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Radiation-Driven Cross Presentation and STING Activation Enhance CAR T Cell Therapy in a Murine Lymphoma Model

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Ph.D. Dissertation

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RADIATION-DRIVEN CROSS PRESENTATION AND STING ACTIVATION ENHANCE CAR T CELL THERAPY IN A MURINE LYMPHOMA MODEL

This diploma thesis was conducted at the Department of Radiation Oncology,

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DECLARATION

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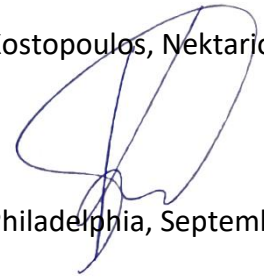

Philadelphia, September 2023

«Είσαι μικρός και δε χωράς

τον αναστεναγμό μου»

~ Γ. Μπιθικώτσης

Dedicated to yiayia Eleni.

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ΠΕΡΙΛΗΨΗ

Τα CAR T λεμφοκύτταρα που αναγνωρίζουν τον κυτταρικό δείκτη CD19 έχουν αλλάξει τη θεραπευτική προσέγγιση των ασθενών με CD19-θετικές υποτροπιάζουσες ή ανθεκτικές αιματολογικές κακοήθειες. Ωστόσο, ένα σημαντικό υποσύνολο αυτών των ασθενών είτε αποτυγχάνει να ανταποκριθεί στην θεραπεία με CAR T κύτταρα είτε τελικά υποτροπιάζει. Συχνά οι ασθενείς έχουν συμπτωματική νόσο που απαιτεί κάποια μορφή θεραπείας κατά τη διάρκεια της περιόδου κατασκευής των κυττάρων CAR T. Η ακτινοθεραπεία είναι μια καθιερωμένη θεραπεία που προάγει τον ανοσολογικά μεσολαβούμενο θάνατο των καρκινικών κυττάρων. Στην παρούσα διατριβή διερευνήθηκε πως η εφαρμογή ακτινοθεραπείας πριν από τη χορήγηση CAR T κυττάρων μπορεί να ενισχύσει την αποτελεσματικότητα των τελευταίων. Χρησιμοποιήθηκαν μοντέλα ποντικών που έφεραν δύο όγκους λεμφώματος A20, έναν ακτινοβολημένο και έναν μη ακτινοβολημένο. Ο συνδυασμός ακτινοθεραπείας/CAR T κυττάρων οδήγησε σε σημαντική συρρίκνωση του όγκου που δεν είχε υποβληθεί σε ακτινοθεραπεία (abscopal tumor), ενώ περισσότερα CAR T κύτταρα εντοπίστηκαν και στους δύο όγκους σε σχέση με την ομάδα που υποβλήθηκε μόνο σε θεραπέια με CAR T. Στην ίδια ομάδα αυξήθηκε η διασταυρούμενη αντιγονοπαρουσίαση (cross presentation) όπως απεδείχθη από την παρουσία περισσότερων ενδογενών T κυττάρων εξειδικευμένων για συγκεκριμένα καρκινικά αντιγόνα. Επιπλέον, θεραπεία μεταφοράς αντιγονοειδικών T κυττάρων απέδειξε το μηχανισμό της διασταυρούμενης αντιγονο-παρουσίασης. Η ακτινοθεραπεία ενεργοποίησε το μονοπάτι cGAS/STING το οποίο συνδέθηκε άμεσα με τα βελτιωμένα αντικαρκινικά αποτελέσματα των κυττάρων CAR-T. Συνολικά, αυτά τα αποτελέσματα υποδηλώνουν ότι η ακτινοθεραπεία μπορεί να χρησιμεύσει ως βέλτιστη θεραπεία γεφύρωσης (bridging therapy) στο πλαίσιο της θεραπείας με CAR T κύτταρα.

Λέξεις-Κλειδιά: λέμφωμα; ανοσοθεραπεία; CAR T-λεμφοκύτταρα; γεφυρωτική ακτινοθεραπεία; διασταυρούμενη αντιγονοπαρουσίαση

ABSTRACT

CD19-targeted chimeric antigen receptor (CAR) T cells have transformed the treatment of patients with relapsed or refractory CD19-positive hematologic malignancies. However, a significant subset of these patients either fails to respond or eventually relapses and, often has symptomatic disease that requires some form of treatment to support them during the manufacture period of CAR T cells. Radiotherapy (RT) is an established curative and palliative cancer treatment which promotes immunologically mediated tumor cell death. We investigated how RT could be applied prior to CAR T cell therapy to enhance its efficacy. We used mice models bearing two A20 lymphoma tumors, one treated with RT and one without. We found that CAR T cells when administrated with RT exhibited improved control of the abscopal, non-RT-treated tumor. More CAR T cells infiltrated both tumors of the group treated with RT and CAR T cells, whereas antigen specific T cells and adoptive T cell transfer therapy validated upregulated cross presentation in the same group. RT induced activation of the cGAS/STING pathway which was directly associated with the enhanced CAR-T cell anti-tumor effects. Overall, these results suggest RT may serve as optimal adjuvant and bridging therapy in the context of CAR T cell therapy.

Key-words: lymphoma; immunotherapy; CART cells; bridging radiation; cross presentation

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4-1BBL	4-1BB Ligand
ABC	Activated B-Like
ACT	Adaptive Cell Therapy
ALL	Acute Lymphoblastic Leukemia
AP-1	Activator Protein-1
APC	Antigen Presenting Cell
ASCT	Autologous Stem Cell Transplant
B-ALL	B- Acute Lymphoblastic Leukemia
BBB	Blood-Brain Barrier
BCL	B Cell Lymphoma
BCMA	B Cell Maturation Antigen
bRT	Bridging Radiation Therapy
bST	Bridging Systemic Therapy
BT	Bridging Therapy
CAR	Chimeric Antigen Receptor
CART-19	CD19-Targeted CAR T cells
CD19	Cluster of Differentiation 19
CD28	Cluster of Differentiation 28
CD38	Cluster of Differentiation 38
CD40	Cluster of Differentiation 40
CD40L	Cluster of Differentiation 40 Ligand
CD80	Cluster of Differentiation 80
CD86	Cluster of Differentiation 86
CD137	Cluster of Differentiation 137
CD275	Cluster of Differentiation 275
cGAMP	Cyclic GMP-AMP

cGAS	Cyclic GMP–AMP Synthase
CML	Chronic Myeloid Leukemia
CP	Cyclophosphamide
CR	Complete Response
CRP	C-Reactive Protein
CRS	Cytokine Release Syndrome
CTL	Cytotoxic T Lymphocyte
CTLA-4	Cytotoxic T-Lymphocyte-Associated Protein 4
DC	Dendritic Cell
DLBCL	Diffused Large B Cell Lymphoma
FACS	Fluorescence Activated Cell Sorting
FDA	Food and Drug Administration
FL	Follicular Lymphoma
GCB	Germinal Center B-cell-like
Gp70	Glycoprotein 70
HL	Hodgkin Lymphoma
ICANS	Immune effector Cell-Associated Neurotoxicity Syndrome
ICI	Immune Checkpoint Inhibitor
IFN-α1	Interferon-α1
IFN-β1	Interferon-β1
IFN-γ	Interferon-γ
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-7	Interleukin-7
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-15	Interleukin-15

IL-18	Interleukin-18
IL-23	Interleukin-23
IPI	International Prognostic Index
irAEs	Immune-relates Adverse Effects
IRF3	Interferon Regulatory Factor 3
IV injection	Intravenous injection
LAG-3	Lymphocyte Activation Gene-3
LDH	Lactate Dehydrogenase
mAb	Monoclonal antibody
MCL	Mantle Cell Lymphoma
MDSC	Myeloid-Derived Suppressor Cell
MHC	Major Histocompatibility Complex
MM	Multiple Myeloma
MRD	Minimal Residual Disease
MTV	Metabolic Tumor Volume
NE	Neurological Events
NFAT	Nuclear Factor of Activated T Cells
NF-κB	Nuclear Factor-κB
NHL	Non-Hodgkin Lymphoma
NK	Natural Killer
ORR	Overall Response Rate
OS	Overall Survival
OX-40L	Ox-40 Ligand
PBS	Phosphate-Buffered Saline
PFS	Progression-Free Survival
PD-1	Programmed cell Death protein 1
PDL-1	Programmed Death-Ligand 1
PMBCL	Primary Mediastinal B-Cell Lymphoma

RT	Radiation Therapy
R/R	Relapsed/Refractory
scFv	Single chain variable Fragment
STING	Stimulator of Interferon Genes
TAA	Tumor-Associated Antigen
TCR	T Cell Receptor
TDLNs	Tumor Draining Lymph Nodes
TGF-β	Transforming Growth Factor-β
TILs	Tumor Infiltrating Lymphocytes
TME	Tumor MicroEnvironment
TNF	Tumor Necrosis Factor
TRAIL	Tumor necrosis factor-Related Apoptosis-Inducing Ligand
TRUCK	T Cell Redirected for Universal Cytokine-Mediated Killing
WHO	World Health Organization

1. Introduction

1.1 Lymphoma

Lymphoma, a complex and diverse group of cancers originating in the lymphatic system, poses significant challenges in the field of oncology. It affects the body's lymphocytes, a type of white blood cell crucial for the immune system's proper functioning. Although scientists have made enormous progress in understanding and curing lymphoma, it remains a prominent global health concern [1]. According to recent data, lymphoma accounts for a substantial number of cancer cases and deaths worldwide. The American Cancer Society projects that only in the United States 1,958,310 new cancer cases and 609,820 cancer deaths will occur in the year of 2023 [2]. Although the leading cancer types are breast, lung and colon in women and prostate, lung and colon in men, it is estimated that there will be approximately 89,380 new cases of lymphoma in 2023 and 21,080 deaths attributed to this disease [2]. These figures reflect the pressing need for comprehensive research and effective treatment strategies to combat lymphoma.

Lymphoma, like leukemia, is a type of cancer that affects the blood and the lymphatic system. Unlike leukemia, which primarily affects the bone morrow and refers to malignant blood cells that travel through the bloodstream, lymphoma arises within the lymphatic system. This intricate network of vessels, lymph nodes and organs plays a pivotal role in immune function. It contains different types of lymphocytes which play a crucial role in the immune system's defense against infections and diseases. The main types of lymphocytes – B cells, T cells and natural killer (NK) cells – are a key component of the adaptive immune response, which involves recognizing and targeting specific pathogens or abnormal cells, maintaining overall health. Sometimes these lymphocytes become malignant leading to lymphoma, a formidable adversary [3].

Lymphoma involves all different types of lymphocytes and it may have a wide range of symptoms. It can be categorized into two primary types: Hodgkin’s Lymphoma and non-Hodgkin's lymphoma. Hodgkin lymphoma (HL), first described in 1832 by the British physician Thomas Hodgkin, is characterized by the presence of specific abnormal cells

called Reed-Sternberg cells [4]. Non-Hodgkin lymphoma (NHL), on the other hand, comprises a diverse group of lymphomas with distinct biological and clinical characteristics and accounts for 90% of lymphoma cases [5]. It is further classified into various subtypes based on the specific lymphocyte involved; about 85-90% of NHL cases are derived from B cells whereas the rest of the cases are derived from T cells and NK cells [6].

Each NHL subtype exhibits unique features, contributing to the complexity of diagnosis and treatment. In 2016, the World Health Organization (WHO) published the updated classification system for NHL, encompassing various subtypes that provide valuable insights into the disease (Appendix A) [7]. Among the most common subtypes identified within the WHO classification are diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma (FL). DLBCL is characterized by the rapid proliferation of large B cells and it accounts for 30-40% of NHL cases [8]. It is further classified into distinct molecular subtypes, including germinal center B-cell-like (GCB) and activated B-cell-like (ABC), each with unique genetic and clinical features [9]. FL, on the other hand, is characterized by the abnormal growth of B cells within lymph nodes, forming small nodules. It often exhibits an indolent (slow growing) nature and can transform into a more aggressive lymphoma over time. By precisely defining and classifying NHL subtypes, the WHO classification system facilitates personalized treatment strategies and enhances our understanding of the disease, ultimately leading to improved patient outcomes [6].

1.2 Frist Line Treatments for Lymphoma

The treatment of lymphoma depends on several factors, including the type, stage, and overall health of the patient [10]. Treatment approaches may include chemotherapy, radiation therapy, immunotherapy, targeted therapy, and stem cell transplantation. Chemotherapy, utilizing powerful drugs to kill cancer cells, is a commonly employed strategy for lymphoma treatment. Radiation therapy (RT), involving high-energy beams to destroy cancer cells, is another modality that can be used alone or in combination with chemotherapy. Immunotherapy, a promising advancement in cancer treatment, aims to enhance the body's immune response against cancer cells. Targeted therapy focuses on specific molecules or pathways involved in cancer growth, allowing for more precise treatment [10].

Chemotherapy plays a crucial role in the treatment of lymphoma, serving as a first line therapy for many patients. The specific chemotherapy regimen and duration of treatment depend on various factors such as the type and stage of lymphoma, overall health of the patient, and treatment goals [10].

Chemotherapy is often administered in cycles, with each cycle consisting of a period of drug administration followed by a rest period to allow the body to recover. Chemotherapy may be given orally, intravenously (IV), or as a combination of both, depending on the specific drugs and treatment plan. The standard treatment for patients with DLBCL is R-CHOP, a regimen that combines several drugs, including the anit-CD20 antibody rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone. More than 60% of patients treated with R-CHOP achieve complete response (CR) [11]. Selective patients with limited-stage, bulky disease might also receive RT following R-CHOP [12].

However, about 30-40% of the patients will be refractory to R-CHOP or relapse after initial response [10]. Patients with relapsed or refractory (r/r) disease after the first-line therapy require salvage chemoimmunotherapy followed by high dose therapy and autologous stem cell transplant (HD-ASCT) to achieve a more intense and targeted treatment effect. But even after second-line therapy where patients achieve a complete response to salvage chemotherapy and make it to ASCT, up to 60-70% will experience relapse disease [13]. The multi-institutional SCHOLAR-1 study provided a benchmark for the poor outcomes of salvage chemotherapy [14]. The study evaluated 636 R/R DLBCL patients who were defined as having progressive or stable disease at best response to chemotherapy or experiencing relapse within 12 months from ASCT. The study reported 26% of objective response rate (ORR) and 7% of complete response (CR). The median overall survival (OS) was at 6.3 months whereas 20% of patients remained alive after 2 years [14]. The SCHOLAR-1 study reports data before the establishment of further treatment options like CAR T cells. Although advancements in supportive care have helped to improve quality of life for patients undergoing treatment, chemotherapy still causes important side effects such as hair loss, nausea, and fatigue. The future of the significant subset of patients with R/R DLBCL is now brighter after the establishment of new therapies, such as CAR T cells.

1.3 CAR T Cell Therapy

It was in the late of 80s when Yoshihisa Kuwana at the Institute for Comprehensive Medical Science in Japan, and Gideon Gross at the Weizmann Institute in Israel independently described the first chimeric receptors and demonstrated that these types of synthetic receptor molecules enable Major Histocompatibility Complex (MHC)-independent target recognition by T cells [15]. Chimeric Antigen Receptor (CAR) T cells overcome the limitations of the T Cell Receptor (TCR), such as the need for expression, MHC identity and co-stimulation and their unique receptor structure gives them fundamental antitumor advantages [15].

CAR receptors are composed of the antigen-binding domains of an antibody fused to TCR signaling machinery. An antibody, secrete or membrane-bound, consists of two identical heavy (H) and two identical light (L) chains. Each chain has constant (C) regions which are responsible for the effector antibody functions and variable (V) regions which recognize and bind antigens with high specificity [16] (Figure 1.1A). A TCR recognizes an antigen only through MHC molecules that carry that antigen. Antigen recognition is associated with CD3 and ζ proteins which transduce the signals that lead to T cell activation [17,18] (Figure 1.1B).

The extracellular antigen-binding domain of a CAR consists of a single chain variable fragment (scFv) of a monoclonal antibody (mAb) while the intracellular part consists of the TCR signaling CD3ζ domain and the TCR costimulatory Cluster of Differentiation 28 (CD28) or 4-1BB (CD137) domain (Figure 1.2). These three domains (one for the recognition of the antigen and two for the initiation of the signal) are fused together and form the CAR [15]. The tumor antigen binds to the scFv and leads to downstream signaling through phosphorylation of CD3ζ and additional signaling cascades initiated from the co-stimulatory domains. Eventually, the CAR T cell gets activated and kills the cancer cell through its cytotoxic effector functions [19]. The most common and safe methods to introduce the CAR transgene cassettes into T cells is transduction with retroviral or lentiviral infection [20].

1.3.1 CAR T Cell constructs

The engineering of CARs has evolved over time and, so far, there are four generations of CAR molecules (Figure 1.2). The first generation of CAR T cells consists of an antibody scFv and an intracellular CD3ζ domain. These minimal structures are able to recognize a specific

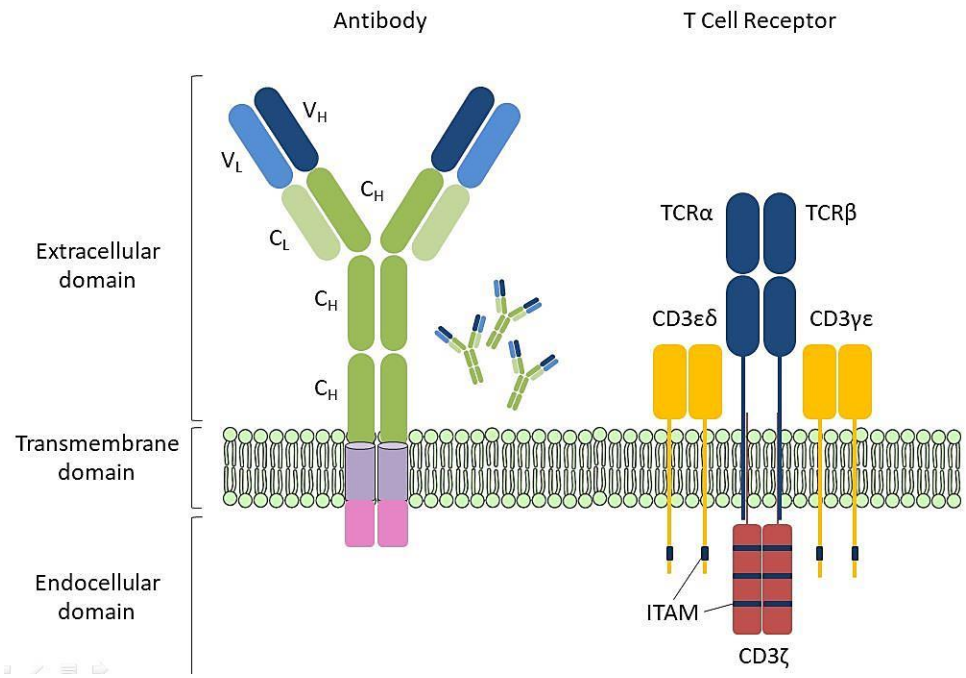

Figure 1.1 Antibody and TCR structure. *(A) Schematic diagram of a membrane-bound antibody on the surface of a B lymphocyte. The antigen-binding sites are formed by the variable VL and VH domains. (B) Schematic diagram of the TCR on the surface of a T lymphocyte. The antigen-binding sites are formed by the α and β chains, the signal is transduced by the CD3 chains.*

antigen independently of MHC and produce interleukine-2 (IL-2). Despite the first excitement, it was soon evident that the signaling and proliferative capability of these first-generation CAR T cells was limited [21]. Thus, a second generation of CAR T was developed. These engineered T cells have the same structure as the first ones with the addition of an intracellular costimulatory domain either CD28 or 4-1BB (CD137) which provides the second signal required for full T cell activation [19]. CD28 is a homodimeric glycoprotein that is constitutively expressed on resting and activated T cells. The extracellular portion of CD28 binds to CD80 (B7-1), CD86 (B7-2) or CD275 (B7-H2) and this interaction induces downstream signaling pathways that activate multiple transcription factors, including nuclear factor of activated T cells (NFAT), activator protein 1 (AP-1) and nuclear factor-κB (NF-κB). These transcription factors induce expression of anti-apoptotic proteins, promoting T cell proliferation and survival but also drive production of various cytokines, such as IL-2, IL-4 and IL-10 [22]. CD137 or 4-1BB is a transmembrane protein that is upregulated upon T cell activation by antigen stimulation. Once expressed, 4-1BB binds to its ligand, 4-1BB ligand (4-1BBL) and downstream signaling leads to NF-κB activation. NF-

κB drives IL-2 production and induces expression of anti-apoptotic proteins that promote T cell survival and proliferation [22]. In the second-generation CAR T cells, the co-stimulatory domain CD28 or 4-1BB results in an enhanced TCR signaling, activation, proliferation, production of cytotoxic cytokines and survival [19]. The third generation of CAR T cells combines CD28 together with 4-1BB costimulatory domains. Also, another costimulatory chain named OX-40 can be used instead of the 4-1BB. OX-40 or CD134 is expressed on activated T cells and binds to its ligand, OX-40 ligand (OX-40L). Costimulatory signals from OX-40 promote T cell division and survival and regulate cytokine production [23]. Assembled signal from both costimulatory domains, CD28 and 4-1BB or OX-40, results in enhanced T cell activation [19].

The fourth generation CAR T cells are genetically engineered to carry two trans-genes, one for the CAR and one for an inducible gene, such as cytokines. These new CAR T cells are also called T cells redirected for universal cytokine-mediated killing (TRUCKs) because they deliver a transgenic product to the targeted tissue. Nuclear factor of activated T cells (NFAT)

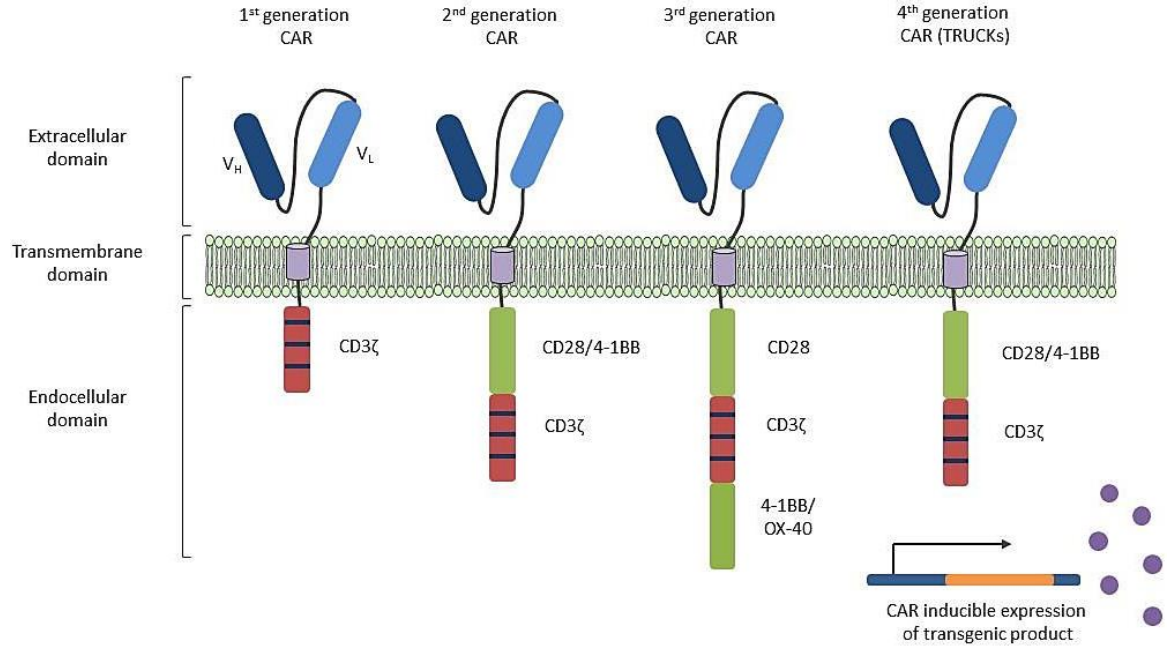

Figure 1.2 Evolution of different chimeric antigen receptor (CAR) generations. *The first generation consisted only of a scFv antibody for target binding linked to the intracellular signaling domain CD3ζ derived from the TCR. Second generation CARs include one co-stimulatory domain, mostly of the CD28 family, whereas third generation CARs contain two co-stimulatory molecules, CD28 and 4-1BB or OX-40. TRUCK T cells are the fourth generation CAR T cells which are additionally modified with a constitutive or inducible expression cassette for a transgenic protein, for instance a cytokine, which is released by the CAR T cell to modulate the T-cell response. scFv, single chain fragment of variable region; TCR, T cell receptor; TRUCK, T cell redirected for universal cytokine-mediated killing.*

has been used to induce cytokines expression, such as IL-12, IL-7, IL-15, IL-18 and IL-23, which improve T cell activation, modulate the immunological and vascular tumor environment and recruit additional immune cells to fight those cancer cells that are not recognized by CAR T cells and they are entering early phase trials [24]. Moreover, TRUCKs or built-in CAR T cells have been engineered to express anti-PD-1 (Programmed cell Death protein 1) or anti-PD-L1 (Programmed Death-Ligand 1) antibodies, which block the PD-1/PD-L1 interaction between tumor and CAR T cells and thus increase CAR T cell effectiveness [25]. Overall, CAR technology keeps advancing and is addressed as a promising tool for lymphoma treatment.

1.3.2 CAR T cell manufacturing

Before the initiation of CAR T cell therapy, patients must be declared eligible based on their disease progression and response to prior therapy regiments. For example, patients with poor-risk genetics who do not respond to first-line chemotherapy have a very high chance not to respond to salvage immunochemotherapy and usually they are evaluated to receive CAR T cell treatment [26]. Candidate patients first undergo leukapheresis, in which autologous T cells are isolated from their peripheral blood. Post leukapheresis, T cells are genetically modified to express the CAR, are activated and expanded *ex vivo* and then infused intravenously back into the patient [27,28] (Figure 1.3). The time between the approval and the infusion of CAR T cells varies per patient but it averages to 3-5 weeks including patient evaluation, insurance approval, manufacturing of the cells after collection and shipping [29,30].

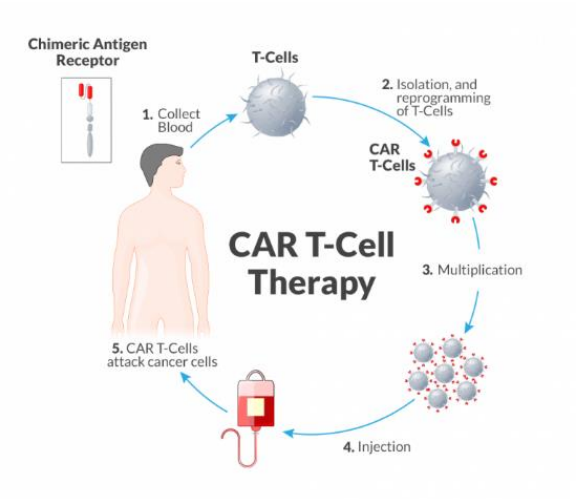

Figure 1.3 ***CAR-T cell manufacturing.*** *T cells are isolated through leukapheresis, are genetically engineered, expanded and re-infused back to the patient.*

During CAR T cell manufacturing is common in clinical protocols that patients receive a lymphodepleting therapy [19]. The most well-established scheme used in clinic is lympho-depleting chemotherapy with fludarabine and cyclophosphamide (CP) [31]. Lymphodepletion prior to CAR T infusion has been associated with a series of benefits including improved

CAR T cell expansion and antitumor efficacy in hematologic malignancies [32]. Lymphodepletion depletes endogenous lymphocytes making space for the infused CAR T cells to expand and target malignant cells. It can also deplete immunosuppressive cells such as regulatory T cells and myeloid derived suppressor cells (MDSCs) whereas it can improve the function of antigen presenting cells (APCs) and the availability of homeostatic cytokines [33]. Overall, lymphodepletion is associated with improved outcomes of CAR T cell therapy and it is critical to occur after the leukapheresis process so it doesn't affect the quality of leukocytes isolated from the CAR T cell candidates [26].

1.3.3 Commercial CAR T Cell Products

CAR T cell therapy has already shown impressive clinical responses in hematological malignancies, mainly B cell lymphoma and leukemia. CD19 constitutes a B-cell surface co-receptor whose signaling is very important for keeping the balance between humoral response and tolerance induction [34]. CD19 biomarker is frequently and highly expressed in B cell malignancies, but also is expressed early during B cell development and later, on differentiated plasma cells. Therefore, CD19 is found at over 95% of all B-cell neoplasms and CAR T cells targeting CD19 has been the lead paradigm for engineered T cell cancer therapies [28,35].

Since 2017, six CAR T cell products have been successfully tested in clinical trials and are currently approved by the Food and Drug Administration (FDA) for use in patients with advanced R/R blood cancers. The commercially available products are *axicabtagene ciloleucel* (axi-cel), *tisagenlecleucel* (tisa-cel), *lisocabtagene maraleucel* (liso-cel) and *brexucabtagene autoleucel* (brexu-cel) targeting CD19, and *idecabtagene vicleucel* (ide-cel) and *ciltacabtagene autoleucel* (celta-cel) targeting B cell maturation antigen (BCMA) [35] (Figure 1.4). Four of them are for patients with B cell lymphomas, two for patients with B cell acute lymphoblastic leukemia (B-ALL) and two for patients with multiple myeloma (MM). All six available products are second generation CAR T cells and their antigen-binding domain targets either CD19 which is a single-chain variable fragment derived from the mouse FMC63 monoclonal antibody, or BCMA which is a mouse 11D5-3 single-chain

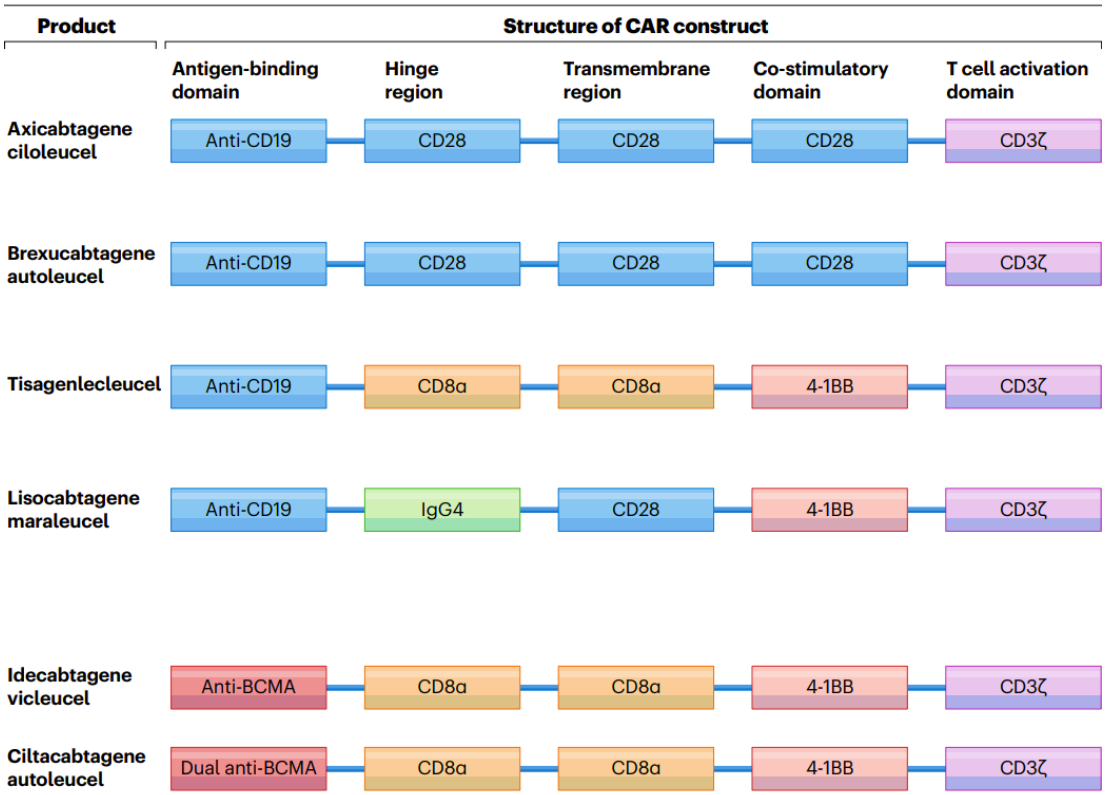

Figure 1.4 ***FDA-approved CAR T cell therapies.*** *A total of six chimeric antigen receptor (CAR) products are currently available commercially, including four for patients with B cell lymphomas, two for patients with B cell acute lymphoblastic leukaemia (B-ALL) and two for those with multiple myeloma (MM). All approved products have a second-generation CAR construct, consisting of an antigenbinding domain, a hinge region, a transmembrane region, a co-stimulatory domain and a T cell activation domain. All CD19-targeted CARs contain the same antigen-binding domain, which is a single-chain variable fragment derived from the mouse FMC63 monoclonal antibody. Axicabtagene ciloleucel and brexucabtagene autoleucel use the same CAR but differ in their manufacturing processes, with production of brexucabtagene autoleucel including an additional step designed to remove malignant cells from the leukapheresis product. Tisagenlecleucel differs from these products in that it contains different hinge and transmembrane domains and includes a 4-1BB domain instead of a CD28 domain for co-stimulation. Lisocabtagene maraleucel is delivered at a defined CD4+ :CD8+ T cell composition. The CAR gene for axicabtagene ciloleucel and brexucabtagene autoleucel is delivered using a gammaretrovirus, whereas those for tisagenlecleucel and lisocabtagene maraleucel are delivered using lentiviruses. Idecabtagene vicleucel includes a mouse 11D5-3 single-chain variable fragment targeting B cell maturation antigen (BCMA). Ciltacabtagene autoleucel has a binding domain consisting of two linked camelid heavy-chainonly variable (VHH) antigen-binding domains targeting BCMA. In both products, the CAR gene is delivered using a lentivirus. FL, follicular lymphoma; HSCT, haematopoietic stem cell transplantation; LBCL, large B cell lymphoma; MCL, mantle cell lymphoma; R/R, relapsed and/or refractory (Cappell & Kochenderfer, 2023;edited).*

variable fragment. Their CAR construct also consists of a hinge region, a transmembrane region, a co-stimulatory domain and a T cell activation domain [35].

All the currently approved CAR T cell therapies originated from pivotal studies that have revolutionized the treatment approach for patients diagnosed with lymphoma (Table 1).

The ZUMA-1 trial [36], an international single-arm multicenter phase I/II study evaluated the efficacy of *axicabtagene ciloleucel* (axi-cel) in 101 patients with r/r large B cell lymphoma (LBCL), primary mediastinal B-cell lymphoma (PMBCL) and transformed follicular lymphoma (FL). The trial demonstrated remarkable efficacy, with an overall response rate (ORR) of 82% and a complete response (CR) rate of 54%. CR continued to exist in 40% of the patients after a median follow-up of 15.4 months whereas the overall rate of survival was 52% at 18 months. These results led to axi-cel approval by the FDA in October 2017 for treatment of patients with r/r BCL after two or more lines of systemic therapy [37]. A 2-years follow up of the ZUMA-1 trial showed an overall survival (OS) of 50.5% [38]. Most recently, in a median follow-up of 63.1 months (5 years), 31% of the patients continued to have response whereas the median OS was 25.8 months with an OS rate at 42.6%. These response rates indicate the significant impact of axi-cel in treating LBCL highlighting the potential for long-term disease control [39]. The durability of responses observed in ZUMA-1 affirmed the therapeutic value of axi-cel and solidified its status as a groundbreaking treatment option for patients with r/r LBCL [36,38–40] .

In May 2018, FDA approved *tisagenlecleucel* tisa-cel for treatment of adults with r/r LBCL after two or more lines of systemic therapy based on the single-arm, open-label, multi-center, phase II clinical trial JULIET [41]. In this trial, 111 patients received tisa-cel infusion and the ORR and CR rates were 52% and 40% respectively. The study also demonstrated a 1-year relapse-free survival rate of 65% [42,43]. A 3-years follow-up (median 40.3 months) demonstrated an ORR at 53% with 39% of the patients having complete response [44].

TRANSCEND was a novel single-arm, multicenter, open-label, phase I trial that led to *lisocabtagene maraleucel* (liso-cel) approval by the FDA in February 2021 for the treatment of adult patients with r/r LBCL after two or more lines of systemic therapy [45]. TRANSCEND was the first study that introduced a different CAR T cell infusion protocol than the previous ones. Unlike axi-cel and tisa-cel, liso-cel manufacturing separately transduces and expands the $CD8^+$ and $CD4^+$ CAR T cells and administrates them back to the patients at a target dose of $50x10^6$ each subpopulation. Out of 269 patients that received liso-cel, 256 were evaluated for response and demonstrated OR and CR rates of 73% and 53% respectively [46]. The 2-year Progression-Free survival (PFS) and OS rates were 40.6% and 50.5% respectively,

Table 1.1 *Patients characteristics, CAR T cell products, efficacy and toxicities of the original clinical studies ZUMA-1, JULIET, TRANSCEND, ZUMA-2, KARMMA and CARTITUDE-1.*

	Study name	ZUMA-1	JULIET	TRANSCEND	ZUMA-2	KARMMA	CARTITUDE-1
Patients	Study design	Single arm Phase I/II	Single arm phase II	Single arm phase I	Single arm phase II	Single arm phase II	Single arm phase Ib/II
	Patients enrolled	119	167	344	74	140	113
	Median age	58 (23-69)	56 (22-76)	63 (54-70)	65 (38-79)	61 (33-78)	61 (56-68)
	Histological type	DLBCL/PMBCL/tFL	R/R LBCL	R/R DLBCL/PMBCL/HGBCL/FL	R/R MCL	R/R MM	R/R MM
	Prior lines of therapy	3 (2-4)	3 (1-6)	3 (1-8)	3 (1-5)	6 (3-16)	6 (4-8)
	N apheresis /infused (%)	111/108	165/111	344/269	74/68	140/128	113/97
CAR T Cell therapy	Name	Axi-cel	Tisa-cel	Liso-cel	Bruxa-cel	Ide-cel	Cilta-cel
	Target CD19/ BCMA dose	$2x10^6$ CAR^+ T cells/kg	$3x10^8$ CAR^+ T cells ($0.1x10^8$ – $6x10^8$)	$100x10^6$ $50x10^6$ $CD8^+$ and $50X10^6$ $CD4^+$ CAR^+ T cells	$2x10^6$ CAR^+ T cells per kg	$150×10^6$, $300×10^6$ or $450×10^6$ CAR^+ T cells	$0·75 × 10^6$ CAR^+ T cells per kg ($0·5×10^6$–$1·0×10^6$)
	Median apheresis to CAR-T infusion time (days)	17	N/A	37 (27-244)	16	N/A	29 (28-33)
	Bridging therapy	Not allowed	104 (92%)	159 (59%)	25 (37%)	112 (88%)	73 (75%)
	Lympho-depletion regimen	500 mg/m² cyclophosphamide and 30 mg/m² fludarabine for 3 days	250 mg/m² cyclophosphamide and 25 mg/m² fludarabine for 3 days or 90mg/m² bendamustine for 2 days	300 mg/m² cyclophosphamide and 30 mg/m² fludarabine for 3 days	500 mg/m² cyclophosphamide and 30 mg/m² fludarabine for 3 days	300 mg/m² cyclophosphamide and 30 mg/m² fludarabine for 3 days	300 mg/m² cyclophosphamide and 30 mg/m² fludarabine for 3 days

	Study name	ZUMA-1	JULIET	TRANSCEND	ZUMA-2	KARMMA	CARTITUDE-1
Efficacy	Median follow-up (months)	51.1	40.3 (8-43.8)	12 (2-16.7)	12.3 (7-32.3)	13.3 (0.2-21.2)	12.4 (6-15.4)
	ORR (%)	74	52	73	93%	73	97
	CR (%)	54	40	53	67%	33	67
	Median PFS (months)	5.9	2.9	6.8	N/A	8.8	NR
	PFS (%)	39% @ 2years	NR	44.1% @ 1 year	61% @ 1 year	N/A	77 @ 1 year
	Median OS (months)	25.8	12	21.1	N/A	19.4	N/A
	OS (%)	44% @ 4 years	49% @ 1 year	57.9% @ 1 year	83% @ 1 year	78% @ 1 year	89 @ 1 year
Toxicity	Grade ≥ 3 CRS, %	11	23	2	15	5	4
	Grade ≥ 3 NT, %	32	11	10	31	3	9

whereas CAR T cells were present in the patients' peripheral blood for up to 4 years [47].

Brexucabtagene autoleucel (brexu-cel) received FDA approval in July 2020 for adult patients with r/r mantle cell lymphoma (MCL) [48]. Its approval was based on the multicenter phase II clinical trial ZUMA-2 enrolled 74 patients in total and evaluated 68 that received brexu-cel at a target dose of $2x10^6$ cells/kg. The first time point of 7 months demonstrated a ORR of 93% and a CR rate of 67% in the first 60 treated patients' analysis [49]. More recently, after a median follow-up of 35.6 months (3 years) the ORR and CR rate among all 68 patients were 91% and 68% respectively. Medians for duration response, PFS and OS were 28.2 months, 25.8 months and 46.6 months respectively [50].

A phase II study, KarMMa, was conducted to evaluate *idecabtagene vicleucel's* (ide-cel) efficacy in individuals with r/r multiple myeloma (MM) [51]. Out of 140 patients enrolled, 128 received ide-cel and the median follow-up time was 13.3 months. Response was recorded

in 73% of the patients while 33% had a CR. The rate of minimal residual disease (MRD)-negative status was 26% and the median PFS rate was 8.8 months. These promising clinical data led ide-cel to its approval by the FDA in March 2021 for treatment of adult patients with r/r MM after four or more prior lines of therapy including an immunomodulatory agent, a proteasome inhibitor, and an anti-CD38 monoclonal antibody [52].

In February 2022, another CAR T cell product, *ciltacabtagene autoleucel* (cilta-cel), joined the group of the FDA-approved commercial drugs. Cilta-cel, like ide-cel, is used for the treatment of adult patients with r/r MM after four or more prior lines of therapy, including a proteasome inhibitor, an immunomodulatory agent, and an anti-CD38 monoclonal [53].

Table 1.2 *CAR T cell products and their approval series for leukemia and lymphoma treatment*

CAR T Cell Product	Diagnosis	Prior lines of therapy, n	FDA Approval Date	Trial
Axi-cel	r/r LBCL	≥ 2	October 2017	ZUMA-1
	r/r FL	≥ 2	April 2021	ZUMA-5
	r/r LBCL	1	April 2022	ZUMA-7
Brexu-cel	r/r MCL	≥ 2	July 2020	ZUMA-2
	r/r ALL	≥ 1	October 2021	ZUMA-3
Tisa-cel	r/r B-ALL	≥ 2	August 2017	ELIANA
	r/r LBCL	≥ 2	May 2018	JULIET
	r/r FL	≥ 2	May 2022	ELARA
Liso-cel	r/r LBCL	≥ 2	February 2021	TRANSCEND
	r/r LBCL	1	June 2022	TRANSFORM
Ide-cel	r/r MM	≥ 4	March 2021	KarMMa
Cilta-cel	r/r MM	≥ 4	February 2022	CARTITUDE-1

CARTITUDE-1, an open label, multicenter, phase 1b/2 clinical study evaluated the product in 97 patients and the median follow up was 12.4 months. The investigators recorded an ORR of 97% and CR of 65%, while PFS was 77% and OS rate was 89% after 12 months. After a median follow-up of 27.7 months, the responses deepened; among all 97 patients, ORR was 97.7% including 82.5% of complete response [54].

Following the groundbreaking studies demonstrating the efficacy of innovative products, subsequent clinical trials have played a crucial role in obtaining further approvals for these

treatments (Table 2). Notably, the FDA approved axi-cel in April 2021 for the treatment of r/r FL following two or more lines of systemic therapy, as supported by the ZUMA-5 trial [55,56]. Similarly, the ZUMA-7 trial evaluated axi-cel in adult patients with r/r LBCL who had failed first-line chemoimmunotherapy or relapsed within 12 months of treatment, leading to FDA approval in April 2022 [57,58]. Tisa-cel received accelerated FDA approval in May 2022 for adult patients with r/r FL after two or more lines of systemic therapy, based on the ELARA study [59,60] and the TRANSFORM study resulted in FDA approval of liso-cel for r/r LBCL after one prior therapy in June 2022 [61,62].

These clinical trials and subsequent approvals signify the remarkable progress made in expanding the therapeutic options of CAR T cells and improving outcomes for patients in need. More clinical studies are being conducted to investigate the use of CAR T cells as first-line treatments [63]. Moreover, promising preclinical and clinical studies are underway to investigate the development and efficacy of allogeneic CAR T cells, which offer an off-the-shelf treatment option (Hu et al., 2022; Jing et al., 2022; Mailankody et al., 2023).

1.3.4 Toxicities

While CAR T cell therapy has demonstrated remarkable effectiveness in the treatment of lymphoma, it is accompanied by certain side effects. These include immune-related adverse events (irAEs) that can occur within the initial weeks of CAR T cell therapy, notably severe cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS) [67]. CRS arises from immune activation following CAR T cell reinfusion, rapid cell expansion, and interaction with antigens, leading to a systemic inflammatory response characterized by excessive production of cytokines and chemokines. CRS represents the most frequently encountered irAE in CAR T cell therapy and manifests as a sepsis-like syndrome [67]. Its symptoms can vary from fatigue, mild to high fever, hypoxia, and multi-organ dysfunction to capillary leak and, in severe cases, even mortality [68,69]. Recent reports highlight the pivotal role of myeloid-derived macrophages in CRS development following CAR T cell infusion. These macrophages become activated upon co-localization with CAR T and tumor cells, secreting core cytokines that contribute to CRS through interactions like CD40-CD40L and a self-enhancing catecholamine loop. All these cytokines contribute to the initiation and escalation of CRS [70]. Neurologic events (NE) stand

as the second most commonly observed toxicity associated with CAR T cell therapy and are likely driven by excessive cytokine release similar to CRS [67]. Managing ICANS is more challenging, as its symptoms can range from mild encephalopathy, confusion, and tremors to more severe conditions such as neurologic aphasia, apraxia, dysgraphia, coma, and even death [68].

Elevated levels of lactate dehydrogenase (LDH) and a high tumor burden prior to CAR T cell infusion have been identified as risk factors for CRS and NE [69]. Patients who experience CRS are often admitted to intensive care units and are effectively managed through the administration of tocilizumab, an anti-IL-6 antibody and the only FDA-approved therapy for CRS [71,72]. Corticosteroids, either alone or in combination with tocilizumab, are also prescribed to alleviate severe CRS symptoms [73]. Notably, in May 2023, the FDA granted approval for the initiation of a phase 1b/2a clinical trial involving CTO1681, a compound that prevents and treats CRS by targeting and reducing NF-κB signaling, resulting in reduced inflammation while still allowing for a functioning immune system. NE pose greater challenges in management, but patients are typically treated with corticosteroids. Alternative approaches for NE treatment involve the use of prophylactic or early steroids and other anti-inflammatory agents such as siltuximab (an IL-6 antagonist), anakinra (an IL-1 receptor antagonist), and dexamethasone, which have demonstrated the ability to penetrate the blood-brain barrier (BBB) [73].

CAR T cell infusion can lead to several other toxicities, including cytopenias and infections. While CD19 is highly expressed in B cell malignancies it is also present in normal B cells, CAR T cells targeting CD19 often cause B cell aplasia, a condition where normal B cells are depleted [74]. However, this loss of normal B cells can be temporary, with recovery occurring over time, or it can be managed with replacement antibody therapy, making CD19 still an optimal target for CAR T cell therapy [28]. Hypogammaglobulinemia is another potential toxicity that can arise after CAR T cell treatment. This condition involves reduced levels of gamma globulins, including immunoglobulins, which are essential for the immune system's proper functioning. Patients experiencing hypogammaglobulinemia may require intravenous immune globulin to support their immune system if they do not show sufficient recovery of immunoglobulin levels on their own [69].

Out of the six pivotal studies, ZUMA-1 [36] reported any grade of CRS in 93% of the patients that received axi-cel. According to the grading system by Lee et al [75], 13% of the patients developed grade 3 or higher CRS, with only one patient experiencing grade 5 CRS. The onset of CRS occurred within a median time of 2 days after infusion, and the syndrome resolved within a median of 8 days. NE were observed in 64% of the patients, with 28% of them experiencing grade 3 or higher events. The median time until NE onset was 5 days, and resolution occurred within a median of 17 days after infusion. In terms of management, 43% of the patients received tocilizumab and 27% received glucocorticoids to address CRS, NE, or both. In a real-world study, based on the ZUMA-1 trial reported similar findings, with 91% of patients developing any grade of CRS and 7% experiencing grade 3 or higher CRS. NE were observed in 69% of the patients in any grade, and 31% developed grade 3 or higher neurotoxicity [76].

In the clinical trial Juliet [42], which evaluated the efficacy of tisa-cel, it was observed that 58% of the patients developed CRS, with 22% experiencing grade 3 or higher CRS as determined by the University of Pennsylvania's grading scale [77]. The syndrome typically manifested within a median time of 2 days after infusion, and the median time for resolution was 7 days. To manage CRS, 14% of the patients received tocilizumab, while 10% received tocilizumab in combination with glucocorticosteroids. NE were reported in 21% of the patients, with 12% of them experiencing grade 3 or higher NE. The median onset of NE occurred on day 6 after infusion, and the median time until NE resolution was on day 14.

The TRANSCEND study [46] involved 269 patients treated with liso-cel, 42% of the them developed CRS of any grade, with a median onset of 5 days. The Penn grading system [77] reported grade 3 or higher CRS in 2% of the patients. Treatment strategies for managing CRS included tocilizumab administration in 10% of the patients, corticosteroids in 2%, and a combination of both in 8%. NE of any grade occurred in 30% of the patients, with a median onset of 9 days, and 10% of the patients experienced grade 3 or higher neurotoxicity.

Among the patients enrolled in ZUMA-2 [49], 91% experienced CRS, with 15% of them having grade 3 or higher CRS based on the grading system proposed by Lee et al [75]. The median time to CRS onset was 2 days, and the median duration until resolution was 4 days. Tocilizumab was administered to manage CRS in 59% of the patients, while glucocorticoids

were given to 22% of them. NE, occurring in 63% of the patients, had a median onset time of 7 days and a median duration of 12 days. Grade 3 or worse neurotoxicity was observed in 31% of the patients. Tocilizumab was used to manage NE in 26% of the patients, and 38% received glucocorticoids.

During the KarMMa trial [51], a total of 128 patients were treated with ide-cel and CRS occurred in 4% of the patients, with a median onset time of 1 day and median duration of 5 days. Grade 3 or higher CRS was graded according to Lee et al [75] and observed in 5% of

Table 1.3 *Toxicity rates between CAR T cell products with CD28 versus 4-1BB co-stimulatory domain*

Study	Costimulatory domain (product)	Treated patients	Any grade CRS	CRS grade ≥ 3	Median onset/duration of CRS (days)	Any grade NE	NE grade ≥ 3	Median onset/duration of NE	Grading system
ZUMA-1	CD28 (axi-cel)	108	93%	13%	2/8	64%	28%	5/17	Lee et al
ZUMA-5	CD28 (axi-cel)	148	82%	7%	4/6	59%	19%	7/14	Lee et al
ZUMA-7	CD28 (axi-cel)	180	92%	6%	3/7	60%	21%	7/9	Lee et al
ZUMA-2	CD28 (brexu-cel)	68	91%	15%	2/11	63%	31%	7/12	Lee et al
ZUMA-3	CD28 (brexu-cel)	55	89%	24%	5/7.5	60%	25%	9/7	Lee et al
Average	CD28	559	89.4 %	13%	3.2/7.9	61.2%	24.8%	7/11.8	
ELIANA	4-1BB (tisa-cel)	75	77%	46%	3/8	40%	13%	N/A	Upenn
JULIET	4-1BB (tisa-cel)	111	58%	22%	3/7	21%	12%	6/14	Upenn
ELARA	4-1BB (tisa-cel)	97	49%	0%	4/4	37.1%	4%	9/2	Lee et al
TRANSCEND	4-1BB (liso-cel)	269	42%	2%	5	30%	10%	9	Lee et al
TRANSFORM	4-1BB (liso-cel)	89	49%	1%	5/4	12%	4%	11/6	Lee et al
KarMMa	4-1BB (ide-cel)	128	84%	5%	1/5	18%	3%	2/3	Lee et al
CARTITUDE-1	4-1BB (cilta-cel)	97	95%	4%	7/4	21%	2%	8/4	Lee et al
Average	4-1BB	866	64.8 %	11.4%	4/5.3	25.6%	6.8%	7.5/5.8	

the patients. CRS management involved the use of tocilizumab in 52% of the patients and glucocorticosteroids in 15% of the patients. NE of any grade were reported in 17% of the patients, with 3% experiencing grade 3 or higher neurotoxicity.

In the CARTITUDE-1 trial [54], CRS was observed in 95% of the patients, with 4% experiencing grade 3 or higher syndrome as graded according to Lee et al criteria [75]. The median time to CRS onset was 7 days, with a median duration of 4 days. NE occurred in 21% of the patients, with 2% experiencing grade 3 or higher events. The median time to neurotoxicity onset was 8 days, with a median duration of 4 days. CRS was managed with tocilizumab in 69% of the patients and corticosteroids in 22% of the patients, while neurotoxicity was managed with corticosteroids and tocilizumab in 9% and 4% of the patients, respectively.

The timing and severity of adverse events can vary among CAR T cell products, often attributed to the specific co-stimulatory domains used in their design. CAR T cells with a CD28 co-stimulatory domain, such as axi-cel and brexu-cel, have been associated with earlier and higher rates of CRS and NE. This is believed to be a result of the rapid expansion and activation of CAR T cells mediated by CD28 signaling. In contrast, CAR T cells incorporating a 4-1BB co-stimulatory domain exhibit a more gradual response, enhanced persistence, and central memory differentiation [22,78].

Comparing only the studies that have led to FDA-approved products, although utilizing different grading systems to assess adverse events, the analysis reveals notable differences (Table 3). CD28 co-stimulatory domain CAR T cell therapies like axi-cel and bruxa-cel demonstrated CRS in any grade in 89.4% of the total patients. Conversely, 4-1BB-based CAR T cells such as tisa-cel, liso-cel, ide-cel, and cilto-cel resulted in CRS with an average occurrence of 64.8%. The occurrence of grade 3 or worse CRS was relatively similar between the CD28 and 4-1BB products, with an average of 11.4% and 13% of the total patients, respectively. The average median onset of CRS was 3.2 days for CD28-based products and 4 days for 4-1BB-based products, while the average median duration was 7.9 and 5.3 days, respectively. When examining NE, CD28-CAR T cells exhibited notably higher rates compared to 4-1BB-CAR T cells, with average incidences of 61.2% and 25.6%, respectively. More importantly, CD28 products caused neurological toxicities in an average of 24.8% of the patients whereas 4-1BB only affected 6.8%. The average median onset and

duration of the events were 7 days and 11.8 days, respectively, for CD28-based products, whereas 4-1BB-based products had an average median onset and duration of 7.5 and 5.8 days, respectively. Overall, it appears that CAR T cell products with a 4-1BB costimulatory domain exhibit lower rates of adverse events compared to CD28-based CAR T cells. However, it is important to note that all CAR T cell products can still result in adverse events despite their clinical effectiveness.

1.3.5 CAR T cell therapy failure

Despite the initially high response rates observed in CAR T cell therapy, a significant proportion of patients, approximately 50-60%, either do not respond or experience temporary responses followed by relapse [79,80]. Several mechanisms have been proposed to explain resistance to CAR T cell therapy. One well-known mechanism is antigen escape, where the cancer cells develop clones that downregulate or completely halt the expression of the targeted antigen, such as CD19. Tumor cells with low or absent CD19 expression can evade recognition by circulating CAR T cells [81]. Tumor cells have been found to employ alternative splicing of the CD19 mRNA, leading to the exclusion of exon 2 which contains the CD19-CAR T cell binding epitope. This alternative splicing mechanism allows tumor cells to escape the cytotoxic effects of CAR T cell therapy [82,83]. Moreover, mutations in tumor cells' genetics can result in alterations to the target antigen's structure, causing it to become unrecognizable to CAR T cells [84]. Also, intratumoral heterogeneity can result in subpopulations of tumor cells that lack the target antigen, thus making them resistant to CAR T-cell attack [85].

Tumors create immunosuppressive microenvironments that impact the infiltration and function of CAR T cells. T cell exhaustion after prolonged exposure to tumor antigens is characterized by the expression of various markers, such as Lymphocyte Activation Gene-3 (LAG-3) and PD-1, and renders CAR T cells less effective in recognizing and eliminating tumor cells [84]. Furthermore, low numbers of tumor-associated macrophages and the presence of inhibitory cytokines like Transforming Growth Factor-beta (TGF-β) and IL-10, have been associated with early relapse following CAR T cell therapy [86,87]. Similarly, high levels of MDSCs, elevated serum ferritin, C-Reactive Protein (CRP), and IL-6 can impair the efficacy of CAR T cells [86]. Tumors can also upregulate immune checkpoint pathways, such

as PD-1 or Cytotoxic T-Lymphocyte-Associated protein 4 (CTLA-4), to inhibit CAR T cell activity, limiting their function [88]. Additionally, tumor metabolic dysfunction can create a hypoxic and nutrient-poor tumor microenvironment, further contributing to CAR T cell failure [89].

Another described mechanism that limits CAR T cell function is their trafficking to the tumor site. Especially in solid tumors, the ability of CAR T cells to traffic and infiltrate the tumors is limited as the immunosuppressive Tumor Microenvironment (TME) and physical tumor barriers such as the tumor stroma limit the penetration and mobility of CAR-T cells [88]. The inclusion of non-human sequences in CAR constructs has been observed to trigger immune responses, including T-cell mediated immunity and the production of anti-CAR antibodies. The development of such antibodies can result in the clearance of the therapeutic CAR-T cells, potentially diminishing the effectiveness of CAR-T cell reinfusion [90].

Patient-related factors also play a role in CAR T cell failure. Several studies have shown that advanced disease stage (III/IV), elevated levels of LDH, baseline CRP, and high International Prognostic Index (IPI) scores are associated with CAR T cell therapy failure [89]. In the ZUMA-1 study, it was observed that patients with high baseline tumor burden as defined by the metabolic tumor volume (MTV) assessed by 18F-fluorodeoxyglucose PET scan, had poorer clinical outcomes [11].

The quality of CAR T cells is also crucial, as it can have a greater impact than the quantity of cells infused. Patients who have undergone multiple lines of cytotoxic chemotherapy may experience lower lymphocyte counts due to the depletion of naïve and effector memory T cells, as well as reduced T cell proliferation capability [91,92]. As a result, CAR T cell candidates with a history of multiple therapies exhibit poorer baseline T cell fitness and persistent cytopenias, resulting in limited CAR T cell expansion and suboptimal treatment responses [93]. In recognition of these challenges, studies such as ZUMA-7, TRANSFORM, and BELINDA have specifically explored the use of CAR T cell therapy as a second-line treatment option, aiming to address the limitations associated with previous therapies and improve patient outcomes. [92].

1.4 Need of bridging therapy

Although CAR T cell therapy has demonstrated promising results and high response rates, one challenge is the relatively lengthy manufacturing period required before CAR T cell infusion. This waiting period, which typically lasts 3-4 weeks, poses a significant concern for patients with aggressive and symptomatic disease who cannot afford to delay treatment [12,89]. In order to bridge this gap and ensure that patients remain stable and eligible for CAR T cell therapy, supplementary therapies – referred as bridging therapies – may be administered.

The importance of bridging therapy (BT) lies in its role as a lifeline for patients, keeping them alive and stable while autologous high-quality CAR T cells are being produced. BT aims to alleviate symptoms and maintain disease control preventing a decline in performance status, organ dysfunction, and any other complications that could potentially jeopardize the patient's eligibility for CAR T cell therapy and impact their likelihood of achieving a positive response [12,94,95]. Notably, in the ZUMA-1 trial, where bridging therapy was not allowed, approximately 10% of the enrolled patients did not receive axi-cel, primarily due to disease progression among other reasons [95].

A range of treatment options could serve as bridging therapies until patients' lymphodepletion and CAR T cell infusion. Corticosteroids, chemotherapy, targeted therapy, and RT can all be utilized as part of this approach [92]. Currently most CAR T cell candidates are patients with R/R B cell lymphoma after a first or second line of chemoimmunotherapy treatment. These chemotherapy-resistant patients quite often demonstrate sensitivity to RT, making it a suitable choice for BT [71].

1.5 Bridging Radiation therapy

Until off-the-shelf CAR T-cell therapy becomes more advanced and approved for regular clinical use, RT will play a significant role as BT for certain patients [94]. Patients that are candidates for CAR T cell therapy might not be in optimal health due to factors as severe cytopenias, which is decrease of the number of blood cells, or infections. Since their eligibility depends on the disease status or organ function, bridging RT can help stabilize patients' condition and maintain eligibility until CAR T cell infusion [96]. Besides that chemotherapy-resistant lymphomas are frequently sensitive to RT, another benefit of RT is

its debulking effectiveness. RT can be a useful tool to decrease the lymphoma tumor burden, particularly among patients with highly chemorefractory disease [89]. As mentioned before, higher baseline tumor burden and MTV were associated with worse ORR and OS and high grades of CRS among CAR T cell patients [11]. Therefore, RT can facilitate effective tumor debulking which will potentially decrease the severity of CRS and improve CAR T cell outcomes. Radiation has lymphodepleting abilities, thus it could also serve solely or in combination with chemotherapy as a lymphodepleting agent giving CAR T cells the needed space to work [97].

RT is known for its complementary immunomodulatory effects and priming the local or systemic immune system. RT generates double-strand DNA damage within tumor cells, leading to their destruction. This process exposes tumor antigens (neoantigens) on the cell surface, which trigger the activation of APCs like dendritic cells (DCs). These primed DCs initiate an adaptive immune response by presenting antigens to $CD8^+$ T cells, crucial for antitumor immunity [98]. Simultaneously, the DNA damage results in the release of fragmented DNA into the cytosol and the formation of micronuclei. This activates the Cyclic GMP–AMP synthase (cGAS)-mediated innate immune pathway, marked by the recognition of the "danger signal" by cGAS, its activation, and the subsequent generation of Cyclic GMP-AMP (cGAMP). This molecule binds to stimulator of interferon genes (STING), which then activates Interferon Regulatory Factor 3 (IRF3), leading to the production of type I interferons (IFNs) [99]. This intricate cascade activates DCs, further enhancing $CD8^+$ T cell priming and promoting immune-mediated tumor control. Additionally, RT's effects extend beyond local tumor treatment; it can induce an "abscopal effect" by inhibiting distant tumors, resulting in a systemic antitumor response that targets both local and distant metastatic tumor cells [99,100].

1.5.1 Radiation therapy prior to CAR T cells in preclinical studies

Through these mechanisms, RT could sensitize the tumor and work synergistically to increase CAR T cell efficacy. Preclinically, two studies have been applied RT before CAR T cell infusion. In a pancreatic model, DeSelm et al. investigated a novel strategy involving the combination of low-dose RT and CAR T cell therapy to address the challenge of tumor antigen heterogeneity. They discovered that exposing tumor cells to low-dose radiation

sensitized them to subsequent elimination by CAR T cells. This sensitization was found to be associated with the activation of Tumor necrosis factor-Related Apoptosis-Inducing Ligand (TRAIL)-mediated death pathways. The CAR T cells, upon engagement with tumor cells, were found to produce TRAIL, a protein capable of inducing apoptosis in cancer cells. This TRAIL production by CAR T cells was instrumental in driving the death of both antigen-positive and antigen-negative tumor cells that had been sensitized by the prior low-dose radiation. This mechanism provides a mechanistic basis for the enhanced efficacy of CAR T cells against heterogeneous tumors when combined with localized radiation therapy [102]

In another preclinical study, Weiss et al. developed a pioneering immunotherapeutic approach for glioblastoma, a challenging brain tumor. They engineered CAR T cells targeting multiple tumor antigens using the NKG2D receptor. Notably, they found that combining these CAR T cells with low-dose local radiotherapy produced a synergistic effect. This combination led to increased migration of CAR T cells to the tumor site and enhanced CAR T-cell activity against irradiated tumor cells. This research shows that teaming up CAR T cell therapy with radiation could be a promising approach to treat glioblastoma more effectively in the future [102].

The synergistic effects of RT with CAR T cells observed in the preclinical models of pancreatic cancer and glioblastoma offer promising avenues for improving therapeutic outcomes. However, it's worth noting that while the synergistic effects of RT and CAR T cell therapy have been demonstrated in these models, their application specifically to murine lymphoma models remains unexplored. So far, there have been only clinical trials focusing on lymphoma treatment and have investigated the use of RT as bridging therapy before CAR T cell therapy in human patients, underscoring the translational relevance of these findings. Research in murine lymphoma models could provide valuable insights into the potential benefits and mechanisms of this combination therapy for treating lymphoma and expand our understanding of its broader applicability in cancer treatment strategies.

1.5.2 Bridging Radiation in Clinic

When choosing bridging RT for lymphoma, patient factors to be considered include prior regiments, side effects and overall response to chemotherapy and other prior treatments [91]. Symptomatic progressive disease that requires palliation to maintain CAR T eligibility

should be evaluated [71]. Important factor is also the overall tumor burden as large volumes of lymphoma (bulky disease) can threaten clinical function or death while awaiting CAR T cell manufacturing [103]. Limited distribution of relapse that can be encompassed in RT fields must also be considered [71].

Some initial clinical trials like ZUMA-1 did not allow bridging therapy [36], however, in practice many patients require therapy for disease control prior to infusion of CAR T-cells and several studies provide early evidence that RT as a bridging treatment can be safe and effective.

Arscott et al. were the first to evaluate the use of RT as a bridge to CAR T cell therapy in a pioneering phase IIA clinical trial. In this study, five patients received RT prior to tisa-cel infusion. The results showed promising outcomes for the bridging RT (bRT) group, with a one-year PFS of 78% and OS of 100%. In contrast, the non-bRT group had a one-year PFS and OS of 44% and 65%, respectively. Notably, no patients in the bRT group developed CRS greater than grade 3, whereas the overall occurrence of grade 3 CRS was 24% [104].

The subsequent studies following Arscott et al.'s pioneering report have consistently supported the notion that bRT does not compromise the efficacy of CAR T cell therapy and may be associated with lower incidences of CAR T-related toxicities. Table 4 provides a comprehensive summary of all the relevant studies conducted to date, where RT was used as bridging therapy prior to the administration of various commercial CAR T cell products. Studies that did not assess the impact of bRT on the outcomes, did not provide data on relevant endpoints of interest, or had a sample size of fewer than four patients have been excluded.

Pinnix et. al. evaluated bridging therapies in 124 patients that received axi-cel. RT was delivered at a median dose of 35.2Gy with median fraction size of 2.5Gy in 11 patients. Patients that received bRT showed a higher ORR of 100% compared to the groups that received bridging systemic therapy (bST) (67%) or noBT at all (82%). The CR in the bRT group was significantly higher than the bST and noBT groups with 82%, 38% and 48%, respectively. The 1-year PFS for patients who received bRT was 44%, which was significantly higher than the 1-year PFS of 25% recorded in the bST cohort. The median PFS for patients who received bRT was 8.9 months compared to 4.7 months for the bST cohort. For the bRT

group the 1-year OS was 63% and the median OS was 3.9 months, with no significant differences from the other cohorts. No patients in the bRT cohort experienced CRS greater than grade 3, in comparison to the bST and noBT groups where 9% and 10% experienced grade 3-5 CRS, respectively. NT of grade 3 or higher occurred in 27% of the RT-bridged patients compared to 49% and 34% in the bST and noBT groups, respectively [105].

In a more recent trial, Ladbury et. al. bridged 12 patients with a median RT dose of 20Gy with a median fraction size of 2.25Gy. The patients that received bRT numerically had the greatest PFS and OS, with 1-year PFS and OS of 75% and 91.7%, respectively, whereas 3.32 years was the median PFS and OS in the same group. In the bRT cohort, 16.7% of the patients developed grade 3 or higher CRS and ICANS, which were no different from those observed in the rest of the groups [106].

The collective evidence from multiple clinical trials indicates that RT can be administered safely as a bridging therapy for patients slated to undergo CAR T cell infusion. These trials collectively demonstrate that utilizing RT as a bridging strategy does not compromise the efficacy of CAR T cells. Instead, it may even correlate with reduced incidences of severe CRS and NE. Additionally, RT as a bridging intervention showed promising outcomes, including improved ORR and CR rates, as well as improved PFS and OS rates. These findings underline the feasibility and safety of using RT as a valuable adjuvant treatment to enhance the clinical effectiveness of CAR T cell therapy for cancer patients in need. However, exploring the synergistic mechanisms between RT and CAR T cell therapy in lymphoma murine models remains crucial. These investigations have the potential to uncover the fundamental factors driving the combined therapeutic effects, providing valuable insights that can pave the way for more effective and precise treatment strategies.

1.5.3 Timing and dose of RT

All the clinical cases described above indicate that RT can be safely and effectively administrated to bridge CAR T cell candidate patients. However, the optimal timing, dose and target are yet unknown. Clinical oncologists suggest that RT should be delivered ideally after leukapheresis [92], and that was the case in most of the patients of the current trials.

Leukocytes are highly radiosensitive; thus RT has the potential to impact circulating blood cells even when delivered in low doses [107]. An adequate lymphocyte count and good fitness

Table 1.4 *Pivotal studies that have used radiation as bridging therapy. *com.: comprehensive*

Author et al	Year	Histological Type	CAR T product	n of bRT Patients	Sites of RT
Arscott	2018	DLBCL (61%), FL (34%), MCL (5%)	Tisa-cel	5	**n/a**
Imber	2019	DLBCL (n=9), TFL (n=4)	Axi-cel, tisa-cel	13	**n/a**
Qu	2019	DLBCL	CD19/CD20 CD19/CD22	6	**n/a**
Yu	2022	r/rABCL	CD19/22 cocktail	31	**Abdomen, neck, brain, head and neck, chest, breast, waist, iliac muscle, calf**
Fan	2023	r/rDLBCL	Tisa-cel	20	**Left lower lobe of lung**
Saifi	2022	r/r NHL	axi-cel, tisa-cel, or bruxa-cel	14	**Mainly bdomen, pelvis, chest**
Ladbury	2023	r/rDLBCL, PMBCL, TFL	Axi-cel or tisa-cel	12	**Neck, head and neck, calf, sinus**
Hubbeling	2023	DLBCL, MCL, BL (Burkitt)	Axi-cel, tisa-cel, liso-cel or brexu-cel	41	**Head and neck, extremities, pelvis, retroperitoneum**
Wright	2020	DLBCL, TFL	Axi-cel or tisa-cel	5	**n/a**
Pinnix	2020	DLBCL, TFL, PMBCL	Axi-cel	11	**Spine, thorax, abdomen/pelvis**
LaRiviere	2019	r/r NHL	Axi-cel or tisa-cel	5	**n/a**
Sim	2019 2021	r/rLBCL	Axi-cel	11	**b/a**
Kuhnl	2021	r/r DLBCL, transformed lymphoma	Axi-cel or tisa-cel	64	**n/a**
Brady	2021	r/rDLBCL, PMBCL	n/a	23	**Abdomen/pelvis, axilla, bone, mediastrium, head and neck, testes**
Jain	2021	r/rDLBCL	Axi-cel	8	**n/a**
Lutfi	2021	DLBCL, PMBCL, TFL	Axi-cel or tisa-cel	14	**n/a**
Niezink	2021	DLBCL, TFL	n/a	19	**n/a**
Dandapani	2020	r/rBCL	n/a	7	**Head and neck, limbs, chest**
Ravella	2021	r/rDLBCL	Axi-cel	5	**n/a**
Gulrayz	2021	SCNSL	Axi-cel or tisa-cel	5	**Whole brain**
Manjunath	2021	r/rMM	CART-BCMA	4	**Scull base, spine, hips, bilateral orbits**

Author et al	Median RT dose (Gy)	Median fractions	ORR %	CR %	PFS %	OS %	CRS G3-5%	NT G3-5%
Arscott	N/A	n/a	n/a	n/a	78% (1-year)	100% (1-year)	0%	24%
Imber	20Gy	4Gy	90% (day 30)	54% (day 30)	n/a	n/a	8%	31%
Qu	40Gy	2Gy	100% (2-months)	33% (2-months)	n/a	n/a	0%	0%
Yu	8Gy	1.8-3Gy	86.2% (6-months)	51.7% (6-months)	46.9% (1-year)	60.5% (1-year)	0%	0%
Fan	36Gy	n/a	45%	n/a	2.7 months	n/a	10%	0%
Saifi	20Gy	4Gy	n/a	n/a	47% (1-year)	67% (1-year)	7%	7%
Ladbury	20Gy	2.25Gy	87.5% (com.*RT) 54.5% (focal RT)	87.5% (com.*RT) 27.3% (focal RT)	75% (1-year)	91.7% (1-year)	16.7%	16.7%
Hubbeling	30Gy	3Gy	79%	61% (20.3 months)	20 months	NR	5%	12%
Wright	37.5Gy	2.2-4Gy	n/a	60%	20% (1-year)	80% (1-year)	0%	0%
Pinnix	35.2Gy	2.5Gy	100%	82%	44% (1-year)	63% (1-year)	0%	27%
LaRiviere	n/a	n/a	n/a	n/a	n/a	n/a	0%	n/a
Sim	20Gy	2-4Gy	81.8%	45.5%	52% (1-year)	57% (1-year)	9%	27%
Kuhnl	20-40Gy	n/a	63% (3-months)	50% (3-months)	58% (1-year)	65% (1-year)	11%	13%
Brady	30Gy (n=17)	n/a	n/a	52.2% (8.8 months)	5.1 months	17.8 months	n/a	n/a
Jain	20Gy	3-4Gy	n/a	12.5% (Day 30	n/a	n/a	14.2%	43%
Lutfi	n/a	n/a	n/a	50%	75.5% (1-year)	51.3% (1-year)	64.3% (G2-4)	42.9% (G2-4)
Niezink	20Gy	4Gy	n/a	n/a	74.1% (1-year)	48.9% (1-year)	n/a	n/a
Dandapani	24Gy	1.8-2.25Gy	86%	n/a	n/a	n/a	n/a	n/a
Ravella	30Gy	3Gy	n/a	25%	20%	60%	n/a	n/a
Gulrayz	4-40Gy	2-20	n/a	85.7% (day 28)	83 days	129 days	20%	20%
Manjunath	22Gy	3-8Gy	n/a	0%	94 days	264 days	25%	25%

T cells are needed for the successful production of CAR T cells; therefore, the optimal timing for bridging RT delivery would be between apheresis and CAR T cell infusion [95]. Although a delay of CAR T administration can occur due to financial or manufacturing issues [91], a shorter palliative course of bRT may be preferred depending on the disease progression, the clinical scenario and the planned date for CAR T cell infusion [94].

Different target sites for bridging radiation have been described in clinic with the most common irradiated site to be the abdomen, followed by extremities, pelvis, groin, chest wall, mediastinum, neck, head and spine [108] (table 4). At their study, Pinnix et. al. reported that comprehensive RT that encompassed all active sites of disease improved PFS and OS rates in comparison to focal RT that excluded all active disease sites [105]. This observation was further supported by following studies. In the case of Ladbury et. al. patients who received comprehensive RT had significantly improved PFS and OS rates in comparison to the patients that received focal RT [106]. The same trend was observed by Hubbeling et. al. but it did not reach the statistical significance [109]. Although these studies support that comprehensive RT may be more effective bridging therapy than focal RT, that is only a hypothesis due to the small patient number.

The optimal dose and regiment also remain unclear. However, RT courses should be carefully chosen to avoid potential RT-related toxicities such as significant lymphopenia and myelosuppression and prevent worsening any short- or long-term side effects [91]. The dose and fractions of delivered RT should be well considered so they debulk and neutralize immunosuppressive metabolic TMEs and prime the immune system to augment the CAR T cell efficacy [89]. Although the ideal dose and fractionation regimens are not yet well-established, preclinical studies support that hypo-fractionated RT is superior to a single dose of RT in promoting antitumor immune responses [110,111]. In clinic, all case reports and retrospective trials described above used fractions of RT and, in general, hypo-fractionated, low dose RT is recommended for bridging therapy since it only needs to be adequate to palliate the disease until CAR T cell infusion [94,108,112]. Currently, in the published studies the median dose of bridging RT ranges between 4 and 40Gy with an average of 25.8Gy split in 2.8 fractions (1.8-4) (table 4) and none of them reported high-grade acute RT toxicities.

Taken as a whole, when considering RT bridging, ideally it should be delivered after leukapheresis if possible, to minimize impact on T cell fitness. A more comprehensive RT treatment may be helpful if it can be delivered safely with minimal toxicity. Hypo-fractionated regimens can often be delivered safely and may result in a more favorable immune microenvironment. Overall, the current evidence suggests that bridging RT can be a safe and effective option for patients with R/R lymphoma in the context of CAR T cell therapy. Of course, prospective clinical trials, multi-institutional collaborations and real-world data are needed to evaluate the role of RT in bridging patients prior to CAR T cell therapy.

1.6 Aim of the study

CD19-targeted chimeric antigen receptor T-cells (CART-19) have revolutionized the treatment of patients with relapsed or refractory B cell lymphoma (r/r BCL). With four CAR T-cell therapies approved by the Food and Drug Administration for treating CD19-positive hematologic malignancies, the field has witnessed remarkable progress [36]. However, despite the clinical success, a significant subset (50-60%) of patients who receive CD19-CAR T cell therapy either fails to respond or eventually relapses [80]. This highlights the need for ongoing research and improvements to enhance treatment outcomes. Additionally, many patients who are eligible for CART-19 therapy often have a progressive or symptomatic disease, necessitating alternative treatment options to support them during the period between leukapheresis (collection of T-cells for CAR modification) and CART-19 cell infusion [94]. Finding effective interim therapies to manage the disease during this manufacturing phase and improve the antitumor effects is crucial for the overall success of the treatment.

Radiotherapy (RT) is a well-established treatment modality in cancer therapy, and approximately half of all patients with solid tumors receive RT at some point during their disease course [113]. RT induces tumor cell death through various mechanisms, including necrosis, apoptosis, autophagy, mitotic catastrophe, and senescence [114]. Additionally, RT activates immunologically active tumor cell death pathways, promoting potent immune modulatory effects. These effects involve the activation of signaling programs, such as the cGAS-STING pathway and the cross priming of tumor-associated antigens (TAAs), leading to the elicitation of anti-tumor $CD8^+$ T cell responses and potential abscopal effects [115].

To date, there are no preclinical studies using RT in combination with CAR T cells for murine lymphoma treatment. However, RT has been used as bridging therapy prior to CAR T cell infusion in clinical reports but the data on its efficacy and dosing protocols are limited. These studies have shown that bridging RT does not increase the risk of CAR T-related toxicities or negatively affect patient outcomes in individuals with R/R LBCL [30,80,105,116,117]. Based on these findings, we hypothesize that strategically applying RT as a bridging therapy can enhance the antitumor effects of CAR T cells, providing a promising avenue for further exploration in improving CAR T cell therapy outcomes.

To investigate our hypothesis, we established and conducted experiments using an A20 B cell lymphoma mouse model in our laboratory. We evaluated the impact on tumor progression of two different RT regimens, 4Gy in 2 fractions or 8Gy as a single dose, which emulate the immune adjuvant doses used in our institute. Subsequently, we implemented the identified most effective RT regimen in combination with CART-19 cell therapy.

Our research endeavors to delve into fundamental inquiries surrounding the mechanisms through which RT augments the effectiveness of CAR T cell therapy. We meticulously investigate the influence of RT on the infiltration patterns of CAR T cells within the intricate tumor microenvironment. Our focus extends to comprehending the intricate processes through which RT bolsters the localization and distribution of these therapeutic cells.

Simultaneously, our study delves into the intricate concept of cross-presentation, seeking to unravel the mechanism by which it sparks a broader immune response via the activation of antigen-specific primed $CD8^+$ T cells. A critical aspect of our investigation is the probing of the cGAS/STING pathway's activation, a response incited by RT. This pathway triggers the production of type I interferons, subsequently activating dendritic cells and leading to the initiation of effector T cell responses.

Our investigation supports the idea that using RT could become an important part of improving CAR T cell immunotherapy. Through our research, we aim to contribute novel insights that hold promise in elevating the clinical and therapeutic applications of CAR T cell immunotherapy.

2. Materials and Methods

2.1 Mouse strains

Six to eight weeks female mice on BALB/c background were purchased from the Jackson Laboratory (Strain #000651). Same age and sex, co-housed adult mice were used in all experiments. The mice were randomly assigned to different experimental groups just after tumor challenge. The mice were checked daily for any clinical signs of pain or distress. Approximately 34 days post A20 tumor challenge and 28 days post CT26 tumor challenge all animals were euthanized using CO_2 for tissue collection. One male and one female mouse on BALB/c background expressing only the CD45.1 allele (also known as Ly5.1) were purchased also from the Jackson Laboratory (Strain #006584). These mice were bred at the University of Pennsylvania and used to generate CD19-CAR T cells. All animals were maintained in the same facility of University of Pennsylvania. All in vivo experiments were performed according to the guidelines of the University of Pennsylvania.

2.2 Cell lines

Non-adherent A20 tumor cells, a $CD19^+$ murine B-cell lymphoma, were acquired from ATCC, propagated in 5% CO_2 at 37°C and cultured in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin and 0.1% β-mercaptoethanol. CT26 colorectal cancer cells were also acquired from ATCC, propagated in 5% CO_2 at 37°C and cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/ ml penicillin and 100 mg/ ml streptomycin and 0.1% β-mercaptoethanol. Platinum-E (Plat-E) retroviral packaging cell line was acquired from Cell Biolabs, propagated in 5% CO_2 at 37°C and cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, 1% HEPES and 1% Glutamax. All cell lines were tested for mycoplasma contamination before tumor challenge in vivo.

2.3 Tumor challenge

For the liquid model, BALB/c mice were injected subcutaneously in the right and left flank with 2×10^6 A20 cells per flank in 100µL of PBS/Matrigel Matrix (ratio 1:1). For the solid tumor models, BALB/c mice were injected subcutaneously on the lower back with 2.5×10^5

CT26 cells/mouse in 100ul of PBS. Once tumors were detectable, progression was monitored every other day by caliper measurements and was expressed in square millimeters by product of two perpendicular dimensions [$((m_1*m_1)*m_2)/2$, where m_1 the smaller and m_2 the bigger dimension. Mice were euthanized when control mice tumors reached approximately 2 cm^3, according to the University of Pennsylvania guidelines. Tumor progression data were analyzed using GraphPad Prism 8.

2.4 Irradiation

Only the right flank tumors were irradiated with either 8Gy or 2 fractions of 4Gy when tumor volumes were approximately 300 mm3, using an XRAD320iX, an x-ray system capable of delivering a precise radiation dosage to small animals such as mice. Irradiation of TDLNs was avoided in order to allow T cell priming. The Department of Radiation Oncology houses an XRad 320IX Biological X-Ray Irradiator (Precision X-Ray) in the Smilow Center for Translational 7 Research. The irradiator is calibrated for absolute dose using the AAPM TG-61 protocol for kV x-ray beam dosimetry (Ma CM, 2001). A20 cells were in vitro irradiated with 2x4Gy or 8Gy using a Precision X-Ray Xrad 320ix Cabinet Cell irradiator.

2.5 Lymphodepleting Chemotherapy

For endogenous lymphocyte depletion prior to CAR T cell infusion or adoptive T cell transfer all mice received one dose of cyclophosphamide 100mg/kg i.p. on day 20 post tumor challenge.

2.6 CD19-CAR T cell manufacturing

The murine CD19bbz CAR construct was generated in a retroviral backbone with GFP. Stably transduced 3rd generation Plat E cells expressing viral envelope and gag/pol were transfected with CAR plasmid to make retroviral supernatants. Splenocytes were harvested from CO_2-euthanized mice, were passed via a 70 µm filter and red cell depleted using ACK-lysis buffer. Mouse T cells were isolated from mice carrying the differential Ptprca pan leukocyte marker commonly known as CD45.1 or Ly5.1, using mouse CD3 negative selection kit (Miltenyi Biotec Inc, CA) in combination with MACS LS column as per the manufacturer's instructions. Flow staining was performed each time on isolated T cells with antibodies to CD3, CD4, CD8 and CD19 to validate the sample purity. Only samples with a

T cell purity > 95% were used for CAR production. Isolated T cells were incubated on day 1 in RPMI-10% FBS media containing 1% HEPES, sodium pyruvate, NEAA, Pen/Strep and 0.05M 2-mercaptoethanol and were activated with Mouse T-Activator CD3/CD28 Dynabeads (Life Technologies, NY) in the presence of IL-2 (100 U/mL) and IL-7 (10 ng/mL). On day 3 of ex vivo T cell culture, retroviral supernatant was spun at 2000xg for 2 hours on non-tissue culture plates coated with 20ug/mL retronectin (Clontech, CA). Cells were counted and adjusted to $1.0x10^6$/mL and were incubated for an additional 24 hours. On day 4, CAR % was assessed by flow cytometry. If the CAR transduced cells were less than 50%, T cells were transduced for a second time. Following the second transduction, T cells were counted and expanded for additional 24 hours before magnetic CD3/CD28 bead removal. On day 5, 2-6 hours after bead removal, T cells were counted, resuspended in cold PBS and tail i.v. injected in recipient mice on day 21 post A20 tumor challenge with a dose of $1x10^6$ per mouse.

2.7 Adoptive T cell therapy

Adoptive T cells transfer was performed 1 day after tumor implantation. Single cell suspension of mice spleen tissue treated with RT and CD19 CAR T cells, or CD19 CAR T cells alone were used to magnetically isolate T lymphocyte population using Pan T cell isolation kit. T cells were counted and plated with a density of $2.5x10^6$/well in 24-well plates previously coated with 2µg/ml of mouse anti-CD3 antibody (clone17A2). Th1 polarizing media, composed by mouse IL-12 (3.3 ng/ml; Peprotech), mouse anti-CD28 (1ug/ml; clone 37.51) and mouse anti–IL-4 (10 µg/ml; clone 11B11) was used to culture T cells. After 48h, fresh polarizing medium was added to the wells (mouse anti IL-12, mouse anti–IL-4 and mouse IL-2 (0.6 ng/ml; Peprotech). The day after, cells were collected and the percentage of AH1-specific $CD8^+$ T cells was determined by Flow cytometry. On day 4, $5×10^6$ T cells were adoptively transferred into recipient mice through IV injection.

2.8 Cell isolation and purification

Tumors and spleens were collected from mice at different time points. Tumors were digested using 600U/mL of collagenase type IV, while spleens were digested using 2 mg/mL of collagenase type D, resuspended in HBSS. Red blood cells were depleted using ACK-lysis buffer. Splenocytes were used to isolate T cells with mouse Pan T Cell isolation kit (Miltenyi

Biotec, 130-095-130) according to the manufacturer's instructions. Tumor cell suspension was used to isolate DCs using CD11c isolation kit (Miltenyi Biotec, 130-125-835).

2.9 Flow cytometry

Cells were washed, stained and subjected to up to 8-parameter flow cytometry on a FACS Canto flow cytometer using BD FACS Diva software, BD Biosciences and data were analyzed using FlowJo version X. LIVE/DEAD (L/D) Fixable Aqua Dead Cell Stain (Life Technologies, Catalog # L34957) was used to gate for living cells. The following monoclonal antibodies against mouse markers were purchased from Biolegend and used to phenotype the T cells: CD45.2 (clone 30-F11), CD8 (clone 53-6.7), CD3 (clone 145-2C11), CD45.1 (clone A20) and CD107. MHC Tetramer/APC–H2Ld gp70 (SPSYVYHQF) was purchased from MBL and used to detect specifically infiltrating AH1 T cells. DC phenotyping was carried out using the following monoclonal antibodies against mouse markers: CD45, CD11b (clone M1/70), CD11c (clone N4-18) and XCR1. We used three gating strategy: L/D, CD45.2, CD3, CD8, AH1 tetramer and L/D, CD3, CD45.1 and L/D, CD45, CD11b, CD11c, XCR1.

2.10 Gene expression analysis

Relative quantification of the expression levels of selected genes was carried out by real time qPCR, using an ABI PRISM Viia7 (AB). Total RNA from in vitro cultured and irradiated A20 cells was extracted using Trizol reagent according to the manufacturer's instructions in different time points post RT: 24, 48, 72, 96 hours. 2 µg of total RNA was used for cDNA synthesis with random primers, using high-capacity cDNA reverse transcription kit (Applied Biosystem, 4368813). 50 ng of cDNA was used for each real time PCR reaction. TaqMan gene expression assays were used to quantify expression levels of mouse cxcl9 (Mm00434946_m1), ccl5 (Mm01302427_m1), ifn-β (Mm00439552_s1), ifn-α1 (Mm03030145_gH). rn18s (Mm03928990_g1) was used as house-keeping gene.

2.11 ELISpot

Ninety-six well multiscreen immobilon-P (MAIP) filtration plates (Millipore) were coated overnight with a 2.5 µg/mL solution of rat anti-mouse IFNγ in sterilized PBS. The assay was carried out using A20 medium. The plates were washed 3 times with sterilized PBS and blocked with R10 for 3 hours before cells were plated. $0.1x10^6$ T cells/well were co-cultured

with $0.1x10^6$ A20 tumor cells with 1 µg/mL of AH1 peptide and 1 µg/mL of anti-MHCI antibody overnight at 37°C. Otherwise, $0.5x10^6$ splenocytes/well were incubated with or without 1 µg/mL of AH1 peptide. After the incubation, the plates were washed 6 times with PBS containing 0.05% Tween-20. Then, plates were incubated with anti-mouse biotin-conjugated anti IFNγ antibody for 3 hours at room temperature, followed by incubation with streptavidin-alkaline phosphatase conjugate for 30 minutes at 37°C. Finally, the plates were washed 3 times with washing buffer and 3 times with PBS and then developed by adding nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate. The spots were measured using an automated ELISpot reader (Autoimmun Diagnostika GmbH) and the numbers were analyzed using GraphPad Prism 8.

2.12 gp70 DNA vaccine

Plasmid-DNA immunization was performed as described previously [118]. Briefly, 50 µg of gp70 in phosphate-buffered saline (PBS) was injected in BALB/c mice intramuscularly and electroporation was performed (2 pulses at 100 mV for 200 ms) immediately after injection. Immunization was repeated once per week for 3 weeks before T cell isolation.

2.13 Statistics

Sample sizes were decided based on pilot experiments and on our experience with similar experiments11,12. Two-way ANOVA was used to compare tumor progression. One-tailed Mann-Whitney test or two-tailed student's t tests were used to compare data sets where indicated. A P value less than 0.05 was considered significant (*P < 0.05, **P < 0.01, ***P < 0.001, ****P<0.0001). All statistical analysis was conducted using GraphPad Prism 8.

2.14 Study approval

All animal studies were approved by the IACUC and the University Laboratory Animal Resources at the University of Pennsylvania. Mice were treated in accordance with University of Pennsylvania guidelines.

3. Results

3.1 Fractionated Irradiation of A20 lymphoma increases abscopal anti-tumor effects

The optimal dose and regimen for radiation therapy (RT) in the context of CAR T cell therapy remain uncertain. Careful selection of RT courses is essential to avoid potential toxicities and enhance CAR T cell efficacy. While the ideal dose and fractionation regimens are not firmly established, preclinical studies suggest that hypo-fractionated RT, delivered in smaller doses over multiple sessions, is more effective in promoting antitumor immune responses compared to a single high dose. In clinical practice, hypo-fractionated, low dose RT is often recommended for bridging therapy to prepare patients for CAR T cell infusion. Based on that, we initially investigated the use of RT in the A20 lymphoma model, focusing on the choice of dose and fractionation regimens. We tested a clinically relevant RT dose used in our institution administrated in a single dose or in fractionation manner. Subcutaneous injection of $2x10^6$ A20 tumor cells was administered in both flanks of the

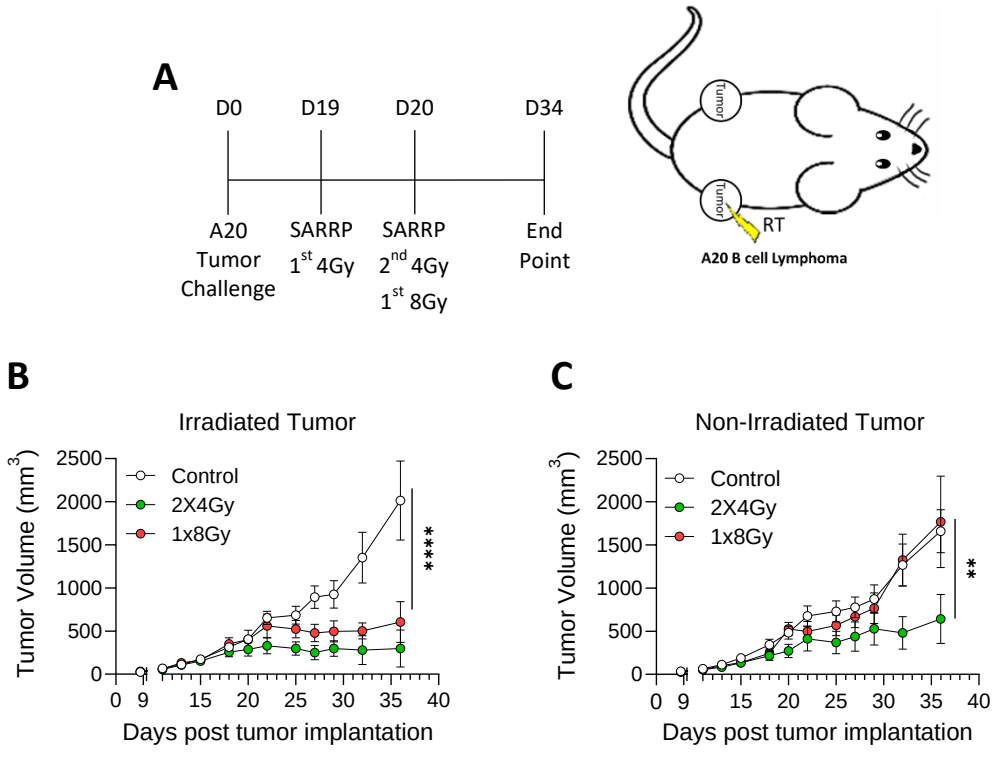

Figure 3.1. ***Fractionated Irradiation of A20 lymphoma increases abscopal anti-tumor effects****. (A) Working model: Timeline and schematic representation of in vivo A20 tumor-bearing mice treated with 2x4Gy or 1x8Gy RT in one of the two tumors. (B-C) A20 tumor growth from irradiated and non-irradiated tumor. Graphs show the mean ± SEM. (**P< 0.01, ****P<0.0001).*

mice, and one tumor was subsequently irradiated either with a single dose of 8Gy or two fractions of 4Gy after 20 days (Figure 3.1A). The impact of RT on tumor progression was monitored in both the irradiated and non-irradiated tumors. While the effects of RT alone on the irradiated tumor were similar regardless of fractionation (Figure 3.1B), fractionated RT demonstrated a noticeable improvement in the anti-tumor response of the abscopal tumors, surpassing the effects of the single dose of RT. (Figure 3.1C).

3.2 Fractionated Irradiation of A20 model increases cytotoxic and antigen-specific T cell infiltration

To gain insights into the tumor microenvironment (TME), we performed Fluorescence Activated Cell Sorting (FACS) analysis. Notably, fractionated RT led to a significant increase in the infiltration of $CD3^+$ and $CD8^+$ T cells in both the primary and abscopal tumors, in comparison to single dose RT (Figure 3.2A and B). Moreover, qPCR analysis demonstrated

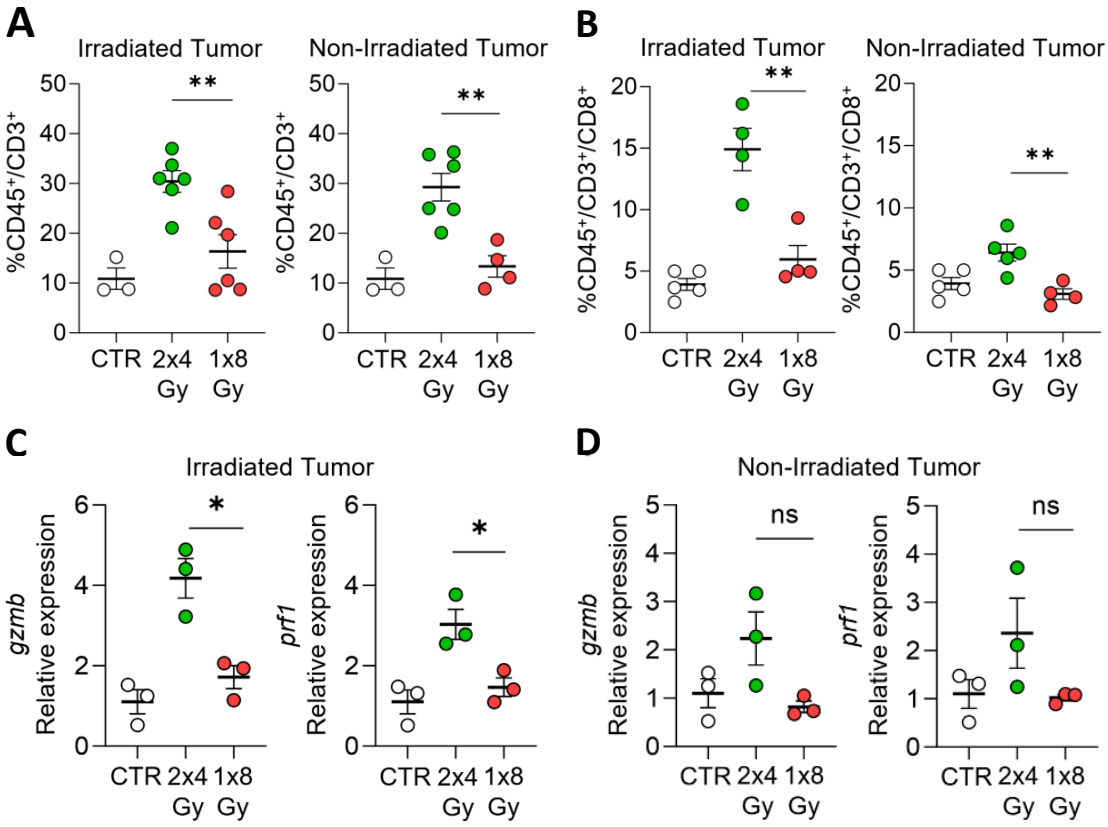

Figure 3.2 ***Fractionated Irradiation of A20 model increases cytotoxic and antigen-specific T cell infiltration.*** *(A) $CD3^+$ and (B) $CD8^+$ T cell infiltration in radiated and non-irradiated tumors after treatment with 1x8Gy or 2x4Gy RT. (C-D) Gene expression of Granzyme B (gzmb) and Perforin 1 (prf1) in irradiated and non-irradiated tumor 5 days post RT. Graphs show the mean ± SEM. (*P <0.05, **P< 0.01).*

elevated expression levels of CTL effector molecules, *Granzyme B (gzmb)* and *Perforin 1*

(prf1), in both tumors of mice treated with fractionated RT (Figure 3.2C and D). We conclude that a dose of 8Gy effectively controlled tumor progression in the irradiated tumor, while only fractionated RT exhibited enhanced cytotoxic T cell infiltration and abscopal effects. Due to the more promising outcomes observed with fractionated RT, we proceeded with this regimen for subsequent experiments.

3.3 Radiation prior to CAR T cell therapy enhances tumor control and increases survival

To explore how RT affects CAR T cell therapy, we utilized the A20 tumor model. Mice were injected with $2x10^6$ A20 tumor cells in both flanks, and after 20 days, one tumor was treated with RT followed by lymphodepleting chemotherapy and intravenous administration of $1x10^6$ CART-19 cells (Figure 3.3A). Notably, both RT and CART-19 therapies individually

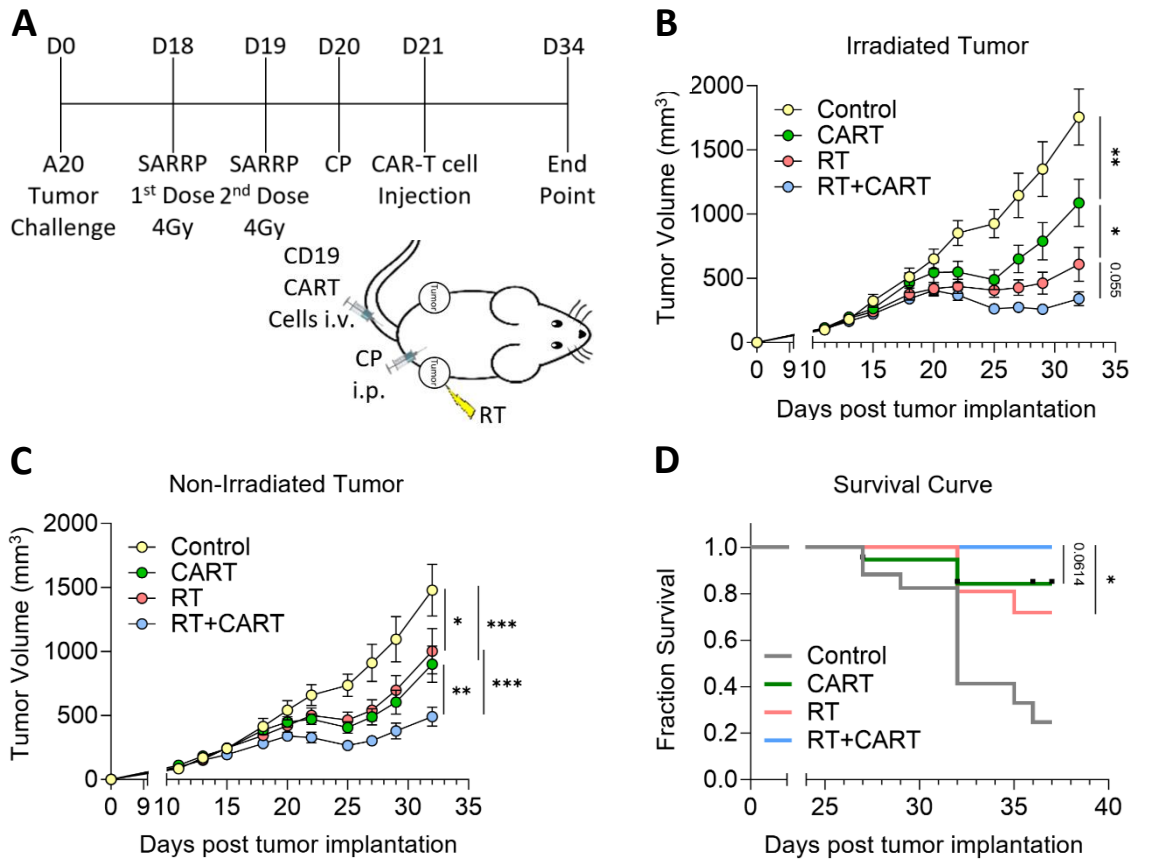

Figure 3.3 ***Radiation prior to CAR T cell therapy enhances tumor control and increases survival.*** *(A) Working model: Timeline and schematic representation of in vivo A20 tumor bearing mice treated with RT followed by lymphodepleting cyclophosphamide (CP) and CD19-CAR T Cell infusion. (B-C) A20 tumor growth from irradiated and non-irradiated tumor. (D) Survival curve after treatment administration. Graphs show the mean ± SEM. (*P <0.05, **P < 0.01, ***P < 0.001, ****P<0.0001).*

exerted significant effects on tumor progression in both the irradiated and abscopal tumors (Figure 3.3B). Interestingly, the combination of the two therapies significantly enhanced the antitumor effects resulting in further reduction of tumor growth in the abscopal tumors (Figure 3.3C). Moreover, RT prior to CAR T cell therapy significantly improved the overall survival of the mice compared to those treated with RT alone, whereas the same group

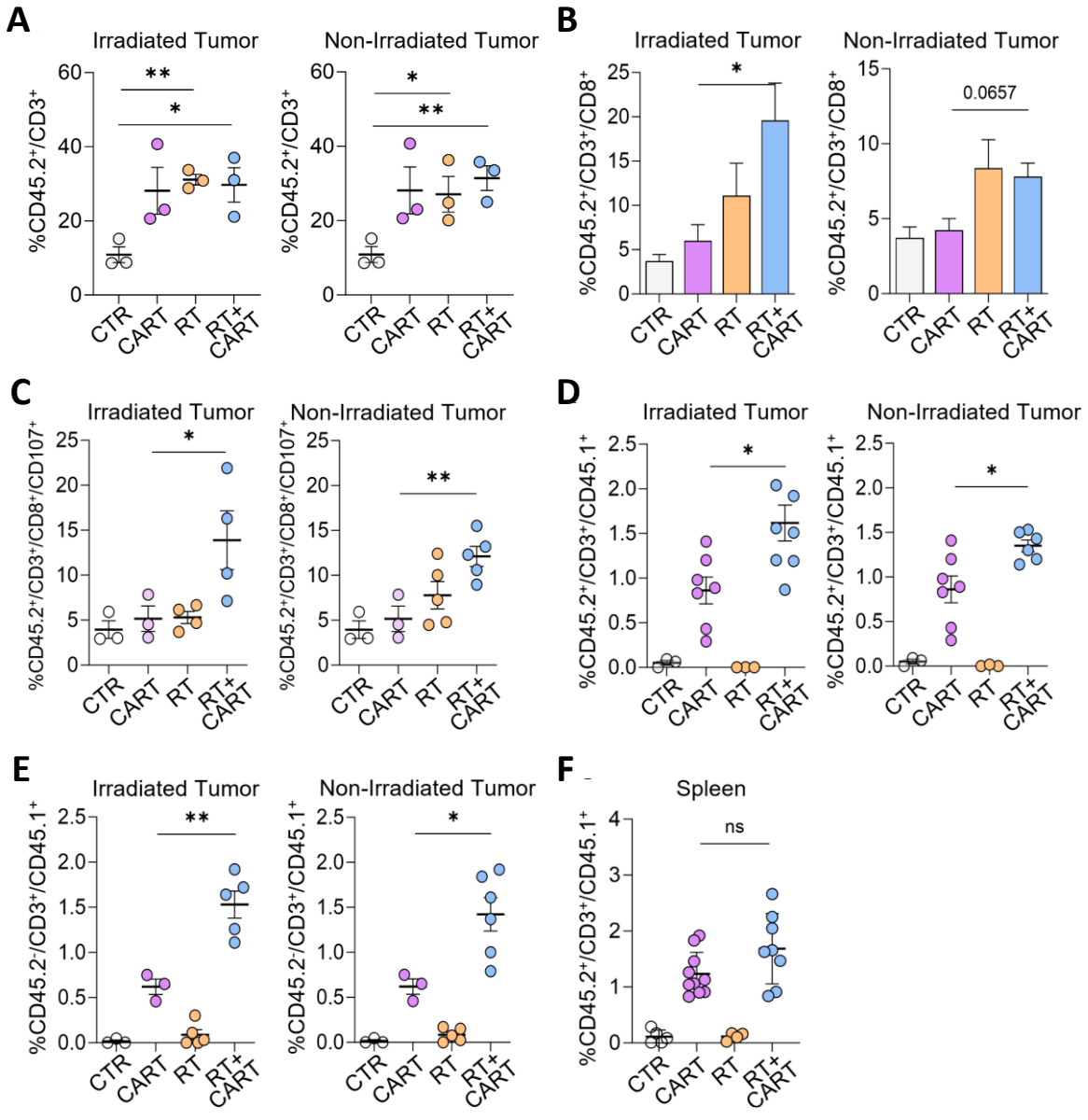

Figure 3.4 ***Radiation prior to CAR T cell therapy enhances CAR T Cell tumor infiltration and anti-tumor response in vivo.*** *(A) CD3+, (B) CD8+ and (C) CD107+ T cell infiltration in irradiated and non-irradiated tumors. (D-E) CD45.1+ T cell infiltration in irradiated and non-irradiated tumor representing CD19-CAR T cells 5 days and 13 days post RT treatment. (F) CD45.1+ T cell infiltration in the spleen. Graphs show the mean ± SEM. (*P <0.05, **P < 0.01).*

also exhibited increased survival compared to the group that received only CAR T cell therapy (Figure 3.3D).

3.4 Radiation prior to CAR T cell therapy enhances CAR T Cell tumor infiltration and anti-tumor response in vivo.

Analysis of the tumor microenvironment after radiation and CAR T cell treatment revealed a higher infiltration of $CD3^+$ T cells in both the irradiated and non-irradiated tumors across all treatment groups (Figure 3.4A). Although more $CD8^+$ T cells infiltrated the tumors of both groups that received RT (Figure 3.4B), only the group that received the combination treatment demonstrated a significant increase in cytotoxic $CD107^+$ T cells (Figure. 3.4C). CART-19 cells were generated from CD45.1 congenic mice and the CD45.1 marker was used to track the adoptively transfer T cells. A significantly higher $CD45.1^+$ T cell population was identified in both irradiated and non-irradiated tumors of mice previously treated with RT followed by CAR T cells (Figure 3.4D and E). A similar trend was observed in the spleens of the same group, with a higher $CD45.1^+$ T cell population (Figure 3.4F). These findings suggest that RT enhances the anti-tumor effects of CAR T cells by promoting the infiltration of endogenous T cells and CAR T cells into the tumor microenvironment.

3.5 RT/CART treatments combination treatment boosts TAAs cross-presentation and TAAs T cell response.

Radiation exerts potent immune modulatory effects, which involve the recruitment of cross-presenting DCs and the promotion of TAA cross-priming with antitumor $CD8^+$ T cell elicitation and abscopal effects. To investigate the involvement of TAA cross-presentation in the adjuvant activity of RT, we analyzed tumors from mice treated with RT followed by CAR T cell therapy. Consistent with our hypothesis, RT treatment significantly increased the infiltration of DCs in the irradiated tumors (Figure 3.5A). We also assessed the expression of three critical genes associated with the antigen presentation pathway - *batf3, xcr1,* and *b2m* - and observed their upregulation in the RT-treated tumors (Figure 3.5B). Notably, the A20 model expresses both CD19 (the target for CAR T cells) and the TAA gp70 which contains the immune-dominant epitope AH1 (SPSYVYHQF). In order to track the

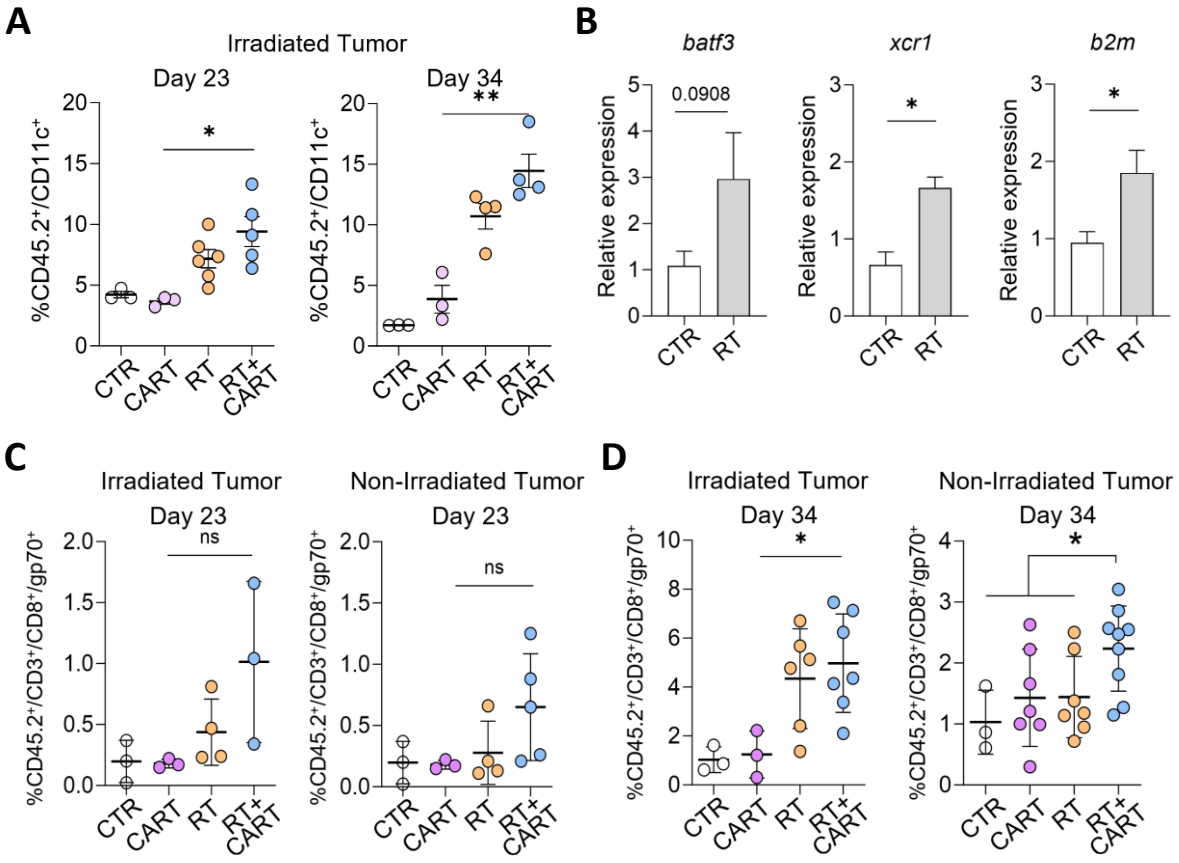

Figure 3.5 ***RT/CART treatments combination treatment boosts TAAs cross-presentation and TAAs T cell response.*** *(A) Dendritic Cell (DC) infiltration in irradiated tumor on day 23 and 34 post tumor challenge. (B) Gene expression of antigen presentation markers batf3, xcr1 and b2m in tumors 5 days post RT. (C-D) Tetramer staining of AH1-specific T cells infiltration in irradiated and non-irradiated tumors on day 23 and 34 post tumor challenge. Graphs show the mean ± SEM. (*P <0.05, **P < 0.01).*

endogenous $CD8^+$ T cells that have been specifically primed against the AH1 TAA, we utilized an AH1 tetramer. A tetramer is a complex of four MHC class I molecules associated with a specific peptide, in our case the AH1 peptide, and bound to a fluorochrome. Interestingly, AH1 tetramer staining from the different treatment groups showed an increase in the infiltration of AH1-specific T cells in the RT-treated tumors, as well as in the abscopal tumors shortly after the treatments (Figure 3.5C). This increase in AH1-specific T cells became significant by the end of the experiment (Figure 3.5D), indicating that RT induced enhanced CAR T cell outcomes by upregulating cross presentation.

3.6 RT enhances CAR T cell therapy efficacy through cross-presentation of tumor associated antigens

To validate the cross-presentation mechanism, we isolated DCs from the irradiated tumors of all groups and co-cultured them with naive T cells isolated from an AH1-immunized mouse spleen, in the presence or absence of an anti-MHCI antibody. The tumor-derived DCs from the mice treated with RT and CAR T cells significantly presented more AH1 antigen to T cells in an MHCI-dependent manner (Figure 3.6A). Furthermore, stimulation of tumor cell suspensions from all groups with AH1 peptide resulted in significantly higher production of IFNγ in the group treated with RT and CAR T cells. These effects were abrogated in the presence of an anti-MHCI antibody (Figure 3.6B). To further validate the

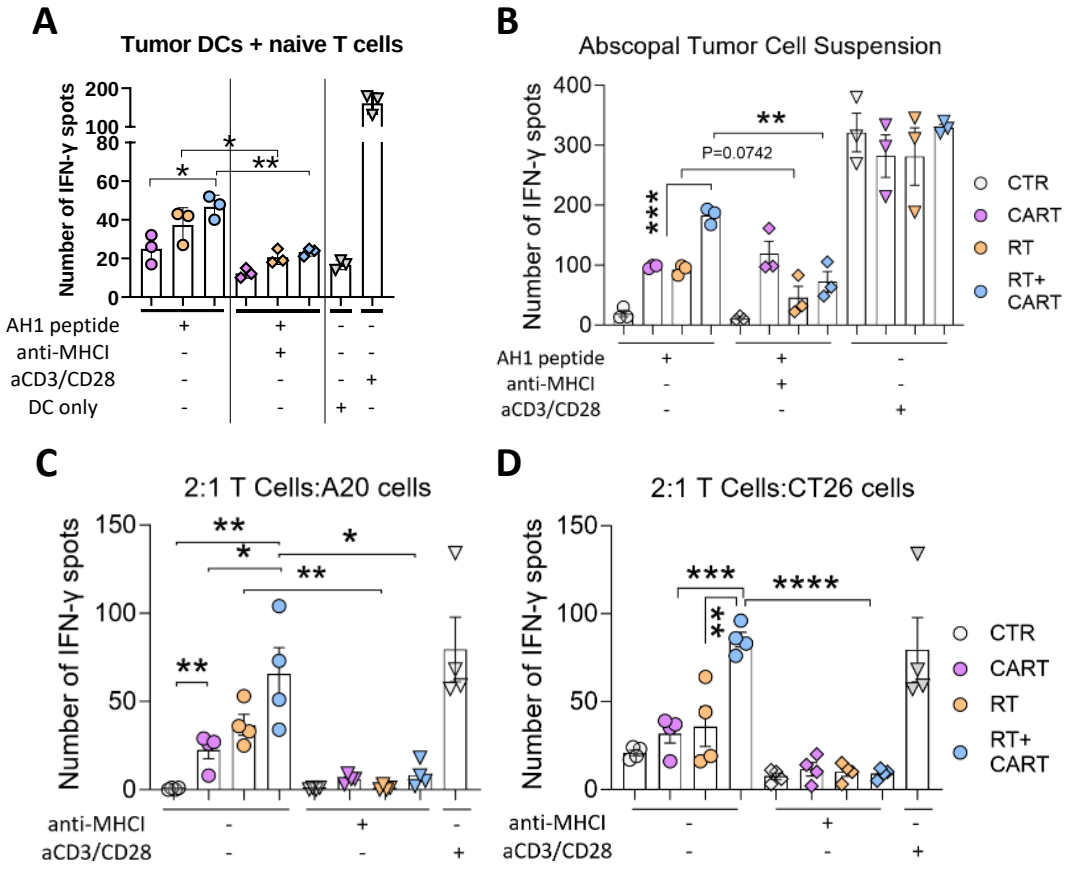

Figure 3.6 ***RT enhances CAR T cell therapy efficacy through cross-presentation of tumor associated antigens.*** *(A) Eli-spot assay displaying IFNγ spots after co-culture of isolated DCs from irradiated tumors with AH1-specific T cells isolated from an AH1-immunized mouse. (B) IFNγ spots of tumor cell suspension stimulated overnight with the AH1 peptide. (C) CD3+ T cells isolated from treated mice stimulated overnight with AH1 peptide and incubated with (C) A20 or (D) CT26 tumor cells. All assays were performed in the presence or absence of an anti-MHC class I antibody. Anti-CD3 and anti-CD28 antibodies were used to stimulate T cells as positive control. Graphs show the mean ± SEM. (*P <0.05, **P < 0.01, ***P < 0.001, ****P<0.0001).*

presence of AH1-primed $CD8^+$ T cells, we isolated T cells from the spleens of the different treatment groups, stimulated them with AH1 peptide and co-cultured in vitro with A20 tumor cells in the presence of anti-CD19 or/and anti-MHCI antibodies. Only the T cells from the group treated with RT and CART showed a significant increase in IFNγ spots, which was diminished when cell interactions were blocked with an anti-CD19 or anti-MHCI antibody (Figure 3.6C).

In another approach to prove cross presentation, we utilized the CT26 cell line, which is a syngeneic BALB/c-derived colorectal carcinoma cell line which similarly to A20 cells expresses the endogenous TAA gp70. However, CT26 lacks CD19 expression which makes it sensitive only to AH1-specific T cells but not to CD19-CART cells. Stimulation of T cells from the combination-treated group with the AH1 peptide and co-culturing them with CT26 cells resulted in increased reactivity and a higher number of IFNγ spots, which was dependent on MHCI (Figure 3.6D). These findings further support the synergistic effects of RT and CAR T cell therapy, demonstrating upregulation of antigen presentation and epitope spreading.

3.7 Adoptive T cell transfer from mice treated with RT followed by CAR T cells in CT26 tumor bearing mice confirms cross presentation concept

To further confirm the role of cross-presentation in the enhanced anti-tumor effects of CAR T cells combined with RT, we conducted a back-to-back adoptive T cell therapy. $CD3^+$ T cells isolated from the spleens of A20 tumor-bearing mice previously treated with CAR T therapy alone or in combination with RT were expanded ex vivo and then intravenously transferred into CT26 tumor-bearing recipient mice (Figure 3.7A). The specificity of the expanded T cells against the AH1 TAA was confirmed prior to transfer (Figure 3.7B). Lymphodepleted CT26 tumor-bearing mice that received T cells from donors treated with RT followed by CAR T cells exhibited significantly slower tumor progression compared to the group that was transferred T cells from donors treated only with CAR T cells (Figure 3.7C). Moreover, the group that received T cells from the combination-treated donors showed increased infiltration of $CD8^+$ T cells (Figure 3.7D) and cytotoxic T cells within the tumors (Figure 3.7E). Importantly, the group that received T cells from donors treated with combination therapy demonstrated significantly higher infiltration of AH1-specific $CD8^+$ T cells into the tumors

(Figure 3.7F). When spleen cell suspensions were stimulated overnight with AH1 peptide, significantly more IFNγ spots were recorded from the mice previously received T cells from

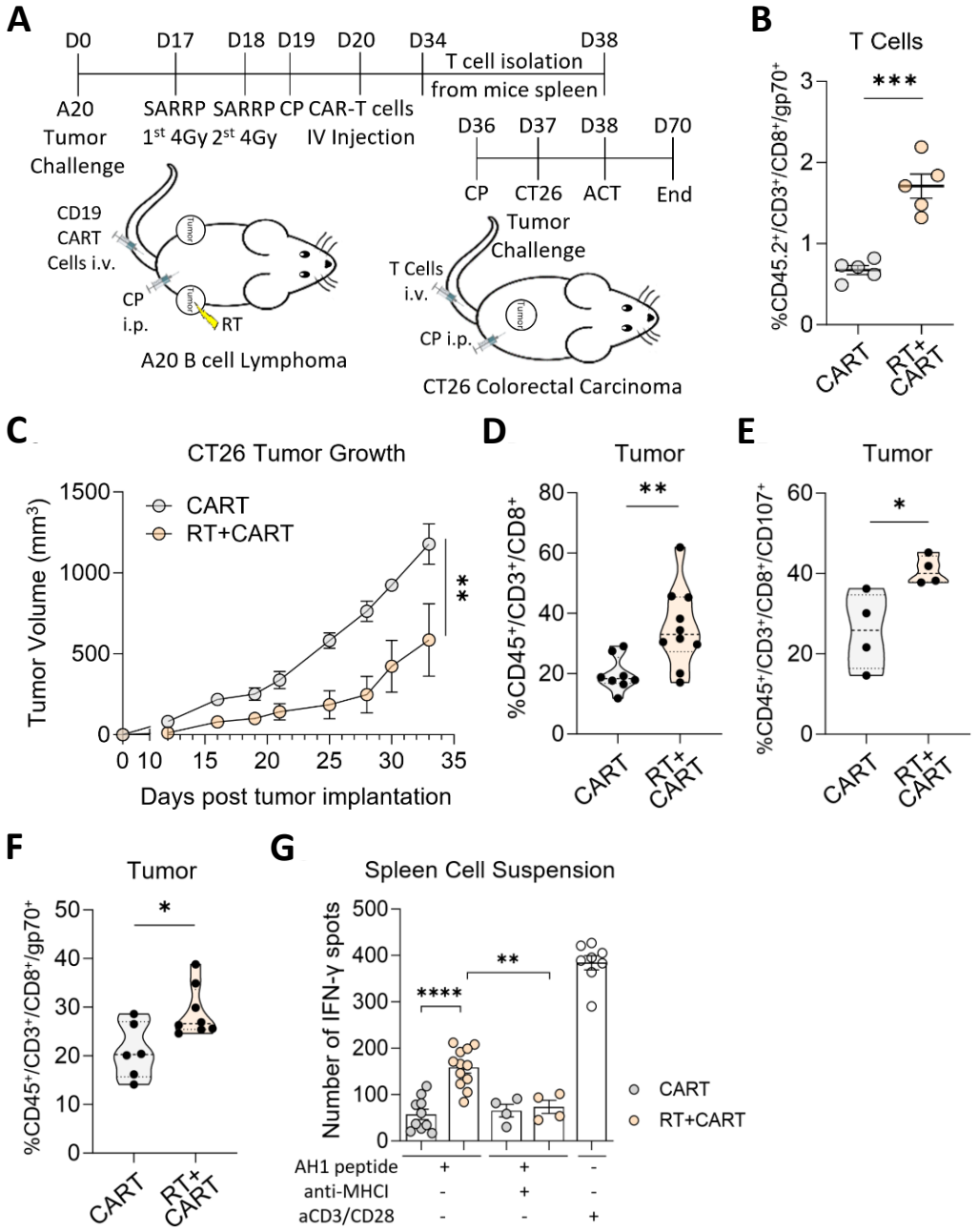

Figure 3.7 ***Adoptive T cell transfer from mice treated with RT followed by CAR T cells in CT26 tumor bearing mice confirms cross presentation concept.*** *(A) Working model: Timeline and schematic representation: CD3+ T cells were isolated from the spleens of A20 tumor-bearing mice treated with RT followed by CAR T cells, expanded ex vivo for four days and adoptively transferred to CT26 tumor-bearing mice. (B) Tetramer staining of AH1-specific T cells from donor mice after ex vivo expansion, before ACT. (C) CT26 tumor growth of recipient mice. (D) CD8+ T cell infiltration in the CT26 tumor. (E) AH1-specific T cell infiltration in the CT26 tumor. (F) CD107+ T cell infiltration in the CT26 tumor (G) IFNγ spots of an Eli-spot assay after overnight AH1 peptide stimulation of splenocytes from mice that received ACT. The assay was performed in the presence or absence of an anti-MHC class I antibody. Anti-CD3 and anti-CD28 antibodies were used to stimulate T cells as positive control. Graphs show the mean ± SEM. (*P <0.05, **P < 0.01, ***P < 0.001, ****P<0.0001).*

RT plus CART cell-treated donors, with this immune reaction being mediated by MHCI (Figure 3.7G).

3.8 Endogenous RT-mediated cross primed T cells eliminate CD19-negative A20 tumor cells

To investigate the impact of combination of RT and CAR T cells on A20 tumor cells in vitro, A20 tumor cells were irradiated with two doses of 4Gy and incubated with CART-19 cells. After 24 hours we observed the significant effects of the single therapies on the tumor cells in comparison to the untreated cells. Notably, A20 cells that were treated with both RT and CAR T cells presented the highest rate of sensitivity (Figure 3.8A).

Besides the CD19 antigen which accounts for the target of the CAR T cells, A20 tumor cells also abundantly express a pan murine A20 marker named B220. CD19 and B220 markers allow for the specific identification and characterization of A20 tumor cells. Interestingly,

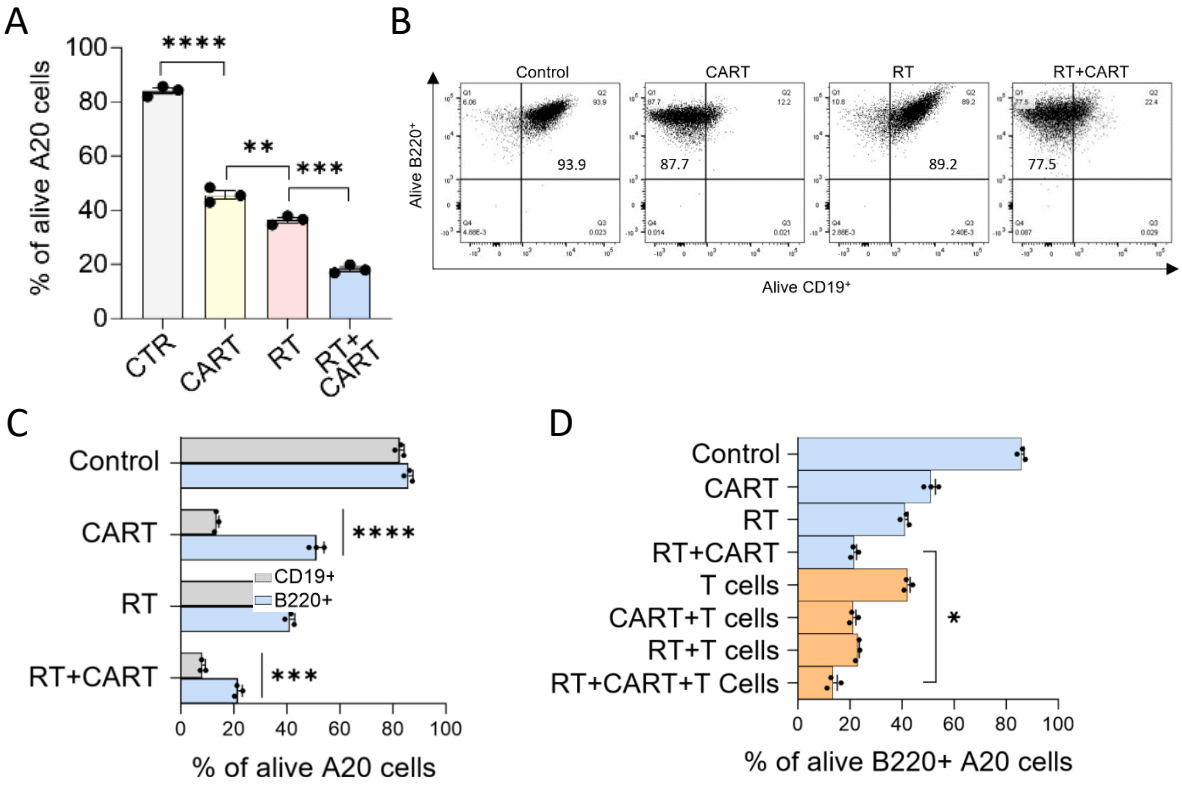

Figure 3.8 ***Endogenous RT-mediated cross primed T cells eliminate CD19-negative A20 tumor cells.*** *(A) Percentage of alive A20 tumor cells after 24 hours of RT, CAR T or RT plus CAR T cell treatment in vitro. (B) Flow cytometry for B220 and CD19 markers after in vitro treatment of A20 tumor cells with RT, CAR T or RT and CAR T cell treatment. (C) B220+ and CD19+ alive A20 cells post treatments. (D) B220+ alive A20 cells after treatment with radiation (RT), CAR T cells (CART) and T cells isolated from CT26-bearing mouse adoptively transferred with T cells from RT+CART donor. Graphs show the mean ± SEM. (*P <0.05, **P < 0.01, ***P < 0.001,****P<0.0001).*

flow cytometry analysis of the treated A20 cells revealed a dramatic loss of the CD19 antigen in the survival populations post CAR T cell treatment which was not observed in the control or the RT-treated groups (Figure 3.8B). Moreover, CAR T cells eliminated most of the $CD19^{+}$ A20 cells leaving a great CD19-negative population alive, whereas RT eliminated the tumor cells regardless of antigen expression (Figure 3.8C). Since we previously demonstrated that RT induced cross presentation, we decided to investigate the impact of endogenous cross primed T cells on A20 cells. We isolated the $CD3^{+}$ T cells from the spleen of a CT26 bearing mouse that had rejected the tumor after ACT from the RT+CART-treated donors. We then incubated these T cells with the previously in vitro wild type or irradiated A20 tumor cells with or without CART-19 cells. Interestingly, although the endogenous T cells alone had a significant impact on the B220 positive tumor cells, when combined with RT and CAR T cells led to an enormous A20 elimination (Figure 3.8D). These results confirm that RT induces cross presentation and endogenous T cells primed against specific TAAs increase CAR T cell efficacy providing adjuvant tumor elimination.

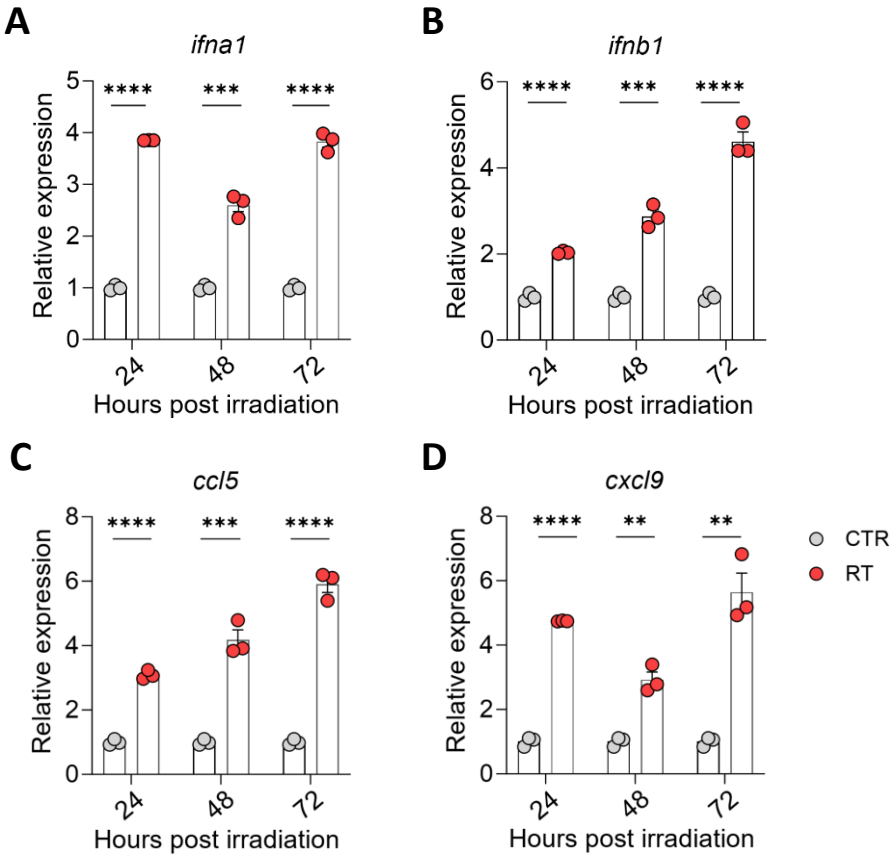

Figure 3.9 ***Radiation upregulates IFN type I and chemokines signaling.*** *(A-D) Expression of interferon a1 (ifna1), interferon b1 (ifnb1), ccl5 and cxcl9 24, 48 and 72 hours after n vitro radiation (RT). Graphs show the mean ± SEM. (***P < 0.001, ****P<0.0001).*

3.9 Radiation upregulates IFN type I and chemokines signaling

To better understand the enhanced tumor infiltration of CAR T cells achieved through the combination of RT and CAR T cell therapy, we investigated the impact of RT on the expression of key chemokines involved in immune cell trafficking within tumors. In an in vitro setting, A20 tumor cells were exposed to RT, and the expression levels of ifn-α1, ifn-β1, ccl5, and cxcl9 were assessed at multiple time points; 24-, 48-, 72-, and 96-hours post-RT. The results revealed a significant upregulation of both interferon type I genes (ifn-α1 and ifn-β1) following RT at all time points compared to untreated cells (Figure 3.9A and B). Additionally, the expression of ccl5 and cxcl9, two chemokines involved in immune cell recruitment, was significantly increased in A20 tumor cells after in vitro RT (Figure 3.9C and D). Consistent findings were observed also in vivo (Figure 3.9E and F). These findings suggest that upregulation of CXCL9 may play a crucial role in facilitating lymphocyte infiltration within the tumor microenvironment, including CAR T cells.

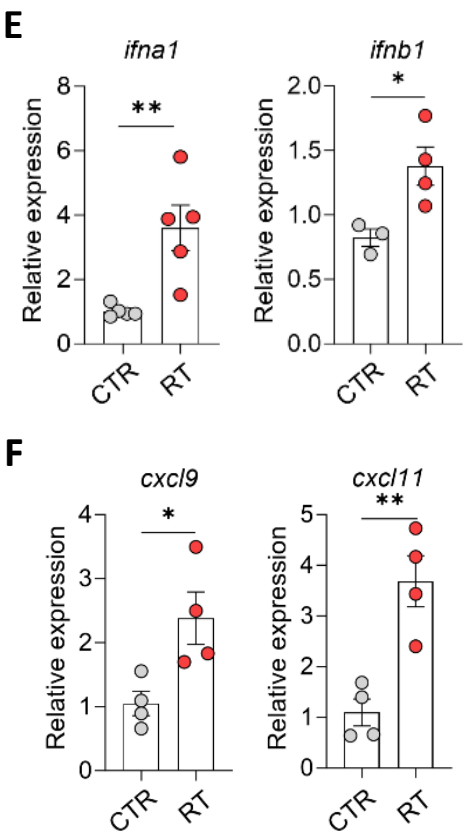

Figure 3.9 (cont.) Radiation upregulates IFN type I and chemokines signaling. *(E-F) Expression of ifna1, ifnβ1, cxcl9 and cxcl11 48h post in vivo IR.*

3.10 Tumor radiation activates STING signaling

Type I interferons initiate a variety of responses including DC activation and maturation, as well as CD8+ T cell activation. Upregulation of the IFN type I genes in the irradiated tumor and induction of cross presentation led to the hypothesis that STING pathway is relevant to the results observed. To investigate the relevance of this mechanism in our observations we performed western blot analysis from proteins isolated from tumors treated with RT or RT with CAR T cells. In line with our hypothesis, we observed a significant enhancement of the STING pathway activation in the irradiated tumors (Figure 3.11A and B). These findings suggest that STIING activation is involved in the adjuvant effects of RT.

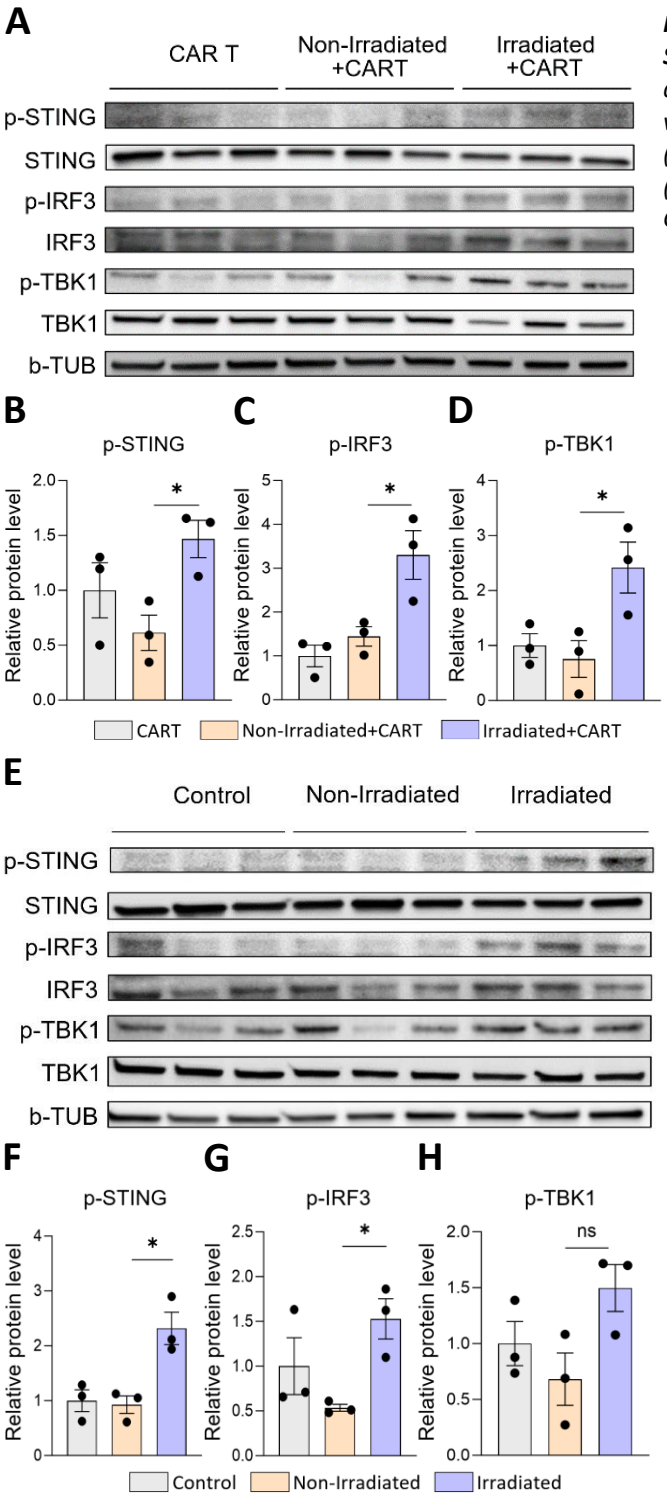

Figure 3.10 Tumor radiation activated STING signaling. *(A) STING-IRF3 pathway activation and (B-D) quantification post in vivo tumor RT and CAR T cell treatment. (E) STING-IRF3 pathway activation and (F-H) quantification post in vivo tumor RT. Graphs show the mean ± SEM. (*P <0.05).*

3.11 Radiation enhances CAR T cell therapy through STING activation

To further validate the relevance of the STING pathway in vivo, we induced block of STING using the STING antagonist H-151. A20 tumor bearing BALB/c mice were treated with RT as

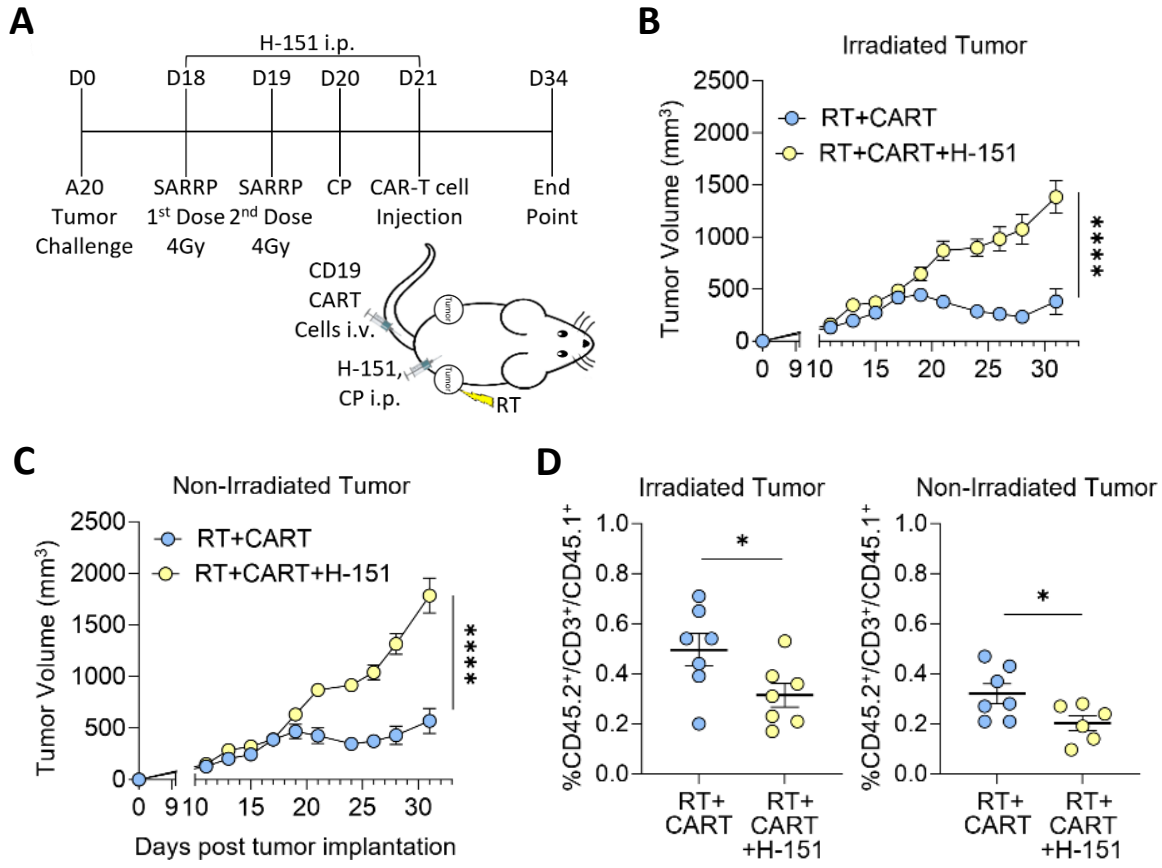

Figure 3.11 Radiation enhances CAR T cell therapy through STING activation. *(A) Working model: Timeline and schematic representation of in vivo A20 tumor-bearing mice treated with RT followed by CD19-CAR T Cell infusion with or without the STING antagonist H-151. (B-C) Tumor growth from irradiated and non-irradiated tumors. (D) CD45.1+ T cell infiltration in irradiated and non-irradiated tumors, representing CD19-CAR T cells. Graphs show the mean ± SEM. (*P <0.05, ****P<0.0001).*

previously described followed by CAR T cell therapy with or without H-151 and tumor progression was monitored (Figure 3.11A). Inhibition of STING significantly abolished the anti-tumor effects of RT on the irradiated tumor and the synergistic effects of the two treatments on the non-irradiated tumor (Figure 3.11B and C). STING blocking also caused a significant decrease in the infiltration of CAR T cells in both irradiated and non-irradiated tumors (Figure 3.11D).

3.12 Radiation-mediated STING activation is needed for cross presentation

Inhibition of STING significantly resulted in less endogenous T cells infiltrating the spleens in comparison to the mice that did not receive inhibition treatment (Figure 3.12A). Evaluation of the TME revealed that less endogenous T cells tended to infiltrate both irradiated and non-irradiated tumors of the mice that were treated with the STING

antagonist (Figure 3.12B). Importantly, the STING antagonist also caused a significant decrease in the infiltration of DCs in both irradiated and non-irradiated tumors (Figure 3.12E). To confirm the role of STING in cross presentation, tumor and spleen suspensions were stimulated overnight with AH1 peptide with or without anti-MHCI antibody.

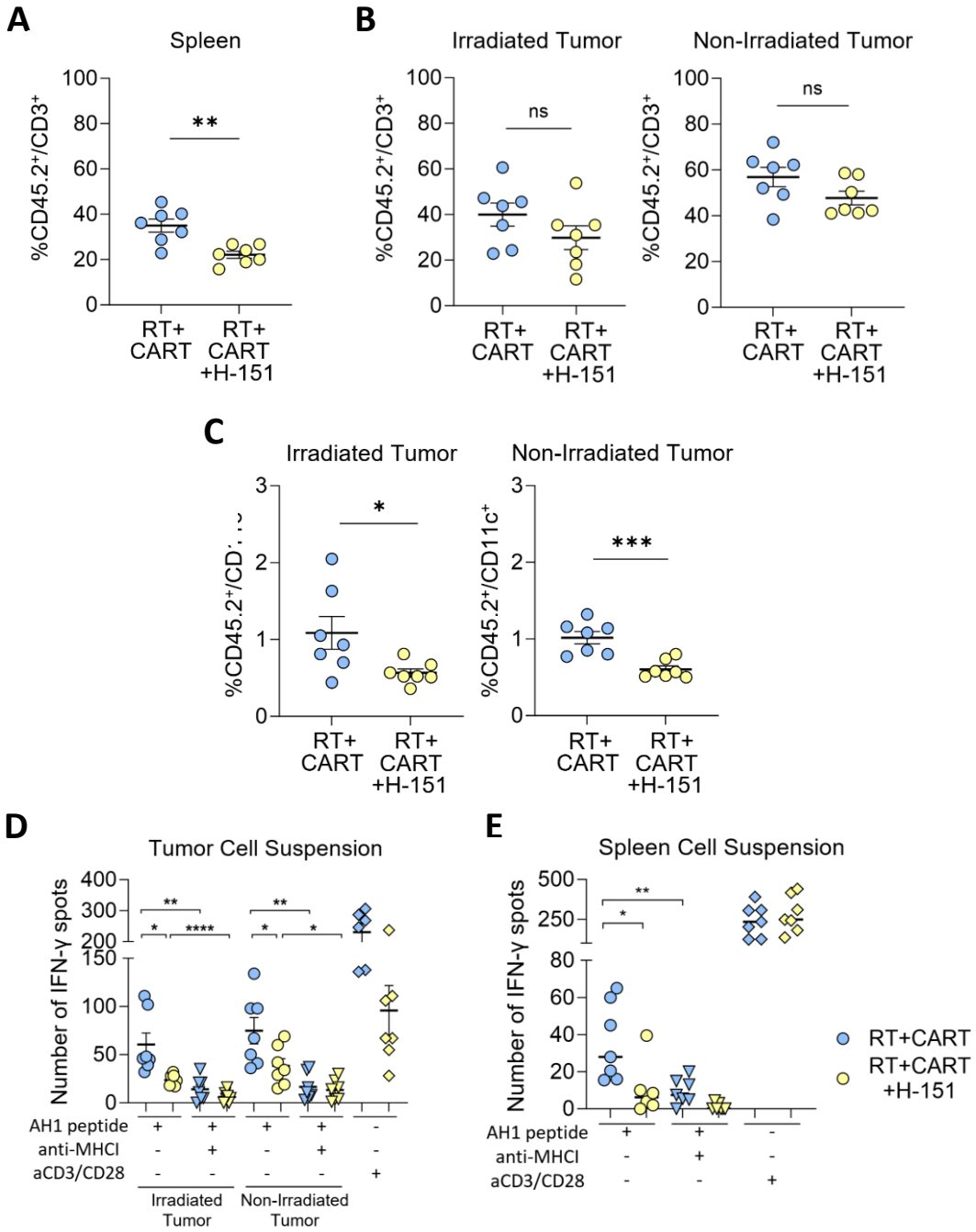

Figure 3.12 Radiation-mediated STING activation is needed for cross presentation. *(A-B) CD3+ T cell infiltration in spleen, irradiated and non-irradiated tumors after treatment with RT plus CAR T cells or RT plus CAR T cells plus the STING inhibitor. (C) CD11c+ dendritic cell (DC) infiltration in irradiated and non-irradiated tumors. (D-E) IFNγ spots after overnight stimulation of tumor or spleen cell suspensions with the AH1 peptide with or without anti-MHCI antibody. Anti-CD3 and anti-CD28 antibodies were used to stimulate T cells as positive control. Graphs show the mean ± SEM. (*P <0.05, **P < 0.01, ***P < 0.001, ****P<0.0001).*

Significantly less IFNγ spots were recorded from the tumors and spleens of mice treated with H-151 in comparison to the mice that received only the combination of RT and CAR T cells. The addition of the anti-MHCI antibody decreased IFNγ spots specifically in the RT plus CAR T cell group, as expected (Fig. 6F and G). These findings provide strong evidence that STING serves as a crucial mediator in RT-induced cross presentation and enhanced anti-tumor effects of CAR T cell therapy.

4. Discussion

Bridging radiation therapy (RT) has been safely used in clinical protocols to maintain performance status for patients that are scheduled for CAR T cell therapy and, to date, bRT has not led to significant RT- or CART-related toxicities or negatively impact outcomes [30,80,104–106,116,117,119]. In preclinical models, DeSelm et. al. used low-dose conditioning immunogenic RT to sensitize pancreatic tumor cells, even tumor cells that they have proceeded to antigen escape, to become susceptible to CAR T cells through TRAIL-mediated death [101]. In a glioblastoma mouse model, RT was shown to synergize with CAR T cells by increasing their anti-tumor effectiveness and infiltration to the tumor site [102]. This doctoral research addresses for the first time the use of RT prior to CAR T cell therapy in a murine A20 lymphoma model and demonstrates that RT provides adjuvant anti-tumor effects that improves CAR T cell outcomes.

In our experiments we decided to use the A20 tumor model, a BALB/c-derived B cell lymphoma cell line. In order to understand how the A20 model responds to RT, we initially tested to deliver RT in a single dose or two equal fractions. The dose was decided to be 8Gy which is a clinically relevant dose used in our institution. Previous rodent studies in different tumor models have demonstrated that fractionated RT leads to enhanced antitumor immune responses in comparison to single dose RT [110,120]. In line to these studies, our model showed that a total dose of 8Gy when delivered in two fractions of 4Gy results in enhanced control of non-irradiated tumor sites, indicating that fractionated RT could mediate an enhanced pro-immunogenic effect and abscopal effects. Indeed, assessing the tumor microenvironment (TME) by multicolor flow cytometry analysis (FACS) we observed a superior immune reaction characterized by an increased infiltration of T cells in both irradiated and abscopal tumors of the group treated with two fractions of RT. The further characterization of the tumor infiltrated T cells showed that they were cytotoxic and specifically primed against the gp70 tumor associated antigen (TAA). The A20 tumor cell line abundantly expresses the ectopic viral antigen gp70 which includes the immune-dominant epitope AH1. Higher infiltration of AH1-specific T cells suggests that

hypo-fractionated RT promoted cross presentation in our model, as it has also been suggested by previous studies [110,121,122].

Our study demonstrated an important synergy between fractionated RT and CAR T cell therapy. RT administration prior to CAR T cell infusion presented enhanced tumor control not only in the site of RT but also in abscopal, non-irradiated sites, and increased the overall survival of the mice as shown in patients too. Bridging RT and CAR T cell therapy synergistic effects may have been introduced in numerous case reports and clinical trials; however our preclinical study is the first one that mechanistically describes how RT could enhance the effectiveness of CAR T cells. The infiltration of $CD8^+$ T cells in the TME is a result of several signaling molecules such as chemokines. It has been shown that upregulation of tumor CXCL9 is associated with higher infiltration of tumor infiltrated lymphocytes (TILs) [87]. Our model exhibited a higher expression of CXCL9 after administration of RT both in vitro and in vivo. Moreover, we observed a greater CAR T cell tumor infiltration after RT treatment. Hence, RT-mediated upregulation of CXCL9 seems to be crucial in the migration of CAR T cells in the tumor. More CAR T cells present in the TME result in greater recognition of the CD19 antigen expressed by the A20 tumor cells, therefore superior anti-tumor effects and tumor control.

Besides CD19, which is expressed in over 95% of B malignancies and is the optimal target for CAR T cells, the A20 cell line also expresses gp70, which can be a target of endogenous AH1 primed T cells. After noticing abscopal effects of the fractionated RT, we decided to further look into this mechanism and how it participates in the enhanced CAR T cell antitumor results that we observed. In the group that received RT followed by CAR T cell infusion, more DCs infiltrated the irradiated tumor. That is probably due to the fact the RT of the tumor results in tumor cell death and release of a pool of TAA. DCs in the TME effectively engulf and process TAA and migrate to tumor draining lymph nodes where they cross present them to naïve $CD8^+$ T cells through MHC class I molecules. Activated, CD8 T cells primed to recognize specific TAA migrate in the TME and effectively eliminate their targets. In our study, we observed a higher infiltration of DCs in the irradiated tumors of the groups that received either RT alone or RT followed by CAR T cells. Importantly, the tumor infiltrated DC expressed critical genes that participate in the cross-presentation

pathway. On top of that, tetramer staining for the AH1 TAA showed an important increase of endogenous AH1-specific CD8 T cells in both irradiated and abscopal tumors of the group that received the combination therapy. In the same group, a greater immune response against the AH1 TAA was recorded when tumor cell suspension, spleen cell suspension or isolated T cells were all stimulated with the AH1 peptide. Importantly, this greater immune response was demolished in all these assays in the presence of an anti-MHC class I antibody, which is the key mediator of cross presentation.

Although these data strongly indicate that RT enhances CAR T cell therapy outcomes through cross presentation, we proceeded with an adoptive T cell transfer (ACT) experiment to validate our observations. As previously said, A20 tumor cells express both CD19 and gp70 among other antigens. CD19 is the target of the CAR T cells whereas gp70 is the TAA recognized by cross-primed endogenous CD8 T cells in our model. The CT26 cell line is a syngeneic BALB/c-derived colorectal carcinoma cell line which expresses the endogenous TAA gp70 similarly to A20 cells. However, CT26 tumor cells lack CD19 expression and, therefore, they cannot be targets of CAR T cells. ACT from mice treated with CAR T cell therapy alone or RT followed by CAR T cell therapy validated the cross-presentation concept. CT26-bearing mice hat received T cells from donors previously treated with RT and CAR T cells presented a superior tumor control which was supported by a better immune response characterized by higher tumor infiltrated cytotoxic CD8 T cells and AH1-specific T cells.

Approximately half of the patients that receive CAR T cell therapy relapse after initial response due to several reasons. One well-known mechanism that results in CAR T cell failure is antigen escape, where cancer cells develop clones that downregulate or completely halt the expression of the targeted antigen, such as CD19 [83,84]. Tumor cells with low or absent CD19 expression can evade recognition by circulating CAR T cells. In our A20 model, CAR T cells display successful tumor killing targeting CD19; however, in vitro, there is a 10-15% population that escapes, loses CD19 expression and survives, whereas RT treatment does not seem to impact tumor cells antigen escape. T cells were then Isolated from a CT26-bearing mouse that rejected the tumor after being transferred with T cells from a RT plus CAR T cell donor. Its T cells also displayed successful A20 killing

independently from CD19 expression. Notably, when cultured together, CD19 CAR T cells specifically target and eliminate CD19 positive A20 tumor cells and cross primed CD8 t cells provide an adjuvant tumor killing by targeting specific TAA. Collectively, our data validate that RT induces DCs to cross present TAA to CD8 T cells which provide a supplement killing mechanism alongside CAR T cell CD19-specific killing.

It has been previously demonstrated that STING is the key protein required for the antitumor effects of RT [122]. RT causes DNA to break which activates the cGAS protein. cGAS protein then binds to STING and the downstream pathway leads to phosphorylation of IRF3 transcription factor which results in the expression of type I IFN genes. IFN type I are essential for DC-mediated cross-priming of $CD8^+$ T cells. Our study verifies this mechanism in the A20 model. Cross presentation of $CD8^+$ T cells was associated with upregulation of IFN type I genes as well as STING-IRF3 pathway activation. To validate that STING is the mediator of the cross-presentation effects that we observed, we treated mice with RT and CAR T cells but blocking STING using the H-151 inhibitor. Indeed, STING inhibition abolished the adjuvant effects of RT to CAR T cell therapy by decreasing DC infiltration in the tumor sites as well as their ability to cross present TAA to CD8 T cells.

Overall, our study is the first one using RT prior to CAR T cell therapy in a murine lymphoma model. We demonstrated that RT can effectively be an adjuvant therapy alongside CAR T cell therapy by providing supplementary tumor killing trough STING-mediated cross presentation of endogenous CD8 T cells. We believe that our study will shed light on the clinical use of RT as bridging therapy and provide innovative knowledge which is going to help CAR T cell immunotherapy improve its clinical and therapeutic applications.

5. Conclusion

Cancer immunotherapies have changed the standard treatments as they lead to long-term disease control. More recently, CAR T cell therapy has become a well-known cancer immunotherapy and has revolutionized the treatment approach of patients with r/r lymphomas. On the other side, radiation is a well-established cancer therapy with a series of immunomodulatory mechanisms. Although radiation has been successfully used in patients as bridging therapy prior to CAR T cell therapy, data are only available from case reports and retrospective clinical trials. This doctoral research demonstrates that radiation can be delivered prior to CD19-CAR T cells and can improve their efficacy by providing adjuvant anti-tumor immunity. Overall, we demonstrated that radiation upregulates the cross-presentation machinery through DNA-sensing STING-IRF3 cascade activation and provides antigen-specific tumor elimination supplementing CAR T cell antitumor effects. In conclusion, this study provides the first evidence of how RT can improve CAR T cell therapy efficacy in murine B cell lymphoma. Subsequent clinical trials will give a better understanding of the specific individualized regimen of bridging radiation that will optimize CAR T cell efficacy in patients and improve its therapeutic applications.

APPENDIX A

2016 WHO classification of mature lymphoid, histiocytic, and dendritic neoplasms

Mature B-cell neoplasms

Chronic lymphocytic leukemia/small lymphocytic lymphoma

Monoclonal B-cell lymphocytosis

B-cell prolymphocytic leukemia

Splenic marginal zone lymphoma

Hairy cell leukemia

Splenic B-cell lymphoma/leukemia, unclassifiable

Splenic diffuse red pulp small B-cell lymphoma

Hairy cell leukemia-variant

Lymphoplasmacytic lymphoma

Waldenström macroglobulinemia

Monoclonal gammopathy of undetermined significance (MGUS), IgM

μ heavy-chain disease

γ heavy-chain disease

α heavy-chain disease

Monoclonal gammopathy of undetermined significance (MGUS), IgG/A

Plasma cell myeloma

Solitary plasmacytoma of bone

Extraosseous plasmacytoma

Monoclonal immunoglobulin deposition diseases

Extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma)

Nodal marginal zone lymphoma

Pediatric nodal marginal zone lymphoma

Follicular lymphoma

In situ follicular neoplasia

Duodenal-type follicular lymphoma

Pediatric-type follicular lymphoma

Large B-cell lymphoma with IRF4 rearrangement

Primary cutaneous follicle center lymphoma

Mantle cell lymphoma

In situ mantle cell neoplasia

Diffuse large B-cell lymphoma (DLBCL), NOS

Germinal center B-cell type

Activated B-cell type

T-cell/histiocyte-rich large B-cell lymphoma

Primary DLBCL of the central nervous system (CNS)

Primary cutaneous DLBCL, leg type

EBV^+ DLBCL, NOS

EBV^+ mucocutaneous ulcer

DLBCL associated with chronic inflammation

Lymphomatoid granulomatosis

Primary mediastinal (thymic) large B-cell lymphoma

Intravascular large B-cell lymphoma

ALK^+ large B-cell lymphoma

Plasmablastic lymphoma

Primary effusion lymphoma

HHV8^{+} DLBCL, NOS

Burkitt lymphoma

Burkitt-like lymphoma with 11q aberration

High-grade B-cell lymphoma, with MYC and BCL2 and/or BCL6 rearrangements

High-grade B-cell lymphoma, NOS

B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and classical Hodgkin lymphoma

Mature T and NK neoplasms

T-cell prolymphocytic leukemia

T-cell large granular lymphocytic leukemia

Chronic lymphoproliferative disorder of NK cells

Aggressive NK-cell leukemia

Systemic EBV^{+} T-cell lymphoma of childhood

Hydroa vacciniforme–like lymphoproliferative disorder

Adult T-cell leukemia/lymphoma

Extranodal NK-/T-cell lymphoma, nasal type

Enteropathy-associated T-cell lymphoma

Monomorphic epitheliotropic intestinal T-cell lymphom

Indolent T-cell lymphoproliferative disorder of the GI tract

Hepatosplenic T-cell lymphoma

Subcutaneous panniculitis-like T-cell lymphoma

Mycosis fungoides

Sézary syndrome

Primary cutaneous $CD30^+$ T-cell lymphoproliferative disorders

Lymphomatoid papulosis

Primary cutaneous anaplastic large cell lymphoma

Primary cutaneous γδ T-cell lymphoma

Primary cutaneous $CD8^+$ aggressive epidermotropic cytotoxic T-cell lymphoma

Primary cutaneous acral $CD8^+$ T-cell lymphoma

Primary cutaneous $CD4^+$ small/medium T-cell lymphoproliferative disorder

Peripheral T-cell lymphoma, NOS

Angioimmunoblastic T-cell lymphoma

Follicular T-cell lymphoma

Nodal peripheral T-cell lymphoma with TFH phenotype

Anaplastic large-cell lymphoma, ALK^+

Anaplastic large-cell lymphoma, ALK^-

Breast implant–associated anaplastic large-cell lymphoma

Hodgkin lymphoma

Nodular lymphocyte predominant Hodgkin lymphoma

Classical Hodgkin lymphoma

Nodular sclerosis classical Hodgkin lymphoma

Lymphocyte-rich classical Hodgkin lymphoma

Mixed cellularity classical Hodgkin lymphoma

Lymphocyte-depleted classical Hodgkin lymphoma

Posttransplant lymphoproliferative disorders (PTLD)

Plasmacytic hyperplasia PTLD

Infectious mononucleosis PTLD

Florid follicular hyperplasia PTLD

Polymorphic PTLD

Monomorphic PTLD (B- and T-/NK-cell types)

Classical Hodgkin lymphoma PTLD

Histiocytic and dendritic cell neoplasms

Histiocytic sarcoma

Langerhans cell histiocytosis

Langerhans cell sarcoma

Indeterminate dendritic cell tumor

Interdigitating dendritic cell sarcoma

Follicular dendritic cell sarcoma

Fibroblastic reticular cell tumor

Disseminated juvenile xanthogranuloma

Erdheim-Chester disease

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