation and proliferation. Approved CAR T-cell therapy involves genetically engineered autologous products, utilizing the patient's CAR T cells to target tumor cell antigens. Currently, four CD19-targeted CAR T-cell therapies are approved for treating r/r B-ALL and r/r B-NHL. Despite its efficacy, around 60% of patients experience disease relapse post-CD19 CAR-T treatment, often due to mechanisms like CD19 antigen loss. Also, in some patients, life-threatening toxicities occur, including severe CRS and ICANS (144, 145). Ongoing clinical trials suggest that CD20 CAR T-cell therapy could be a promising treatment for r/r NHL, even in cases of CD19-negative disease post-CD19 CAR-T cell relapse (Table 1). The structure of the clinically tested CD20 CAR T-cells is presented in Figure 3.

Phase 1/2 clinical trials utilizing second- and third-generation CAR constructs have confirmed the feasibility and efficacy of autologous anti-CD20 CAR-T cells in r/r $CD20^+$ B-NHL (146). Particularly noteworthy is the efficacy of CD20 CAR T-cell therapy in treating r/r B-NHL patients who had previously failed chemotherapy, including R-CHOP. Studies indicate that CD20-targeted CAR T cells exhibit effectiveness even in cases of low antigen expression, proposing their potential utility for patients with CD20-downregulated B-NHL refractory to CD20 mAb therapy (28, 147). A comprehensive overview of ongoing and completed clinical trials for single CD20 CAR T-cell therapy in hematologic malignancies can be found in Table 2 of a recent review (148).

CD20 is also a pivotal target in CAR-T cell immunotherapies designed to mitigate antigen escape risks. Strategies targeting both CD19 and CD20 include bispecific/tandem CARs, co-administration of CD19 and CD20-directed CAR-T cells as well as sequential treatment with CD19 and CD20-directed CAR-T cells. Tan CAR7 T cells are bispecific CAR T cells composed of tandem extracellular domains targeting CD20 and CD19 tumor antigens linked in frame to the tisa-cel backbone, capable of activation via binding to either CD19 or CD20 tumor antigens, or both (149). Long-term remissions were observed following the use of Tan CAR7 T cells in r/r B-NHL with a safety profile that included CRS but few cases of high-grade CRS (150, 151). In a recent phase 1 dose-escalation trial, autologous CD19/CD20 bispecific CAR-T cells derived from naïve and memory T cells demonstrated safety and strong efficacy (90% ORR, 70% CR rate) in patients with r/r B-NHL (152). Beyond bispecific CD19/CD20 CAR T-cells, ongoing clinical trials explore sequential CD20 CAR-T after CD19 CAR-T infusion and combined infusion of CD19 and CD20-specific CAR-T cells for r/r B-ALL or DLBCL. However, a phase 2 trial combining anti-CD19 and anti-CD20 CAR-T cells in r/r DLBCL showed limited long-term responses (153). Recently, a combinatorial CAR-T cell approach targeting three antigens, CD19, CD20, and CD22, demonstrated efficacy in preclinical models, including leukemic cells that do not express CD19, thereby showcasing the promising potential for treating CD19-negative relapses (154). This approach is now undergoing testing in a clinical trial (NCT05418088).

Resistance to CD20-directed immunotherapies

Despite substantial progress in CD20-targeting immunotherapies, the issue of resistance and post-treatment relapse remains prominent. Resistance to CD20-targeted therapies encompasses a spectrum of mechanisms, ranging from alterations in CD20 antigen levels to compromised immune system effector functions, and extending to diverse mechanisms of immune evasion (Figure 4). One of the main causes of resistance is the loss of the CD20 antigen on the surface of the target cell, which can be caused by changes in the expression of the *MS4A1* gene, including silenced expression and alternative splicing

(28, 155–158). A recent study has shown that the gene encoding CD20 in both healthy and malignant B cells is alternatively spliced into four 5'-UTRs variants, of which especially variants V3 and V4 support robust translation. It has also been demonstrated that resistance to the BsAbs therapy targeting CD20 results from the V3-to-V1 shift (28). A potential strategy to combat this resistance through the use of phosphorodiamidate morpholino oligomers or antisense oligonucleotides was presented in preclinical studies (28). Other mechanisms of antigen loss which may also cause resistance include the internalization of the CD20-mAb complex by cancer cells through endocytosis as well as the transfer of membrane fragments containing CD20 from a cancer cell to an effector cell called trogocytosis (159–161).

Resistance to CD20-directed immunotherapies may also be caused by impaired effector functions of the immune system, such as CDC and ADCC. Therapies targeting CD20 may cause complement depletion and overexpression of its inhibitors CD55 and CD59 (44, 162). Additionally, downregulation of the complement component C1qA was associated with the resistance of DLBCL cells to rituximab *in vitro* (163). Potential strategies to overcome these mechanisms may include the use of inhibitors of complement regulatory proteins as well as the use of the new asymmetric CD55-binding bispecific antibodies (164). Moreover, rituximab-coated tumor cells were shown to significantly downregulate CD16 (FcγRIII), leading to impaired ADCC (165). Mutations that modify the binding of the Fc fragment of antibodies to FcγR can be used to increase the effector functions of antibodies (166, 167). However, as previously mentioned, this approach may not always be optimal when utilizing BsAbs, as it carries an increased risk of premature activation of T cells, cytokine release, and tissue damage.

Additionally, genetic alterations within signaling pathways can also contribute to resistance to CD20-directed therapies, especially in the context of T cell activation, which is crucial for the activity of BsAbs and CAR-T cells (168, 169). Moreover, the tumor microenvironment can play a role in resistance by creating an immunosuppressive milieu. Tumor cells and immunosuppressive cells in the tumor microenvironment, e.g. myeloid-derived suppressive cells (MDSCs), tumor-associated macrophages (TAMs), and regulatory T cells (Tregs), can secrete suppressive cytokines that inhibit the activity of effector cells (T cells, NK cells, phagocytes), thereby reducing the effectiveness of immunotherapy (170, 171). It has also been shown that some proteins secreted by the tumor may have a suppressive effect, including galectin-1, which inhibited CD20 mAb-induced phagocytosis in the lymphoma microenvironment (172). Moreover, overexpression of PD-L1 by tumor cells can contribute to resistance to CD20-targeting therapies by dampening the activity of effector T cells induced by these therapies. Tumor cells may increase PD-L1 expression in response to treatment, leading to T cell exhaustion and reduced efficacy of CD20-targeting therapies (173). Combining CD20-targeting therapies with immune checkpoint inhibitors is a potential strategy to overcome resistance and is currently tested in clinical trials, as discussed in more detail in section *Combination therapies with CD20 immunotherapeutics*.

Due to the multitude of resistance mechanisms, it is crucial to actively search for new methods that can increase the effectiveness of immunotherapy. Research efforts encompass the identification of novel therapeutic targets beyond CD20, the refinement of patient stratification, and the incorporation of combination therapeutic strategies. A recent review summarizes potential solutions to overcome resistance to CAR-T therapy (174).

Combination therapies with CD20 immunotherapeutics

To enhance their efficacy, anti-CD20 mAbs are commonly administered in combination with other drugs. One notable combination is the R-CHOP regimen, which integrates rituximab with cyclophosphamide, doxorubicin, vincristine, and prednisone, and has been extensively employed in treating patients with DLBCL and MCL. Similarly, R-pola-CHP (rituximab, polatuzumab, cyclophosphamide, doxorubicin) regimen is an approved treatment for advanced-stage DLBCL. Other regimens include a combination of rituximab, dexamethasone, high-dose cytarabine and a platinum-based agent (R-DHAP) used in the treatment of MCL, and the addition of lenalidomide to rituximab (R-lenalidomide) which has shown promising results, particularly in patients with r/r FL. Combinations such as bendamustine and rituximab (BR) and fludarabine, cyclophosphamide, and rituximab (FCR) have demonstrated efficacy in the treatment of CLL. Furthermore, novel combinations of rituximab with targeted agents have shown significant potential. Rituximab in combination with venetoclax, a BCL-2 inhibitor, as well as with idelalisib, a PI3K inhibitor, has been approved for the treatment of CLL. Furthermore, the R-GemOx regimen, which combines rituximab with gemcitabine and oxaliplatin, has exhibited notable efficacy in r/r B-NHL in phase 2 clinical trial (175), and is currently being compared to a similar regimen using glofitamab instead of rituximab (glofit-GemOx) in a phase 3 clinical trial (176). In the treatment of $CD20^+$ B-ALL, rituximab is added to standard chemotherapy regimens in patients with a Philadelphia chromosome-negative (Ph-) B-ALL. In patients with Philadelphia chromosome-positive (Ph+) $CD20^+$ B-ALL, rituximab is combined with chemotherapy and BCR-ABL1 tyrosine kinase inhibitors, such as imatinib and dasatinib.

Several clinical trials have investigated the efficacy of other combination therapies involving CD20-targeting in patients with B-NHLs. Among these trials, the combination of R-DHAP regimen with temsirolimus, an mTOR inhibitor, has shown encouraging results, demonstrating improved outcomes in patients with r/r DLBCL (47). Temsirolimus has also demonstrated effectiveness in combination with rituximab alone in patients with r/r MCL in phase 2 clinical trial (177). Moreover, checkpoint inhibitors are also being tested in phase 1 and 2 clinical trials in combination with anti-CD20 therapies. The addition of atezolizumab to an R-CHOP regimen in previously untreated DLBCL patients resulted in 77,5% CR (178). It is also tested in combination with BsAbs glofitamab and mosunetuzumab in phase 1/2 clinical trials in

patients with NHL (NCT02500407, NCT03533283) (179). Additionally, pembrolizumab (anti-PD1 mAb) was evaluated in combination with rituximab in a single-arm phase 2 clinical trial and resulted in 67% ORR with 50% CR among patients with r/r FL (180).

Notably, drugs that are used in combinations with CD20-targeting immunotherapies may have a bidirectional impact on CD20 antigen expression and thus the effectiveness of these therapies. Prednisolone, a glucocorticosteroid present in many chemotherapeutic regimens, was shown to upregulate CD20 on some primary B-ALL samples *in vitro* (4). On the other hand, some drugs that are used together with anti-CD20 mAbs, such as BTK inhibitor ibrutinib, PI3Kδ inhibitor idelalisib, or SYK inhibitor dasatinib, were shown to downregulate CD20 and demonstrated inhibitory effects on cytotoxic effector cells (181–184). These drugs decreased the efficacy of anti-CD20 mAbs *in vitro* (183, 184). It may also be one of the reasons for the failure of an attempt to improve ibrutinib efficacy in CLL by the addition of rituximab, as demonstrated in a randomized clinical trial showing no improvement in PFS in the rituximab+ibrutinib group versus ibrutinib alone (185). This highlights the need for further research on the drug-induced changes in cellular signaling and related CD20 regulation. Understanding these relationships may be important for selecting the most effective therapies and improving therapeutic results. Interestingly, several classes of CD20-upregulating drugs were described, including aurora kinase inhibitors, FOXO1 inhibitors, and chromatin modulators, enabling the increase in anti-CD20-mAbs efficacy in preclinical settings (13). Combining these drugs with CD20-targeting therapies could be a potentially valuable strategy to overcome resistance, however, it requires further evaluation in a clinical setting.

Concluding remarks

A breakthrough in the treatment of B cell malignancies is evident with recent approvals of CD19 CAR-T cells and BsAbs, particularly those targeting CD20xCD3, offering effective treatment and potential cure for r/r patients. Over the past 25 years, CD20, an early target in immunotherapy, has demonstrated remarkable effectiveness. However, the widespread use of cytotoxic T cell-based therapies appeared with new challenges such as treatment-related complications and side effects. Effective management requires the accumulation of comprehensive knowledge and experience, including identifying risk factors for CRS, ICAN, and refining treatment guidelines. These improvements are crucial for the widespread use of these innovative drugs.

With diverse treatment modalities emerging, from naked mAbs to BsAbs and CAR-T cells, understanding determinants of activity and resistance mechanisms for the specific types of treatment are crucial for their optimal selection and clinical efficacy. Decent levels of CD20 are essential for the efficacy of all types of CD20-directed immunotherapies, however, recent preclinical reports emphasize that different types of anti-CD20 therapies require different amounts of CD20 protein on the cell surface to be effective. While a certain level of reduction in CD20 compromises the activity of anti-CD20 mAbs and BsAbs, it may still be adequate for the effectiveness of CD20 CAR-T cells (28). Although the CD20 CAR-T constructs currently being tested in the clinic show great efficacy, further refinements to the CD20 CAR constructs, including changes around the scFv sequence, have shown significant superiority in preclinical models and offer the prospect of even better outcomes for patients (186).

Key directions for CD20 immunotherapy improvement also include combination strategies with small molecule drugs and simultaneous targeting of multiple immunotherapy targets to enhance precision and minimize relapse risks. Simultaneous targeting of CD20 with other antigens like CD19 and CD22 demonstrates efficacy in preclinical models (154) and ongoing clinical trials. Noteworthy, a better understanding of the determinants of response and resistance will be critical for patient selection and future rational combinations.

Author contributions

AD: Conceptualization, Writing – original draft, Writing – review & editing, Visualization, Investigation. KD: Conceptualization, Writing – original draft, Writing – review & editing, Investigation. MF: Conceptualization, Investigation, Writing – original draft, Writing – review & editing, Funding acquisition, Supervision, Visualization.

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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.

Publisher's note

References

1. Sędek Ł, Bulsa J, Sonsala A, Twardoch M, Wieczorek M, Malinowska I, et al. The immunophenotypes of blast cells in B-cell precursor acute lymphoblastic leukemia: how different are they from their normal counterparts? *Cytometry B Clin Cytom*. (2014) 86:329–39. doi: 10.1002/cyto.b.v86.5

2. Thomas DA, O'Brien S, Jorgensen JL, Cortes J, Faderl S, Garcia-Manero G, et al. Prognostic significance of CD20 expression in adults with *de novo* precursor B-lineage acute lymphoblastic leukemia. *Blood*. (2009) 113:6330–7. doi: 10.1182/blood-2008-04-151860

3. Jeha S, Behm F, Pei D, Sandlund JT, Ribeiro RC, Razzouk BI, et al. Prognostic significance of CD20 expression in childhood B-cell precursor acute lymphoblastic leukemia. *Blood*. (2006) 108:3302–4. doi: 10.1182/blood-2006-04-016709

4. Dworzak MN, Schumich A, Printz D, Pötschger U, Husak Z, Attarbaschi A, et al. CD20 up-regulation in pediatric B-cell precursor acute lymphoblastic leukemia during induction treatment: setting the stage for anti-CD20 directed immunotherapy. *Blood*. (2008) 112:3982–8. doi: 10.1182/blood-2008-06-164129

5. Sędek Ł, Theunissen P, Sobral da Costa E, van der Sluijs-Gelling A, Mejstrikova E, Gaipa G, et al. Differential expression of CD73, CD86 and CD304 in normal vs. leukemic B-cell precursors and their utility as stable minimal residual disease markers in childhood B-cell precursor acute lymphoblastic leukemia. *J Immunol Methods*. (2019) 475:112429. doi: 10.1016/j.jim.2018.03.005

6. Worch J, Makarova O, Burkhardt B. Immunreconstitution and infectious complications after rituximab treatment in children and adolescents: what do we know and what can we learn from adults? *Cancers*. (2015) 7:305–28. doi: 10.3390/cancers7010305

7. Maury S, Chevret S, Thomas X, Heim D, Leguay T, Huguet F, et al. Rituximab in B-lineage adult acute lymphoblastic leukemia. *New Engl J Med*. (2016) 375:1044–53. doi: 10.1056/NEJMoa1605085

8. Klein C, Lammens A, Schäfer W, Georges G, Schwaiger M, Mössner E, et al. Epitope interactions of monoclonal antibodies targeting CD20 and their relationship to functional properties. *MAbs*. (2013) 5:22–33. doi: 10.4161/mabs.22771

9. Rougé L, Chiang N, Steffek M, Kugel C, Croll TI, Tam C, et al. Structure of CD20 in complex with the therapeutic monoclonal antibody rituximab. *Science*. (2020) 367:1224–30. doi: 10.1126/science.aaz9356

10. Kläsener K, Jellusova J, Andrieux G, Salzer U, Böhler C, Steiner SN, et al. CD20 as a gatekeeper of the resting state of human B cells. *Proc Natl Acad Sci U S A*. (2021) 118:e2021342118. doi: 10.1073/pnas.2021342118

11. Li H, Ayer LM, Lytton J, Deans JP. Store-operated cation entry mediated by CD20 in membrane rafts. *J Biol Chem*. (2003) 278:42427–34. doi: 10.1074/jbc.M308802200

12. Walshe CA, Beers SA, French RR, Chan CHT, Johnson PW, Packham GK, et al. Induction of cytosolic calcium flux by CD20 is dependent upon B Cell antigen receptor signaling. *J Biol Chem*. (2008) 283:16971–84. doi: 10.1074/jbc.M708459200

13. Pavlasova G, Mraz M. The regulation and function of CD20: an "enigma" of B-cell biology and targeted therapy. *Haematologica*. (2020) 105:1494–506. doi: 10.3324/haematol.2019.243543

14. Shimizu R, Kikuchi J, Wada T, Ozawa K, Kano Y, Furukawa Y. HDAC inhibitors augment cytotoxic activity of rituximab by upregulating CD20 expression on lymphoma cells. *Leukemia*. (2010) 24:1760–8. doi: 10.1038/leu.2010.157

15. Laidlaw BJ, Cyster JG. Transcriptional regulation of memory B cell differentiation. *Nat Rev Immunol*. (2021) 21:209–20. doi: 10.1038/s41577-020-00446-2

16. Himmelmann A, Riva A, Wilson GL, Lucas BP, Thevenin C, Kehrl JH. PU.1/Pip and basic helix loop helix zipper transcription factors interact with binding sites in the CD20 promoter to help confer lineage- and stage-specific expression of CD20 in B lymphocytes. *Blood*. (1997) 90:3984–95. doi: 10.1182/blood.V90.10.3984

17. Thévenin C, Lucas BP, Kozlow EJ, Kehrl JH. Cell type- and stage-specific expression of the CD20/B1 antigen correlates with the activity of a diverged octamer DNA motif present in its promoter. *J Biol Chem*. (1993) 268:5949–56. doi: 10.1016/S0021-9258(18)53411-6

18. Wojciechowski W, Li H, Marshall S, Dell'Agnola C, Espinoza-Delgado I. Enhanced expression of CD20 in human tumor B cells is controlled through ERK-dependent mechanisms. *J Immunol*. (2005) 174:7859–68. doi: 10.4049/jimmunol.174.12.7859

19. Słabicki M, Lee KS, Jethwa A, Sellner L, Sacco F, Walther T, et al. Dissection of CD20 regulation in lymphoma using RNAi. *Leukemia*. (2016) 30:2409–12. doi: 10.1038/leu.2016.230

20. Grzelak L, Roesch F, Vaysse A, Biton A, Legendre R, Porrot F, et al. IRF8 regulates efficacy of therapeutic anti-CD20 monoclonal antibodies. *Eur J Immunol*. (2022) 52:1648–61. doi: 10.1002/eji.202250037

21. Pyrzynska B, Dwojak M, Zerrouqi A, Morlino G, Zapala P, Miazek N, et al. FOXO1 promotes resistance of non-Hodgkin lymphomas to anti-CD20-based therapy. *Oncoimmunology*. (2018) 7:e1423183. doi: 10.1080/2162402X.2017.1423183

22. Kawabata KC, Ehata S, Komuro A, Takeuchi K, Miyazono K. TGF-β-induced apoptosis of B-cell lymphoma Ramos cells through reduction of MS4A1/CD20. *Oncogene*. (2013) 32:2096–106. doi: 10.1038/onc.2012.219

23. Seitz V, Butzhammer P, Hirsch B, Hecht J, Gütgemann I, Ehlers A, et al. Deep sequencing of MYC DNA-binding sites in Burkitt lymphoma. *PLoS One*. (2011) 6:e26837. doi: 10.1371/journal.pone.0026837

24. Yu D, Dews M, Park A, Tobias JW, Thomas-Tikhonenko A. Inactivation of Myc in murine two-hit B lymphomas causes dormancy with elevated levels of interleukin 10 receptor and CD20: implications for adjuvant therapies. *Cancer Res*. (2005) 65:5454–61. doi: 10.1158/0008-5472.CAN-04-4197

25. Tomita A, Hiraga J, Kiyoi H, Ninomiya M, Sugimoto T, Ito M, et al. Epigenetic regulation of CD20 protein expression in a novel B-cell lymphoma cell line, RRBL1, established from a patient treated repeatedly with rituximab-containing chemotherapy. *Int J Hematol*. (2007) 86:49–57. doi: 10.1532/IJH97.07028

26. Scialdone A, Hasni MS, Damm JK, Lennartsson A, Gullberg U, Drott K. The HDAC inhibitor valproate induces a bivalent status of the CD20 promoter in CLL patients suggesting distinct epigenetic regulation of CD20 expression in CLL in vivo. *Oncotarget*. (2017) 8:37409–22. doi: 10.18632/oncotarget.v8i23

27. Frys S, Simons Z, Hu Q, Barth MJ, Gu JJ, Mavis C, et al. Entinostat, a novel histone deacetylase inhibitor is active in B-cell lymphoma and enhances the anti-tumour activity of rituximab and chemotherapy agents. *Br J Haematol*. (2015) 169:506–19. doi: 10.1111/bjh.13318

28. Ang Z, Paruzzo L, Hayer KE, Schmidt C, Torres Diz M, Xu F, et al. Alternative splicing of its 5′-UTR limits CD20 mRNA translation and enables resistance to CD20-directed immunotherapies. *Blood*. (2023) 142:1724–39. doi: 10.1182/blood.2023020400

29. Almagro JC, Daniels-Wells TR, Perez-Tapia SM, Penichet ML. Progress and challenges in the design and clinical development of antibodies for cancer therapy. *Front Immunol*. (2017) 8:1751. doi: 10.3389/fimmu.2017.01751

30. Fouda GE, Bavbek S. Rituximab hypersensitivity: from clinical presentation to management. *Front Pharmacol*. (2020) 11:572863. doi: 10.3389/fphar.2020.572863

31. Schieber T, Li A, Lundberg J, Wiczer T, Voorhees T. Successful obinutuzumab administration after rituximab discontinuation due to intolerance in patients with hematologic disorders. *Blood Adv*. (2023) 7:3431–4. doi: 10.1182/bloodadvances.2022009548

32. Ali SB, Yuson C, Hissaria P. Rituximab hypersensitivity reactions and tolerance of ofatumumab therapy. *Clin Exp Rheumatol*. (2021) 39:648–50. doi: 10.55563/clinexprheumatol/z3mbin

33. Coiffier B, Lepretre S, Pedersen LM, Gadeberg O, Fredriksen H, van Oers MHJ, et al. Safety and efficacy of ofatumumab, a fully human monoclonal anti-CD20 antibody, in patients with relapsed or refractory B-cell chronic lymphocytic leukemia: a phase 1-2 study. *Blood*. (2008) 111:1094–100. doi: 10.1182/blood-2007-09-111781

34. Ko S, Jo M, Jung ST. Recent achievements and challenges in prolonging the serum half-lives of therapeutic IgG antibodies through Fc engineering. *BioDrugs*. (2021) 35:147–57. doi: 10.1007/s40259-021-00471-0

35. Weiner GJ. Rituximab: mechanism of action. *Semin Hematol*. (2010) 47:115–23. doi: 10.1053/j.seminhematol.2010.01.011

36. Lim SH, Vaughan AT, Ashton-Key M, Williams EL, Dixon SV, Chan HTC, et al. Fc gamma receptor IIb on target B cells promotes rituximab internalization and reduces clinical efficacy. *Blood*. (2011) 118:2530–40. doi: 10.1182/blood-2011-01-330357

37. Deans JP, Li H, Polyak MJ. CD20-mediated apoptosis: signalling through lipid rafts. *Immunology*. (2002) 107:176–82. doi: 10.1046/j.1365-2567.2002.01495.x

38. Hamlin PA, Musteata V, Park SI, Burnett C, Dabovic K, Strack T, et al. Safety and efficacy of engineered toxin body MT-3724 in relapsed or refractory B-cell non-Hodgkin's lymphomas and diffuse large B-cell lymphoma. *Cancer Res Commun*. (2022) 2:307–15. doi: 10.1158/2767-9764.CRC-22-0056

39. Kimball AS, Webb TJ. The roles of radiotherapy and immunotherapy for the treatment of lymphoma. *Mol Cell Pharmacol*. (2013) 5:27–38.

40. Grillo-López AJ, White CA, Dallaire BK, Varns CL, Shen CD, Wei A, et al. Rituximab: the first monoclonal antibody approved for the treatment of lymphoma. *Curr Pharm Biotechnol*. (2000) 1:1–9. doi: 10.2174/1389201003379059

41. Pierpont TM, Limper CB, Richards KL. Past, present, and future of rituximab-the world's first oncology monoclonal antibody therapy. *Front Oncol*. (2018) 8:163. doi: 10.3389/fonc.2018.00163

42. Yelvington BJ. Subcutaneous rituximab in follicular lymphoma, chronic lymphocytic leukemia, and diffuse large B-cell lymphoma. *J Adv Pract Oncol*. (2018) 9:530–4.

43. Pérez-Callejo D, González-Rincón J, Sánchez A, Provencio M, Sánchez-Beato M. Action and resistance of monoclonal CD20 antibodies therapy in B-cell Non-Hodgkin Lymphomas. *Cancer Treat Rev*. (2015) 41:680–9. doi: 10.1016/j.ctrv.2015.05.007

44. Kennedy AD, Beum PV, Solga MD, DiLillo DJ, Lindorfer MA, Hess CE, et al. Rituximab infusion promotes rapid complement depletion and acute CD20 loss in chronic lymphocytic leukemia1. *J Immunol*. (2004) 172:3280–8. doi: 10.4049/jimmunol.172.5.3280

45. Leonard JP, Trneny M, Izutsu K, Fowler NH, Hong X, Zhu J, et al. AUGMENT: A phase III study of lenalidomide plus rituximab versus placebo plus rituximab in

relapsed or refractory indolent lymphoma. *J Clin Oncol*. (2019) 37:1188–99. doi: 10.1200/JCO.19.00010

46. Johnston PB, LaPlant B, McPhail E, Habermann TM, Inwards DJ, Micallef IN, et al. Everolimus combined with R-CHOP-21 for new, untreated, diffuse large B-cell lymphoma (NCCTG 1085 [Alliance]): safety and efficacy results of a phase 1 and feasibility trial. *Lancet Haematol*. (2016) 3:e309–316. doi: 10.1016/S2352-3026(16)30040-0

47. Witzens-Harig M, Viardot A, Keller U, Wosniok J, Deuster O, Klemmer J, et al. The mTOR inhibitor temsirolimus added to rituximab combined with dexamethasone, cytarabine, and cisplatinum (R-DHAP) for the treatment of patients with relapsed or refractory DLBCL - results from the phase-II STORM trial. *Hemasphere*. (2021) 5:e636. doi: 10.1097/HS9.0000000000000636

48. Lin TS. Ofatumumab: a novel monoclonal anti-CD20 antibody. *Pharmgenomics Pers Med*. (2010) 3:51–9. doi: 10.2147/PGPM

49. Teeling JL, French RR, Cragg MS, van den Brakel J, Pluyter M, Huang H, et al. Characterization of new human CD20 monoclonal antibodies with potent cytolytic activity against non-Hodgkin lymphomas. *Blood*. (2004) 104:1793–800. doi: 10.1182/blood-2004-01-0039

50. Barth MJ, Mavis C, Czuczman MS, Hernandez-Ilizaliturri FJ. Ofatumumab exhibits enhanced *in vitro* and *in vivo* activity compared to rituximab in preclinical models of mantle cell lymphoma. *Clin Cancer Res*. (2015) 21:4391–7. doi: 10.1158/1078-0432.CCR-15-0056

51. Cleary KLS, Chan HTC, James S, Glennie MJ, Cragg MS. Antibody distance from the cell membrane regulates antibody effector mechanisms. *J Immunol*. (2017) 198:3999–4011. doi: 10.4049/jimmunol.1601473

52. Kumar A, Planchais C, Fronzes R, Mouquet H, Reyes N. Binding mechanisms of therapeutic antibodies to human CD20. *Science*. (2020) 369:793–9. doi: 10.1126/science.abb8008

53. Desikan SP, Keating MJ, Ferrajoli A, Jain N, Ohanian M, Pemmaraju N, et al. Early treatment with ofatumumab in patients with high-risk CLL. *Blood*. (2022) 140:9876–8. doi: 10.1182/blood-2022-167284

54. van Imhoff GW, McMillan A, Matasar MJ, Radford J, Ardeshna KM, Kuliczkowski K, et al. Ofatumumab versus rituximab salvage chemoimmunotherapy in relapsed or refractory diffuse large B-cell lymphoma: the ORCHARRD study. *J Clin Oncol*. (2017) 35:544–51. doi: 10.1200/JCO.2016.69.0198

55. Maloney DG, Ogura M, Fukuhara N, Davis J, Lasher J, Izquierdo M, et al. A phase 3 randomized study (HOMER) of ofatumumab vs rituximab in iNHL relapsed after rituximab-containing therapy. *Blood Advances*. (2020) 4:3886–93. doi: 10.1182/bloodadvances.2020001942

56. Jabbour E, Richard-Carpentier G, Sasaki Y, Konopleva M, Patel K, Roberts K, et al. Hyper-CVAD regimen in combination with ofatumumab as frontline therapy for adults with Philadelphia chromosome-negative B-cell acute lymphoblastic leukaemia: a single-arm, phase 2 trial. *Lancet Haematol*. (2020) 7:e523–33. doi: 10.1016/S2352-3026(20)30144-7

57. Byrd JC, Brown JR, O'Brien S, Barrientos JC, Kay NE, Reddy NM, et al. Ibrutinib versus ofatumumab in previously treated chronic lymphoid leukemia. *N Engl J Med*. (2014) 371:213–23. doi: 10.1056/NEJMoa1400376

58. Sandhu S, Mulligan SP. Ofatumumab and its role as immunotherapy in chronic lymphocytic leukemia. *Haematologica*. (2015) 100:411–4. doi: 10.3324/haematol.2015.124107

59. Flinn IW, Hillmen P, Montillo M, Nagy Z, Illés Á, Etienne G, et al. The phase 3 DUO trial: duvelisib vs ofatumumab in relapsed and refractory CLL/SLL. *Blood*. (2018) 132:2446–55. doi: 10.1182/blood-2018-05-850461

60. Mössner E, Brünker P, Moser S, Püntener U, Schmidt C, Herter S, et al. Increasing the efficacy of CD20 antibody therapy through the engineering of a new type II anti-CD20 antibody with enhanced direct and immune effector cell-mediated B-cell cytotoxicity. *Blood*. (2010) 115:4393–402. doi: 10.1182/blood-2009-06-225979

61. Ferrara C, Stuart F, Sondermann P, Brünker P, Umaña P. The carbohydrate at FcgammaRIIIa Asn-162. An element required for high affinity binding to non-fucosylated IgG glycoforms. *J Biol Chem*. (2006) 281:5032–6. doi: 10.1074/jbc.M510171200

62. Patz M, Isaeva P, Forcob N, Müller B, Frenzel LP, Wendtner CM, et al. Comparison of the in *vitro* effects of the anti-CD20 antibodies rituximab and GA101 on chronic lymphocytic leukaemia cells. *Br J Haematol*. (2011) 152:295–306. doi: 10.1111/j.1365-2141.2010.08428.x

63. Herter S, Herting F, Mundigl O, Waldhauer I, Weinzierl T, Fauti T, et al. Preclinical activity of the type II CD20 antibody GA101 (obinutuzumab) compared with rituximab and ofatumumab in *vitro* and in xenograft models. *Mol Cancer Ther*. (2013) 12:2031–42. doi: 10.1158/1535-7163.MCT-12-1182

64. Alduaij W, Ivanov A, Honeychurch J, Cheadle EJ, Potluri S, Lim SH, et al. Novel type II anti-CD20 monoclonal antibody (GA101) evokes homotypic adhesion and actin-dependent, lysosome-mediated cell death in B-cell Malignancies. *Blood*. (2011) 117:4519–29. doi: 10.1182/blood-2010-07-296913

65. Bologna L, Gotti E, Manganini M, Rambaldi A, Intermesoli T, Introna M, et al. Mechanism of action of type II, glycoengineered, anti-CD20 monoclonal antibody GA101 in B-chronic lymphocytic leukemia whole blood assays in comparison with rituximab and alemtuzumab. *J Immunol*. (2011) 186:3762–9. doi: 10.4049/jimmunol.1000303

66. Illidge T, Klein C, Sehn LH, Davies A, Salles G, Cartron G. Obinutuzumab in hematologic Malignancies: lessons learned to date. *Cancer Treat Rev*. (2015) 41:784–92. doi: 10.1016/j.ctrv.2015.07.003

67. Chu Y, Awasthi A, Lee S, Edani D, Yin C, Hochberg J, et al. Obinutuzumab (GA101) vs. rituximab significantly enhances cell death, antibody-dependent cytotoxicity and improves overall survival against CD20+ primary mediastinal B-cell lymphoma (PMBL) in a xenograft NOD-scid IL2Rgnull (NSG) mouse model: a potential targeted agent in the treatment of PMBL. *Oncotarget*. (2020) 11:3035–47. doi: 10.18632/oncotarget.v11i32

68. Goede V, Fischer K, Engelke A, Schlag R, Lepretre S, Montero LFC, et al. Obinutuzumab as frontline treatment of chronic lymphocytic leukemia: updated results of the CLL11 study. *Leukemia*. (2015) 29:1602–4. doi: 10.1038/leu.2015.14

69. Tobinai K, Klein C, Oya N, Fingerle-Rowson G. A review of obinutuzumab (GA101), a novel type II anti-CD20 monoclonal antibody, for the treatment of patients with B-cell Malignancies. *Adv Ther*. (2017) 34:324–56. doi: 10.1007/s12325-016-0451-1

70. Townsend W, Hiddemann W, Buske C, Cartron G, Cunningham D, Dyer MJS, et al. Obinutuzumab versus rituximab immunochemotherapy in previously untreated iNHL: final results from the GALLIUM study. *Hemasphere*. (2023) 7:e919. doi: 10.1097/HS9.0000000000000919

71. Le Gouill S, Ghesquières H, Oberic L, Morschhauser F, Tilly H, Ribrag V, et al. Obinutuzumab vs rituximab for advanced DLBCL: a PET-guided and randomized phase 3 study by LYSA. *Blood*. (2021) 137:2307–20. doi: 10.1182/blood.2020008750

72. Sehn LH, Martelli M, Trněný M, Liu W, Bolen CR, Knapp A, et al. A randomized, open-label, Phase III study of obinutuzumab or rituximab plus CHOP in patients with previously untreated diffuse large B-Cell lymphoma: final analysis of GOYA. *J Hematol Oncol*. (2020) 13:71. doi: 10.1186/s13045-020-00900-7

73. Freeman CL, Sehn LH. A tale of two antibodies: obinutuzumab versus rituximab. *Br J Haematol*. (2018) 182:29–45. doi: 10.1111/bjh.15232

74. Michot JM, Bouabdallah R, Vitolo U, Doorduijn JK, Salles G, Chiappella A, et al. Avadomide plus obinutuzumab in patients with relapsed or refractory B-cell non-Hodgkin lymphoma (CC-122-NHL-001): a multicentre, dose escalation and expansion phase 1 study. *Lancet Haematol*. (2020) 7:e649–59. doi: 10.1016/S2352-3026(20)30208-8

75. Cartron G, Watier H. Obinutuzumab: what is there to learn from clinical trials? *Blood*. (2017) 130:581–9. doi: 10.1182/blood-2017-03-771832

76. Witzig TE, Gordon LI, Cabanillas F, Czuczman MS, Emmanouilides C, Joyce R, et al. Randomized controlled trial of yttrium-90-labeled ibritumomab tiuxetan radioimmunotherapy versus rituximab immunotherapy for patients with relapsed or refractory low-grade, follicular, or transformed B-cell non-Hodgkin's lymphoma. *J Clin Oncol*. (2002) 20:2453–63. doi: 10.1200/JCO.2002.11.076

77. Morschhauser F, Radford J, Van Hoof A, Vitolo U, Soubeyran P, Tilly H, et al. Phase III trial of consolidation therapy with yttrium-90-ibritumomab tiuxetan compared with no additional therapy after first remission in advanced follicular lymphoma. *J Clin Oncol*. (2008) 26:5156–64. doi: 10.1200/JCO.2008.17.2015

78. Sachpekidis C, Jackson DB, Soldatos TG. Radioimmunotherapy in non-Hodgkin's lymphoma: retrospective adverse event profiling of Zevalin and Bexxar. *Pharm (Basel)*. (2019) 12:141. doi: 10.3390/ph12040141

79. Kaminski MS, Tuck M, Estes J, Kolstad A, Ross CW, Zasadny K, et al. 131I-tositumomab therapy as initial treatment for follicular lymphoma. *N Engl J Med*. (2005) 352:441–9. doi: 10.1056/NEJMoa041511

80. Kaminski MS, Zelenetz AD, Press OW, Saleh M, Leonard J, Fehrenbacher L, et al. Pivotal study of iodine I 131 tositumomab for chemotherapy-refractory low-grade or transformed low-grade B-cell non-Hodgkin's lymphomas. *J Clin Oncol*. (2001) 19:3918–28. doi: 10.1200/JCO.2001.19.19.3918

81. Iagaru A, Mittra ES, Ganjoo K, Knox SJ, Goris ML. 131I-Tositumomab (Bexxar) vs. 90Y-Ibritumomab (Zevalin) therapy of low-grade refractory/relapsed non-Hodgkin lymphoma. *Mol Imaging Biol*. (2010) 12:198–203. doi: 10.1007/s11307-009-0245-9

82. Green DJ, Press OW. Whither radioimmunotherapy: to be or not to be? *Cancer Res*. (2017) 77:2191–6. doi: 10.1158/0008-5472.CAN-16-2523

83. Chow VA, Rajendran JG, Fisher DR, Appelbaum FR, Cassaday RD, Martin PS, et al. A phase II trial evaluating the efficacy of high-dose Radioiodinated Tositumomab (Anti-CD20) antibody, etoposide and cyclophosphamide followed by autologous transplantation, for high-risk relapsed or refractory non-hodgkin lymphoma. *Am J Hematol*. (2020) 95:775–83. doi: 10.1002/ajh.25818

84. Shadman M, Li H, Rimsza L, Leonard JP, Kaminski MS, Braziel RM, et al. Continued excellent outcomes in previously untreated patients with follicular lymphoma after treatment with CHOP plus rituximab or CHOP plus 131I-tositumomab: long-term follow-up of phase III randomized study SWOG-S0016. *J Clin Oncol*. (2018) 36:697–703. doi: 10.1200/JCO.2017.74.5083

85. Schaefer NG, Huang P, Buchanan JW, Wahl RL. Radioimmunotherapy in non-Hodgkin lymphoma: opinions of nuclear medicine physicians and radiation oncologists. *J Nucl Med*. (2011) 52:830–8. doi: 10.2967/jnumed.110.085589

86. Chamarthy MR, Williams SC, Moadel RM. Radioimmunotherapy of non-Hodgkin's lymphoma: from the "magic bullets" to "radioactive magic bullets." *Yale J Biol Med*. (2011) 84:391–407.

87. Gazyvaro | European Medicines Agency (2023). Available online at: https://www.ema.europa.eu/en/medicines/human/EPAR/gazyvaro.

88. MabThera | European Medicines Agency (2023). Available online at: https://www.ema.europa.eu/en/medicines/human/EPAR/mabthera.

89. Lang DSP, Hagger C, Pearson A. Safety of rapid rituximab infusion in adult cancer patients: a systematic review. *Int J Nurs Pract*. (2011) 17:357–69. doi: 10.1111/j.1440-172X.2011.01950.x

90. Makatsori M, Kiani-Alikhan S, Manson AL, Verma N, Leandro M, Gurugama NP, et al. Hypogammaglobulinaemia after rituximab treatment-incidence and outcomes. *QJM*. (2014) 107:821–8. doi: 10.1093/qjmed/hcu094

91. Brinkmann U, Kontermann RE. The making of bispecific antibodies. *mAbs*. (2017) 9:182–212. doi: 10.1080/19420862.2016.1268307

92. Ma J, Mo Y, Tang M, Shen J, Qi Y, Zhao W, et al. Bispecific antibodies: from research to clinical application. *Front Immunol*. (2021) 12:626616. doi: 10.3389/fimmu.2021.626616

93. Sun Y, Yu X, Wang X, Yuan K, Wang G, Hu L, et al. Bispecific antibodies in cancer therapy: Target selection and regulatory requirements. *Acta Pharm Sin B*. (2023) 13:3583–97. doi: 10.1016/j.apsb.2023.05.023

94. Wu J, Fu J, Zhang M, Liu D. Blinatumomab: a bispecific T cell engager (BiTE) antibody against CD19/CD3 for refractory acute lymphoid leukemia. *J Hematol Oncol*. (2015) 8:104. doi: 10.1186/s13045-015-0195-4

95. Engelberts PJ, Hiemstra IH, de Jong B, Schuurhuis DH, Meesters J, Beltran Hernandez I, et al. DuoBody-CD3xCD20 induces potent T-cell-mediated killing of Malignant B cells in preclinical models and provides opportunities for subcutaneous dosing. *EBioMedicine*. (2020) 52:102625. doi: 10.1016/j.ebiom.2019.102625

96. van der Horst HJ, de Jonge AV, Hiemstra IH, Gelderloos AT, Berry DRAI, Hijmering NJ, et al. Epcoritamab induces potent anti-tumor activity against Malignant B-cells from patients with DLBCL, FL and MCL, irrespective of prior CD20 monoclonal antibody treatment. *Blood Cancer J*. (2021) 11:1–8. doi: 10.1038/s41408-021-00430-6

97. Mhibik M, Gaglione EM, Eik D, Herrick J, Le J, Ahn IE, et al. Cytotoxicity of the CD3×CD20 bispecific antibody epcoritamab in CLL is increased by concurrent BTK or BCL-2 targeting. *Blood Advances*. (2023) 7:4089–101. doi: 10.1182/bloodadvances.2022009517

98. Hutchings M, Mous R, Clausen MR, Johnson P, Linton KM, Chamuleau MED, et al. Dose escalation of subcutaneous epcoritamab in patients with relapsed or refractory B-cell non-Hodgkin lymphoma: an open-label, phase 1/2 study. *Lancet*. (2021) 398:1157–69. doi: 10.1016/S0140-6736(21)00889-8

99. Kater AP, Christensen JH, Bentzen HH, Niemann CU, Hutchings M, Chen J, et al. Subcutaneous epcoritamab in patients with relapsed/refractory chronic lymphocytic leukemia: preliminary results from the epcore CLL-1 trial. *Blood*. (2021) 138:2627. doi: 10.1182/blood-2021-146563

100. *Research C for DE and. FDA grants accelerated approval to epcoritamab-bysp for relapsed or refractory diffuse large B-cell lymphoma and high-grade B-cell lymphoma*. FDA (2023). Available at: https://www.fda.gov/drugs/drug-approvals-and-databases/fda-grants-accelerated-approval-epcoritamab-bysp-relapsed-or-refractory-diffuse-large-b-cell.

101. Tepkinly | European Medicines Agency (2023). Available online at: https://www.ema.europa.eu/en/medicines/human/EPAR/tepkinly.

102. Smith EJ, Olson K, Haber LJ, Varghese B, Duramad P, Tustian AD, et al. A novel, native-format bispecific antibody triggering T-cell killing of B-cells is robustly active in mouse tumor models and cynomolgus monkeys. *Sci Rep*. (2015) 5:17943. doi: 10.1038/srep17943

103. Zhu M, Olson K, Kirshner JR, Khaksar Toroghi M, Yan H, Haber L, et al. Translational findings for odronextamab: From preclinical research to a first-in-human study in patients with CD20+ B-cell Malignancies. *Clin Trans Science*. (2022) 15:954–66. doi: 10.1111/cts.13212

104. Bannerji R, Arnason JE, Advani RH, Brown JR, Allan JN, Ansell SM, et al. Odronextamab, a human CD20×CD3 bispecific antibody in patients with CD20-positive B-cell Malignancies (ELM-1): results from the relapsed or refractory non-Hodgkin lymphoma cohort in a single-arm, multicentre, phase 1 trial. *Lancet Haematol*. (2022) 9:e327–39. doi: 10.1016/S2352-3026(22)00072-2

105. Weinstock M, Elavalakanar P, Bright S, Ambati SR, Brouwer-Visser J, Pourpe S, et al. Complete responses to odronextamab in two patients with diffuse large B-cell lymphoma refractory to chimeric antigen receptor T-cell therapy. *Br J Haematol*. (2022) 199:366–70. doi: 10.1111/bjh.18383

106. *Odronextamab BLA for Treatment of Relapsed/Refractory Follicular Lymphoma (FL) and Diffuse Large B-cell Lymphoma (DLBCL) Accepted for FDA Priority Review*. Regeneron Pharmaceuticals Inc (2023). Available at: https://investor.regeneron.com/news-releases/news-release-details/odronextamab-bla-treatment-relapsedrefractory-follicular/.

107. *Odronextamab Receives EMA Filing Acceptance for Treatment of Relapsed/Refractory Follicular Lymphoma and Diffuse Large B-cell Lymphoma*. Regeneron Pharmaceuticals Inc (2023). Available at: https://investor.regeneron.com/news-releases/news-release-details/odronextamab-receives-ema-filing-acceptance-treatment/.

108. Sun LL, Ellerman D, Mathieu M, Hristopoulos M, Chen X, Li Y, et al. Anti-CD20/CD3 T cell–dependent bispecific antibody for the treatment of B cell Malignancies. *Sci Trans Med*. (2015) 7:287ra70–287ra70. doi: 10.1126/scitranslmed.aaa4802

109. Spiess C, Merchant M, Huang A, Zheng Z, Yang NY, Peng J, et al. Bispecific antibodies with natural architecture produced by co-culture of bacteria expressing two distinct half-antibodies. *Nat Biotechnol*. (2013) 31:753–8. doi: 10.1038/nbt.2621

110. Budde LE, Sehn LH, Matasar M, Schuster SJ, Assouline S, Giri P, et al. Safety and efficacy of mosunetuzumab, a bispecific antibody, in patients with relapsed or refractory follicular lymphoma: a single-arm, multicentre, phase 2 study. *Lancet Oncol*. (2022) 23:1055–65. doi: 10.1016/S1470-2045(22)00335-7

111. Bartlett NL, Assouline S, Giri P, Schuster SJ, Cheah CY, Matasar M, et al. Mosunetuzumab monotherapy is active and tolerable in patients with relapsed/refractory diffuse large B-cell lymphoma. *Blood Advances*. (2023) 7:4926–35. doi: 10.1182/bloodadvances.2022009260

112. Budde LE, Olszewski AJ, Assouline S, Lossos IS, Diefenbach C, Kamdar M, et al. Mosunetuzumab with polatuzumab vedotin in relapsed or refractory aggressive large B cell lymphoma: a phase 1b/2 trial. *Nat Med*. (2023) 30:229–39. doi: 10.1038/s41591-023-02726-5

113. Lunsumio | European Medicines Agency (2023). Available online at: https://www.ema.europa.eu/en/medicines/human/EPAR/lunsumio#ema-inpage-item-overview.

114. *Research C for DE and. FDA D.I.S.C.O. Burst Edition: FDA approval of Lunsumio (mosunetuzumab-axgb) for adult patients with relapsed or refractory follicular lymphoma after two or more lines of systemic therapy*. FDA (2023). Available at: https://www.fda.gov/drugs/resources-information-approved-drugs/fda-disco-burst-edition-fda-approval-lunsumio-mosunetuzumab-axgb-adult-patients-relapsed-or.

115. Bacac M, Colombetti S, Herter S, Sam J, Perro M, Chen S, et al. CD20-TCB with obinutuzumab pretreatment as next-generation treatment of hematologic Malignancies. *Clin Cancer Res*. (2018) 24:4785–97. doi: 10.1158/1078-0432.CCR-18-0455

116. Hutchings M, Morschhauser F, Iacoboni G, Carlo-Stella C, Offner FC, Sureda A, et al. Glofitamab, a novel, bivalent CD20-targeting T-cell–engaging bispecific antibody, induces durable complete remissions in relapsed or refractory B-cell lymphoma: A phase I trial. *JCO*. (2021) 39:1959–70. doi: 10.1200/JCO.20.03175

117. Dickinson MJ, Carlo-Stella C, Morschhauser F, Bachy E, Corradini P, Iacoboni G, et al. Glofitamab for relapsed or refractory diffuse large B-cell lymphoma. *New Engl J Med*. (2022) 387:2220–31. doi: 10.1056/NEJMoa2206913

118. Ferhanoglu B, Gulbas Z, Uzay A, Özcan M, Ozkalemkas F, Dal MS, et al. Glofitamab in relapsed/refractory diffuse large B cell lymphoma: real world data. *Blood*. (2022) 140:6704–5. doi: 10.1182/blood-2022-165385

119. Dickinson M, Viardot A, Marks R, Topp MS, Morschhauser F, Jacobs B, et al. Glofitamab + Pola-R-CHP in patients with previously untreated diffuse large B-cell lymphoma (DLBCL): Results from a phase Ib study. *JCO*. (2023) 41:7549–9. doi: 10.1200/JCO.2023.41.16_suppl.7549

120. *Research C for DE and. FDA grants accelerated approval to glofitamab-gxbm for selected relapsed or refractory large B-cell lymphomas*. FDA (2023). Available at: https://www.fda.gov/drugs/drug-approvals-and-databases/fda-grants-accelerated-approval-glofitamab-gxbm-selected-relapsed-or-refractory-large-b-cell.

121. Columvi | European Medicines Agency (2023). Available online at: https://www.ema.europa.eu/en/medicines/human/EPAR/columvi#ema.

122. Baliga R, Li K, Manlusoc M, Hinton P, Ng D, Tran M, et al. High avidity IgM-based CD20xCD3 bispecific antibody (IGM-2323) for enhanced T-cell dependent killing with minimal cytokine release. *Blood*. (2019) 134:1574. doi: 10.1182/blood-2019-131650

123. Hernandez GH, So J, Logronio KA, Kotturi MF, Kim WS, Armand P, et al. Pharmacodynamics and biomarker correlates of imvotamab (IGM-2323), the first-in-class CD20xCD3 bispecific igM antibody with dual mechanisms of action, in patients with advanced B cell Malignancies. *Blood*. (2022) 140:6436–8. doi: 10.1182/blood-2022-163235

124. Hart KC, Logronio K, Li M, Yakkundi P, Manlusoc M, Li K, et al. Abstract 4179: High valency of IGM-2323, a CD20xCD3 IgM bispecific T cell engager, displaces rituximab binding and induces potent B lymphoma cell killing. *Cancer Res*. (2022) 82:4179. doi: 10.1158/1538-7445.AM2022-4179

125. Logronio KA, Joglekar M, Oyasu M, Yakkundi P, Leabman MK, Hernandez G, et al. Combination of imvotamab and loncastuximab tesirine shows enhanced anti-tumor activity in a preclinical model of non-Hodgkin's lymphoma. *Hematological Oncol*. (2023) 41:552–3. doi: 10.1002/hon.3164_413

126. Budde E, Gopal AK, Kim WS, Flinn IW, Cheah CYY, Nastoupil L, et al. A phase 1 dose escalation study of Igm-2323, a novel anti-CD20 x anti-CD3 IgM T cell engager (TCE) in patients with advanced B-cell Malignancies. *Blood*. (2021) 138:132. doi: 10.1182/blood-2021-153355

127. Chu SY, Lee SH, Rashid R, Chen H, Chan EW, Phung S, et al. Immunotherapy with long-lived anti-CD20 × Anti-CD3 bispecific antibodies stimulates potent T cell-mediated killing of human B cell lines and of circulating and lymphoid B cells in monkeys: A potential therapy for B cell lymphomas and leukemias. *Blood*. (2014) 124:3111. doi: 10.1182/blood.V124.21.3111.3111

128. Moore GL, Bernett MJ, Rashid R, Pong EW, Nguyen DHT, Jacinto J, et al. A robust heterodimeric Fc platform engineered for efficient development of bispecific antibodies of multiple formats. *Methods*. (2019) 154:38–50. doi: 10.1016/j.ymeth.2018.10.006

129. Patel K, Michot JM, Chanan-Khan A, Ghesquieres H, Bouabdallah K, Byrd JC, et al. Safety and anti-tumor activity of plamotamab (XmAb13676), an anti-CD20 x anti-CD3 bispecific antibody, in subjects with relapsed/refractory non-Hodgkin's lymphoma. *Blood*. (2021) 138:2494. doi: 10.1182/blood-2021-144350

130. Patel K, Riedell PA, Tilly H, Ahmed S, Michot JM, Ghesquieres H, et al. A phase 1 study of plamotamab, an anti-CD20 x anti-CD3 bispecific antibody, in patients with relapsed/refractory non-Hodgkin's lymphoma: recommended dose safety/efficacy update and escalation exposure-response analysis. *Blood*. (2022) 140:9470–2. doi: 10.1182/blood-2022-159586

131. Kellner C, Hallack D, Glorius P, Staudinger M, Mohseni Nodehi S, de Weers M, et al. Fusion proteins between ligands for NKG2D and CD20-directed single-chain variable fragments sensitize lymphoma cells for natural killer cell-mediated lysis and enhance antibody-dependent cellular cytotoxicity. *Leukemia*. (2012) 26:830–4. doi: 10.1038/leu.2011.288

132. Lutz S, Klausz K, Albici AM, Ebinger L, Sellmer L, Teipel H, et al. Novel NKG2D-directed bispecific antibodies enhance antibody-mediated killing of Malignant B cells by NK cells and T cells. *Front Immunol*. (2023) 14:1227572. doi: 10.3389/fimmu.2023.1227572

133. Nalivaiko K, Hofmann M, Kober K, Teichweyde N, Krammer PH, Rammensee HG, et al. A recombinant bispecific CD20×CD95 antibody with superior activity against normal and Malignant B-cells. *Mol Ther*. (2016) 24:298–305. doi: 10.1038/mt.2015.209

134. Hörner S, Moustafa-Oglou M, Teppert K, Hagelstein I, Kauer J, Pflügler M, et al. IgG-based bispecific anti-CD95 antibodies for the treatment of B cell-derived Malignancies and autoimmune diseases. *Cancers*. (2022) 14:3941. doi: 10.3390/cancers14163941

135. Brandl M, Große-Hovest L, Holler E, Kolb HJ, Jung G. Bispecific antibody fragments with CD20 × CD28 specificity allow effective autologous and allogeneic T-cell activation against Malignant cells in peripheral blood and bone marrow cultures from patients with B-cell lineage leukemia and lymphoma. *Exp Hematol*. (1999) 27:1264–70. doi: 10.1016/S0301-472X(99)00072-7

136. Otz T, Große-Hovest L, Hofmann M, Rammensee HG, Jung G. A bispecific single-chain antibody that mediates target cell-restricted, supra-agonistic CD28 stimulation and killing of lymphoma cells. *Leukemia*. (2009) 23:71–7. doi: 10.1038/leu.2008.271

137. Dickinson M, Carlo-Stella C, Morschhauser F, Bachy E, Corradini P, Iacoboni G, et al. Glofitamab in patients with relapsed/refractory (R/R) diffuse large B-cell lymphoma (DLBCL) and ≥ 2 prior therapies: Pivotal phase II expansion results. *JCO*. (2022) 40:7500–0. doi: 10.1200/JCO.2022.40.16_suppl.7500

138. Labrijn AF, Janmaat ML, Reichert JM, Parren PWHI. Bispecific antibodies: a mechanistic review of the pipeline. *Nat Rev Drug Discovery*. (2019) 18:585–608. doi: 10.1038/s41573-019-0028-1

139. Schuster SJ, Bartlett NL, Assouline S, Yoon SS, Bosch F, Sehn LH, et al. Mosunetuzumab induces complete remissions in poor prognosis non-Hodgkin lymphoma patients, including those who are resistant to or relapsing after chimeric antigen receptor T-cell (CAR-T) therapies, and is active in treatment through multiple lines. *Blood*. (2019) 134:6. doi: 10.1182/blood-2019-123742

140. Thieblemont C, Phillips T, Ghesquieres H, Cheah CY, Clausen MR, Cunningham D, et al. Epcoritamab, a novel, subcutaneous CD3xCD20 bispecific T-cell–engaging antibody, in relapsed or refractory large B-cell lymphoma: dose expansion in a phase I/II trial. *JCO*. (2023) 41:2238–47. doi: 10.1200/JCO.22.01725

141. Budde LE, Assouline S, Sehn LH, Schuster SJ, Yoon SS, Yoon DH, et al. Single-agent mosunetuzumab shows durable complete responses in patients with relapsed or refractory B-cell lymphomas: phase I dose-escalation study. *JCO*. (2022) 40:481–91. doi: 10.1200/JCO.21.00931

142. Budde LE, Olszewski AJ, Assouline S, Lossos IS, Diefenbach C, Kamdar M, et al. Mosunetuzumab plus polatuzumab vedotin demonstrates a favorable safety profile and efficacy in patients (Pts) with relapsed or refractory (R/R) large B-cell lymphoma (LBCL): primary analysis of a phase Ib/II study. *Blood*. (2023) 142:613. doi: 10.1182/blood-2023-174209

143. Falchi L, Carlo-Stella C, Morschhauser F, Hutchings M, Bachy E, Cartron G, et al. Glofitamab monotherapy in pts with relapsed/refractory (R/R) large B-cell lymphoma (LBCL): Extended follow-up and landmark analyses from a pivotal phase II study. *JCO*. (2023) 41:7550–0. doi: 10.1200/JCO.2023.41.16_suppl.7550

144. Marhelava K, Krawczyk M, Firczuk M, Fidyt K. CAR-T cells shoot for new targets: novel approaches to boost adoptive cell therapy for B cell-derived Malignancies. *Cells*. (2022) 11(11):1804. doi: 10.3390/cells11111804

145. Ruella M, Korell F, Porazzi P, Maus MV. Mechanisms of resistance to chimeric antigen receptor-T cells in haematological Malignancies. *Nat Rev Drug Discovery*. (2023) 22:976–95. doi: 10.1038/s41573-023-00807-1

146. Till BG, Jensen MC, Wang J, Qian X, Gopal AK, Maloney DG, et al. CD20-specific adoptive immunotherapy for lymphoma using a chimeric antigen receptor with both CD28 and 4-1BB domains: pilot clinical trial results. *Blood*. (2012) 119:3940–50. doi: 10.1182/blood-2011-10-387969

147. Majzner RG, Mackall CL. Tumor antigen escape from CAR T-cell therapy. *Cancer Discovery*. (2018) 8:1219–26. doi: 10.1158/2159-8290.CD-18-0442

148. Tan Su Yin E, Hu YX, Huang H. The breakthrough and the future: CD20 chimeric antigen receptor T-cell therapy for hematologic Malignancies. *ImmunoMedicine*. (2022) 2:e1039. doi: 10.1002/imed.1039

149. Schneider D, Xiong Y, Wu D, Nölle V, Schmitz S, Haso W, et al. A tandem CD19/CD20 CAR lentiviral vector drives on-target and off-target antigen modulation in leukemia cell lines. *J Immunother Cancer*. (2017) 5:42. doi: 10.1186/s40425-017-0246-1

150. Tong C, Zhang Y, Liu Y, Ji X, Zhang W, Guo Y, et al. Optimized tandem CD19/CD20 CAR-engineered T cells in refractory/relapsed B-cell lymphoma. *Blood*. (2020) 136:1632–44. doi: 10.1182/blood.2020005278

151. Zhang Y, Wang Y, Liu Y, Tong C, Wang C, Guo Y, et al. Long-term activity of tandem CD19/CD20 CAR therapy in refractory/relapsed B-cell lymphoma: a single-arm, phase 1-2 trial. *Leukemia*. (2022) 36:189–96. doi: 10.1038/s41375-021-01345-8

152. Larson SM, Walthers CM, Ji B, Ghafouri SN, Naparstek J, Trent J, et al. CD19/CD20 bispecific chimeric antigen receptor (CAR) in naive/memory T cells for the treatment of relapsed or refractory non-Hodgkin lymphoma. *Cancer Discovery*. (2023) 13:580–97. doi: 10.1158/2159-8290.CD-22-0964

153. Sang W, Shi M, Yang J, Cao J, Xu L, Yan D, et al. Phase II trial of co-administration of CD19- and CD20-targeted chimeric antigen receptor T cells for relapsed and refractory diffuse large B cell lymphoma. *Cancer Med*. (2020) 9:5827–38. doi: 10.1002/cam4.3259

154. Fousek K, Watanabe J, Joseph SK, George A, An X, Byrd TT, et al. CAR T-cells that target acute B-lineage leukemia irrespective of CD19 expression. *Leukemia*. (2021) 35:75–89. doi: 10.1038/s41375-020-0792-2

155. Brouwer-Visser J, Fiaschi N, Deering RP, Dhanik A, Cygan KJ, Zhang W, et al. Baseline biomarkers of T-cell function correlate with clinical responses to odronextamab (REGN1979), and loss of CD20 target antigen expression identified as a mechanism of treatment resistance. *Blood*. (2020) 136:10–1. doi: 10.1182/blood-2020-137499

156. Schuster SJ, Huw LY, Bolen CR, Assouline SE, Bartlett NL, Budde LE, et al. Characterization of CD20 expression loss as a mechanism of resistance to mosunetuzumab in patients with relapsed/refractory B-cell non-Hodgkin lymphomas. *JCO*. (2022) 40:7526–6. doi: 10.1200/JCO.2022.40.16_suppl.7526

157. Reinert J, Beitzen-Heineke A, Wethmar K, Stelljes M, Fiedler W, Schwartz S. Loss of CD22 expression and expansion of a CD22dim subpopulation in adults with relapsed/refractory B-lymphoblastic leukaemia after treatment with Inotuzumab-Ozogamicin. *Ann Hematol*. (2021) 100:2727–32. doi: 10.1007/s00277-021-04601-0

158. Liu SQ, Grantham A, Landry C, Granda B, Chopra R, Chakravarthy S, et al. A CRISPR screen reveals resistance mechanisms to CD3-bispecific antibody therapy. *Cancer Immunol Res*. (2021) 9:34–49. doi: 10.1158/2326-6066.CIR-20-0080

159. Beers SA, French RR, Chan HTC, Lim SH, Jarrett TC, Vidal RM, et al. Antigenic modulation limits the efficacy of anti-CD20 antibodies: implications for antibody selection. *Blood*. (2010) 115:5191–201. doi: 10.1182/blood-2010-01-263533

160. Beum PV, Peek EM, Lindorfer MA, Beurskens FJ, Engelberts PJ, Parren PWHI, et al. Loss of CD20 and bound CD20 antibody from opsonized B cells occurs more rapidly because of trogocytosis mediated by fc receptor-expressing effector cells than direct internalization by the B cells. *J Immunol*. (2011) 187:3438–47. doi: 10.4049/jimmunol.1101189

161. Taylor RP, Lindorfer MA. Fcγ-receptor–mediated trogocytosis impacts mAb-based therapies: historical precedence and recent developments. *Blood*. (2015) 125:762–6. doi: 10.1182/blood-2014-10-569244

162. Golay J, Zaffaroni L, Vaccari T, Lazzari M, Borleri GM, Bernasconi S, et al. Biologic response of B lymphoma cells to anti-CD20 monoclonal antibody rituximab in *vitro*: CD55 and CD59 regulate complement-mediated cell lysis. *Blood*. (2000) 95:3900–8. doi: 10.1182/blood.V95.12.3900

163. Li J, Zhu Z, Zhu Y, Li J, Li K, Zhong W. METTL3-mediated m6A methylation of C1qA regulates the Rituximab resistance of diffuse large B-cell lymphoma cells. *Cell Death Discovery*. (2023) 9:1–12. doi: 10.1038/s41420-023-01698-2

164. Lee SM, Min SW, Kwon HS, Bae GD, Jung JH, Park HI, et al. Effective clearance of rituximab-resistant tumor cells by breaking the mirror-symmetry of immunoglobulin G and simultaneous binding to CD55 and CD20. *Sci Rep*. (2023) 13:18275. doi: 10.1038/s41598-023-45491-8

165. Bowles JA, Weiner GJ. CD16 polymorphisms and NK activation induced by monoclonal antibody-coated target cells. *J Immunol Methods*. (2005) 304:88–99. doi: 10.1016/j.jim.2005.06.018

166. Cheney CM, Stephens DM, Mo X, Rafiq S, Butchar J, Flynn JM, et al. Ocaratuzumab, an Fc-engineered antibody demonstrates enhanced antibody-dependent cell-mediated cytotoxicity in chronic lymphocytic leukemia. *MAbs*. (2014) 6:749–55. doi: 10.4161/mabs.28282

167. Liu R, Oldham RJ, Teal E, Beers SA, Cragg MS. Fc-engineering for modulated effector functions-improving antibodies for cancer treatment. *Antibodies (Basel)*. (2020) 9:64. doi: 10.3390/antib9040064

168. Arenas EJ, Martínez-Sabadell A, Rius Ruiz I, Román Alonso M, Escorihuela M, Luque A, et al. Acquired cancer cell resistance to T cell bispecific antibodies and CAR T targeting HER2 through JAK2 down-modulation. *Nat Commun*. (2021) 12:1237. doi: 10.1038/s41467-021-21445-4

169. Martínez-Sabadell A, Morancho B, Rius Ruiz I, Román Alonso M, Ovejero Romero P, Escorihuela M, et al. The target antigen determines the mechanism of acquired resistance to T cell-based therapies. *Cell Rep*. (2022) 41:111430. doi: 10.1016/j.celrep.2022.111430

170. Tie Y, Tang F, Wei Yq, Wei Xw. Immunosuppressive cells in cancer: mechanisms and potential therapeutic targets. *J Hematol Oncol*. (2022) 15:61. doi: 10.1186/s13045-022-01282-8

171. Silveira CRF, Corveloni AC, Caruso SR, Macêdo NA, Brussolo NM, Haddad F, et al. Cytokines as an important player in the context of CAR-T cell therapy for cancer: Their role in tumor immunomodulation, manufacture, and clinical implications. *Front Immunol*. (2022) 13:947648. doi: 10.3389/fimmu.2022.947648

172. Lykken JM, Horikawa M, Minard-Colin V, Kamata M, Miyagaki T, Poe JC, et al. Galectin-1 drives lymphoma CD20 immunotherapy resistance: validation of a preclinical system to identify resistance mechanisms. *Blood*. (2016) 127:1886–95. doi: 10.1182/blood-2015-11-681130

173. Xu-Monette ZY, Zhou J, Young KH. PD-1 expression and clinical PD-1 blockade in B-cell lymphomas. *Blood*. (2018) 131:68–83. doi: 10.1182/blood-2017-07-740993

174. Glover M, Avraamides S, Maher J. How can we engineer CAR T cells to overcome resistance? *BTT*. (2021) 15:175–98. doi: 10.2147/BTT.S252568

175. Mounier N, El Gnaoui T, Tilly H, Canioni D, Sebban C, Casasnovas RO, et al. Rituximab plus gemcitabine and oxaliplatin in patients with refractory/relapsed diffuse large B-cell lymphoma who are not candidates for high-dose therapy. A phase II Lymphoma Study Association trial. *Haematologica*. (2013) 98:1726–31. doi: 10.3324/haematol.2013.090597

176. Hertzberg M, Ku M, Catalani O, Althaus B, Simko S, Gregory GP. A phase III trial evaluating glofitamab in combination with gemcitabine plus oxaliplatin versus rituximab in combination with gemcitabine and oxaliplatin in patients with relapsed/refractory (R/R) diffuse large B-cell lymphoma (DLBCL). *JCO*. (2021) 39:TPS7575–TPS7575. doi: 10.1200/JCO.2021.39.15_suppl.TPS7575

177. Ansell SM, Tang H, Kurtin PJ, Koenig PA, Inwards DJ, Shah K, et al. Temsirolimus and rituximab in patients with relapsed or refractory mantle cell lymphoma: a phase 2 study. *Lancet Oncol*. (2011) 12:361–8. doi: 10.1016/S1470-2045(11)70062-6

178. Younes A, Burke JM, Cheson BD, Diefenbach CS, Ferrari S, Hahn UH, et al. Safety and efficacy of atezolizumab with rituximab and CHOP in previously untreated diffuse large B-cell lymphoma. *Blood Adv*. (2022) 7:1488–95. doi: 10.1182/bloodadvances.2022008344

179. Hutchings M, Gritti G, Sureda A, Terol MJ, Dyer MJ, Iacoboni G, et al. CD20-TCB, a novel T-cell-engaging bispecific antibody, can be safely combined with the anti-PD-L1 antibody atezolizumab in relapsed or refractory B-cell non-Hodgkin lymphoma. *Blood*. (2019) 134:2871. doi: 10.1182/blood-2019-123978

180. Nastoupil LJ, Chin CK, Westin JR, Fowler NH, Samaniego F, Cheng X, et al. Safety and activity of pembrolizumab in combination with rituximab in relapsed or refractory follicular lymphoma. *Blood Adv*. (2022) 6:1143–51. doi: 10.1182/bloodadvances.2021006240

181. Pavlasova G, Borsky M, Seda V, Cerna K, Osickova J, Doubek M, et al. Ibrutinib inhibits CD20 upregulation on CLL B cells mediated by the CXCR4/SDF-1 axis. *Blood*. (2016) 128:1609–13. doi: 10.1182/blood-2016-04-709519

182. Sandova V, Pavlasova GM, Seda V, Cerna KA, Sharma S, Palusova V, et al. IL4-STAT6 signaling induces CD20 in chronic lymphocytic leukemia and this axis is repressed by PI3Kδ inhibitor idelalisib. *Haematologica*. (2021) 106:2995–9. doi: 10.3324/haematol.2021.278644

183. Winiarska M, Bojarczuk K, Pyrzynska B, Bil J, Siernicka M, Dwojak M, et al. Inhibitors of SRC kinases impair antitumor activity of anti-CD20 monoclonal antibodies. *MAbs*. (2014) 6:1300–13. doi: 10.4161/mabs.32106

184. Bojarczuk K, Siernicka M, Dwojak M, Bobrowicz M, Pyrzynska B, Gaj P, et al. B-cell receptor pathway inhibitors affect CD20 levels and impair antitumor activity of anti-CD20 monoclonal antibodies. *Leukemia*. (2014) 28:1163–7. doi: 10.1038/leu.2014.12

185. Burger JA, Sivina M, Jain N, Kim E, Kadia T, Estrov Z, et al. Randomized trial of ibrutinib vs ibrutinib plus rituximab in patients with chronic lymphocytic leukemia. *Blood*. (2019) 133:1011–9. doi: 10.1182/blood-2018-10-879429

186. Chen X, Chen LC, Khericha M, Meng X, Salvestrini E, Shafer A, et al. Rational protein design yields a CD20 CAR with superior antitumor efficacy compared with CD19 CAR. *Cancer Immunol Res*. (2023) 11:150–63. doi: 10.1158/2326-6066.CIR-22-0504

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