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**Adoptive transfer of T cells transduced
with an HLA-independent T-cell receptor
against tyrosinase-related protein 2**

Dissertation

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Declaration in lieu of oath

I hereby declare that I have written the present dissertation independently and have used no sources or aids other than those stated. All passages taken from other works, whether quoted directly or paraphrased, have been clearly marked as such. This dissertation has not been submitted to any other university or examination board in the same or similar form.

Mainz, 17.12.2025

Alice Mary-Ann Bury

Abbreviations

ABC – Avidin-Biotin Complex
ACT – Adoptive Cell Therapy
AEC – 3-Amino-9-ethylcarbazole
BLI – Bioluminescence Imaging
bp – Base Pairs
BSA – Bovine Serum Albumin
CAR – Chimeric Antigen Receptor
CPS – counts per second
CD – Cluster of Differentiation
CIITA – Class II Transactivator
CTL – Cytotoxic T Lymphocyte
cTCR – chimeric T-Cell Receptor
DMEM – Dulbecco's Modified Eagle Medium
DMSO – Dimethyl Sulfoxide
DNA – Deoxyribonucleic Acid
dNTPs – Deoxynucleotide Triphosphates
EGFP – Enhanced Green Fluorescent Protein
ELISpot – Enzyme-Linked Immunospot
FACS – Fluorescence-Activated Cell Sorting
FCS – Fetal Calf Serum
FLUC – Firefly Luciferase
HB – Helix Breaker
HLA – Human Leukocyte Antigen
IFN- γ – Interferon Gamma
IL – Interleukin
IVIS – *In Vivo* Imaging System
KO – Knockout
LB – Lysogeny Broth
LN – Lymph Node

LTR – Long Terminal Repeat
MHC – Major Histocompatibility Complex
mTCR – murine T-Cell Receptor
mTRBC – murine T-Cell Receptor Beta Chain
NEAA – Non-Essential Amino Acids
NLUC – NanoLuc Luciferase
NSG – NOD scid gamma (mouse strain)
ORF – Open Reading Frame
PAC – Puromycin N-Acetyl-Transferase
PBS – Phosphate Buffered Saline
PCR – Polymerase Chain Reaction
PD-1 – Programmed Cell Death Protein 1
RPMI – Roswell Park Memorial Institute Medium
RT – Room Temperature
SDM – site-directed mutagenesis
sgRNA – single guide RNA
TBI – Total Body Irradiation
TCR – T-Cell Receptor
TGF- β – Transforming Growth Factor Beta
Th – T Helper Cell
TNF- α – Tumor Necrosis Factor Alpha
TNTC – Too Numerous To Count
TRP2 – Tyrosinase-Related Protein 2
V β – Variable Region Beta Chain
WPRE – Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element

Summary

Adoptive T-cell receptor (TCR) therapy has shown promise in melanoma and other tumor types, but efficacy is often compromised when tumors evade detection by downregulating HLA class I molecules, impairing antigen presentation. Strategies that enable T-cell responses independent of classical HLA-mediated presentation are therefore needed, particularly in solid tumors, in which antigen loss and heterogeneity are major barriers.

A promising target for TCR-based immunotherapy of melanoma is Tyrosinase-related protein 2 (TRP2). As a melanocyte differentiation antigen, TRP2 contributes to melanin biosynthesis and may have anti-apoptotic functions, while its restricted expression in normal tissues reduces the risk of off-tumor toxicity. Exploiting naturally occurring, HLA-independent TCRs against TRP2 offers a strategy to circumvent immune evasion through HLA downregulation while maintaining antigen-specific tumor recognition.

In this dissertation, the efficacy and safety of a naturally occurring, HLA-independent $\alpha\beta$ TCR against human and murine TRP2 were evaluated *in vivo* using xenograft and syngeneic melanoma models. The TCR, originally identified in a melanoma patient, was previously cloned, codon-optimized, and engineered with murine constant regions.

In the xenograft model, NSG mice bearing HLA-deficient Ma-Mel-86b melanoma cells, either with endogenous TRP2 expression or virally transduced to overexpress TRP2, were treated with human anti-TRP2 TCR-T cells. Control mice received T cells transduced with either an HLA-A*02:01-restricted anti-NY-ESO-1 TCR or firefly luciferase alone. In the syngeneic model, C57BL/6 mice carrying murine melanoma cells (B78.H1) stably expressing human TRP2 received murine anti-TRP2 TCR-T cells with endogenous TCR α - and β -chains disrupted by CRISPR/Cas9 editing. In both models, tumor cells expressed NanoLuc luciferase, T cells expressed firefly luciferase, and IL-2 was given to support T-cell activity. Treatment efficacy was assessed by *in vivo* bioluminescence imaging, histology, and physical examination.

Anti-TRP2 TCR-T cells consistently localized to tumors and persisted *in vivo*, eliminating TRP2-overexpressing tumors in both models while only delaying growth in natural TRP2-expressing tumors. Safety assessments revealed no systemic toxicity or relevant on-target, off-tumor effects. In the syngeneic model, localized vitiligo-like depigmentation occurred without evidence of internal organ toxicity.

These results demonstrated the apparent safety and anti-tumor activity of the HLA-independent TRP2-specific TCR but also pointed to limited efficacy against naturally expressing tumors, likely due to inflammation-induced dedifferentiation and antigen downregulation. *In vitro*, the receptor tyrosine kinase inhibitor tivozanib preserved TRP2 expression under TNF- α - or IFN- γ -induced stress. Future studies will be needed to evaluate whether combining TCR therapy with tivozanib or other drugs that are able to reverse inflammation-induced dedifferentiation can improve control of tumors with natural TRP2 expression *in vivo*.

In summary, this dissertation demonstrates *in vivo* antitumor activity and an apparent favorable safety profile of an HLA-independent TRP2-specific TCR. While tumors overexpressing TRP2 were effectively eliminated, efficacy against tumors with natural TRP2 expression was limited. More broadly, the results illustrated how HLA-independent TCRs bypass classical antigen presentation and may guide the development of safe and more versatile TCR therapies for solid tumors.

Zusammenfassung

Die adoptive T-Zellrezeptor(TCR)-Therapie hat bei Melanomen und anderen Tumorarten vielversprechende Ergebnisse gezeigt, doch die Wirksamkeit wird häufig dadurch eingeschränkt, dass Tumoren der Erkennung entgehen, indem sie HLA-Klasse-I-Moleküle herunterregulieren und so die Antigenpräsentation beeinträchtigen. Daher werden Strategien benötigt, die T-Zellantworten unabhängig von der klassischen HLA-vermittelten Präsentation ermöglichen, insbesondere bei soliden Tumoren, bei denen Antigenverlust und -heterogenität zentrale Hürden darstellen.

Ein vielversprechendes Ziel für die TCR-basierte Immuntherapie des Melanoms ist das Tyrosinase-related protein 2 (TRP2). Als Melanozyten-Differenzierungsantigen trägt TRP2 zur Melaninbiosynthese bei und kann antiapoptotische Funktionen besitzen, während seine eingeschränkte Expression in gesundem Gewebe das Risiko von Off-Tumor-Toxizitäten reduziert. Die Nutzung natürlich vorkommender, HLA-unabhängiger TCRs gegen TRP2 bietet eine Strategie, um Immunflucht durch HLA-Herunterregulation zu umgehen und gleichzeitig eine antigen-spezifische Tumorerkennung aufrechtzuerhalten.

In dieser Dissertation wurden Wirksamkeit und Sicherheit eines natürlich vorkommenden, HLA-unabhängigen $\alpha\beta$ -TCRs gegen humanes und murines TRP2 *in vivo* in Xenograft- und syngenem Melanommodellen untersucht. Der TCR, ursprünglich aus dem Blut einer Melanompatientin identifiziert, war zuvor kloniert, codonoptimiert und mit murinen Konstantregionen ausgestattet worden.

Im Xenograft-Modell wurden NSG-Mäuse, die HLA-defiziente Ma-Mel-86b-Melanomzellen mit entweder endogener TRP2-Expression oder viraler TRP2-Überexpression trugen, mit humanen anti-TRP2-TCR-T-Zellen behandelt. Kontrollmäuse erhielten T-Zellen, die entweder mit einem HLA-A*02:01-restringiert anti-NY-ESO-1-TCR oder mit Firefly-Luciferase allein transduziert waren. Im syngenem Modell erhielten C57BL/6-Mäuse mit murinen Melanomzellen (B78.H1), die stabil humanes TRP2 exprimierten, murine anti-TRP2-TCR-T-Zellen, bei denen die endogenen TCR- α - und - β -Ketten mittels CRISPR/Cas9 ausgeschaltet worden waren. In beiden Modellen exprimierten die Tumorzellen NanoLuc-Luciferase, die T-Zellen Firefly-Luciferase, und IL-2 wurde zur Unterstützung der T-Zellaktivität verabreicht. Die Behandlungseffekte wurden mittels *in vivo* Biolumineszenzbildgebung, Histologie und körperlicher Untersuchung der Mäuse beurteilt.

Anti-TRP2-TCR-T-Zellen lokalisierten sich konsistent an den Tumoren und persistierten *in vivo*. Sie eliminierten TRP2-überexprimierende Tumoren in beiden Modellen vollständig, verzögerten jedoch lediglich das Wachstum natürlich TRP2-exprimierender Tumoren. Untersuchungen zur Sicherheit der Therapie zeigten weder systemische Toxizität noch relevante on-Target, off-Tumor-Effekte. Im syngenem Modell trat eine lokalisierte, vitiligoartige Depigmentierung auf, ohne Hinweise auf Schäden innerer Organe.

Diese Ergebnisse belegen die Sicherheit und anti-tumorale Aktivität des HLA-unabhängigen TRP2-spezifischen TCRs, weisen jedoch zugleich auf eine begrenzte Wirksamkeit gegenüber natürlich TRP2-exprimierenden Tumoren hin, vermutlich bedingt durch entzündungsinduzierte Dedifferenzierung und Antigen-Downregulation. *In vitro* konnte der Rezeptor-Tyrosin-Kinase-Inhibitor Tivozanib die TRP2-Expression unter TNF- α - oder IFN- γ -induziertem Stress stabilisieren. Zukünftige Studien sollten untersuchen, ob die Kombination von TCR-Therapie mit Tivozanib oder anderen Wirkstoffen, die entzündungsbedingte Dedifferenzierung rückgängig machen können, die Kontrolle natürlich TRP2-exprimierender Tumoren *in vivo* verbessert.

Zusammenfassend zeigte diese Dissertation eine *in vivo*-antitumorale Aktivität sowie ein offenbar günstiges Sicherheitsprofil eines HLA-unabhängigen, TRP2-spezifischen TCR. Während Tumoren mit TRP2-Überexpression wirksam eliminiert wurden, war die Wirksamkeit gegenüber natürlich TRP2-exprimierenden Tumoren begrenzt. Darüber hinaus verdeutlichen die Ergebnisse, wie HLA-unabhängige TCRs die klassische Antigenpräsentation umgehen und zur Entwicklung sicherer und vielseitigerer TCR-Therapien für solide Tumoren beitragen können.

1 Introduction

1.1 T-cell immunity

The immune system protects the body from infections and malignant transformation through coordinated cellular and humoral mechanisms. It consists of the innate immune system, which delivers rapid but nonspecific responses, and the adaptive immune system [1]. Central to adaptive immunity are T cells, specialized lymphocytes that recognize and respond to antigens via the T-cell receptor (TCR) [2]. The TCR interacts with peptide antigens presented by major histocompatibility complex (MHC) molecules, called Human Leukocyte Antigen (HLA) in humans, on antigen-presenting cells. A typical TCR consists of two protein chains linked by disulfide bonds. The most common type, the alpha-beta ($\alpha\beta$) TCR, is made up of one α and one β chain. Each chain has a variable (V) region, which binds antigens, and a constant (C) region. Together, the variable regions form a unique antigen-binding site, allowing T cells to recognize diverse antigens. A less common type, the gamma-delta ($\gamma\delta$) TCR, consists of one γ and one δ chain [3]. While structurally similar to $\alpha\beta$ TCRs, $\gamma\delta$ TCRs often recognize non-peptide antigens independently of HLA presentation [4].

The Cluster of Differentiation (CD) is a classification system used to identify and distinguish different types of immune cells based on specific cell surface proteins they express. CD3 is a key marker found on all T cells, making it a defining characteristic for identifying T lymphocytes. It is a protein complex associated with the TCR and plays a critical role in T cell activation and signaling [5]. CD4⁺ and CD8⁺ T cells are the two main subsets of T lymphocytes, each with distinct immune functions. CD4⁺ T cells, also known as helper T cells or Th cells, coordinate and regulate immune responses. Upon antigen recognition, they activate and differentiate into subsets such as Th1, Th2, Th17, and Treg, each with specific roles. They secrete cytokines like IL-2, IFN- γ , IL-4, IL-10, and TGF- β , that modulate the activity of immune cells, including macrophages, B cells, and CD8⁺ T cells [6]. CD8⁺ T cells, also known as cytotoxic T Lymphocytes (CTLs), specialize in killing infected or cancerous cells. After recognizing antigens presented by MHC class I molecules, they activate, proliferate, and differentiate into cytotoxic effector cells. They secrete cytokines such as IFN- γ and TNF- α and use perforin and granzymes to induce apoptosis in target cells [7].

1.1.1 T-cell development

T cells originate from bone-marrow precursors that migrate to the thymus, where they mature. Immature T cells, known as thymocytes, progress through distinct stages to develop a functional T-cell receptor. At the double-negative (DN) stage, thymocytes lack CD4 and CD8 co-receptors, and the TCR β -chain undergoes rearrangement. Successful β -chain rearrangement leads to the formation of a pre-TCR with a surrogate α -chain. Thymocytes then express both CD4 and CD8 co-receptors, entering the double-positive (DP) stage, where the TCR α -chain rearranges, forming a complete $\alpha\beta$ TCR [1]. Functional TCRs are tested through two selection processes. Positive selection ensures T cells recognize self-MHC molecules with moderate affinity, while negative selection eliminates T cells that bind too strongly to self-peptides, preventing autoimmune responses. Thymocytes that fail either test undergo apoptosis. Surviving cells differentiate into either CD4⁺ helper T cells or CD8⁺ cytotoxic T cells, depending on whether they interact with MHC class II or MHC class I molecules, respectively. These processes ensure that mature T cells are both functional and self-tolerant [8].

The diversity of TCRs arises from a complex gene rearrangement process called V(D)J recombination. This mechanism involves the random combination of gene segments to create a unique TCR for each T cell. TCR genes are organized into loci encoding the α and β chains (for $\alpha\beta$ TCR) or γ and δ chains (for $\gamma\delta$ TCR). Each locus contains multiple segments: the V (variable), D (diversity) only in the β and δ chains, J (joining) and C (constant) segments. For the α chain, one V segment and one J segment are randomly selected and joined together, followed by the addition of a C segment. For the β chain, one V, one D, and one J segment are randomly combined, followed by the addition of a C segment [3]. This recombination is mediated by the RAG1 and RAG2 (Recombination Activating Genes) enzymes, which recognize specific recombination signal sequences flanking each segment. They bring these segments together, cleave the DNA, and join them to form a continuous coding sequence [9]. Additional diversity is introduced at the junctions of the V, D, and J segments through the addition or deletion of nucleotides by terminal deoxynucleotidyl transferase (TdT). This results in the formation of unique complementary-determining regions (CDRs), which are the specific parts of the TCR that interact with antigens. The combination of different V, D, and J segments, along with the pairing of α and β

chains, creates an immense repertoire of possible TCRs. This combinatorial and junctional diversity enables the immune system to recognize a vast array of antigens [10]. The process of V(D)J rearrangement and formation of a $\alpha\beta$ TCR is illustrated in Figure 1.

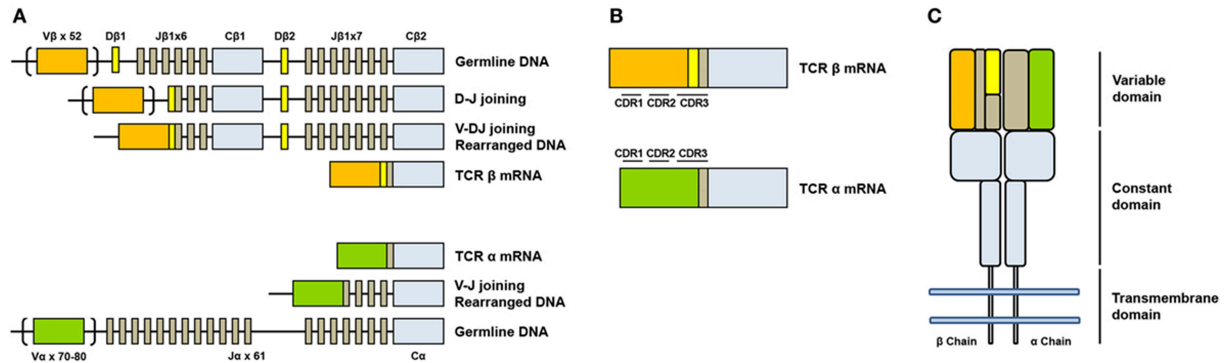


Figure 1: Somatic V(D)J rearrangement in TCR alpha and beta chains.

(A) The genomic layout and somatic recombination process of the TCRB and TCRA loci ensure antigen receptor diversity. This is achieved through sequential rearrangement of V, D, and J segments for TCR beta chains, and V and J segments for TCR alpha chains. The diversity generated through these rearrangements, known as combinatorial diversity, is further enhanced by the addition or deletion of nucleotides at the junction sites, contributing to junctional diversity. (B) Successful rearrangements produce functional transcripts for both TCR beta and alpha chains. (C) The T-cell receptor (TCR) consists of two main subunits: TCR alpha and TCR beta. Each subunit contains a constant region and a variable region, with the latter playing a crucial role in recognizing antigens. Adapted from De Simone et al., 2018 [11].

1.1.2 Antigen recognition and presentation

T-cell antigens are typically processed oligopeptides presented via MHC class I or MHC class II molecules. Classical MHC class I molecules are heterodimers composed of a polymorphic heavy chain and the invariant light chain β 2-microglobulin (B2M). The heavy chain contains three extracellular domains (α 1, α 2, α 3) and a transmembrane and cytoplasmic region. Its membrane-distal domains (α 1 and α 2) form a narrow peptide-binding groove that binds 8–10 amino acid peptides, while the membrane-proximal domain serves as the interaction site for the CD8 co-receptor on cytotoxic T cells. These molecules present endogenous peptides, usually derived from intracellular proteins degraded by the proteasome and transported into the endoplasmic reticulum (ER) via the TAP transporter. In the ER, peptides bind to MHC class I molecules, and the peptide-MHC complex is transported to the cell surface. Because MHC class I is expressed on nearly all nucleated cells, CD8⁺ T cells can monitor them for viral infections or cancer-related abnormalities [12], [13], [14].

In contrast, MHC class II molecules consist of non-identical α and β chains that together form an open-ended peptide-binding groove. This allows the presentation of longer, typically 12–25 amino acid peptides derived from exogenous proteins that have been internalized and processed within endosomal compartments of professional antigen-presenting cells (APCs) such as dendritic cells, macrophages, and B cells. The CD4 co-receptor primarily contacts the membrane-proximal domains, stabilizing the interaction between the TCR and peptide–MHC class II complexes. Newly synthesized MHC class II molecules are transported to endosomal compartments, where the invariant chain (Ii) is removed, allowing peptides to bind. The peptide–MHC class II complex is then transported to the cell surface for recognition by CD4⁺ T cells [12], [13], [14]. [Figure 2](#) shows an overview of the MHC class I and Class II antigen presentation pathways.

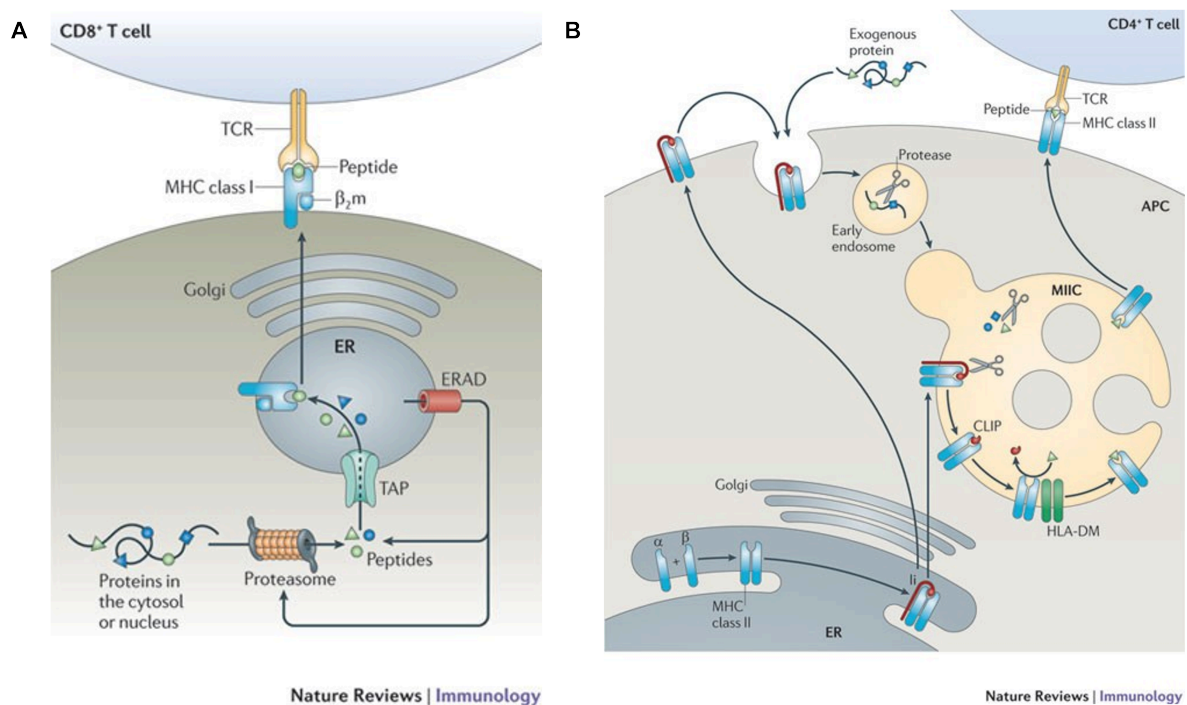


Figure 2: Overview of MHC class I and class II antigen presentation pathways.

Intracellular and extracellular antigens are processed and presented to CD8⁺ and CD4⁺ T cells, respectively, via distinct pathways. (A) In the MHC class I pathway, intracellular antigens are degraded by the proteasome, and peptides are transported into the endoplasmic reticulum (ER) by the transporter associated with antigen presentation (TAP). These peptides are loaded onto MHC class I molecules, which are then transported through the Golgi to the cell surface for recognition by CD8⁺ T cells. (B) Conversely, in the MHC class II pathway, MHC class II molecules associate with the invariant chain (Ii) in the ER and are transported to the MHC class II compartment (MIIC). Here, Ii is degraded, leaving the class II-associated Ii peptide (CLIP) in the peptide-binding groove. HLA-DM facilitates the exchange of CLIP for antigenic peptides derived from endocytosed extracellular proteins. The peptide–MHC class II complexes are then delivered to the plasma membrane for presentation to CD4⁺ T cells. Both pathways are essential for initiating adaptive immune responses. Adapted from Neefjes et al., 2011 [15].

In *Homo sapiens*, HLA molecules exhibit three key genetic properties: polymorphism, polyallelism, and polygeny, which collectively enhance immune diversity. Polymorphism refers to the vast variety of HLA gene variants (alleles) across the population, making them some of the most diverse genes in the human genome. This variation in peptide-binding grooves allows HLA molecules to present a wide range of pathogen-derived peptides to T cells, strengthening population-level immunity. Polyallelism emphasizes the extensive number of alleles at each HLA gene locus, leading to unique haplotype combinations in individuals. With two alleles inherited per gene, each person expresses a diverse set of HLA molecules, improving the immune system's ability to recognize and respond to various antigens. Polygeny refers to the presence of multiple HLA genes within an individual, specifically HLA-A, -B, and -C for Class I molecules and HLA-DR, -DP, and -DQ for Class II molecules. Each gene produces distinct HLA proteins, allowing for the presentation of a broader array of peptides and increasing responsiveness to diverse pathogens. In addition to these classical HLA molecules, there are non-classical HLA molecules such as HLA-E, -F, and -G (Class I) and HLA-DM and -DO (Class II). These non-classical molecules play roles in immune regulation, tolerance, and stress responses. Unlike classical HLA molecules, non-classical ones exhibit limited polymorphism and are often inducibly expressed or restricted to specific cell types. Together, these genetic properties enable the immune system to recognize a wide variety of antigens, ensuring both individual and population-level immune resilience [12], [16], [17], [18].

Two additional notable types of receptors that present antigens to T cells are MR1 molecules and CD1 molecules. MR1 (MHC class I-related protein) presents small metabolite antigens derived from bacterial vitamin B2 (riboflavin) biosynthesis pathways. These metabolites are commonly produced by certain bacteria and yeast and are presented to Mucosal-Associated Invariant T (MAIT) cells. MAIT cells are essential for mucosal immunity in tissues such as the lungs, intestines, and liver, and are particularly effective in responding to bacterial and fungal infections [19]. In contrast, CD1 molecules (CD1a, CD1b, CD1c, and CD1d) specialize in presenting lipid-based antigens rather than peptide antigens. These lipids are often derived from bacterial cell walls or self-lipids. CD1 molecules present these antigens to Natural Killer T (NKT) cells, a unique subset of T cells that express a semi-invariant T-cell receptor and share features with both T cells and NK cells. NKT cells act as a bridge between innate and adaptive immunity, enabling rapid immune responses to lipid-based antigens [20], [21].

Natural killer (NK) cells are cytotoxic lymphocytes of the innate immune system that can eliminate virus-infected and malignant cells in the absence of prior antigen priming. Rather than using a clonally rearranged antigen receptor, NK cells integrate signals from inhibitory receptors that sense self MHC class I molecules and activating receptors that recognize stress-induced ligands. Inhibitory receptors, such as KIRs (Killer-cell Immunoglobulin-like Receptors) and NKG2A/CD94, recognize MHC class I molecules on healthy cells, signaling NK cells to avoid attacking. However, many viruses and tumors evade immune detection by downregulating MHC class I, which NK cells recognize as a "missing-self" signal, triggering cytotoxicity. Activating receptors, like NKG2D, detect stress-induced ligands such as MIC (MHC class I Chain-Related Molecules) and ULBP (UL16 Binding Proteins), prompting NK cell activation. Additionally, CD16 (Fc γ RIII) enables NK cells to mediate antibody-dependent cellular cytotoxicity (ADCC) by binding to antibody-coated target cells. NK cells primarily eliminate targets through the release of perforin and granzymes to induce cell death and secrete cytokines like IFN- γ to modulate immune responses [22], [23].

1.2 Malignant melanoma

Cutaneous malignant melanoma is the most aggressive and deadliest form of skin cancer originating from melanocytes, the pigment-producing cells in the skin. If not detected and treated early, it can metastasize to other parts of the body. In 2022, approximately 25,000 new cases were diagnosed in Germany, resulting in around 3,000 deaths from this disease [24].

Malignant melanoma risk factors include excessive UV exposure from the sun or tanning devices, as well as fair skin, light hair, light eyes, and a high number of moles or atypical moles. A family history of melanoma further increases susceptibility. Preventive measures include using sunscreen, wearing protective clothing, and avoiding peak sun exposure hours [25]. Treatment options for metastatic melanoma include surgical metastasectomy, radiotherapy, photodynamic therapy, immunotherapy, targeted therapy, and chemotherapy. Dacarbazine, approved in 1974, remains the standard chemotherapy drug but offers a 5-year survival rate of only 2–6%. In contrast, newer immunotherapies, including cytokine therapy, immune checkpoint inhibitors, and TIL (tumor-infiltrating lymphocyte) therapy, have shown promising results, particularly in advanced melanoma cases [26], [27].

1.2.1 Immune evasion strategies

Tumors use various strategies to evade immune surveillance, including downregulating tumor-associated antigens, upregulating immune checkpoint molecules like programmed death-ligand 1 (PD-L1), and modulating the tumor microenvironment to suppress immune responses, enabling their growth and metastasis. A key immune escape mechanism is the loss or downregulation of MHC molecules, which reduces recognition by cytotoxic T lymphocytes. This can result from genetic mutations in MHC-related genes (e.g., B2M, TAP1, TAP2), epigenetic silencing of antigen-processing machinery, or immunosuppressive cytokines such as TGF- β . Tumors may also selectively downregulate specific MHC alleles, reducing T-cell recognition while retaining others to prevent NK cell activation. This mechanism is observed in cancers such as melanoma, non-small cell lung cancer, and HPV-associated cervical cancer. Therapeutic strategies to counteract this include using IFN- γ to upregulate MHC expression, epigenetic drugs to reactivate silenced MHC genes, and NK cell-based therapies to target MHC-deficient tumors. Restoring or bypassing MHC expression is viewed as critical for improving the effectiveness of cancer immunotherapies [28].

1.2.2 Malignant melanoma model Ma-Mel-86

The group of [REDACTED] and [REDACTED] (Department of Dermatology, University Hospital Essen) established a human melanoma model derived from patient Ma-Mel-86, who had stage IV malignant melanoma. From lymph node metastases excised at different time points during disease progression, four stable tumor cell lines were generated: Ma-Mel-86a, -86b, -86c, and -86f. These lines differ in antigen expression, particularly with respect to HLA class I molecules and melanosomal differentiation antigens. Ma-Mel-86a expresses all HLA class I molecules but lacks melanosomal differentiation antigens such as tyrosinase, gp100, and TRP2 due to a stable dedifferentiated c-Jun^{high}/MITF^{low} phenotype [29]. In contrast, Ma-Mel-86b and Ma-Mel-86f are HLA class I-deficient as a result of biallelic mutations in the B2M gene. These lines express melanosomal differentiation antigens. Ma-Mel-86c shows a partial loss of HLA class I haplotype and retains melanosomal differentiation antigens [30], [31]. [Figure 3](#) presents an overview of the disease progression, indicating time points for collecting metastatic tumor tissue and PBMCs from this patient.

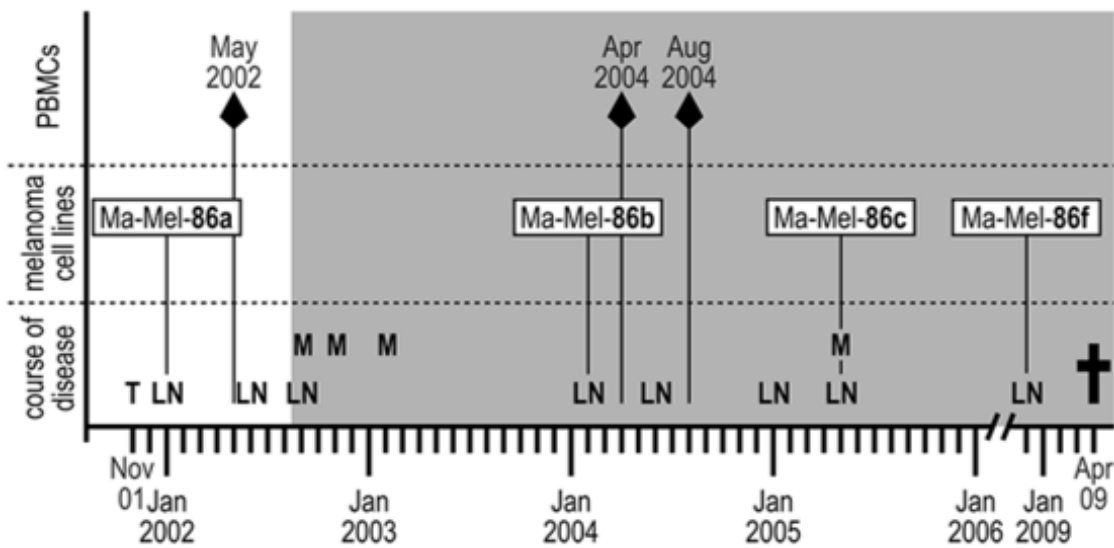


Figure 3: The melanoma model Ma-Mel-86.

The Ma-Mel-86a, -86b, -86c, and -86f melanoma cell lines were derived from four distinct lymph node (LN) metastases surgically excised from patient Ma-Mel-86 at different time points. Peripheral blood mononuclear cells (PBMCs) from the same patient were collected in May 2002, April 2004, and August 2004. Despite presenting with advanced-stage disease, including metastases (M) in the brain, breast, and intestines, the patient survived nearly eight years following the initial diagnosis of the primary tumor (T). Stage IV disease is represented in gray. Adapted from Schrörs et al., 2017 [31].

The anti-tumoral T-cell response in the Ma-Mel-86 melanoma model was then analyzed by stimulating PBMCs, isolated from the patient's blood in 2004, with the established tumor cell lines in autologous mixed lymphocyte-tumor cell cultures (MLTCs). This approach revealed several HLA-restricted, melanoma-specific T-cell responses, including recognition of the melanosomal differentiation antigens gp100 and TRP2. In addition, HLA-independent $\alpha\beta$ T cells were identified in the patient's blood. These HLA-independent CD8⁺ T cells did not express NK cell differentiation markers, such as CD16 and CD56. Their antigen recognition could be blocked by anti-CD3 antibodies but not by antibodies against HLA class I or class II. The T cells were capable of recognizing and lysing the HLA class I-negative melanoma cell line Ma-Mel-86b. Notably, two distinct $\alpha\beta$ TCRs from T cell clones 2C/417 and 11C/171 were identified that specifically target TRP2. However, only one of them (2C/417) also recognized the murine TRP2 homolog [32], [33]; this TCR was therefore selected for the animal experiments described in this dissertation.

Further investigations into TRP2-specific HLA-independent $\alpha\beta$ T-cell responses were conducted by [REDACTED] and [REDACTED] using blood samples from other patients with metastatic melanoma and umbilical cord blood donations. Isolated T cells from these blood samples were stimulated with HLA class I and class

II-negative tumor cell lines. These studies identified HLA-independent, TRP2-reactive T cells in three additional melanoma patients and in one umbilical cord blood donation. TRP2 was expressed as intact surface molecule, and its recognition by these TCRs was independent of HLA presentation [34], [35].

In the same Ma-Mel-86 melanoma model, HLA-independent TCRs have been identified that recognize the tumor-associated antigens colony stimulating factor 2 receptor alpha (CSF2RA) and PD-L1. These HLA-independent T-cell receptors offer significant potential for adoptive T-cell therapy in cancer immunotherapy. By isolating autologous T cells, equipping them with HLA-independent, TAA-reactive TCRs, and reinfusing them into the patient, the need for HLA matching can be bypassed. Unlike HLA-restricted therapies, which are limited by HLA allele diversity, HLA-independent TCRs present a universal strategy for targeting tumors across diverse patient populations.

1.2.3 The melanoma-associated antigen tyrosinase-related protein 2

Tyrosinase-related protein 2 (TRP2), also known as dopachrome tautomerase (DCT) or TYRP2, is a melanocyte differentiation antigen involved in melanin biosynthesis and has additionally been reported to exhibit anti-apoptotic properties [36]. As a type II membrane protein regulated by the microphthalmia-associated transcription factor (MITF) [37], TRP2 is primarily expressed in melanocytes and melanoma cells, making it an attractive target for immunotherapy [38], [39], [40], [41]. Strobel et al. reported TRP2 overexpression in 50% of melanoma tissues, highlighting its potential as an immunogenic target [42].

TRP2 in human and mouse is a ~519-residue, melanosome-associated glycoprotein that exhibits 83% sequence identity and 90% similarity between the two species. It shares the defining structural architecture of the tyrosinase family: an N-terminal signal peptide for secretory targeting, a large intramelanosomal domain with 15 conserved cysteine residues (forming multiple disulfide bonds), and a single transmembrane helix near the C-terminus anchoring the protein to the membrane [43]. A representative 3D structure is shown in [Figure 4](#). According to GeneCards, the intramelanosomal domain is extensively N-glycosylated (at least six Asn sites) [44]. Notably, TRP2 binds Zn^{2+} ions in its active site (rather than the Cu^{2+} found in tyrosinase) [45]. By homology, mouse and human TRP2 are expected to share their overall fold: a monomeric, disulfide-stabilized metalloprotein with a binuclear Zn(II) active site and an EGF-like cysteine-rich subdomain [43].

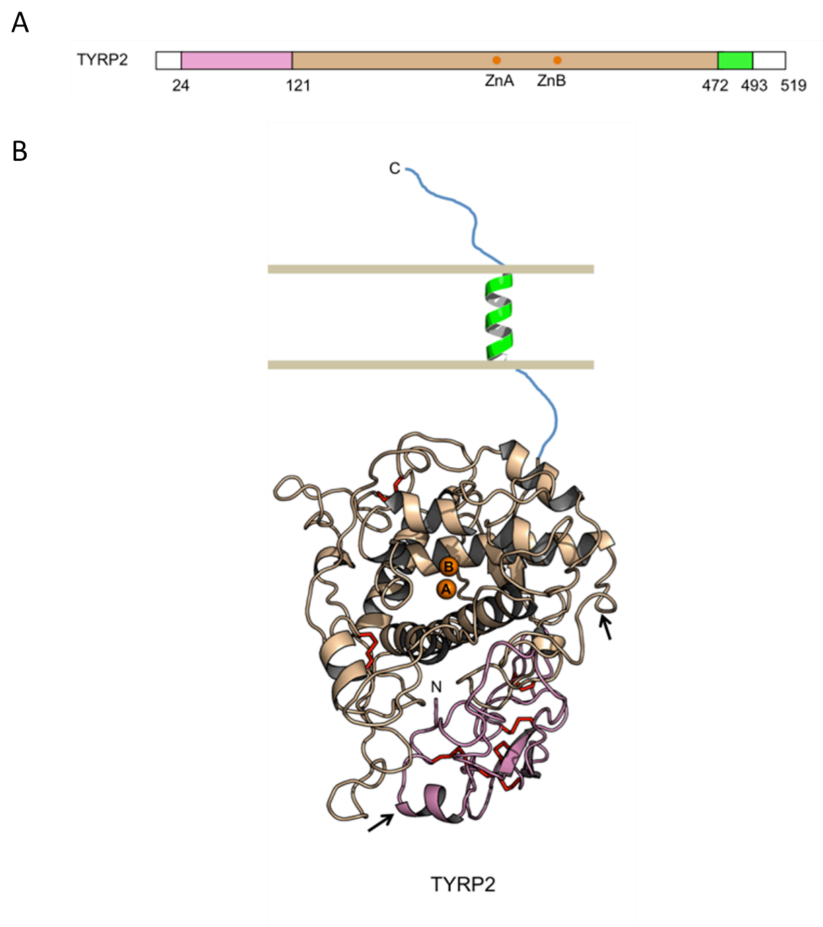


Figure 4: Structural organization of human TRP2 (TYRP2).

(A) Schematic representation of TRP2 (TYRP2) showing the N-terminal signal peptide (pink), the cysteine-rich and tyrosinase-like domain (beige), the single transmembrane helix (green), and the C-terminal cytosolic tail. The tyrosinase-like region contains the binuclear metal-binding site, while zinc ions are indicated as colored spheres (orange). (B) The structure is based on a homology model derived from the TYRP1 crystal structure. Adapted from Lai et al., 2018 [46].

Because the TCR evaluated in this study recognizes TRP2 in an HLA-independent manner, structural motifs and post-translational modifications such as glycosylation could influence antigen recognition. Although the exact mechanism by which the HLA-independent anti-TRP2 TCR recognizes its target has yet to be defined, preliminary studies by the Wölfel group (personal communication, [REDACTED]) provide important insights. Using site-directed mutagenesis of individual amino acids and exon deletion constructs of the TRP2 sequence, followed by IFN- γ ELISpot assays, they demonstrated that both zinc-binding sites are essential for TCR recognition. Furthermore, exon 2 was identified as critical for antigen recognition, as its deletion completely abrogated T-cell reactivity.

1.3 Adoptive cell therapy

Adoptive cell therapy (ACT) refers to treatment strategies in which autologous or allogeneic immune cells are expanded and often genetically modified *ex vivo* and subsequently reinfused into the patient to mediate tumor rejection. Most current clinical approaches employ T cells, which can be enriched from tumor tissue or peripheral blood and equipped with defined receptors to enhance their specificity and effector function [47]. [Figure 5](#) provides an overview of the three main ACT strategies currently being explored and used in the clinic: tumor-infiltrating lymphocyte (TIL) therapy, TCR-T-cell therapy, and chimeric antigen receptor (CAR)-T-cell therapy.

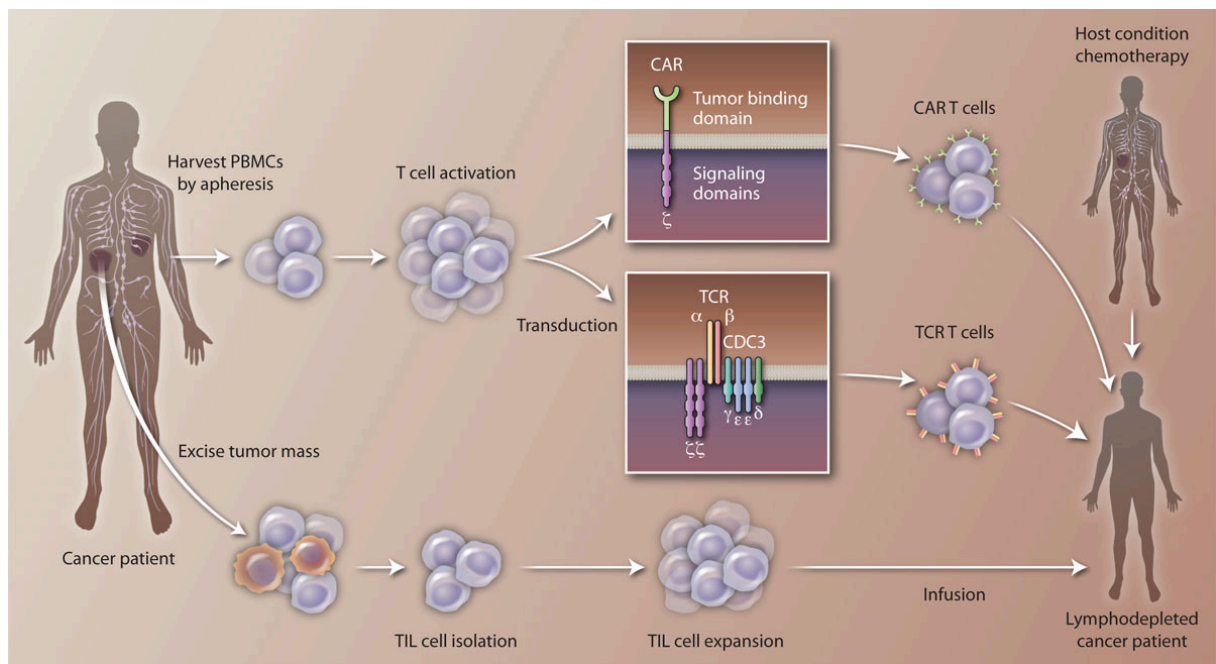


Figure 5: Overview of adoptive cell therapy approaches.

Adoptive cell therapy encompasses three main strategies: TILs, TCR-engineered T cells, and CAR-engineered T cells. TILs are isolated, enriched, and expanded from surgically resected tumor biopsies. In contrast, TCR- and CAR-engineered T cells are generated from peripheral blood lymphocytes by introducing specific receptors via viral or non-viral gene transfer methods. Prior to cell infusion, patients typically undergo a lymphodepleting chemotherapy regimen to optimize therapeutic efficacy. Adapted from June et al., 2015 [48].

1.3.1 Chimeric antigen receptors

Chimeric antigen receptor (CAR) technology enables T cells to recognize surface antigens in an HLA-independent manner and has markedly expanded the therapeutic options for patients with B-cell malignancies [49]. First conceptualized by Zelig Eshhar as "T-bodies", CARs combine the antigen specificity of antibodies with T-cell effector

functions, bypassing the need for MHC presentation [50]. Michel Sadelain advanced the field with methods for T-cell transduction, enabling stable CAR integration and large-scale T-cell production [51]. Carl June further refined this technology, leading to the development of the CD19-directed genetically modified autologous T-cell immunotherapy tisagenlecleucel, the first CAR T-cell therapy approved in Germany [52], [53]. Figure 6 shows the structure of a second-generation CAR compared to the canonical TCR.

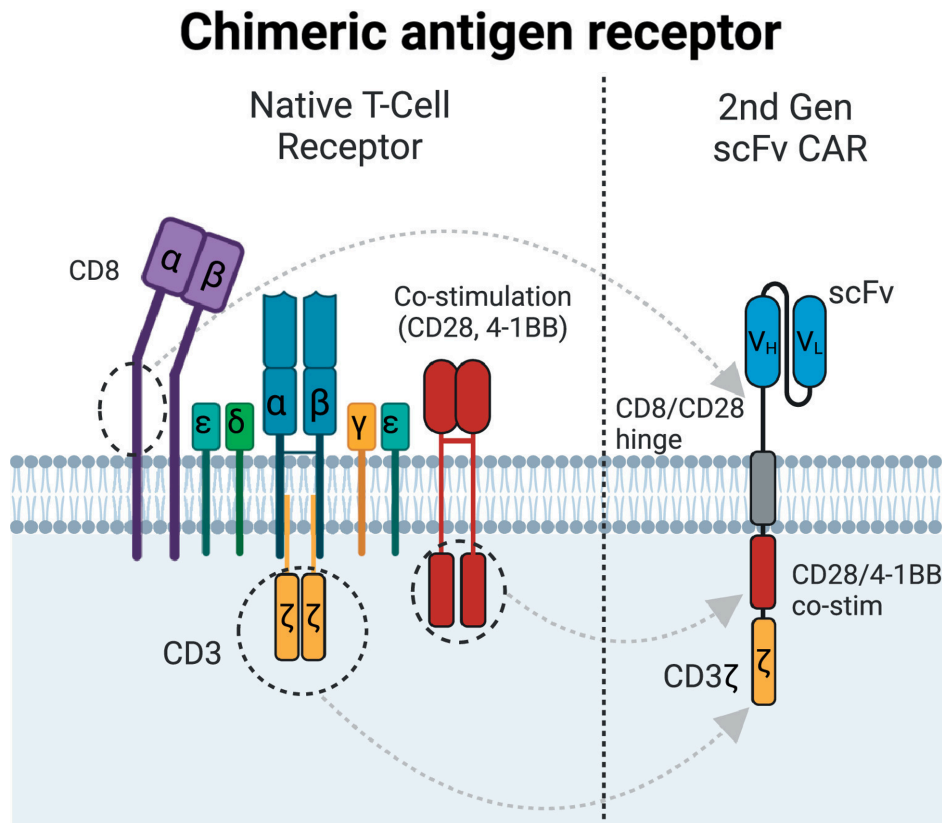


Figure 6: Structure of second-generation CARs compared to the canonical T-cell receptor.

Second-generation CARs (right panel) are engineered artificial single-chain receptors combining distinct protein domains derived from natural components of the T-cell receptor (left panel). These CARs typically consist of an antibody-derived single-chain variable fragment (scFv) for antigen binding (blue), a hinge and transmembrane domain (commonly from CD8 or CD28, shown in black), an intracellular co-stimulatory domain (from CD28 or 4-1BB, shown in red), and a CD3-zeta (CD3ζ) signaling domain (gold). Adapted from Nix and Wiita, 2024 [54].

The European Commission granted tisagenlecleucels approval on August 27, 2018, for the treatment of relapsed/refractory B-cell acute lymphoblastic leukemia (ALL). It is now also approved for the treatment of relapsed/refractory diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma (FL) [55]. Building on the success of tisagenlecleucel, additional CAR T-cell therapies targeting CD19 for the treatment of mantle cell lymphoma and chronic lymphocytic leukemia (CLL), as well as ther-

apies targeting B-cell maturation antigen (BCMA) for the treatment of multiple myeloma, have been approved [56]. CAR T-cell therapy has been highly successful in treating hematological malignancies, largely due to the high accessibility of malignant cells and the consistent, nearly exclusive expression of target antigens. Despite these advancements, CAR T-cell therapy has failed to show efficacy in treating solid tumors, primarily due to obstacles such as the immunosuppressive tumor microenvironment, a dense extracellular matrix, and poor vascularization, all of which prevent CAR T-cells from effectively infiltrating the tumor and reaching their targets [57].

1.3.2 Tumor-infiltrating lymphocytes

One approach to overcome these limitations is tumor-infiltrating lymphocyte (TIL) therapy, first described by Steven Rosenberg in 1988 [58]. Here, TILs are isolated from a patient's resected tumor, expanded in large numbers in a laboratory setting, and then reintroduced into the patient's body to target and destroy cancer cells. In February 2024, the U.S. Food and Drug Administration (FDA) granted accelerated approval to lifileucel, the first TIL therapy approved for solid tumors. This decision was based on clinical trials showing a 31.4% objective response rate in patients with advanced melanoma resistant to prior treatments. The success of TIL therapy in melanoma has encouraged research into its potential applications for other solid tumors, including ovarian, lung, and pancreatic cancers [59]. TIL therapy typically involves a diverse T-cell population capable of targeting multiple tumor antigens, including mutated tumor-specific antigens, neoantigens, and overexpressed self-antigens. However, extensive *in vitro* stimulation during the expansion process often leads to T-cell exhaustion, marked by the expression of inhibitory receptors such as PD-1, TIM-3, and LAG-3. Despite this exhaustion, TILs generally retain cytotoxic capabilities and exhibit a mix of effector and central/effector memory phenotypes. The expanded TIL population remains heterogeneous, containing both highly active anti-tumor cells and others with reduced functionality [60], [61]. TIL therapy utilizes the patient's natural anti-tumor T cells, that can infiltrate tumors without requiring genetic engineering. However, variability in TIL quantity and functionality exists, as some patients have low levels of TILs or a weakened immune system. These differences significantly impact the effectiveness of the therapy and overall treatment outcomes [62].

1.3.3 T-cell receptors

Alongside CAR and TIL therapies, TCR-T-cell therapy offers distinct advantages. In contrast to CAR-T, canonical TCR-T cells can recognize intracellular antigens presented on MHC molecules and exhibit higher sensitivity to low antigen expression compared to CAR-T cells, making them effective in solid tumor environments with variable antigen density [63], [64]. Unlike TIL therapy, TCR-T therapy allows for the precise engineering of T-cell receptors, enhancing specificity and affinity for tumor antigens and addressing the variability often seen in TIL-derived cells [47]. On August 2, 2024, the U.S. Food and Drug Administration (FDA) approved its first TCR-engineered T-cell therapy, afamitresgene autoleucel, for the treatment of metastatic or unresectable synovial sarcoma. Afamitresgene autoleucel is an autologous T-cell therapy where T cells from a patient's peripheral blood are collected and genetically engineered using a lentiviral vector to express an affinity-enhanced TCR. This TCR specifically targets a fragment of melanoma-associated antigen A4 (MAGE-A4) presented by HLA-A*02 [65], [66]. The MAGE gene family represents a group of cancer-germline antigens primarily expressed in germ cells of the testis and various malignant solid tumors but not in other normal adult tissues. This selective expression profile makes MAGE and other cancer-germline antigens highly attractive targets for cancer immunotherapy [67].

1.3.4 T-cell activation, exhaustion, and memory differentiation markers

In adoptive T-cell therapy, the functional state, activation profile, and differentiation status of transferred T cells critically influence therapeutic efficacy and persistence [68]. Upon antigen encounter, T cells undergo activation driven by TCR signaling strength, co-stimulation, and cytokine exposure, accompanied by metabolic reprogramming [69]. Early activation markers include CD69 [70], and CD25 (IL-2R α), enabling responsiveness to IL-2-mediated STAT5 signaling and proliferation. Sustained antigen stimulation can lead to expression of inhibitory receptors such as PD-1, CTLA-4, and TIGIT, which mark the onset of an exhaustion program associated with diminished effector function and altered metabolic fitness [71], [72].

T-cell differentiation into effector and memory subsets is shaped by antigen exposure, cytokines (e.g., IL-7 and IL-15), and transcriptional regulators. In murine T cells, memory phenotypes are commonly defined by CD44, CD62L, and IL-7R α (CD127) expression, distinguishing naive (CD44⁻CD62L⁺), central memory (TCM;

CD44⁺CD62L⁺), effector memory (TEM; CD44⁺CD62L⁻), and terminal effector subsets [73], [74]. In human T cells, analogous subsets are identified by CD45RA, CD45R0, CCR7, CD62L, CD27, CD28, IL-7R α , CD57 and CD95 expression (e.g., naive: CD45RA⁺ CCR7⁺ CD27⁺ CD28⁺ CD95⁻ CD62L⁺; TCM: CD45RA⁻ CCR7⁺ CD27⁺ CD28⁺ CD62L⁺ CD95⁺; TEM: CD45RA⁻ CCR7⁻ CD62L⁻ CD57⁺) [75], [76]. These phenotypes have practical relevance for cell therapy: less-differentiated and central-memory-biased T cells exhibit enhanced proliferative capacity and long-term persistence, whereas terminal effector or exhausted populations display potent early cytotoxicity but limited durability [77].

Because the functional state of transferred T cells influences anti-tumor efficacy, persistence, and potential toxicities, this work includes immunophenotyping of engineered murine T cells to assess activation (CD25, CD69), inhibitory receptor expression (PD-1, CTLA-4, TIGIT), and differentiation status (CD44/CD62L/IL-7R α). These analyses enabled evaluation of the T-cell phenotype of the transferred T-cell product prior to infusion and at the experimental endpoint in the syngeneic model.

1.3.5 Adverse effects of adoptive cell therapy

Adoptive cell therapy with engineered T-cell receptors (TCR-T) has emerged as a promising cancer immunotherapy, showing success across multiple malignancies in clinical trials [78]. However, the clinical application of TCR-T is still limited by potentially severe adverse effects. These can be broadly grouped into four categories: (i) systemic immune activation toxicities, such as cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS); (ii) off-target toxicities, resulting from cross-reactivity with unrelated peptides; (iii) on-target/off-tumor toxicities, caused by recognition of the intended antigen on healthy tissues; and (iv) therapy-related risks, including secondary T-cell malignancies and toxicities from conditioning regimens.

One common complication is cytokine release syndrome (CRS), a systemic inflammatory response caused by the rapid release of cytokines from activated T cells. While CRS is most widely recognized as a major side effect of CAR-T therapy, it has also been observed in patients treated with TCR-T. Symptoms range from mild flu-like signs to severe conditions such as high fever, hypotension, and multi-organ dysfunction [79]. Another major risk is immune effector cell-associated neurotoxicity syndrome (ICANS), which can manifest as encephalopathy, seizures, confusion, and aphasia. ICANS is a known complication of T-cell-based immunotherapies, most prominently CAR-T cell therapy, and typically occurs early after cell infusion, often in temporal association with cytokine release syndrome. It is thought to result from systemic

inflammation, endothelial activation, and transient disruption of the blood–brain barrier. Clinical symptoms range from mild cognitive impairment and dysgraphia to seizures and severe encephalopathy, while most cases are reversible with supportive care and corticosteroid treatment [80], [81]. Together with CRS, ICANS belongs to systemic immune activation toxicities, which differ mechanistically from toxicities caused by antigen cross-reactivity.

Severe toxicities can arise from unintended cross-reactivity of engineered TCRs. In one clinical trial targeting the MAGE-A3 peptide 112–120 (KVAELVHFL) presented by HLA-A*02, TCR cross-recognition of the related antigens MAGE-A9 and MAGE-A12, which are expressed in the brain, resulted in fatal neurotoxicity [49], [82]. In another trial, a TCR directed against a different MAGE-A3 epitope (EVDPIGHLY) presented on HLA-A*01 caused lethal cardiac toxicity due to off-target recognition of a structurally similar peptide from the muscle protein titin (ESDPIVAQY) [83].

In addition to off-target effects, another major risk arises when the intended antigen itself is expressed on normal tissues. This so-called on-target/off-tumor toxicity occurs when TCR-T cells attack healthy cells that share the targeted antigen, leading to tissue damage and severe side effects [84]. A well-documented example is a clinical trial targeting MART-1 (Melanoma Antigen Recognized by T cells 1). Patients receiving high-avidity TCRs specific for HLA-A*02:01-restricted MART-1 peptide 26-35 experienced toxicities such as dermatitis, uveitis, and hearing impairment due to MART-1 expression in healthy melanocytes [85]. Similar on-target/off-tumor effects are well recognized in CAR-T therapy. For example, anti-CD19 CAR-T cells invariably deplete healthy B cells along with malignant ones [86]. In contrast to TCR-T, however, B-cell depletion is clinically manageable through immunoglobulin replacement [87], underscoring that the impact of on-target/off-tumor reactivity depends strongly on the physiological role of the antigen.

Another emerging concern is the potential risk of secondary T-cell malignancies following genetically modified T-cell therapy. Although rare, regulatory agencies now mandate lifelong monitoring of treated patients. These malignancies may arise if viral vectors used to introduce TCR or CAR constructs integrate into the host genome at sites that disrupt normal gene function, potentially driving malignant transformation. Additionally, selective expansion of T-cell clones with growth advantages or genomic instability from rapid proliferation could contribute to this risk. However, direct evidence linking transgenic modifications to secondary malignancies remains scarce [88]. In most reported CAR-T therapy cases, no signs of oncogenic retroviral integration were detected. Instead, factors such as prior chemotherapy exposure or Epstein-Barr virus (EBV) infection are considered more likely con-

tributors to these malignancies [89], [90], [91]. Lastly, lymphodepleting chemotherapy, commonly used alongside CAR-T and TCR-T therapy to enhance efficacy, carries its own risks, including severe cytopenias and increased susceptibility to infections [92].

1.3.6 Genetic engineering strategies

Genetic engineering is essential for T-cell therapy, regardless of the specific immune modulatory receptor being introduced. Viral vector-based transduction remains the most common method, with lentiviral vectors being highly effective due to their ability to stably integrate transgenes into the host genome, ensuring long-term expression in both dividing and non-dividing cells. Similarly, γ -retroviral vectors enable genome integration but are effective only in actively proliferating T cells. While viral methods offer high efficiency and durable gene expression, they carry risks such as insertional mutagenesis and require a complex manufacturing process [93]. Non-viral methods include electroporation or plasmid DNA transfection for receptor gene delivery. Transposon-based systems like Sleeping Beauty and PiggyBac use transposase enzymes to integrate genes into the genome [94], while CRISPR/Cas9 gene editing offers site-specific integration, such as targeting the T-cell receptor alpha chain locus (TRAC), reducing the risk of insertional mutagenesis [95]. However, these approaches generally have lower efficiency compared to viral vectors [96]. Another option is mRNA-based transient gene expression, which avoids genome integration and minimizes long-term risks but requires repeated infusions for sustained therapeutic effects [97].

While viral vectors currently dominate clinical applications due to their efficiency and stability, non-viral and gene-editing technologies are emerging as safer and more precise alternatives [98]. The latest applications are advancing *in vivo* CAR-T and TCR-T cell manufacturing by exploring targeted delivery and efficient *in vivo* reprogramming, enabling direct T-cell engineering within the body and eliminating the cost and complexity of ex vivo methods [99], [100], [101].

1.4 Mouse models for preclinical testing of adoptive cell therapies

Mouse models are essential tools in preclinical cancer research, offering valuable insights into the tumor microenvironment, immune responses, and therapeutic outcomes of T-cell therapies. Both immunocompetent and immunodeficient mouse models play critical roles, each with distinct advantages and limitations.

The NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl} (NSG) mouse strain is one of the most widely used immunodeficient models in cancer research. It carries three key mutations: the NOD background impairs innate immunity by affecting macrophages, dendritic cells, and the complement system; the scid mutation prevents the development of functional B and T cells; and the IL2R γ -null mutation disrupts cytokine signaling (IL-2, IL-4, IL-7, IL-9, IL-15, IL-21), significantly reducing NK cell activity. These features make NSG mice highly permissive for human tumor and immune cell engraftment, enabling detailed studies of human-specific TCR-T therapies. In subcutaneous solid tumor models, NSG mice are effective platforms for testing TCR-T-cell therapies. Human tumor pieces or tumor cell lines are engrafted subcutaneously, followed by the intravenous injection of genetically engineered human TCR-T cells to study tumor-T cell interactions. Researchers can evaluate key metrics such as tumor growth inhibition, T-cell infiltration, and adverse effects [102], [103].

However, the immunodeficiency of NSG mice introduces limitations. The lack of murine immune cells prevents studying interactions between engineered T cells and other immune components, such as macrophages or endogenous T cells. Additionally, NSG mice fail to replicate the complex immunosuppressive microenvironment of human tumors, potentially limiting the translatability of findings. The risk of xenogeneic graft-versus-host disease (GvHD) from infused human T cells can further restrict experimental duration and complicate data interpretation. Moreover, the absence of supportive cytokines like IL-7 and IL-15 in NSG mice can impair the persistence and functionality of TCR-T cells [104], [105].

Complementary, syngeneic models involve transplanting murine tumor cells into genetically matched immunocompetent mice, such as implanting B78.H1 murine melanoma cells into C57BL/6 mice. These models are ideal for studying T-cell-based therapies within a fully functional immune system. By preserving murine immune components, syngeneic models enable the analysis of immune cell interactions, T-cell activation, trafficking, and tumor infiltration. Additionally, tumors in these models grow within an intact immune-suppressive microenvironment, more closely mimicking the physiological conditions of human cancers. This setup allows us to evaluate TCR-T-cell persistence, memory formation, and differentiation over time [103], [106]. Thus, combining xenograft and syngeneic models offers a comprehensive approach to studying and optimizing TCR-T-cell therapies in a pre-clinical setting.

1.5 Overview of the experimental approach

To investigate the safety and efficacy of HLA-independent TCR therapy targeting TRP2, adoptive transfer experiments were conducted using T cells engineered to express an anti-TRP2 TCR. This TCR was originally identified, cloned, and characterized by [REDACTED] as part of her dissertation analyzing the anti-tumor TCR repertoire of the malignant melanoma patient Ma-Mel-86 [33]. Her studies demonstrated cross-reactivity of this TCR to the murine TRP2 homolog, making it a suitable candidate for *in vivo* experiments. Due to this cross-reactivity, it is possible to assess the efficacy, safety, and potential on-target/off-tumor effects in other organs or tissues that naturally express TRP2.

TCR-T-cell therapy was evaluated in a subcutaneous melanoma mouse model using both xenograft and syngeneic approaches. For xenograft experiments, immunodeficient NSG mice were implanted with the human melanoma cell line Ma-Mel-86b_KO. This cell line originates from the same melanoma patient from whom the anti-TRP2 TCR was isolated, and was established from that patient's tumor biopsy in collaboration with [REDACTED] and [REDACTED]. It was later genetically modified in the Wölfel group to create a fully HLA class I and class II-deficient variant. For syngeneic experiments, the murine melanoma cell line B78.H1 was used in immunocompetent mice, enabling the evaluation of TCR-T therapy in the presence of a functional immune system. B78.H1 cells are an amelanotic subclone of the B16 melanoma line that lack MHC class I expression due to defects in the antigen presentation machinery, making them particularly suitable for assessing HLA-independent TCR-mediated tumor control. Subcutaneous implantation of tumor cells is a widely used method in melanoma mouse models to study tumor growth, immune response, and therapeutic efficacy. In this approach, melanoma cells are injected into the subcutaneous space, typically in the flank region of the mice. This model allows for easy monitoring of tumor progression, measurement of tumor volume with calipers, and non-invasive imaging techniques such as *in vivo* bioluminescence imaging (BLI).

Human and murine primary T cells were engineered using distinct protocols to optimize TCR transduction and minimize production time. Human T cells, derived from buffy coats, underwent unspecific activation with an anti-CD3-antibody (OKT-3) for 48 hours, followed by retroviral transduction of a tricistronic TCR construct and antigen-specific stimulation with irradiated tumor cells overexpressing TRP2. As described by Kropp et al. the TCR constructs were designed to contain codon-optimized murine constant domains, preventing mispairing with human endogenous TCRs [107]. Murine T cells, isolated from the spleen, underwent unspecific activation

for 24 hours in the presence of Concanavalin A (Con A). Con A is an antigen-independent mitogen and can be used as an alternative T-cell stimulus. Activation was followed by CRISPR/Cas9-mediated knockout of endogenous murine TCRs to prevent mispairing with transduced TCR chains.

For genome editing, RNP-mediated CRISPR/Cas9 delivery via electroporation was employed, providing a transient approach that avoids DNA integration associated with vector-based CRISPR systems, minimizes off-target effects, and enables rapid editing [108]. To ensure specificity, guide RNAs were designed to target only non-optimized endogenous sequences, leaving codon-optimized transduced TCRs unaffected. CRISPR/Cas9-mediated knockout and retroviral transduction were performed on the same day, streamlining the process and reducing production time.

For *in vivo* experiments, a dual-luciferase BLI system was utilized, with T cells expressing firefly luciferase (FLUC) and tumor cells expressing NanoLuciferase (NLUC). This non-invasive approach enabled longitudinal monitoring of tumor engraftment, tumor progression or regression following T-cell therapy, and the distribution and migration of T cells to tumor sites and other organs. These luciferases exhibit distinct biochemical properties that influence their application *in vivo*. FLUC, derived from *Photinus pyralis*, is an ATP-dependent enzyme that catalyzes the oxidation of D-luciferin in the presence of ATP, oxygen, and Mg^{2+} , producing yellow-green light (~560 nm) [109]. In contrast, NLUC, derived from *Oplophorus gracilirostris*, is ATP-independent and catalyzes the oxidation of furimazine, emitting blue light (~460 nm) [110].

These differences in substrate specificity enable the specific visualization of distinct cell populations within the same animal. After injecting the appropriate substrate into the animals, the light signal specifically shows the location of the cells that express the respective luciferase. Importantly, the tissue penetration capacity of bioluminescent signals is wavelength-dependent, with longer wavelengths penetrating deeper into tissues [111]. Because the tumor cells are located just under the skin, the blue light from NLUC can be easily detected. In contrast, T cells travel to deeper organs like the lungs and spleen, making the longer-wavelength light from FLUC more suitable for detecting these cells inside the body.

Furthermore, the ATP dependence of FLUC provides an additional functional read-out, as only viable T cells generate a detectable signal, enabling the monitoring of T-cell viability and persistence *in vivo*. In contrast, the ATP-independent activity of NLUC allows for the detection of both live and dying tumor cells, ensuring that a loss of NLUC signal more reliably indicates the elimination of tumor cells. This dual-reporter strategy thus enables precise longitudinal assessment of tumor burden and T-cell dynamics following adoptive T-cell therapy.

For *in vivo* tracking of T cells, retroviral expression constructs encoding the chimeric TCR (cTCR) α - and β -chains together with FLUC were generated using the Gateway recombination-based cloning system (Thermo Fisher Scientific). The Gateway system enables high-efficiency, sequence-independent transfer of open reading frames (ORFs) between compatible plasmids without requiring restriction digestion or ligation. Specifically, pDONR221 served as the entry (donor) vector and pMX-DEST as the retroviral destination vector. The pMX backbone contains 5' and 3' long terminal repeats (LTRs), which function as strong viral promoters driving transgene expression after genomic integration [112].

The cTCR constructs used in this study incorporated several design elements to enable coordinated expression of multiple proteins from a single open reading frame. The multicistronic constructs used in this study incorporated self-cleaving 2A peptides, which mediate ribosomal skipping and co-translational separation of adjacent proteins [113]. Among these, P2A generally provides the highest cleavage efficiency [114], while T2A also ensures robust processing; using different 2A elements within the same cassette reduces sequence repetitiveness and limits homologous recombination [113]. To further optimize protein maturation and folding, the constructs additionally included a helix-breaker (HB) sequence, which disrupts unwanted α -helical structures [115], and a furin (Fur) cleavage site, which enables proteolytic processing in the Golgi to generate a defined N-terminus for the downstream protein [116], [117]. Together, these elements support near-equimolar expression of the TCR α - and β -chains and linked reporter genes, ensuring proper stoichiometry and functionality of the expressed proteins. The B-P2A-A design, in which the β -chain precedes the α -chain, has been shown to improve surface expression of engineered TCRs [118].

For Gateway cloning, the gene of interest (GOI) was amplified by PCR using primers flanked by attB1 and attB2 recombination sequences. The PCR product was recombined with pDONR221 in a BP Clonase II reaction, producing an Entry Clone in which the GOI is flanked by attL1 and attL2 sites. pDONR221 contains a ccdB negative selection marker and a kanamycin resistance gene for positive selection in standard *E. coli*. Final expression constructs were obtained through an LR Clonase II reaction between the Entry Clone and pMX-DEST (available in a puromycin-resistant version #35 and

a non-selectable version #3). During the LR reaction, the GOI replaces the toxic *ccdB* cassette in the pMX-DEST vector by recombination into the attR1/attR2 sites. Transformants were selected on ampicillin-containing plates, and correct insertion was verified by PCR spanning vector-insert junctions and by Sanger sequencing.

Tumor cells were transduced with NLUC using a lentiviral transduction system. The pLenti constructs generated for this study were driven by the EF1 α promoter, a strong and ubiquitously active mammalian promoter that supports stable and uniform transgene expression across different tumor cell types [119]. Each construct encoded NLUC and enhanced green fluorescent protein (EGFP), separated by self-cleaving 2A peptides, enabling equimolar expression of both reporters from a single transcript. EGFP served as a fluorescent marker to assess transduction efficiency by flow cytometry and to isolate highly expressing cells by fluorescence-activated cell sorting (FACS). A puromycin resistance gene (PAC) was included to allow antibiotic selection, further enriching for stably transduced populations. To enhance transcriptional output and transgene stability, all constructs contained the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) at the 3' end, which increases mRNA stability and nuclear export, thereby strengthening NLUC and EGFP expression [120]. One construct additionally encoded the full-length human TRP2 gene upstream of the reporter cassette, allowing controlled overexpression of the target antigen on tumor cells. The use of this multicistronic design enabled coordinated expression of TRP2, NLUC, and EGFP from a single promoter. This design facilitated reliable generation of homogeneous, stable cell lines in which bioluminescence accurately reflected tumor burden.

To evaluate potential T-cell-mediated toxicities and adverse effects, organs including skin, liver, lungs, spleen, brain, eyes, intestines, and tumors (if present) were harvested at the conclusion of each T-cell transfer experiment and processed for immunohistochemical analysis in collaboration with [REDACTED]. Blood samples were collected throughout the experiments to assess the presence and persistence of transgenic T cells over time. At the experimental endpoint, blood, spleen, and lymph nodes were additionally analyzed by flow cytometry to determine T-cell differentiation status and exhaustion phenotypes.

1.6 Aim of the study

The overarching aim of this study was to evaluate the therapeutic potential of a naturally occurring, HLA-independent TCR targeting the melanocytic differentiation antigen TRP2 for use in adoptive T cell therapy of melanoma. This TCR was originally derived from a melanoma patient with HLA class I-deficient disease and offers the unique advantage of antigen recognition independent of classical MHC presentation, thereby addressing a key mechanism of immune evasion in solid tumors.

To assess the feasibility, efficacy, and safety of this TCR-based immunotherapy, a comprehensive set of *in vitro* and *in vivo* experiments was conducted. First, molecular biology techniques were employed to generate a series of retroviral and lentiviral vectors enabling TCR expression in T cells and TRP2 expression in tumor cells, respectively. These constructs also incorporated bioluminescent reporter genes, FLUC and NLUC, to allow tracking of transduced T cells and tumor cells, respectively, in *in vivo* studies. These tools were used to engineer human and murine T cells for functional validation and adoptive transfer. *In vivo* efficacy was tested using a xenograft model in immunodeficient NSG mice bearing either TRP2-overexpressing or naturally TRP2-positive melanoma tumors. In parallel, a syngeneic mouse model was established to evaluate therapeutic efficacy, safety, and immune dynamics in an immunocompetent setting.

Functional characterization included Interferon- γ ELISpot and cytotoxicity assays, bioluminescence-based tumor monitoring, flow cytometric phenotyping of engineered T cells, and histopathological analyses of host tissues. Special emphasis was placed on identifying potential off-tumor effects, such as T-cell mediated toxicities and depigmentation.

Together, this study aimed to establish proof-of-concept for the use of HLA-independent TCRs in adoptive cell therapy, with a particular focus on TRP2 as a model antigen for targeting melanoma, and to provide preclinical insights that could guide future translational development of this approach.

2 Material

2.1 Animals

All animal experiments were approved by local authorities (state investigation office Rhine-land-Palatinate, approval ID G21-0-092 and conducted according to the German Animals Protection Law for care and use of experimental animals. All animals were raised and kept in individually ventilated cages under specific hygienic conditions in the Translational Animal Research Centre (TARC) at the University Medical Center (UMC) of the Johannes Gutenberg University Mainz.

2.1.1 NSG mice

NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl} (NSG) mice were obtained from the Translational Animal Research Center (TARC), Mainz, and used for xenograft experiments involving subcutaneous transplantation of human melanoma cells and T-cell transfer studies.

2.1.2 C57BL/6

C57BL/6 wild-type mice were obtained from the Translational Animal Research Center (TARC), Mainz, and used for syngeneic experiments involving subcutaneous transplantation of murine melanoma cells and T-cell transfer studies, and as donor animals for splenocytes in these syngeneic studies.

2.1.3 Lewis rats

Additionally, 8-10 weeks old female Lewis Rats obtained from Janvier Labs, Laval, France, were used to produce T-cell growth factor (TCGF) medium.

2.2 Cell lines

2.2.1 Ma-Mel-86 cell lines

The Ma-Mel-86 cell lines were kindly provided by [REDACTED] (Department of Dermatology, University Hospital Essen). The Ma-Mel-86b cells naturally express TRP2 but lack HLA class I due to biallelic mutations in the *B2M* gene. These cells were retrovirally transduced by [REDACTED] to overexpress the costimulatory molecules CD80 and CD83 [35]. Further genetic modification was performed by

using TALEN technology to introduce biallelic frameshift mutations in the *CIITA* gene, resulting in a lack of HLA class II expression [34]. This modified cell line Ma-Mel-86b_KO^{CIITA}#98.41.2 is hereafter referred to as 86b_KO in this study.

Ma-Mel-86a cells, in contrast, naturally lack TRP2 expression but express both HLA class I and class II. These cells were genetically modified using CRISPR/Cas9 technology to introduce biallelic frameshift mutations in both the *B2M* and *CIITA* genes, leading to the loss of HLA class I and class II expression. This led to the generation of the cell line Ma-Mel-86a_KO^{B2M/CIITA}#2.5#2/3, which is hereafter referred to as 86a_KO in this study.

Table 1: List of modified tumor cell lines used in this study

Cell line	Modifications	Abbreviated name
Ma-Mel-86b_KO ^{CIITA} #98.41.2	retrovirally transduced with CD80 and CD83, TALEN-mediated knock out of <i>CIITA</i>	86b_KO
Ma-Mel-86b_KO ^{CIITA} #98.41.2	retrovirally transduced with CD80 and CD83, TALEN-mediated knock out of <i>CIITA</i> , retrovirally transduced with human full-length TRP2	86b_KO.hTRP2
Ma-Mel-86b_KO ^{CIITA} .NLUC.EGFP#8	retrovirally transduced with CD80 and CD83, TALEN-mediated knock out of <i>CIITA</i> , lentivirally transduced with NLUC and EGFP	86b_KO.NLUC.EGFP
Ma-Mel-86b_KO ^{CIITA} .hTRP2.NLUC.EGFP#1.2	retrovirally transduced with CD80 and CD83, TALEN-mediated knock out of <i>CIITA</i> , lentivirally transduced with human full-length TRP2, NLUC, and EGFP	86b_KO.hTRP2.NLUC.EGFP
Ma-Mel-86a_KO ^{B2M/CIITA} #2.5#2/3	CRISPR/Cas9-mediated knock out of <i>B2M</i> and <i>CIITA</i>	86a_KO
B78.H1.hTRP2.NLUC.EGFP#4	lentivirally transduced with human full-length TRP2, NLUC, and EGFP	B78.H1.hTRP2.NLUC.EGFP

2.2.2 B78.H1 cell line

B78.H1 cells are a subclone of the murine B16 melanoma cell line (C57BL/6 background) characterized by their lack of melanin production (amelanotic phenotype) and an absence of detectable tyrosinase activity [121]. These cells are also deficient in both classical (Class Ia: *Kb* and *Db*) and non-classical (Class Ib: *Q8* and *Q9*, encoding *Qa-2* molecules) MHC expression, even under stimulation with IFN- γ . The loss of MHC expression is due to a lack of transcription for these loci and associated deficiencies in components of the antigen presentation pathway, such as *TAP2* (Transporter Associated with Antigen Processing) and *LMP7* (a proteasome subunit). This combination of defects results in a complete absence of MHC class I molecules on the cell surface, distinguishing B78.H1 cells from other B16 melanoma variants [122], [123].

2.2.3 D05-Mel

The D05-Mel cell line, a human melanoma model, was kindly provided by CW Schmidt (Queensland Institute of Medical Research, Queensland, Australia). The HLA-A*02-positive D05-Mel cell line was previously utilized to investigate TCR-mediated immune responses against melanoma-specific antigens, including NY-ESO-1, a well-known cancer/testis antigen. The study demonstrated that T cells expressing NY-ESO-1-specific TCRs effectively recognized and lysed D05-Mel cells [124]. In this study D05-Mel cells are used as target cells for T cells transduced with an anti-NY-ESO-1-TCR recognizing the peptide SLLMWITQC presented on HLA-A*02.

2.2.4 Phoenix-Ampho

Phoenix-Ampho (Ph-A) cells are a derivative of the HEK293T human embryonic kidney cell line that have been engineered to stably express the components required for the production of amphotropic retroviral particles. They were developed in the laboratory of Dr. Garry Nolan at Stanford University to enable high-efficiency retroviral packaging for gene transfer into a wide range of mammalian cell types, including both human and murine cells. These cells express the viral gag, pol, and amphotropic envelope (env) proteins, allowing the production of replication-incom-

petent retroviruses when transfected with a retroviral vector containing the gene of interest [125]. The Phoenix-Ampho cell line used in this study was kindly provided by Dr. H. Echchannaoui (University Medical Center of the Johannes Gutenberg University, Mainz, Germany).

2.2.5 293FT

293FT cells are a fast-growing, highly transfectable derivative of the HEK293T human embryonic kidney cell line that were engineered for high-efficiency production of lentiviral particles. They are stably transfected with the SV40 large T antigen, which enables episomal replication of plasmids containing the SV40 origin of replication, thereby significantly enhancing protein and viral particle production. Developed by Invitrogen (Thermo Fisher Scientific), 293FT cells are commonly used in combination with third-generation lentiviral packaging systems to generate replication-incompetent lentiviruses for stable gene transfer into both dividing and non-dividing mammalian cells.

2.2.6 NIH-3T3

The NIH-3T3 cell line is a widely utilized fibroblast cell line derived from mouse embryonic tissue. Established in 1962 by George Todaro and Howard Green at the New York University School of Medicine, these cells originated from NIH Swiss mouse embryos [126].

2.2.7 Primary human T cells

Primary human T cells were obtained from buffy coat-derived PBMCs. Buffy Coats were provided by the Transfusion Center of the UMC Mainz. Cells were usually cultivated at a density of $0.5 - 2 \times 10^6$ cells/well in a 24-well plate in Panserin complete medium supplemented with 100 IU/mL IL-2, 10 ng/mL IL-7, and 10 ng/mL IL-15.

2.2.8 Primary murine T cells

Primary murine T cells were obtained from freshly isolated splenocytes. Mice for organ harvesting were provided by the Translational Animal Research Center (TARC) Mainz. Cells were usually cultivated at a density of $0.5 - 2 \times 10^6$ cells/well in a 24-well plate in RPMI Mouse T cell complete medium supplemented with 10% TCGF.

2.3 Bacteria

One Shot Stbl3 Chemically Competent *E. coli* bacteria from Invitrogen™ were used for Plasmid amplification. According to the manufacturer, the Stbl3 strain is derived from the HB101 *E. coli* strain with a hybrid K-12 and B strain genome and exhibits the following genotype: $F^-mcrB\ mrr\ hsdS20(r_B^-, m_B^-)\ recA13\ supE44\ ara-14\ galK2\ lacY1\ proA2\ rpsL20(StrR)\ xyl-5\ \lambda-leu\ mtl-1$.

2.4 Cell culture reagents, chemicals, and buffers

Table 2: Composition of cell culture media

Cell culture medium	Composition
RPMI complete	RPMI 1640 Medium 10% FCS 1% Pen/Strep
DMEM complete	DMEM Medium 10% FCS 1% Pen/Strep 1% L-Glutamine 25 mM Hepes
Panserin complete	Panserin 413 Medium 10% Human Serum 1% Pen/Strep
RPMI Mouse T cell Medium	RPMI 1640 Medium 10% FCS 1% Pen/Strep 2 mM L-Glutamine 20 mM Hepes

Table 3: List of materials and reagents used for cell culture and molecular biology applications

Reagent	Manufacturer
Gibco™ RPMI 1640 Medium	Thermo Fisher Scientific (Schwerte, DE)

Reagent	Manufacturer
Gibco™ DMEM	Thermo Fisher Scientific (Schwerte, DE)
Panserin 413	PAN-Biotech GmbH (Aidenbach, DE)
Gibco™ Opti-MEM™	Thermo Fisher Scientific (Schwerte, DE)
Human serum	AG Wölfel (Mainz, DE)
FCS	PAA Laboratories GmbH (Pasching, A)
Penicillin-Streptomycin	Life Technologies (Darmstadt, DE)
HEPES Buffer (1 M)	Lonza Group Ltd (Basel, CH)
L-Glutamine	Thermo Fisher Scientific (Schwerte, DE)
EDTA (Versen) 1%	Merck (Darmstadt, DE)
Histopaque 1077	Sigma-Aldrich (St. Louis, MO, USA)
Dimethylsulfoxide (DMSO)	Merck KGaA (Darmstadt, DE)
BSA	BD Biosciences (Heidelberg, DE)
FACS Clean™,	BD Biosciences (San Jose, CA, USA)
FACS Flow™,	Merck (Darmstadt, DE)
FACS Rinse™	Merck (Darmstadt, DE)
Formaldehyde	Sigma-Aldrich (St. Louis, MO, USA)
FuGENE® 6 Transfection Reagent	Promega GmbH (Walldorf, DE)
Hexadimethrine bromide (Polybrene)	Sigma-Aldrich (St. Louis, MO, USA)
Puromycin	Sigma-Aldrich (St. Louis, MO, USA)
Trypanblau	Schülke&Mayr (Norderstedt, DE)
Dulbecco's Phosphate Buffered Saline	Sigma-Aldrich (St. Louis, MO, USA)
Agarose Standard	Carl Roth GmbH + Co. KG (Karlsruhe, DE)
TBE Buffer (10x)	SERVA Electrophoresis GmbH (Heidelberg, DE)

Reagent	Manufacturer
TAE Buffer (50x)	SERVA Electrophoresis GmbH (Heidelberg, DE)
Ampuwa® Water	Life Technologies (Darmstadt, DE)
1 kb DNA Ladder	New England Biolabs (Ipswich, MA, USA)
100 bp DNA Ladder	New England Biolabs (Ipswich, MA, USA)
dNTPs	Honeywell Riedel-de Haën AG (Seelze, DE)
Ethanol	Sigma-Aldrich (St. Louis, MO, USA)
Gel Loading Dye Purple (6x)	New England Biolabs (Ipswich, MA, USA)
Isopropanol	Sigma-Aldrich (St. Louis, MO, USA)
Bacto™ Agar	Becton Dickinson (Franklin Lakes, NJ, USA)
NEB® 10-beta-Stable Outgrowth Medium	New England Biolabs (Ipswich, MA, USA)
Kanamycin	Sigma-Aldrich (St. Louis, MO, USA)
Ampicillin	Sigma-Aldrich (St. Louis, MO, USA)
G418-BC	Merck (Darmstadt, DE)
Hygromycin B	Roche (Basel, CH)
Glycerol	BIOTIUM (Hayward, USA)
Lipofectamin 2000	Thermo Fisher Scientific (Schwerte, DE)
peqGREEN	peqLab (Erlangen, DE)
D-Luciferin	Abcam (Cambridge, UK)
Furimazin	Promega (Madison, WI, USA)
Sulfate Latex Beads, 4% w/v, 5 µm	Thermo Fisher Scientific (Schwerte, DE)

2.5 Buffers

Table 4: List of buffers used in this study

Buffer	Composition
Red Blood Cell (RBC) Lysis Buffer	NH ₄ Cl 8,29 g KHCO ₃ 1 g EDTA 0,0372 g H ₂ O ad 1 l pH = 7.29
FACS Buffer	0.1% BSA 2 mM EDTA in PBS (1x)
FACS-Fixingsolution	1% Formalin 2 mM EDTA in PBS (1x)
ELISpot Buffer	NaHCO ₃ 2,9 g Na ₂ CO ₃ 1,6 g Na-Azid 0,2% (w/v) d.H ₂ O ad 1 l
Acetate-Buffer	Acetic acid (0.2 N) 4,6 ml Sodium acetate (0.2 N) 11 ml d.H ₂ O 46,9 ml
Avidin-Peroxidase-Complex	10 ml PBS (1x) 0.1% Tween 20 1 Drop Solution A (ABC Kit, Vectastain) 1 Drop Solution B (ABC Kit, Vectastain)
TAE-Buffer	TAE (50x) 2 ml d.H ₂ O ad 1 l
TBE-Buffer	TBE (10x) 50 ml d.H ₂ O ad 1 l
LB-Medium	Bacto TM Trypton 10 g Yeast extract 5g

Buffer	Composition
	NaCl 10g d.H ₂ O ad 1l
LB-Agar	LB-Medium 1 l Agar 15 g
Peroxidase-Substrate Solution	1 tablet AEC solved in: Dimethyformamide 2,5 ml Acetate Buffer ad 50 ml
Trypanblue (Tb)	Solution A: 0,2% Tb-Solution (1 g Tb in 500 ml d.H ₂ O) Solution B: 4,5% NaCl-Solution (22,5 g NaCl in 500 ml d.H ₂ O) 3 Parts Solution A in 1 Part Solution B
RF1 Buffer	Rubidiumchlorid 100 mM Mangan(II)-chlorid Tetrahydrat 50 mM Kaliumacetat 30 mM Calciumchlorid Dihydrat 10 mM Glycerol 15% w/v pH: 5.8
RF2 Buffer	MOPS 10 mM Rubidiumchlorid 10 mM Calciumchlorid Dihydrat 75 mM Glycerol 15% w/v pH = 6.8
4% Paraformaldehyde (PFA)	Paraformaldehyde (powder) 20 g solved in 500 ml PBS (1x) at 60°C then stored at 4°C pH = 6.9

2.6 Kits

Table 5: List of commercially available kits used in this study

Kit	Manufacturer
Mix2Seq	Eurofins Scientific (Luxemburg, LUX)
Monarch PCR & DNA Cleanup Kit (5 µg)	New England Biolabs (Ipswich, MA, USA)
NucleoSpin Gel and PCR Clean-up	Macherey-Nagel (Düren, DE)
Qubit dsDNA BR Assay Kit	Thermo Fisher Scientific (Schwerte, DE)
NucleoSpin Plasmid (NoLid)	Macherey-Nagel (Düren, DE)
Monarch DNA Gel Extraction Kits	New England Biolabs (Ipswich, MA, USA)
Human T Cell Nucleofector® Kit	Lonza (Basel, CH)
Murine T Cell Nucleofector® Kit	Lonza (Basel, CH)
NEBuilder HiFi DNA Assembly (Cloning Kit or Master Mix)	New England Biolabs (Ipswich, MA, USA)
BD Cytofix/Cytoperm Fixation/ Permeabilization kit	BD Biosciences (Franklin Lakes, NJ, USA)

2.7 Laboratory devices

Table 6: List of laboratory devices used in this study

Device	Manufacturer
Amaxa Nucleofector® 2b Device	Lonza (Basel, CH)
Autoklave: VX-150	Systec (Wettenberg, DE)
Autoklave: DSL-20/9/9-2-FDGD	Systec (Wettenberg, DE)
BD FACS Canto II	BD Biosciences (Heidelberg, DE)

Centrifuge 5424; 5415 R	Eppendorf (Hamburg, DE)
CTL-ImmunoSpot® S6 Micro Analyzer	C.T.L. Europe GmbH (Bonn, DE)
CO ₂ -Incubator BBD6220	Heraeus (Hanau, DE)
Gammacell 2000	Molsgaarg Medical (Gansloe, DNK)
Gel Electrophoresis Chamber	PEQLAB (Erlangen, DE)
Heating Block Thermomixer Comfort, 5436	Eppendorf (Hamburg, DE)
HERA freeze (-80 °C)	Thermo Fisher Scientific (Schwerte, DE)
Bacterial Incubator: UT6420	Heraeus (Hanau, DE)
Microscope Eclipse TS100; TMS	Nikon (Alzenau, DE)
Microwave: MWS 2819	Bauknecht (Neunkirchen, DE)
Multifuge Rotina 420	Hettich (Tuttlingen, DE)
Qubit® 2.0 Fluorometer	Thermo Fisher Scientific (Schwerte, DE)
Thermocycler T1, Tgradient	Biometra (Göttingen, DE)
UVsolo TS Imaging System	Biometra (Göttingen, DE)
VF2 (Vortex Mixer)	Janke & Kunkel GmbH u. Co.KG IKAL-abortechnik (Staufen, DE)
Water Bath: UT 6420	Heraeus (Hanau, DE)
Cytek Aurora Flow Cytometer	Cytek (Fremont, CA, USA)
IVIS Spectrum CT Imaging System	Revvity (Waltham, MA, USA)
FLUOstar Omega	BMG LABTECH (Ortenberg, DE)

2.8 Software

Table 7: List of software used in this study

Software	Software provider
BD FACS DIVA Software	Becton Dickinson (Franklin Lakes, NJ, USA)

Software	Software provider
FlowJo Version 10.9.0	Becton Dickinson (Franklin Lakes, NJ, USA)
GraphPad Prism	Dotmatics (Boston, MA, USA)
Microsoft Office 365 (Word, Excel, PowerPoint)	Microsoft (Redmond, WA, USA)
Geneious	Dotmatics (Boston, MA, USA)
SpectroFlo (Cytex Aurora)	Cytex (Fremont, CA, USA)
Living Image™ Software	Revvity (Waltham, MA, USA)
ImmunoSpot® software	C.T.L. Europe GmbH (Bonn, DE)
Omega Data Analysis Software	BMG LABTECH (Ortenberg, DE)

2.9 Cytokines

Table 8: List of cytokines used in this study

Cytokines	Manufacturer
IL-2 (Proleukin S)	Novartis, CH
IL-7	Miltenyi Biotec, DE
IL-15	Miltenyi Biotec, DE
TCGF (undefined mix of cytokines in supernatant)	AG Wölfel, DE

2.10 Antibodies

Table 9: List of antibodies used in this study

Antibody	Dilution	Manufacturer
Anti-human IFN- γ mAb, clone 1-D1K	500 ng/50 μ L (10 μ g/mL)	Mabtech (Nacka Strand, SWE)
Anti-human IFN- γ mAb, clone 7-B6-1, biotin	2 μ g/mL	Mabtech (Nacka Strand, SWE)

Antibody	Dilution	Manufacturer
Anti-murine IFN- γ mAb, clone AN18	50 ng/50 μ L (1 μ g/mL)	Mabtech (Nacka Strand, SWE)
Anti-murine IFN- γ mAb, clone XMG1.2, biotin	2 μ g/mL	Leinco Technologies (St. Louis, MO, USA)
Anti-hCD3, clone OKT-3	30 ng/mL	AG Wölfel
Anti-hCD3, clone REA613	1:100	Miltenyi Biotec, DE
Anti-hCD4, clone 13B8.2	1:20	BD Biosciences (Franklin Lakes, NJ, USA)
Anti-hCD8, clone B9.11	1:20	BD Biosciences (Franklin Lakes, NJ, USA)
Anti-hVb1, clone BL37.2	1:5	BD Biosciences (Franklin Lakes, NJ, USA)
Anti-hVb3, clone CH92	1:5	BD Biosciences (Franklin Lakes, NJ, USA)
Anti-hCD16, clone 3G8	1:100	BD Biosciences (Franklin Lakes, NJ, USA)
Anti-hCD56, clone N901	1:100	BD Biosciences (Franklin Lakes, NJ, USA)
Anti-mTRBC, clone H57-597	1:200	BioLegend (San Diego, CA, USA)
Anti-mCD3, clone 145-2C11	1:100	Thermo Fisher Scientific (Schwerte, DE)
Anti-mCD4, clone RM4-5	1:100	BD Biosciences (Franklin Lakes, NJ, USA)
Anti-mCD3, clone 17A2	1:200	BioLegend (San Diego, CA, USA)
Anti-mCD8, clone 53-6.7	1:200	BioLegend (San Diego, CA, USA)
Anti-m/hCD44, clone IM7	1:200	BioLegend (San Diego, CA, USA)

Antibody	Dilution	Manufacturer
Anti-mCD25/IL2 receptor alpha, clone PC61	1:200	BD Biosciences (Franklin Lakes, NJ, USA)
Anti-mCD62L/L-Selectin, clone MEL-14	1:200	BD Biosciences (Franklin Lakes, NJ, USA)
Anti-mCD69, clone H1.2F3	1:200	BioLegend (San Diego, CA, USA)
Anti-mCTLA4/CD152, clone UC10-4B9	1:200	BioLegend (San Diego, CA, USA)
Anti-mPD1, clone 29F.1A12	1:200	BioLegend (San Diego, CA, USA)
Anti-mTIGIT/Vstm3, clone 1G9	1:200	BioLegend (San Diego, CA, USA)
Anti-mIL7RA/CD127, clone A7R34	1:200	BioLegend (San Diego, CA, USA)
Viability Red	1:1000	Thermo Fisher Scientific (Schwerte, DE)

2.11 Enzymes

Table 10: List of Enzymes and associated Buffers used in this study

Enzyme	Manufacturer
Gateway BP Clonase II Enzyme Mix	Thermo Fisher Scientific (Schwerte, DE)
Gateway LR Clonase II Enzyme Mix	Thermo Fisher Scientific (Schwerte, DE)
OneTaq® 2X MM w /standard Buffer	New England Biolabs (Ipswich, MA, USA)
Proteinase K	Thermo Fisher Scientific (Schwerte, DE)
Q5® High-Fidelity DNA Polymerase	New England Biolabs (Ipswich, MA, USA)
T4 DNA Ligase	New England Biolabs (Ipswich, MA, USA)
Cas9	Integrated DNA Technologies (Coralville, IA, USA)
SmaI	New England Biolabs (Ipswich, MA, USA)

Enzyme	Manufacturer
SgrDI	New England Biolabs (Ipswich, MA, USA)
CutSmart Buffer	New England Biolabs (Ipswich, MA, USA)
10X Buffer for T4 DNA Ligase	New England Biolabs (Ipswich, MA, USA)

2.12 Primer

Table 11: List of primers used in this study

Primer name	Primer sequence
attB1_aTRP2-cTCR.for	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCCACCAT GGGAATCAGGCTCCTCTGTCGTGTG-3'
aTRP2-cTCR-FUR-HB-T2A.rev	5'-GACTTCCCCTGCCCTCTCCGGATCCGGACCGCTTGGCCCCG CTGCTCCACAGCCGCA-3'
attB1-KOS-AatII-NY-4/ 76.TCR.p1.for	5'-GGGGACAAGTTTGTACAAAAAAGCAGACGTCGCCACCATG GGCTTCAGGCTCCTCTGCTGTGTG-3'
aNY-ESO-1-cTCR-FUR-HB- T2A.rev	5'-GACTTCCCCTGCCCTCTCCGGATCCGGACCGCTTGGCCCCGA CTGCTCCACAGCCGCA-3'
p1-P2A-EGFP.for	5'-AACAAGCTGGAGACGTGGAGGAGAACCCGGGACCTATGG TGAGCAAGGGCGAGGAGCTGT-3'
p2-EGFP-T2A-PAC.rev	5'-GAGGCGCACCGTGGGCTTGTACTCGGTCATTGGGCCGGGA TTTTCTCCACGTCCCCGCATGTTAGAAGACTTCCCCTGCCCTC TCCGGATCCCTTGTACAGCTCGTCCATGCCGAGAGTG-3'
Fur_HB_T2A_GFP.for	5'-GGGCCAAGCGGTCCGGATCCGGAGAGGGCAGGGGAAGTC TTCTAACATGCGGAGACGTGGAGGAGAACCCCGGACCTATGG TGAGCAAGGGCGAGGAGCTG-3'
GFP-2xSTOP-attB2.rev	5'-GGGGACCACTTTGTACAAGAAAGCTGGGTA TTACTATCTAGATCCGGTGGAACC-3'
P2A-hLuc+.for	5'-CTACTAACTTCAGCCTGCTGAAACAAGCTGGAGACGTGGA GGAGAACCCCGGACCTATGGCCGATGCTAAGAACATTAAGA AGGGCCCTG-3'
hLuc+-P2A.rev	5'-CCGGGTTCTCCTCCACGTCTCCAGCTTGTTTCAGCAGGCTG AAGTTAGTAGCTCCGCTTCCCACGGCGATCTTGCCGCCTTTCT

Primer name	Primer sequence
	TAG-3'
HB.T2A_FLUC.for	5'-GGGCCAAGCGGTCCGGATCCGGAGAGGGCAGGGGAAGTC TTCTAACATGCGGAGACGTGGAGGAGAACCCCGGACCTATGG CCGATGC-3'
SDM_FLUC.for	5'-GCTTCCAGAGCATGTATACATTCGTGACATCTC-3'
FLUC_2x STOP_attB2.rev	5'-GGGGACCACTTTGTACAAGAAAGCTGGGTA TTATCACACGGCGATCTTGCCGCCTTCTT-3'
attB1-KOS.FLUC.for	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTC GCCACCATGGCCGATGCTAAGAACATTAAGAAG-3'
FLUC-FUR-HB-T2A.rev	5'-GAAGACTTCCCCTGCCCTCTCCGGATCCGGACCGC TTGGCCCGCACGGCGATCTTGCCGCCTTTC-3'
mTRAC.p670.rev	5'-CTGGCATGTGAAGCTGGTCTGGTTGGAC-3'
seq.mTRAC.p57.for	5'-CCTGTGCCTGTTACCCGACTTCG-3'
seq.mTRBC.p293.for	5'-GCCTGAGCGAAGAGGACAAGTG-3'
mTRBC.p618.rev	5'-GACAGGCAGTAGCTGTAGTTGCTC-3'
seq.FLUC.p572.for	5'-CATTGCCCTGATCATGAACAGCTCTG-3'
pLenti-SgrDI-TRP2.for	5'-GACCCAAGCTGGCTAGTTAAGCTTGATCGTCGACGGCCA TGAGCCCTCTTTGGTGGGGGTTTC-3'
TRP2+CYT-HB-P2A.rev	5'-CAGCTTGTTTCAGCAGGCTGAAGTTAGCGGAGCCGCCGGA GCCGCCGCCCGCTTGGCCCGGGCTTCTTCTGTGTATCTCTTG CTGCTTAAATG-3'
P2A-NLuc.for	5'-GCTACTAACTTCAGCCTGCTGAAACAAGCTGGAGACGTGG AGGAGAACCCCGGACCTATGGTCTTCACACTCGAAGATTTCGT TGGGGAC-3'
NLuc-P2A.rev	5'-AGGTCCCGGGTTCTCCTCCACGTCTCCAGCTTGTTTCAGCA GGCTGAAGTTAGTAGCTCCGCTTCCCGCCAGAATGCGTTCGC ACAGCCG-3'
pFUR-HB-P2A-NLUC.for	5'-CGGGCCAAGAGGGGCGGCGGCTCCGGCGGCTCCGCTACT AACTTCAGTCTGCTGAAGCAGGCTGGAGACGTGGAGGAGAAC CCTGGACCTATGGTCTTCACACTCGAAGATTTCGTTGGGGACT GG-3'
TRP2.p694.for	5'-CAGCGACTCATTGGCAATGAGTC-3'

Primer name	Primer sequence
TRP2.p767.rev	5'-ACATCACACTCGTTCCTCCCAGTG-3'
EGFP.p804.rev	5'-CGGATCTTGAAGTTCACCTTGATGCCG-3'
seq.EGFP.p1.for	5'-ATGGTGAGCAAGGGCGAGGAGCTG-3'
NLUC.p251.for	5'-CTGTGGATGATCACTTTAAGG-3'
NLUC.p336.rev	5'-GAAATAGTCGATCATGTTCCGGCG-3'
seq puro.ORE.STOP.rev	5'-TTAGTAGAAGGGGAGGTTGCGGGGCG-3'
PAC.p312.for	5'-CGCGCAGCAACAGATGGAAGGC-3'
EF1a._p1131.for	5'-CTCAAGCCTCAGACAGTGGTTCAAAG-3'
pMX-seq_206.for	5'-TTACACAGTCCTGCTGACCACC-3'

2.13 CRISPR RNAs

Table 12: List of crRNAs used in this study to knock out the endogenous mouse TCR α - and β -chain constant regions

Name	Sequence	Target
CD.Cas9.ZFGH3888.AC	5'- UUC GGA GUC CCA UAA CUG ACG UUU UAG AGC UAU GCU-3'	mTRAC (TCR α - chain constant region)
CD.Cas9.VQHS7791.AD	5'- GGU CCG UGC UGA CCC CAC UGG UUU UAG AGC UAU GCU-3'	mTRBC (TCR β - chain constant region)

The bolded sequence represents the DNA-binding region of the crRNA, while the unformatted portion binds to the trans-activating CRISPR RNA (tracrRNA) to form the crRNA:tracrRNA duplex, collectively referred to as the guide RNA (gRNA).

3 Methods

3.1 Cellular biology methods

3.1.1 Cell culture and maintenance

All cell lines were cultured in an incubator at 37°C with 5% CO₂ and 95% humidity and all cell culture-related methods, except from cell counting, were performed under sterile conditions with laminar flow.

Adherent cell lines were maintained in 5–25 mL of RPMI complete medium and routinely passaged at a 1:5 to 1:10 ratio when confluency exceeded 80%. The culture medium was discarded for passaging, and the adherent cells were washed with PBS. Cells were detached by adding 2–5 mL of PBS containing 0.05% EDTA (depending on the flask size) and incubating for 3–5 minutes. The effect of EDTA was neutralized by adding 10 mL of RPMI complete medium, and the detached cells were centrifuged at $650 \times g$ for 5 minutes at room temperature (RT). The resulting cell pellets were resuspended in RPMI complete medium, counted, and reseeded at densities appropriate for the cell type and proliferation rate.

Human T cells were cultured in Panserin complete T-cell medium at a standard density of 0.5×10^6 to 1×10^6 cells/mL. Murine T cells were maintained in RPMI complete mouse T-cell medium under the same density conditions.

Cell lines used for viral particle production included Phoenix-Ampho cells for retroviral particles and 293FT cells for lentiviral particles. These cell lines were cultured in DMEM complete medium. For passaging, the culture medium was discarded, and 2–5 mL of TrypLE Express was added to detach the cells. After 30–60 seconds, TrypLE Express was neutralized with 10 mL of DMEM complete medium, and the detached cells were centrifuged at $650 \times g$ for 5 minutes (RT). The cell pellets were resuspended in DMEM complete medium, counted, and seeded at densities appropriate for their intended use.

3.1.2 Cell counting using trypan blue exclusion

A cell suspension was mixed at a 1:2 ratio with Trypan Blue and pipetted into a Fuchs-Rosenthal counting chamber with a chamber depth of 0.2 mm. Unstained (viable) cells in at least two of the 16 grid squares were counted, and the average cell number was calculated. Stained (non-viable) cells were excluded from the count. The cell concentration per milliliter was determined using the following formula:

$$\text{Cell conc. (cells/mL)} = \frac{\text{Average cell count per grid square}}{\text{Chamber depth (0.2 mm)}} \times \text{Dilution factor} \times 1000 \mu\text{L}$$

3.1.3 Cryopreservation and thawing of cells

Cells were harvested and counted prior to cryopreservation. After centrifugation at $650 \times g$ for 5 minutes (RT), the supernatant was discarded, and the cells were resuspended in a cryopreservation medium consisting of 10% DMSO in FCS. The cell suspension was aliquoted into 2 mL cryovials, with 1 mL per vial, and placed in a Mr. Frosty freezing container at -80°C for controlled-rate freezing. After 24 hours, the frozen cryovials were transferred to a liquid nitrogen storage tank for long-term preservation.

To recover cryopreserved cells, a cryovial was retrieved from liquid nitrogen storage and thawed in a 37°C water bath. The thawed cell suspension was immediately transferred to a 15 mL centrifuge tube containing 10 mL of culture medium and centrifuged at $650 \times g$ for 5 minutes (RT). The supernatant was discarded, and the cell pellet was resuspended in fresh medium. The resulting cell suspension was counted and transferred to a cell culture flask for subsequent cultivation.

3.1.4 Flow cytometry

Flow cytometry was used to analyze the expression of cell surface proteins or intracellular EGFP. Surface proteins were labeled via antibody staining, and positive and negative populations were identified using direct or indirect immunofluorescence. In direct immunofluorescence, antigen-specific antibodies are directly conjugated to fluorophores. In indirect immunofluorescence, fluorophore-conjugated secondary antibodies bind to primary antibodies that are specific to surface proteins but not conjugated to fluorophores. Single cells were passed through a flow cytometer, where they encountered a laser beam that excited the fluorophores, causing them to emit light. The emitted fluorescence was detected, providing information on whether antigen-specific primary or secondary antibodies were bound to the cell surface.

The antibodies used and their dilutions are listed in [Table 9](#). For antibody staining, single-cell suspensions were prepared at a concentration of 1×10^5 cells/100 μ L of medium and mixed with antibodies. The mixture was incubated for 20–30 minutes at 4°C or RT, based on the manufacturer’s recommendations for the antibodies used. After staining, cells were washed with 2 mL of FACS buffer, centrifuged at $650 \times g$ for 5 minutes, and the supernatant was discarded.

For directly conjugated antibodies, the cells were fixed at a concentration of 1×10^5 cells/100 μ L of fixation solution and stored at 4°C for up to 5 days before measurement. For non-conjugated primary antibodies, cells were resuspended in a solution of secondary antibodies and incubated for 30 minutes at 4°C. Afterward, cells were washed with 2 mL of FACS buffer, centrifuged at $650 \times g$ for 5 minutes, and the supernatant was discarded. The cells were then fixed at a concentration of 1×10^5 cells/100 μ L of fixation solution and stored at 4°C for up to 5 days prior to analysis.

3.1.4.1 Quantification of T cells in peripheral blood using counting bead

To determine absolute T-cell numbers in peripheral blood, flow cytometric analysis with counting beads (Latex Counting Beads, Invitrogen) was performed. Whole blood samples were collected from mice, and the volume of each sample was recorded in microliters (μ L). Red blood cells were lysed using RBC lysis buffer, followed by washing steps. The remaining cells were stained with fluorochrome-conjugated antibodies specific for relevant T-cell markers.

After staining, cells were fixed in 200 μ L of fixation buffer, and 10 μ L of counting beads were added to each sample. In parallel, a reference tube containing 10 μ L of beads in 200 μ L fixation buffer without cells was prepared and fully acquired on the flow cytometer to determine the total bead count per sample volume.

Flow cytometry was then performed on each cell-containing sample until 10,000 beads were acquired. Marker-positive cell events were recorded, and a correction factor was calculated by dividing the total number of beads per sample (from the reference tube) by 10,000. To calculate the absolute number of marker-positive cells per μ L of blood, the number of marker-positive events in the sample was divided by the original blood volume (in μ L) and multiplied by the correction factor. This allows for the determination of marker-positive T-cell concentration per microliter of blood. Flow cytometry data were analyzed using FlowJo software.

3.1.5 Single-cell sorting and clonal expansion

Flow cytometry can be adapted to include a sorting mechanism, allowing the physical separation and purification of specific cells based on their optical properties. In this study, EGFP expression was used to distinguish and isolate cells with higher transgene expression levels. A single-cell suspension at 10^6 cells/mL was prepared, and the top <5% of EGFP-expressing cells were gated and sorted into 96-well plates at a density of one cell per well. For establishing clonal populations of transduced 86b_KO cell lines, irradiated NIH-3T3 feeder cells (10,000 rad; 5,000 cells/well) were added; feeder cells were not used for B78.H1 sorting.

Following sorting, plates were screened by microscopy. Based on Poisson distribution, only ~10% of the wells were expected to contain growth, and wells containing a single visible colony were selected to ensure monoclonality. After 2–3 weeks, colony outgrowth was assessed, and monoclonal cultures were transferred first to 24-well plates and subsequently to 6-well plates for further expansion and downstream applications.

3.1.6 Isolation of buffy coat-derived PBMCs to obtain primary human T cells

To obtain primary T cells, PBMCs were isolated from the buffy coat provided by the Transfusion Center of the UMC Mainz. The buffy coat was diluted at a ratio of 1:3 with PBS. For density gradient centrifugation, 13 ml of Histopaque was placed in a 50 ml centrifuge tube and overlaid with 35 ml of PBS-diluted buffy coat. This was followed by centrifugation at $800 \times g$ for 20 minutes without brake. The layer of PBMCs, now located in the lower third of the 50 ml centrifuge tube, was aspirated using a rod pipette and collected. The PBMC layer was diluted at a ratio of 1:2 with PBS and centrifuged for 8 minutes at $650 \times g$. The supernatant was discarded, and the cell pellet was resuspended in 50 ml PBS. This was followed by centrifugation for 5 minutes at $450 \times g$. The supernatant was decanted, and the cell pellet was again resuspended in 50 ml PBS and centrifuged for 5 minutes at $650 \times g$. If the cell pellet was colorless at this stage, the PBMC isolation was complete. If the cell pellet was red, an erythrocyte lysis was performed.

The isolated PBMCs were either maintained in culture or cryopreserved for later use. For cultivation, cells were counted and plated at 2×10^6 cells/well (1×10^6 cells/mL) in a 24-well plate in Panserin complete medium supplemented with 30 ng/mL OKT3, 100 IU/mL IL-2, 10 ng/mL IL-7, and 10 ng/mL IL-15. Following 48 hours of incubation, cells were collected and washed thoroughly. This culture protocol effectively enriched the T-cell population, resulting in a predominance of CD3-positive T cells, with minimal contamination from other cell types.

3.1.6.1 Red blood cell (RBC) lysis

For RBC lysis, the cell pellet was resuspended in 5 ml RBC lysis buffer and incubated in a water bath at 37°C for 5 minutes. Subsequently, 45 ml PBS was added, and the sample was centrifuged for 5 minutes at $650 \times g$. If the cell pellet was still not colorless, the RBC lysis was repeated.

3.1.7 Retroviral transduction

3.1.7.1 Retroviral particle production

For human T-cell transduction, Ph-A cells were co-transfected with three plasmids. The essential viral genes required for retrovirus assembly and packaging were distributed across two helper plasmids: pHIT60, encoding the MLV-derived gag and pol genes, and pCOLT/GALV, encoding the gibbon ape leukemia virus (GALV) envelope protein [127]. Additionally, an expression plasmid encoding the gene(s) of interest, packaged into pMX-IRES-Puro_Dest #35 or pMX-Dest #3, was co-transfected into the cells.

For murine T cells, Ph-A cells were co-transfected with two plasmids. The viral genes gag, pol and env are encoded by the pCL-Eco plasmid. This vector features the human cytomegalovirus enhancer-promoter fused with the Moloney murine leukemia virus long terminal repeat at the TATA box in the 5' U3 region, forming the pCL promoter [128]. Additionally, an expression plasmid encoding the gene(s) of interest, packaged into pMX-IRES-Puro_Dest #35 or pMX-Dest #3, was co-transfected into the cells.

Retroviral particles were produced using the packaging cell line HEK-293 Phoenix-Ampho. On Day 0, 10 cm cell culture dishes were coated with 0.01% poly-L-lysine solution (6 mL per dish) for 1 hour at room temperature. Following incubation, the poly-L-lysine solution was removed, and the dishes were rinsed twice with 6 mL of PBS. To prevent drying, PBS was retained in the dishes until cell plating. Meanwhile,

Ph-A cells were detached using TrypLE Express dissociation reagent, pooled, and counted. A total of 4×10^6 cells were seeded in 6 mL of complete DMEM per poly-L-lysine-coated dish and incubated at 37°C. On Day 1, 30 minutes prior to transfection, the medium was replaced with 10 mL of pre-warmed DMEM without Penicillin-Streptomycin (Pen/Strep). Two solutions were prepared in non-polystyrene tubes for the transfection mix. For the transfection of human T cells, Mix A contains 1 mL Opti-MEM, 9 µg retroviral vector plasmid, 4 µg pHIT60, and 4 µg pCOLT/GALV. For the transduction of murine T cells, Mix A contains 1 mL Opti-MEM, 10 µg retroviral vector plasmid, and 7 µg pCL-Eco. For both transfections, Mix B contains 1 mL Opti-MEM and 50 µL Lipofectamine (the optimal amount of Lipofectamine should be pre-determined by titration with each new lot). Mix A and Mix B were incubated separately for 5 minutes at room temperature, then combined gently (without vortexing) and further incubated for 20 minutes at room temperature. The transfection mixture was added dropwise to the Ph-A cells, and the dishes were gently swirled to ensure even distribution. Plates were incubated at 37°C for 6–8 hours or overnight if the transfection was initiated late. After incubation, the medium was replaced with 10 mL of fresh DMEM without Pen/Strep, and cells were further incubated at 37°C.

At 48 hours after transfection, the retroviral supernatant was harvested and centrifuged at $900 \times g$ for 10 minutes to remove cells and sterile-filtered through a 0.45 µm filter after centrifugation. The prepared retroviral supernatant was then ready for subsequent transduction experiments.

3.1.7.2 Retroviral transduction of human T cells

On day 0, fresh PBMCs were isolated from buffy coat using density gradient centrifugation as described in chapter 3.1.6, counted and plated at 2×10^6 cells/well (1×10^6 cells/mL) in a 24-well plate in Panserin complete medium supplemented with 30 ng/mL OKT3, 100 IU/mL IL-2, 10 ng/mL IL-7, and 10 ng/mL IL-15. For retronectin coating, non-tissue culture-treated 24-well plates were coated on day 1 with retronectin at a final concentration of 20 µg/mL in PBS (1 mL/well) and incubated overnight at 4°C. Plates were prepared only for the number of wells needed for transduction. On day 2, the retronectin solution was removed from the wells, pooled for reuse (up to four times), and replaced with 1 mL/well of 2% BSA in PBS. After a 30-minute incubation at room temperature, the BSA solution was removed, and wells were washed twice with PBS. PBS was left in the wells until the retroviral supernatant was added to prevent drying. Retroviral supernatant (1–2 mL/well) was added, and the plates were centrifuged for 2 hours at $2000 \times g$ at 32°C. During centrifugation, PBMCs (predominantly CD3⁺ T cells at this point) were harvested, washed, and counted for trans-

duction. Control wells without retroviral supernatant were included as untransduced controls. After centrifugation, the viral supernatant was removed, and 2×10^6 T cells/well (1×10^6 cells/mL) were added in Panserin complete medium supplemented with 100 IU/mL IL-2, 10 ng/mL IL-7, and 10 ng/mL IL-15. Plates were centrifuged at $650 \times g$ for 5 minutes and incubated at 37°C . After 24h of incubation (day 3), T cells were removed from the retronectin-coated wells and transferred to tissue culture-treated 24-well plates. For puromycin selection, 2×10^6 T cells/well were plated and treated with 1 $\mu\text{g/mL}$ puromycin. If no selection was performed, $1\text{--}1.5 \times 10^6$ T cells/well were plated. Antigen-specific stimulation was carried out by adding $0.1\text{--}0.2 \times 10^6$ irradiated (10,000 rad) antigen-specific stimulator cells per 1×10^6 T cells/well and incubated at 37°C . Flow cytometry analysis was performed on day 6 after transduction to assess transduction efficiency.

3.1.7.3 CRISPR/Cas9-mediated knockout of endogenous murine TCR and retroviral transduction

TCR engineering of primary murine T cells was performed in two sequential steps: (1) CRISPR–Cas9 RNP-mediated knockout of the endogenous TCR α - and β -chain constant regions, followed by (2) retroviral transduction with vectors encoding the chimeric TCR. To prevent mispairing between endogenous murine TCR chains and the transduced chimeric TCR (cTCR), the genes encoding the TCR α (mTRAC) and TCR β (mTRBC) chains were knocked out using a ribonucleoprotein (RNP)-based CRISPR/Cas9 approach. Splenocytes were freshly isolated from C57BL/6 mice using a standard mechanical dissociation protocol and filtered through a $70\text{ }\mu\text{m}$ cell strainer. Erythrocytes were lysed using RBC lysis buffer, and cells were washed with PBS and resuspended in complete mouse T cell medium. Cells were seeded at a density of 3×10^6 cells/mL (12×10^6 cells/well) in a 6-well plate in a total volume of 4 mL, supplemented with 10% T-cell growth factor (TCGF; supernatant from ConA-stimulated rat splenocytes) and $2\text{ }\mu\text{g/mL}$ Concanavalin A (ConA, Sigma-Aldrich), and incubated for 24 hours at 37°C and 5% CO_2 .

In parallel, non-tissue culture-treated 6-well plates were coated with 2 mL per well of retronectin ($20\text{ }\mu\text{g/mL}$ in PBS) and incubated overnight at 4°C . On the following day, retronectin solution was removed, collected for reuse (stored at -20°C , reused up to four times), and replaced with 2 mL/well of 2% BSA in PBS to block non-specific binding. After 30 minutes at room temperature, wells were washed 1–2 times with PBS (avoiding drying of the coated surface), and 2.5 mL of retroviral supernatant was added to each well. Plates were centrifuged at $2000 \times g$ for 2 hours at 32°C to facilitate viral adherence and either held at 37°C until use or used immediately.

For electroporation, the Lonza Mouse T Cell Nucleofector Kit (Catalog #: VPA-1006) was used. Splenocytes (predominantly CD3-positive T cells at this point) were collected by centrifugation ($700 \times g$, 5 min), and the supernatant was retained as preconditioned medium. Cells were washed twice in PBS ($300 \times g$, 10 min) and resuspended at a concentration of 5×10^6 cells per 100 μ L nucleofection buffer (82 μ L Nucleofector Solution + 18 μ L Supplement B per reaction), kept on ice until use.

The crRNAs targeting murine *Trac* and *Trbc* were selected based on optimal on- and off-target scores, as predicted with tools from IDT, Benchling and CRISPOR, and validated experimentally in the lab. The specific sequences are provided in [2.13](#). For RNP complex formation, 1 μ L crRNA and 1 μ L tracrRNA (both 100 μ M, IDT) were mixed per target and heated to 95°C for 5 minutes, then cooled to room temperature to form the duplex. Each crRNA:tracrRNA duplex (1.2 μ L) was combined with 1.7 μ L Cas9 enzyme (Alt-R S.p. Cas9 Nuclease V3, IDT) and 2.1 μ L Opti-MEM, then incubated at room temperature for 10–20 minutes to form the RNP complex.

For each electroporation, 5 μ L (4 μ M) of RNP targeting mTRAC and 5 μ L (4 μ M) targeting mTRBC were combined with 1 μ L electroporation enhancer (IDT) in a 0.2 mL microtube. Then, 100 μ L of the T-cell suspension was added, mixed gently, and the mixture was transferred into a 2 mm gap electroporation cuvette. Air bubbles were avoided, and cuvettes were gently tapped to ensure uniform distribution of the mixture. Electroporation was performed using the Nucleofector 2b Device (Lonza) with program X-001. Immediately after electroporation, 500 μ L of pre-warmed preconditioned medium was added to each cuvette, followed by a 5-minute incubation at 37°C. Cells were then transferred into 1 mL of pre-warmed preconditioned medium and further diluted with complete mouse T cell medium to a final volume of 4 mL.

Following electroporation, the cells were transferred onto retronectin-coated wells containing pre-centrifuged retroviral particles. A portion of the electroporated cells was set aside as an untransduced KO-only control. Cells were centrifuged at $700 \times g$ for 5 minutes to enhance contact with the viral particles and incubated overnight at 37°C, 5% CO₂. A second round of transduction was performed the following day to increase transduction efficiency, after which cells were further expanded and used for downstream *in vitro* assays or adoptive transfer.

3.1.8 Lentiviral transduction

Lentiviral transduction was employed to stably modify tumor cells with reporter and antigen-expression constructs. Tumor cells were engineered to express NLUC for bioluminescence-based tracking, and in selected experiments, full-length human TRP2 to generate antigen-overexpressing variants for *in vivo* studies.

Lentiviral particles were produced using HEK-293FT cells. A total of 1×10^6 cells were seeded in 6 mL of complete DMEM medium in 10 cm culture dishes one day prior to transfection. On the day of transfection, approximately 4 hours before the procedure, the old medium was removed and replaced with 6 mL of fresh DMEM complete medium. HEK-293FT cells were co-transfected with three plasmids, each encoding essential components for lentivirus production. The packaging plasmid pSPAX2 supplies the HIV-1 gag and pol genes, while the envelope plasmid pMD2.G encodes the vesicular stomatitis virus G (VSV-G) envelope protein [129]. The expression plasmid, pLenti-EF1 α -PAC_WPRE #4, contained the gene(s) of interest. For transfection, a mixture was prepared for each dish. A total of 18 μ L of TransIT-LT1 reagent (Mirus) was diluted in 270 μ L of DMEM medium without FBS or antibiotics and incubated at room temperature for 5 minutes. Subsequently, a second mixture consisting of 20 μ L DMEM medium, 1.8 μ g of pSPAX2, 300 ng of pMD2.G, and 3 μ g of the expression plasmid were added to the diluted TransIT-LT1, and the solution was incubated for another 20 minutes. The transfection mixture was then added to the cells, and the dishes were gently shaken to ensure even distribution. The plates were incubated overnight at 37°C. Approximately 16 hours after transfection, the medium was replaced with 4 mL of RPMI complete medium. The supernatant containing lentiviral particles was harvested 30–36 hours later and centrifuged at $800 \times g$ for 10 minutes to remove residual producer cells. The resulting supernatant was filtered through a 0.45 μ m filter to ensure the removal of any remaining producer cells and debris. The filtered lentiviral supernatant was diluted at a ratio of 1:2 with RPMI complete medium, and 5 μ g/mL polybrene was added to enhance transduction efficiency. The prepared lentiviral mixture was either applied directly to adherent target cells, which had been seeded one day prior to transduction at a confluence of 60–70%, or used to transduce freshly harvested suspension target cells. For the latter, 1×10^6 target cells were pelleted, resuspended in 4 mL of the filtered and diluted lentiviral supernatant, and subsequently plated in 6-well plates, with 5×10^5 cells in 2 mL of lentiviral supernatant per well. Spinoculation was performed by centrifuging the plates at $800 \times g$ for 90 minutes. After a 24-hour incubation, the cells were harvested, centrifuged at $650 \times g$ for 5 minutes, and washed to remove the lentiviral supernatant. The cells were then resuspended in RPMI complete medium supplemented with 1 μ g/mL puromycin for selection of successfully transduced cells. Transduction efficiency was assessed via flow cytometry approximately 2 weeks after transduction.

The pLenti vector constructs included the Woodchuck Hepatitis Virus Post-transcriptional Regulatory Element (WPRE), a commonly used sequence to enhance transgene expression. As shown by Higashimoto et al., WPRE improves transcript termination by reducing readthrough transcription from retroviral long terminal repeats (LTRs), particularly in gamma-retroviral vectors. This results in increased levels of total viral mRNA and higher vector titers. The effect is attributed not to enhanced transcription or mRNA stability but rather to improved polyadenylation efficiency, making WPRE a valuable tool for optimizing gene expression in retroviral and lentiviral systems [120].

3.1.9 IFN- γ ELISpot assay

An enzyme-linked immunospot (ELISpot) assay was performed to assess antigen-specific T-cell reactivity by quantifying interferon-gamma (IFN- γ) secretion upon co-culture with target cells. The assay was conducted using ELISpot plates equipped with polyvinylidene fluoride (PVDF) membranes (Multiscreen Filter Plates, Merck Millipore). PVDF membranes were activated by adding 20 μ L of freshly prepared 35% ethanol to each well. The ethanol was immediately removed, and wells were washed three times with 150 μ L of sterile phosphate-buffered saline (PBS) to remove residual ethanol and prepare the membrane for antibody coating. Subsequently, 50 μ L of PBS containing anti-IFN- γ capture antibody (10 μ g/mL anti-human IFN- γ mAb clone 1-D1K; Mabtech or 1 μ g/mL anti-murine IFN- γ mAb clone AN18; Mabtech) was added to each well. Plates were sealed and incubated overnight at 4°C in the dark to allow for optimal antibody binding.

On the following day, plates were washed three times with 150 μ L PBS to remove unbound antibody. Target cells were harvested, washed once with complete RPMI-1640 medium (RPMI + 10% FBS + 1% Penicillin/Streptomycin), and centrifuged at 650 $\times$ g for 5 minutes. After counting, target cells were resuspended at the desired concentration in RPMI complete medium and seeded into the ELISpot wells in 50 μ L volumes. Effector cells (human or murine T cells) were also washed in complete RPMI, and their percentage of cTCR⁺ cells was determined via flow cytometry prior to plating. This percentage was used to calculate the number of total effector cells needed per well to yield the desired number of cTCR⁺ cells (typically 10,000–20,000 cTCR⁺ cells/well in 50 μ L RPMI). For instance, if the transduction efficiency was 50%, 20,000 total T cells were seeded to achieve 10,000 cTCR⁺ T cells. Effector and target cells were co-cultured in a final volume of 100–150 μ L/well. Control wells containing only effector cells were included to assess background cytokine secretion.

Plates were incubated for 18-20 hours at 37°C and 5% CO₂. After incubation, wells were washed six times with PBS containing 0.05% Tween-20 (PBST) to remove non-adherent cells and cytokines. A biotinylated anti-IFN- γ detection antibody (human: clone 7-B6-1 from Mabtech; mouse: clone XMG1.2 from Leinco Technologies) was diluted to 2 μ g/mL in PBS supplemented with 0.5% bovine serum albumin (BSA). 60 μ L of the detection antibody solution was added per well, and plates were incubated for 2 hours at 37°C. During the secondary incubation, an avidin-peroxidase complex was prepared using the VECTASTAIN® Elite® ABC Kit (Vector Laboratories). One drop each of reagent A and B was added to 10 mL of PBS containing 0.1% Tween-20, mixed well and incubated for 30 minutes at room temperature in the dark prior to use. Following the 2-hour antibody incubation, plates were washed six times with PBST and then incubated for 1 hour at room temperature with 95 μ L of the freshly prepared avidin-peroxidase solution per well in the dark.

The chromogenic substrate was prepared by dissolving a 3-amino-9-ethylcarbazole (AEC) tablet in 2.5 mL N,N-dimethylformamide (DMF), followed by the addition of 47.5 mL of ELISpot buffer, filtration through a 0.40 μ m filter, and final supplementation with 25 μ L of 30% H₂O₂. After removal of the enzyme solution, plates were washed three times with PBST and three times with PBS. 100 μ L of the AEC substrate solution was then added to each well and incubated for 10–20 minutes at room temperature until visible dark red spots formed. The reaction was stopped by rinsing the plates thoroughly with tap water. Plates were dried at 50°C for 1–2 hours or left to air-dry overnight before analysis.

Spot formation, corresponding to individual IFN- γ -secreting cells, was quantified using an automated ELISpot reader (e.g., ImmunoSpot® Analyzer, CTL) equipped with ImmunoSpot® software.

3.1.10 Production of TCGF medium

TCGF (T-cell growth factor) medium, enriched with cytokines and growth factors, was prepared to support the long-term *in vitro* culture of murine T cells. This medium was produced by culturing splenocytes isolated from female Lewis rats (8–10 months old). Rats were euthanized via CO₂ inhalation, and splenocytes were harvested through mechanical dissociation. On average, 250 million splenocytes were obtained per spleen. The cells were counted and cultured at a concentration of 7 million cells/mL in T75 flasks containing a total volume of 50 mL complete mouse T cell medium, freshly supplemented with 5 μ g/mL ConA. Cultures were incubated at 37°C with 5% CO₂ for 24 hours. After incubation, the cells were harvested and centrifuged at 320 $\times$ g for 10 minutes at RT, and the cell-free supernatant was col-

lected and stored at 4°C. The splenocytes were then resuspended in 50 mL of fresh rat medium and cultured for an additional 24 hours under identical conditions. The supernatant was collected as before, and the two batches were pooled. To the pooled supernatant, methyl- α -D-mannopyranoside was added at a final concentration of 1 g per 100 mL. After the solute dissolved completely, the supernatant was sterile-filtered and stored as aliquots at -20°C until use.

3.1.11 Isolation and cultivation of mouse splenocytes

To isolate splenocytes, the mouse was euthanized via cervical dislocation, and the flank was sprayed with 70% ethanol. Using sterile scissors and forceps, an incision was made on the left side of the mouse to expose and remove the spleen. The spleen was processed using a 100 μ m cell strainer placed over a 50 mL Falcon tube, pre-wetted with 5 mL of Mouse T cell medium. The spleen was ground on a metal grid in a Petri dish containing 5 mL of Mouse T cell medium using the plunger of a 5 mL syringe. The suspension was then filtered through the 100 μ m strainer. The grid and Petri dish were washed with an additional 10 mL of Mouse T cell medium, and the cell suspension was centrifuged at $650 \times g$ for 5 minutes at RT. The resulting cell pellet was resuspended in 5–10 mL of erythrocyte lysis buffer and incubated at 37°C for 5 minutes to lyse red blood cells. The cells were then washed with 25 mL of Mouse T cell medium and centrifuged again at $650 \times g$ for 5 minutes. The pellet was resuspended in Mouse T cell medium, and the cells were counted. After counting, 12×10^6 splenocytes were seeded per well in a 6-well plate with 4 mL of Mouse T cell medium supplemented with 10% TCGF and 2 μ g/mL Concanavalin A (Con A) for 24 hours. This culture protocol effectively enriched the T-cell population, resulting in a predominance of CD3-positive T cells, with minimal contamination from other cell types.

3.1.12 Bioluminescence-based killing assay

To assess the cytolytic activity of transgenic T cells, a bioluminescence-based assay was performed using firefly luciferase (FLUC)-expressing tumor cell lines. FLUC catalyzes the oxidation of D-luciferin in the presence of ATP, oxygen, and Mg^{2+} , producing luminescence proportional to the number of viable cells. Upon co-culture with T cells, target cell lysis leads to a reduction in luminescence intensity.

FLUC-expressing tumor cells were seeded in triplicate at 10,000 cells per well (100 μ L of 1×10^5 cells/mL) in black, flat-bottom 96-well plates. Transduced T cells were added at varying effector-to-target (E:T) ratios in an additional 100 μ L of RPMI complete medium. D-luciferin was added at a final concentration of 0,25 mg/mL, and plates were centrifuged at $650 \times g$ for 2 minutes before incubation for 24 hours at 37°C. Luminescence was recorded at defined time points using the FLUOstar Omega microplate reader maintained at 37°C.

Wells containing tumor cells only served as maximum luminescence controls (0% cell lysis), while tumor cells treated with a lethal dose of digitonin (30 μ g/mL) served as minimum luminescence controls (100% cell lysis). Tumor cell lysis [%] was calculated using the formula:

$$\text{Specific lysis} = \frac{(1 - (\text{CPS}_{\text{experimental}} - \text{CPS}_{\text{MIN}}))}{(\text{CPS}_{\text{MAX}} - \text{CPS}_{\text{MIN}})} \times 100$$

CPS = counts per second (of raw light)

$\text{CPS}_{\text{experimental}}$ = luminescence from co-culture (effector + target)

CPS_{MAX} = luminescence from target cells alone (no lysis/maximal luminescence)

CPS_{MIN} = luminescence from target cells treated with digitonin (maximum lysis/minimal luminescence)

3.2 Microbiology and molecular biology methods

3.2.1 Cryopreservation and thawing of E. coli

For long-term storage, E. coli cultures were cryopreserved in 15% glycerol. A total of 750 μ L of bacterial over night culture was mixed with 250 μ L of 60% glycerol, transferred to a 2 mL cryovial, and frozen in a Mr. Frosty freezing container at -80°C. To revive cryopreserved bacteria, a disposable inoculation loop was used to scrape frozen bacteria from a 2 mL cryovial. The scraped bacteria were transferred into 5 mL of LB medium supplemented with 100 μ g/mL Kanamycin or 100 μ g/mL Ampicillin.

3.2.2 Preparation of LB agar plates

LB agar was melted and cooled to approximately 50 °C before the addition of antibiotics at a final concentration of 100 μ g/mL (kanamycin or ampicillin). Approximately 25 mL of agar was poured per plate and allowed to solidify. Plates were either used immediately or stored at 4 °C for up to two weeks, depending on the application.

3.2.3 Generation of chemically competent One Shot Stbl3 *E. coli*

E. coli bacteria were streaked onto LB agar plates without antibiotics using a dilution streak method and incubated overnight at 37°C. The following day, a single colony was inoculated into 3 mL of sterile LB medium and incubated overnight at 37°C with shaking at 225 rpm. Buffers RF1 and RF2 were pre-chilled at 4°C overnight. All subsequent steps were performed on ice. One milliliter of the overnight culture was added to 1000 mL of LB medium and incubated until the culture reached an OD₆₀₀ of 0.5–0.6. The bacterial culture was then transferred to 50 mL centrifuge tubes, incubated on ice for 10–15 minutes, and centrifuged at 2700 × g and 4°C for 10 minutes. The supernatant was discarded, and the bacterial pellet was gently resuspended in 33 mL of cold RF1 buffer. After centrifugation at 580 × g and 4°C for 15 minutes, the supernatant was removed, and the pellet was carefully resuspended in 4 mL of cold RF2 buffer. The suspension was incubated on ice for 15 minutes. The bacteria were aliquoted into 50 µL portions, immediately frozen in liquid nitrogen or on dry ice, and stored at -80°C.

3.2.4 Transformation of chemically competent One Shot Stbl3 *E. coli*

Chemically competent One Shot Stbl3 *E. coli* were thawed on ice prior to use. For transformation of plasmid DNA, approximately 1 ng DNA in ≤5 µL volume was added to 50 µL bacteria. For Gateway BP/LR reactions, 50–150 ng of reaction product (≤5 µL) was added instead. Samples were incubated on ice for 30 minutes, followed by heat shock at 42 °C for 30 seconds and a 2-minute incubation on ice. Subsequently, 250 µL pre-warmed SOC medium was added, and cells were incubated at 37 °C with shaking (225 rpm) for 60 minutes. Then, 50–250 µL of the culture was plated on LB-agar plates containing the appropriate antibiotic and incubated at 32 °C for 18–20 hours.

3.2.5 Colony PCR

Individual bacterial colonies were picked from an LB agar plate and resuspended in 100 µL of LB medium containing the appropriate antibiotic. For the colony PCR, 10 µL of this bacterial suspension was transferred into a 0.2 mL reaction tube and centrifuged at 10,000 × g for 10 minutes. The supernatant was removed, and the bacterial pellet was resuspended in a PCR reaction mixture consisting of 10 µL OneTaq Hot Start Reaction Buffer, 8 µL nuclease-free water, 1 µL forward primer (10 nM), and 1 µL reverse primer (10 nM). The mixture was briefly vortexed and centrifuged to collect the contents at the bottom of the tube. The PCR program consisted of the following

steps: initial denaturation at 98°C for 2 minutes; 30–35 cycles of denaturation at 98°C for 10 seconds, primer annealing at a temperature determined by the melting temperature of the primers for 20–30 seconds, and elongation at 68°C for 60 seconds per kilobase of the target DNA sequence. The reaction was held at 4°C upon completion.

3.2.6 PCR amplification using Q5 high-fidelity DNA polymerase

PCR amplification of the gene of interest was performed using Q5 High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA). Each 50 µL reaction contained 35.5 µL nuclease-free water, 5 µL 10× Q5 Reaction Buffer, 5 µL Q5 High GC Enhancer, 1 µL dNTP mix (10 mM each), 1 µL of each gene-specific primer, 1 µL plasmid DNA (5 ng), and 0.5 µL Q5 DNA Polymerase (2 U/µL; final 1 U per reaction). Amplification was carried out in a thermal cycler using a two-step touchdown protocol to improve specificity. This approach was necessary because the primers carried 5′ overhangs for Gateway or NEBuilder assembly, meaning only the 3′ region of each primer was complementary to the template. Initial high-stringency cycles ensured specific annealing of the template-matched region, while subsequent lower-stringency cycles facilitated efficient amplification once the overhang had been incorporated. The higher annealing temperature (T_{m1}) was chosen based on the melting temperature of the template-complementary 3′ primer region, and the lower temperature (T_{m2}) was typically set 4–6 °C below T_{m1} .

After an initial denaturation at 98 °C for 30 s, seven cycles were performed at the higher annealing temperature (T_{m1}), followed by 25 cycles at the lower annealing temperature (T_{m2}). Each cycle consisted of denaturation at 98 °C for 10 s, annealing at the respective temperature for 20–30 s, and extension at 72 °C for 30 s to 1 min depending on amplicon length (calculated at approximately 30 s per 1 kb), with a final extension at 72 °C for 2–5 min. PCR products were analyzed by agarose gel electrophoresis, and bands of the expected size were purified directly or gel-extracted for downstream Gateway recombination or NEBuilder assembly.

3.2.7 Site-directed mutagenesis PCR

For amplification of a FLUC fragment from the plasmid pSSV9_psiCheck #0714 (kindly provided by [REDACTED], University of Heidelberg) combined with site-directed mutagenesis (SDM), the standard Q5 High-Fidelity DNA Polymerase protocol (New England Biolabs) was modified. SDM required the introduction of a point mutation within the FLUC sequence, therefore a mutagenic primer

was designed with a higher annealing temperature than the flanking amplification primers (including an additional temperature offset of approximately +3 °C to accommodate the mismatch), ensuring preferential binding of the SDM primer during the first amplification cycles.

Each 50 µL reaction contained 1 ng pSSV9_psiCheck template DNA, 10 µL 5× Q5 Reaction Buffer, 10 µL 5× Q5 High GC Enhancer, 1 µL dNTP mix (10 mM each), 1 µL forward primer 1 (5 µM), 4 µL mutagenic forward primer 2 (5 µM), 1 µL reverse primer (5 µM), and 0.5 µL Q5 High-Fidelity DNA Polymerase (2000 U/mL; 1 U per reaction), adjusted to 50 µL with nuclease-free water. Reactions were gently mixed, briefly centrifuged, and run on a thermal cycler with the following program: initial denaturation at 98 °C for 2 min; 35 cycles of 98 °C for 10 s, 66 °C for 20 s, and 72 °C for 30 s; followed by a hold at 4 °C.

Because the goal of this reaction was to generate a mutated FLUC amplicon and remove the parental plasmid template, PCR products were separated by agarose gel electrophoresis and the FLUC band was specifically gel-extracted prior to downstream cloning. This step ensured elimination of template plasmid carry-over and enriched for the mutagenized FLUC fragment.

3.2.8 Purification of PCR products

PCR products were purified using the Macherey-Nagel™ NucleoSpin™ Gel and PCR Clean-up Kit following the manufacturer's protocol.

3.2.9 Plasmid isolation

Plasmid DNA was isolated using the Macherey-Nagel™ NucleoSpin™ Plasmid (NoLid) Kit for overnight cultures up to 10 mL and the QIAGEN Plasmid Plus Midi Kit for overnight cultures up to 100 mL, according to the manufacturer's instructions.

3.2.10 Gel extraction

DNA fragments were extracted from agarose gels using the Monarch DNA Gel Extraction Kit from New England Biolabs, following the protocol provided by the manufacturer.

3.2.11 DNA quantification using Qubit fluorometry

DNA concentrations were measured using the Qubit 2.0 Fluorometer (Thermo Fisher Scientific) in combination with the Qubit dsDNA BR (Broad Range) Assay Kit, following the manufacturer's protocol. This fluorescence-based method utilizes a DNA-selective dye that specifically binds to double-stranded DNA (dsDNA). Upon binding, the dye emits a fluorescence signal that is proportional to the amount of dsDNA in the sample. For each measurement, a working solution was prepared by diluting the Qubit reagent in the supplied buffer. DNA standards and samples (1–10 μL) were then mixed with the working solution to a final volume of 200 μL , vortexed briefly, and incubated at room temperature for 2 minutes. Fluorescence was subsequently measured using the Qubit 2.0 instrument, and concentrations were calculated automatically based on the standard curve generated from the provided DNA standards.

3.2.12 DNA sequencing

DNA sequencing was performed using the Mix2Seq Kit (Eurofins Genomics), following the manufacturer's instructions.

3.2.13 Agarose gel electrophoresis

DNA samples were prepared by diluting 10–500 ng of DNA, depending on fragment size (typically ~10–50 ng for PCR products and up to 500 ng for plasmid DNA), with nuclease-free water to a final volume of 10 μL . Subsequently, 2 μL of 6x Purple Loading Dye was added and mixed thoroughly.

To prepare the agarose gel, 1–2 g agarose was dissolved in 100 mL 0.5x TBE buffer in an Erlenmeyer flask and heated in a microwave until fully dissolved. Afterward, 1 μL PegGreen was added, briefly reheated to ensure uniform distribution, and evaporated water was replenished by weighing the flask and adding distilled water if necessary. The melted agarose was poured into a casting tray and allowed to solidify at room temperature. Gel concentration (1–2%) was chosen according to the expected DNA fragment size (1% for ≥ 1 kb, 2% for < 500 bp).

DNA samples and a molecular weight ladder were loaded into the wells, and electrophoresis was performed at ~12.8 V/cm in a standard gel chamber. DNA bands were visualized using a UVsolo TS Imaging System (Biometra).

3.2.14 Restriction enzyme digestion

Plasmid digestion was performed using high-fidelity restriction enzymes from New England Biolabs under standard conditions. For digestion of pLenti-EF1a_NLUC_PAC_WPRE (#6.6), 5 µg of plasmid DNA (1.8 µg/µL; 3 µL) was incubated with SmaI (12 U/µL; 4 µL initial + 4 µL after 1 h; 96 U total) in a 70 µL reaction volume containing 7 µL CutSmart buffer (10x) and nuclease-free water. The reaction was incubated at 25 °C for 1 hour, followed by the addition of the second enzyme aliquot and a further 1 hour incubation at 25 °C. For digestion of pLenti-EF1a_NLUC_EGFP_PAC_WPRE (#6.6.5), 5 µg of plasmid DNA (1.8 µg/µL; 3 µL) was treated with SgrDI (12 U/µL; 4 µL initial + 4 µL after 1.5 h; 96 U total). The reaction was incubated at 25 °C for 1.5 hours, followed by the addition of the second enzyme aliquot and an additional 1.5 hours of incubation. In both cases, digested DNA was resolved by agarose gel electrophoresis and extracted using the NEB Monarch DNA Gel Extraction Kit. DNA concentrations were determined using a Qubit 2.0 Fluorometer prior to downstream applications.

3.2.15 DNA assembly using NEBuilder® HiFi DNA Assembly

Seamless DNA fragment assembly was performed using the NEBuilder® HiFi DNA Assembly Kit (New England Biolabs), which enables the efficient and accurate joining of multiple DNA fragments in a single-tube, isothermal reaction. This method relies on short sequence overlaps (typically 15–40 bp) between adjacent fragments, which were incorporated during PCR amplification or fragment design. For each reaction, DNA fragments were mixed in equimolar or vector:insert ratios of 1:2 in a final volume of 10 µL. The mixture included 5 µL of NEBuilder HiFi DNA Assembly Master Mix and was incubated at 50 °C for 2 hours. Following assembly of pLenti constructs, 5–10 µL of the reaction mixture was transformed into chemically competent *E. coli* cells using heat shock and plated on ampicillin-supplemented LB agar. Correct assembly was confirmed by colony PCR and Sanger sequencing.

3.2.16 Gateway cloning

The Gateway recombination cloning system (Thermo Fisher Scientific) was used for site-specific transfer of DNA fragments between plasmid vectors, based on the att site-mediated recombination mechanism derived from bacteriophage lambda. This approach enables the efficient and directional insertion of a gene of interest (GOI) into various destination vectors without the need for restriction enzyme digestion or lig-

ation. To generate the Entry Clone, the GOI was first amplified by PCR, as described in chapter 3.2.6, using primers flanked by attB1 and attB2 recombination sequences. 150 ng of the PCR product was combined with 150 ng of the donor vector pDONR221, which contains attP sites, and 1 μ L of the BP Clonase II enzyme mix in a final volume of 5 μ L. The reaction was incubated at 25 °C for 1-3 hours or over night, followed by the addition of 1 μ L Proteinase K and incubation at 37 °C for 10 minutes to inactivate the recombinase enzymes, and subsequently transformed into *E. coli* cells. Colonies were selected on freshly prepared kanamycin-containing agar plates, and correct clones were verified by colony PCR and Sanger sequencing. To generate the expression clone, 150 ng of the confirmed Entry Clone was combined with 150 ng of the destination vector pMX-DEST and 1 μ L of the LR Clonase II enzyme mix in a final volume of 5 μ L. The LR reaction facilitates recombination between the attL sites in the entry vector and the attR sites in the destination vector, replacing the ccdB cassette with the GOI. The reaction was incubated at e.g., 25 °C for 1-3 hours, followed by the addition of 1 μ L Proteinase K and incubation at 37 °C for 10 minutes to inactivate the recombinase enzymes, and transformed into *E. coli*. Positive clones were selected on freshly prepared ampicillin-containing plates, and the presence and orientation of the insert were confirmed by colony PCR and sequencing. The resulting expression constructs were used for retroviral packaging and mammalian cell transfection.

3.3 Animal experimental procedures

3.3.1 Subcutaneous engraftment of a tumor cell suspension

The mice were individually removed from their cages and anesthetized with isoflurane (1.5% v/v). The fur at the injection site was removed by shaving with an electric clipper, and the skin was sterilized using a 70% ethanol solution. The skin was then gently lifted at the injection site with blunt forceps, and $0.2\text{--}1 \times 10^6$ tumor cells suspended in 50–100 μ L PBS were injected subcutaneously. Following the injection, the animals were returned to their cages.

3.3.2 *In vivo* bioluminescence imaging (BLI)

For BLI, mice were anesthetized with isoflurane (1.5% v/v) and received an intraperitoneal injection of 100 μ L substrate solution in PBS or dH₂O. Imaging was initiated immediately after substrate administration while animals remained under anesthesia. Each imaging session was limited to a maximum of 15 minutes, after which mice were returned to their cages.

For visualization of tumor cells expressing NanoLuc luciferase, Furimazine (Nano-Glo substrate) was diluted 1:40 in PBS and injected. For visualization of T cells expressing firefly luciferase, D-luciferin was administered at 2.5 mg per 100 μ L dH₂O.

3.3.3 Sublethal whole-body irradiation (TBI)

C57BL/6 animals received a sublethal whole-body irradiation dose of 5 Gray (Gy). For this procedure, mice were placed in their cages within a small-animal irradiation system. The irradiation process lasted no longer than 10 minutes per cage, and animals were observed for general well-being before being returned to their housing.

3.3.4 T-cell transfer

Mice were first warmed in their cages using infrared lamps. For the T-cell transfer, each animal was individually restrained. A maximum of 20×10^6 T cells, suspended in 100 μ l PBS, were administered via intravenous injection into one of the lateral tail veins using a 30G needle. In addition, 7.2×10^5 units of IL-2 in 100 μ l PBS were injected intraperitoneally. After the injections, animals were returned to their cages.

4 Results

4.1 Generation of vector constructs for tumor and T-cell *in vivo* bioluminescence imaging

To perform preclinical evaluation of HLA-independent cTCR-transduced T cells targeting TRP2, vector constructs were generated for both TCR expression in T cells and TRP2 antigen expression in tumor cells. Following successful validation, these constructs were employed in subsequent xenograft and syngeneic T-cell transfer experiments using subcutaneous melanoma models.

4.1.1 Retroviral vectors for bioluminescent T-cell imaging

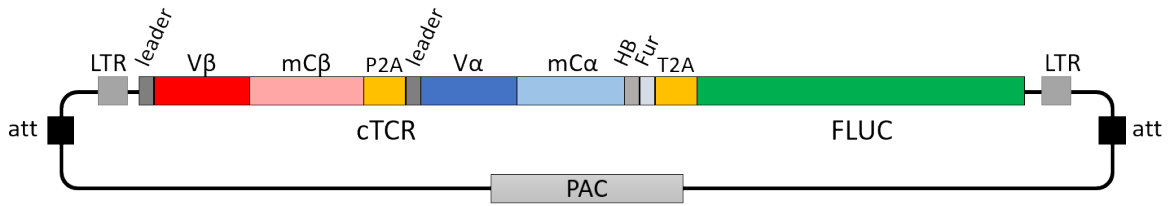
To enable *in vivo* BLI of TCR-transduced T cells, a series of retroviral vector constructs encoding chimeric T-cell receptors (cTCRs) and a firefly luciferase (FLUC) reporter were generated using Gateway recombination as described in chapter 3.2.16, and the NEBuilder HiFi DNA Assembly method as described in chapter 3.2.15. Initial constructs were based on existing pDONR221 entry clones containing either anti-TRP2 or anti-NY-ESO-1 cTCRs in a bicistronic configuration (TRBV_mTRBC_P2A_TRAV_mTRAC) that had previously been recombined into pMX-DEST vectors with or without a puromycin resistance gene (#35 and #3, respectively). For BLI-capable constructs, new entry clones encoding cTCR and FLUC sequences were created.

PCR amplification of the cTCR fragments was performed as described in chapter 3.2.6 using forward primers containing an attB1 site and FLUC-reverse primers encoding a T2A linker followed by a helix breaker (HB) and a furin (FUR) cleavage site. FLUC was amplified using a forward primer incorporating a FUR_HB_T2A-linker sequence and a reverse primer containing an attB2 site and two distinct consecutive stop codons. For the generation of cTCR-FLUC constructs, an internal BsrGI restriction site identified within the FLUC coding sequence was eliminated by site-directed mutagenesis as described in chapter 3.2.7. To achieve this, a three-primer PCR strategy was employed to introduce a silent point mutation at the restriction site, preserving the encoded amino acid sequence.

For the control construct containing FLUC and EGFP (pMX-puro_FLUC-EGFP #3.6.C), the FLUC coding sequence was amplified from the pSSV9_psiCheck plasmid (kindly provided by [REDACTED], University of Heidelberg) using primers incorporating P2A linker sequences at the 5' end and T2A linker at the 3' end of FLUC. EGFP was PCR-amplified from linearized pcDNA3.1_EGFP (#18) using a forward primer containing FUR-HB-T2A and a reverse primer containing an attB2 site and two distinct consecutive stop codons.

All PCR products were gel-purified as described in chapter [3.2.10](#), or column-purified and quantified using a Qubit fluorometer as described in chapter [3.2.11](#). Constructs were assembled via NEBuilder HiFi DNA Assembly and re-amplified using attB1/attB2-flanked primers, followed by PCR column-purification and Qubit quantification. Assembled inserts were recombined into pDONR221 using BP Clonase reactions. Positive bacterial colonies were identified by colony PCR as described in chapter [3.2.5](#), and plasmids were confirmed by Sanger sequencing using the Mix2Seq kit from Eurofins Genomics. For final expression vectors, LR clonase reactions were performed with pMX-DEST vectors containing or lacking puromycin resistance. [Figure 7](#) shows a schematic representation of the tricistronic vector constructs generated for TCR-T-cell engineering. The final vector constructs used for retroviral transduction included pMX-puro_anti-TRP2 cTCR-FLUC #1.1.G, pMX-no-sel_anti-TRP2 cTCR-FLUC #1.1.M, pMX-puro_anti-NY-ESO-1 cTCR-FLUC #4D, and pMX-no-sel_anti-NY-ESO-1 cTCR-FLUC #4.A.

pMX-puro-DEST_cTCR-FLUC (~9000 bp)



pMX-no-sel-DEST_cTCR-FLUC (~8000 bp)

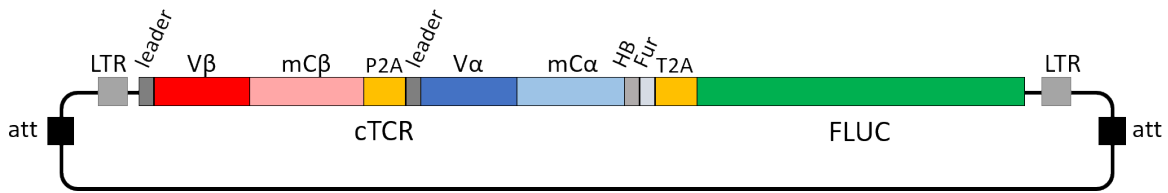


Figure 7: Schematic representation of tricistronic vector constructs used for TCR-T-cell engineering.

The construct begins with a 5' long terminal repeat (LTR) followed by a leader sequence, the variable region of the TCR β -chain, and a murinized β -chain constant domain. A P2A sequence separates this from a second leader sequence, which precedes the variable region of the TCR α -chain and its murinized constant domain. The α -chain is followed by a helix-breaker (HB), a furin cleavage site (Fur), a T2A sequence, and the FLUC gene. The cassette ends with a 3' LTR. The tricistronic construct was inserted into two pMX destination vectors: one containing a puromycin resistance cassette (top) and one without (bottom).

Final tricistronic vectors encoding both TCR α - and β -chains and the FLUC reporter (TRBV_mTRBC_P2A_TRAV_mTRAC_T2A_FLUC) were successfully generated. These were subsequently used for retroviral transduction of human and murine T cells.

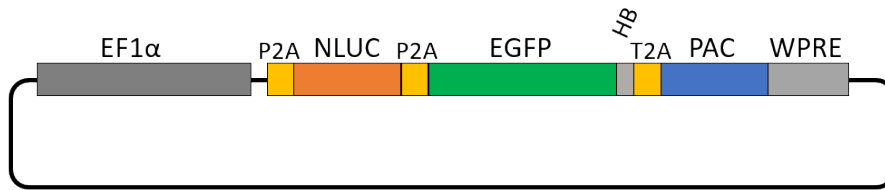
4.1.2 Generation of lentiviral constructs for antigen overexpression and tumor cell imaging

To enable antigen expression and BLI of tumor cells *in vivo*, two lentiviral vector constructs were generated (Figure 8). The vector pLenti-EF1a_hTRP2-NLUC-EGFP-PAC-WPRE (#6.6.5.1) was designed to drive high-level expression of full-length human TRP2, NanoLuciferase (NLUC), and EGFP, along with a puromycin resistance gene for selection. In parallel, the construct pLenti-EF1a_NLUC-EGFP-PAC-WPRE (#6.6.5) was generated to allow natural TRP2 expression while co-expressing NLUC and EGFP. Both vectors were optimized for stable genomic integration into tumor cells, facilitating long-term expression of the respective transgenes.

The cloning strategy involved a stepwise approach. Initially, the lentiviral backbone was modified to include NLUC and EGFP. A pre-existing pLenti-NLUC construct (#6.6) was linearized by restriction enzyme digestion with SmaI as described in chapter 3.2.14, and an EGFP fragment was PCR-amplified from the linearized pcDNA3.1-EGFP (#18) vector using primers encoding a P2A sequence at the 5' end and a PAC-T2A-HB sequence at the 3' end. The linearized backbone and EGFP insert were assembled using NEBuilder HiFi DNA Assembly at an insert-to-vector ratio of 2:1. Following bacterial transformation as described in chapter 3.2.4, positive colonies were identified by colony PCR and confirmed by Sanger sequencing, resulting in the construct pLenti-EF1a_NLUC-EGFP-PAC-WPRE (#6.6.5).

In the second step, full-length human TRP2 was integrated into the generated NLUC-EGFP (#6.6.5) construct. The vector was linearized using SgrDI, and the TRP2 coding sequence was PCR-amplified from the pre-existing pDONR221_TRP2 (#3) entry clone. The insert and vector were assembled via NEBuilder HiFi DNA Assembly, followed by bacterial transformation, colony screening by PCR, and sequence verification. The final construct, pLenti-EF1a_hTRP2-NLUC-EGFP-PAC-WPRE (#6.6.5.1), was confirmed to contain the complete and correct TRP2 insert.

pLenti-EF1 α _NLUC-EGFP-PAC #6.6.5 (9465 bp)



pLenti-EF1 α _TRP2-NLUC-EGFP-PAC #6.6.5.1 (11033 bp)

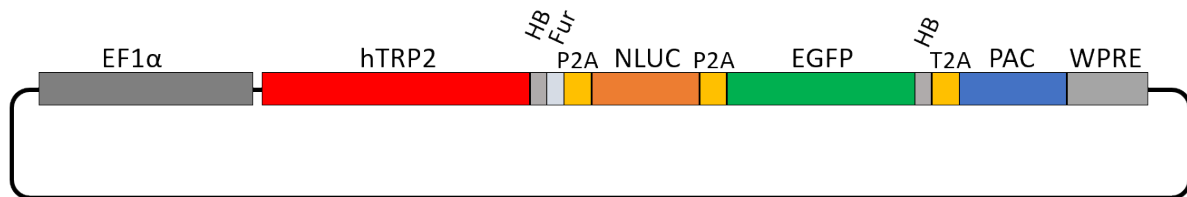


Figure 8: Structure of lentiviral vectors #6.6.5 and #6.6.5.1 for reporter and TRP2 expression.

Both constructs are driven by the EF1 α promoter and contain a reporter cassette composed of NLUC, EGFP, and the puromycin resistance gene (PAC), separated by self-cleaving peptides. In vector #6.6.5, NLUC is followed by P2A, EGFP, a helix-breaker (HB), T2A, and PAC, and the cassette ends with a WPRE element. Vector #6.6.5.1 additionally contains the full-length human TRP2 gene upstream of the reporter cassette, followed by a helix-breaker, a furin cleavage site (Fur), and a P2A sequence. This configuration enables co-expression of TRP2 together with luminescent and fluorescent reporters and allows antibiotic selection.

In conclusion, a comprehensive set of retroviral and lentiviral vector constructs was successfully developed to support both T-cell engineering and tumor cell modification for *in vivo* studies. Retroviral vectors enabled stable expression of cTCRs and a bioluminescent reporter for T-cell tracking, while lentiviral constructs facilitated overexpression of full-length TRP2 and nanoluciferase in melanoma cells. All vectors were validated by sequencing and colony PCR, ensuring correct assembly and expression cassette integrity. The modular design and cloning strategies allowed for parallel development of multiple constructs with selection markers, providing the basis for subsequent xenograft and syngeneic mouse experiments.

4.2 Establishment of genetically modified tumor cell lines for *in vivo* experiments

To generate tumor cell lines suitable for *in vivo* modeling, lentiviral transduction was used to stably modify the human melanoma cell line 86b_KO and the murine melanoma cell line B78.H1, enabling expression of TRP2 and/or NLUC. Transductions were performed as described in chapter 3.1.8, and transduction efficiency was monitored via EGFP fluorescence using flow cytometry as described in chapter 3.1.4. Stable integration was ensured through puromycin selection (1–2 µg/ml).

These transgenic tumor cell lines were used for antigen-specific T-cell recognition and enabled longitudinal monitoring of tumor burden via BLI, forming the foundation for subsequent xenograft and syngeneic *in vivo* experiments.

To obtain clonal populations with high transgene expression, single-cell sorting and clonal expansion were performed as described in chapter 3.1.5. Cells within the top 5% EGFP fluorescence intensity were sorted and seeded into 96-well plates at one cell per well. For 86b_KO cells, irradiated NIH-3T3 feeder cells supported clonal expansion; this was not required for B78.H1 clones. Expanded clones were screened by flow cytometry for high EGFP intensity and vector transcript expression to identify lines suitable for *in vivo* application.

Two human melanoma clones, 86b_KO.hTRP2.NLUC.EGFP#1.2 and 86b_KO.NLUC.EGFP#8, were selected for subsequent xenograft studies based on their EGFP expression profiles. Flow cytometry histograms illustrating EGFP fluorescence for these clones are shown in [Figure 9](#).

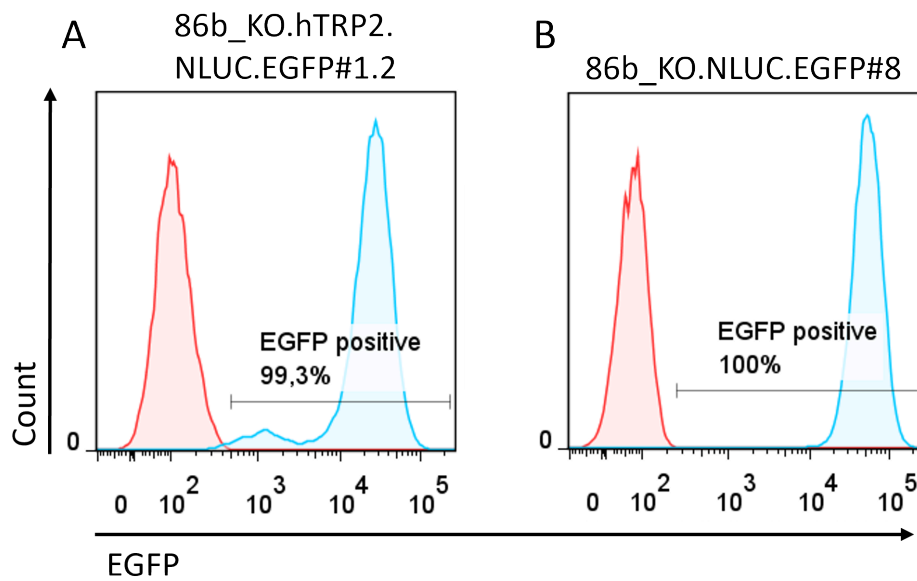


Figure 9: EGFP expression profiles of transduced 86b_KO clone populations selected for xenograft experiments.

(A) Clone 86b_KO.hTRP2.NLUC.EGFP#1.2 (blue), generated by transduction with pLenti-EF1a-hTRP2-NLUC-EGFP-PAC #6.6.5.1, compared to untransduced parental 86b_KO cells (red). (B) Clone 86b_KO.NLUC.EGFP#8 (blue), generated by transduction with pLenti-EF1a-NLUC-EGFP-PAC #6.6.5, compared to unmodified parental cells (red).

Murine B78.H1 melanoma cells were lentivirally transduced to stably express human TRP2 in combination with NLUC. Transduction efficiency was monitored via EGFP fluorescence, and cell sorting as well as single-cell clonal expansion was performed to isolate high-expressing populations.

Clone B78.H1.hTRP2.NLUC.EGFP#4 was selected for use in syngeneic *in vivo* experiments based on its high EGFP expression, as shown in [Figure 10](#). *In vitro* bioluminescence measurements further confirmed functional NLUC expression in the clone population (data not shown).

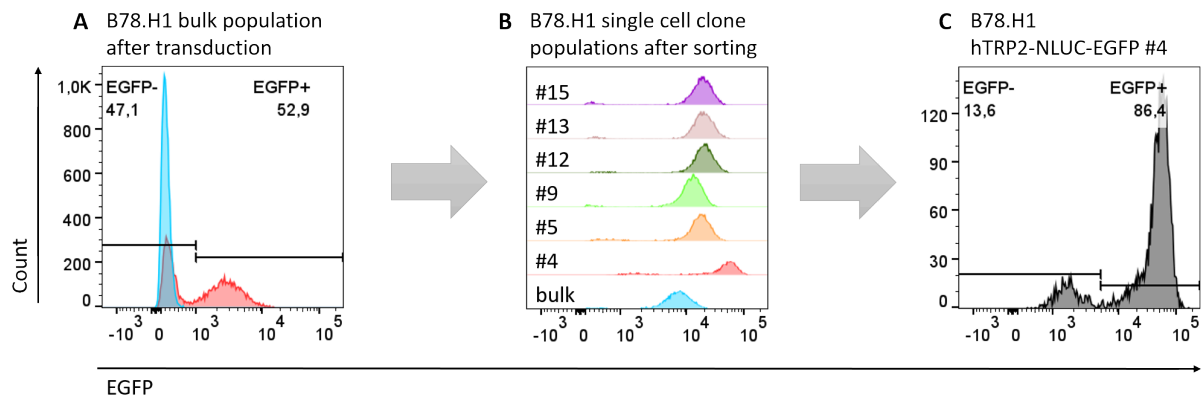


Figure 10: EGFP expression in transduced and subcloned B78.H1 cell populations.

(A) Flow cytometric analysis of bulk transduced B78.H1 cells (red) and untransduced cells (blue), showing EGFP fluorescence profiles. (B) EGFP fluorescence profiles of six single-cell-derived clone populations (#4, #5, #9, #12, #13, #15) compared with the bulk transduced population (blue). (C) Representative EGFP fluorescence profile of subclone B78.H1.hTRP2.NLUC.EGFP#4.

In summary, stable TRP2- and NLUC-expressing melanoma cell lines were successfully established through lentiviral transduction and single-cell clonal expansion. Both human (86b_KO) and murine (B78.H1) melanoma lines were modified to promote antigen-specific T-cell targeting and *in vivo* tumor burden monitoring via BLI. Clonal selection based on EGFP fluorescence intensity enabled the isolation of high-expressing, homogenous populations suitable for *in vivo* application. These engineered tumor cell lines formed the essential platform for subsequent xenograft and syngeneic T-cell therapy studies.

4.3 Xenograft experiments

4.3.1 Generation of transduced human primary T cells for T-cell transfer

To generate transduced human T cells for adoptive transfer, PBMCs were isolated from three buffy coats as described in chapter [3.1.6](#) and screened by flow cytometry for CD3⁺CD4⁺, CD3⁺CD8⁺, and CD16⁺CD56⁺ lymphocyte subsets. As NK cells can mount cytotoxic responses against HLA class I/II-deficient tumor cells, minimizing their presence was essential to attribute any observed antitumor activity solely to the transduced T cells. A buffy coat with a high proportion of CD8⁺ T cells and a low proportion of NK cells was selected for downstream use. On the day of isolation, the selected buffy coat contained 15.7% CD3⁺CD8⁺, 49.6% CD3⁺CD4⁺, and 7.21% CD16⁺CD56⁺ lymphocytes.

PBMCs were cultivated and transduced with retroviral vectors as described in chapter [3.1.7](#). For the first xenograft experiment, treatment T cells were transduced with the construct pMX-puro_anti-TRP2-cTCR-FLUC #1.1.G, and control T cells with pMX-puro_FLUC-EGFP #3.6.C. Transduction efficiency was analyzed by flow cytometry on day 6 after transduction, the day of adoptive cell transfer ([Figure 11](#)). In treatment T cells, 61% of CD3⁺ cells were mTRBC⁺, indicating successful cTCR transduction. CD3⁺ T cells comprised 58.2% CD4⁺ and 38.9% CD8⁺ cells, with 1.58% CD16⁺CD56⁺ NK cells remaining. Control T cells showed 87.3% EGFP positivity, confirming high transduction efficiency.

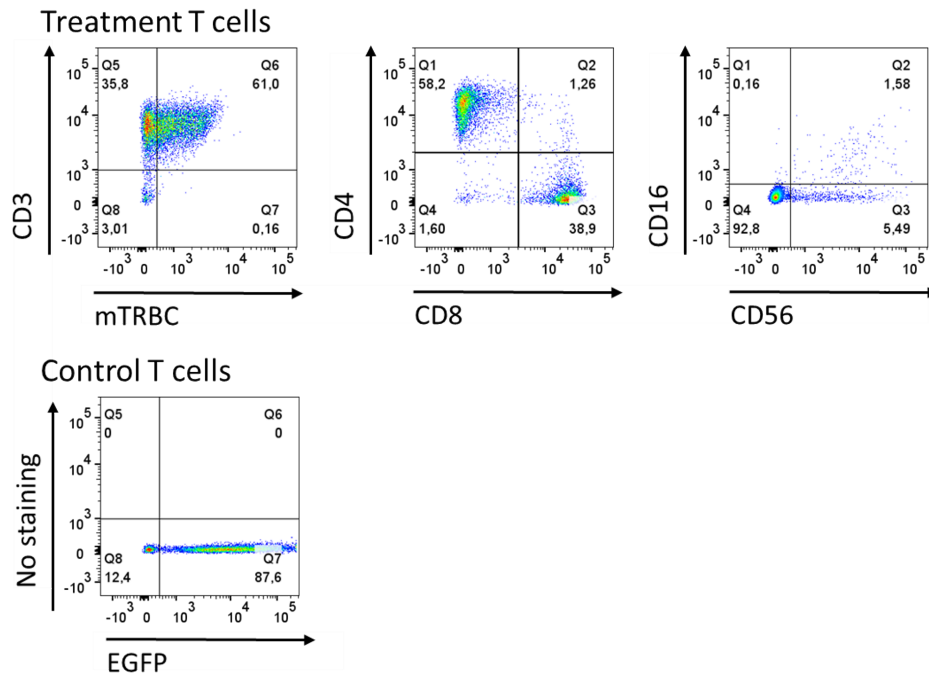


Figure 11: Flow cytometric analysis of transduced human T cells on the day of adoptive transfer.

Transduced T cells were analyzed for transgene expression prior to adoptive transfer. Top row: T cells stained for murinized TCR β (mTRBC) following transduction with pMX-puro_anti-TRP2-cTCR-FLUC #1.1.G. Bottom row: T cells assessed for EGFP fluorescence following transduction with pMX-puro_FLUC-EGFP #3.6.C.

To ensure robust cTCR expression, transduced T cells were maintained under puromycin selection during expansion. Treatment T cells additionally received antigen-specific stimulation with irradiated tumor cells on day 1 after transduction. However, following adoptive transfer, selection pressure was no longer applied *in vivo*. To assess the stability of cTCR expression in the absence of puromycin, a small fraction of transduced T cells was cultured in parallel with or without puromycin for eight days, and cTCR or EGFP expression was monitored by flow cytometry.

Within two days of culturing transduced T cells without puromycin, cTCR expression, measured by mTRBC staining, dropped from 61% to 36.5%, whereas expression remained relatively stable at 56.1% under continuous puromycin selection. This divergence continued over time: by day 14 after transduction (corresponding to day 13 after the last antigen-specific stimulation and day 8 without puromycin), the frequency of cTCR⁺ cells had dropped to 10.2% without selection and to 26.6% with ongoing puromycin treatment. In contrast, control T cells transduced with the FLUC-EGFP vector maintained EGFP expression levels above 90% throughout the 14-day culture

period, increasing slightly under puromycin (from 87.6% on day 6 to 96.7% on day 14), and remaining stable at 90.2% without selection. These findings show that cTCR expression declines in absence of puromycin, while the control construct remains more stable. The corresponding results are shown in [Figure 12](#).

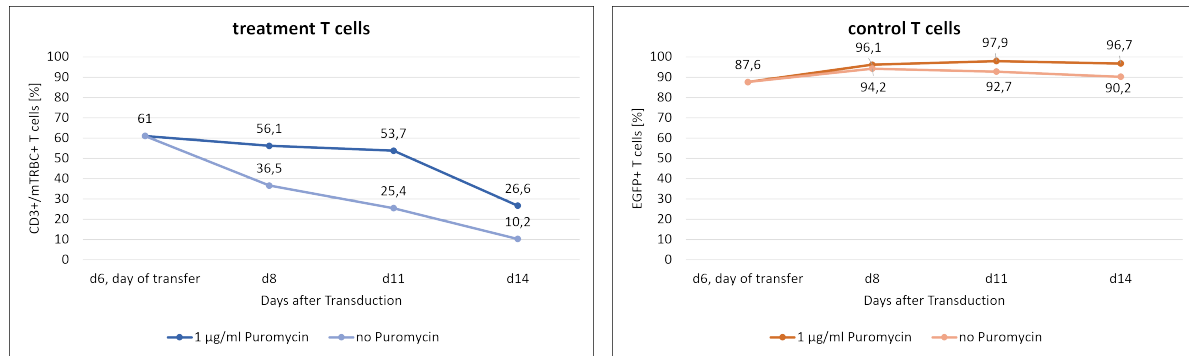


Figure 12: Stability of cTCR surface expression in transduced T cells.

Flow cytometric assessment of cTCR surface expression in transduced T cells cultured in the presence or absence of puromycin. cTCR surface expression was detected using mTRBC staining. For comparison, EGFP expression was measured in control T cells transduced with the FLUC-EGFP construct under the same culture conditions.

To evaluate the antigen specificity of transduced T cells *in vitro*, an IFN- γ ELISpot assay was performed on day 14 after transduction, corresponding to day 13 after the first and final antigen-specific stimulation. For this assay, T cells maintained under puromycin selection were used. As shown in [Figure 13](#), transduced T cells demonstrated strong reactivity toward 86b_KO cells with natural TRP2 expression, as well as TRP2-overexpressing variants used for antigen-specific stimulation (86b_KO.hTRP2) and for *in vivo* inoculation (86b_KO.hTRP2.NLUC.EGFP). T cells cultured alone did not secrete IFN- γ , and control T cells showed no reactivity toward any of the tested tumor cell lines.

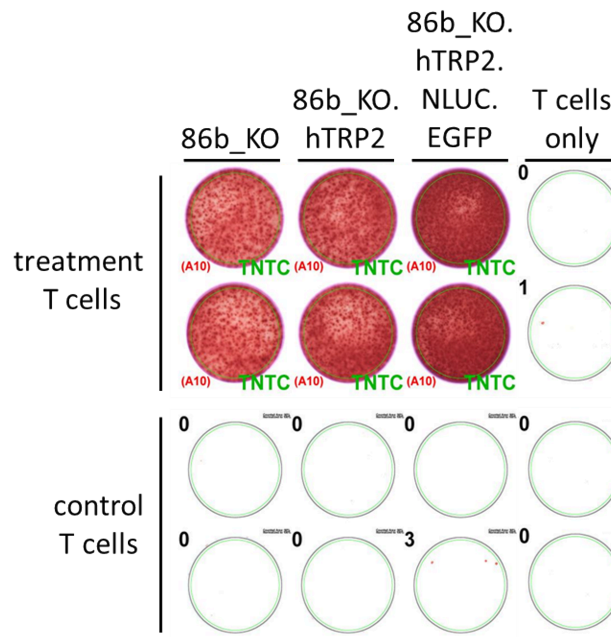


Figure 13: IFN- γ ELISpot assay for assessing antigen-specific activation of transduced T cells.

Indicated tumor cell lines (50,000 cells/well) were co-cultured with either 20,000 CD3⁺mTRBC⁺ cTCR-transduced T cells or EGFP⁺ control T cells. IFN- γ secretion was assessed by ELISpot. Wells containing T cells alone served as controls. TRP2-expressing tumor cell lines included 86b_KO, 86b_KO.hTRP2, and 86b_KO.hTRP2.NLUC.EGFP. TNTC ('too numerous to count') indicates wells in which spot density exceeded the software's capacity to accurately identify individual spots.

4.3.2 Evaluation of HLA-independent anti-TRP2-cTCR T cells in NSG mice with TRP2-overexpressing tumors

The first *in vivo* experiment was conducted in NSG mice, which lack functional T, B, and NK cells, providing a highly controlled environment to evaluate the direct effects of human TCR-transduced T cells on tumor growth. To ensure high antigen availability and create an idealized model for efficacy testing, NSG mice were subcutaneously inoculated on day 0 with 1×10^6 86b_KO.hTRP2.NLUC.EGFP tumor cells. Tumor engraftment was monitored by BLI on post-inoculation days 1 and 6. On day 7, mice received a total of 1×10^7 transduced T cells via tail vein injection along with 7.2×10^5 IU IL-2 intraperitoneally; in the treatment group, approximately 6.1×10^6 of these cells expressed the cTCR. Prior to injection, T cells were washed and resuspended in PBS.

Successful T-cell transfer was confirmed by BLI on day 2 after transfer. Tumor BLI and T-cell BLI were subsequently performed on a weekly basis to monitor tumor progression, T-cell localization and persistence. Blood samples were collected at designated time points for flow cytometric analysis. The experiment concluded 36 days after tumor inoculation (29 days after ACT). The experimental timeline is illustrated in [Figure 14](#).

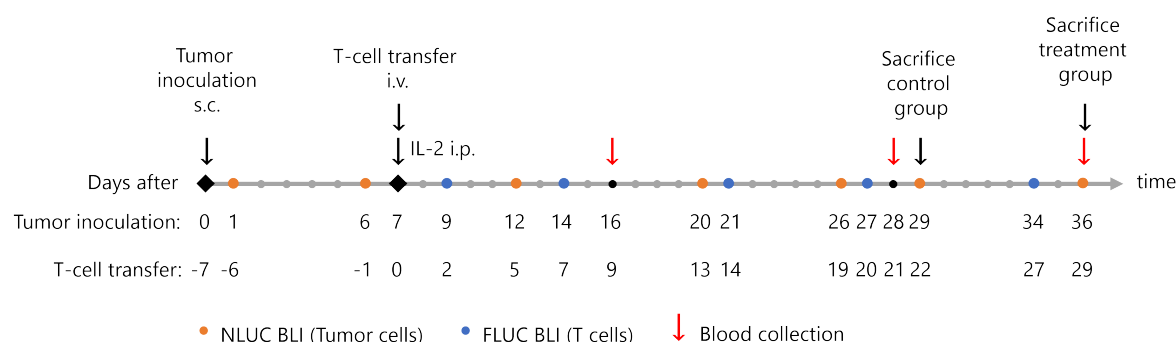


Figure 14: Experimental timeline of the first xenograft experiment using TRP2-overexpressing 86b_KO tumor cells.

The upper row indicates days after tumor inoculation and the lower row indicates days after T-cell transfer. Tumor BLI time points are shown in orange, and T-cell BLI time points in blue. Red arrows mark blood collection time points for flow cytometric analysis.

Representative bioluminescence images of tumor-bearing NSG mice throughout the first xenograft experiment are shown in [Figure 15](#). In BLI images, the signal intensity for both tumor and T cells is displayed as a color-coded radiance scale, with lower values indicating weaker bioluminescence signals and higher values reflecting stronger signals. This scale enables a semi-quantitative comparison of cell presence and viability across different locations and time points during the experiment. On day 6 after inoculation, one day prior to ACT, all mice (except for one control mouse) displayed visible tumor-associated bioluminescence signals, confirming successful engraftment. By day 12 (day 5 after ACT), signal intensity had visibly decreased in the treatment group, while continuing to increase in control mice. Tumor bioluminescence in control animals increased progressively throughout the study. In contrast, treated mice exhibited a complete loss of visible signal by day 20 (day 13 after ACT), with no recurrence observed through day 26 or at the experimental endpoint.

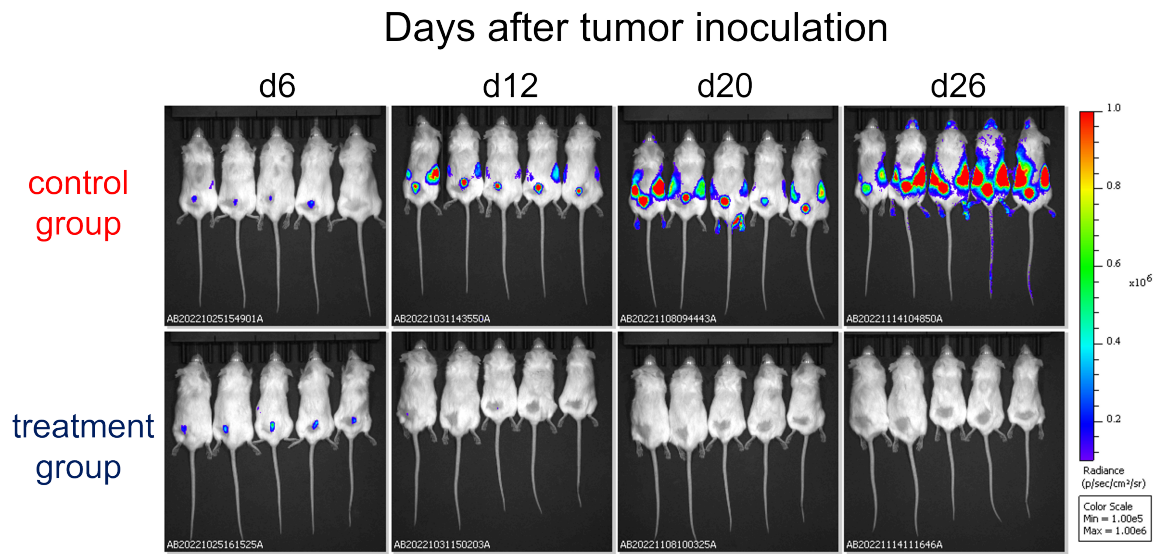


Figure 15: *In vivo* bioluminescence imaging of tumor cells in the first xenograft experiment.

Tumor bioluminescence was recorded on days 6, 12, 20, and 26 after tumor inoculation (left to right). The control group is shown in the upper row (mouse IDs from left to right: 733, 734, 737, 738, 739) and the treatment group in the lower row (mouse IDs from left to right: 735, 736, 740, 741, 742). For each imaging time point, mice received 100 μ L furimazine (1:40 dilution in PBS) via intraperitoneal injection immediately prior to imaging.

As presented in [Figure 16 A](#), manual tumor measurements using a digital caliper revealed continuous tumor growth in mice receiving control T cells. In contrast, tumors in mice treated with anti-TRP2-cTCR-transduced T cells regressed entirely and became non-palpable by day 13 after ACT (day 20 after inoculation). These findings were supported by quantification of NLUC signal intensity at the tumor site ([Figure 16 B](#)), which demonstrated a progressive increase in tumor burden in control mice, whereas treated mice exhibited a rapid decline in signal. By day 20, the average radiance in treated animals had dropped to $\sim 1 \times 10^3$ p/s/cm²/sr, a level comparable to the background signal, confirming the elimination of NLUC-expressing tumor cells.

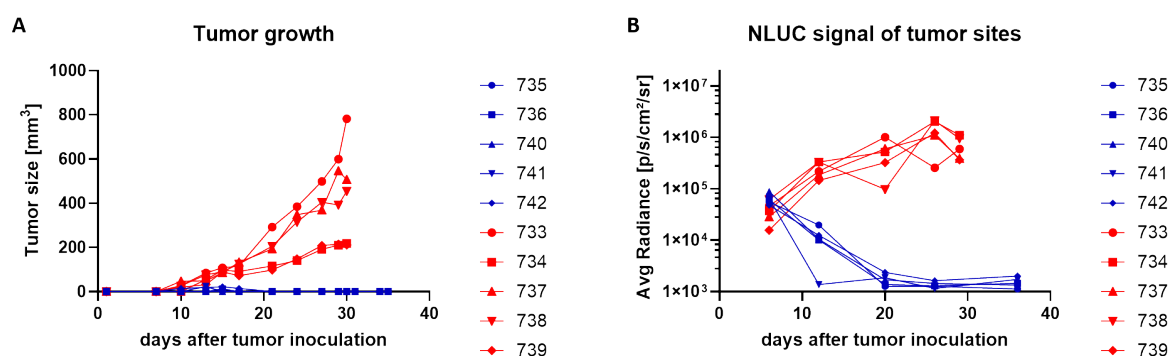


Figure 16: Tumor size and bioluminescence measurements in the first xenograft experiment.

(A) Tumor volume, measured by digital caliper and plotted as mm^3 over time for individual mice. Red lines represent control mice; blue lines represent mice receiving anti-TRP2-cTCR-transduced T cells. (B) Quantification of NanoLuc-derived bioluminescence at the tumor site, expressed as average radiance [photons/s/cm²/sr] and plotted on a logarithmic scale. Average radiance was calculated as the sum of radiance values from all pixels within the ROI divided by the number of pixels (photons/s/cm²/sr). Each curve represents an individual mouse, with red curves corresponding to control mice and blue curves to treated mice.

To gain additional insight, BLI of FLUC-expressing T cells was used to monitor their localization and persistence over time. Due to differences in transduction efficiency, construct-dependent FLUC expression levels, and the use of equal total cell numbers for adoptive transfer, control T cells exhibited a stronger overall BLI signal than treatment T cells.

As presented in [Figure 17](#), on day 2 after ACT, the BLI signal in treatment mice was predominantly localized to the lungs and primary tumor sites. By day 7, anti-TRP2-cTCR T cells had accumulated primarily at the tumor site. In most treated animals, tumor-localized signal persisted throughout the study, gradually declining toward the end. An exception was observed in mouse 741, which showed increased signal intensity on day 20 at the lungs, spleen, and tumor site. In control mice, BLI signal was initially widespread across the body (day 2), then gradually declined and became restricted mostly to the lungs and spleen by day 14. Toward day 20 of the experiment, overall signal intensity increased again. Control T cells also reached the tumor site, but their accumulation was less pronounced than in other regions of the body, such as the spleen.

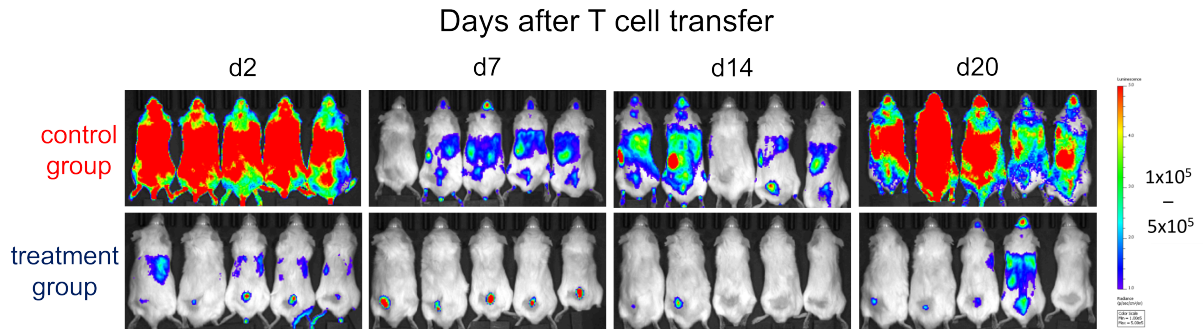


Figure 17: *In vivo* bioluminescence imaging of FLUC-expressing T cells.

BLI of FLUC-expressing T cells on days 2, 7, 14, and 20 after adoptive transfer. Control mice are shown in the upper row (left to right: 733, 734, 737, 738, 739) and treatment mice in the lower row (left to right: 735, 736, 740, 741, 742). Mice received D-luciferin (2.5 mg/100 μ L in dH₂O) via intraperitoneal injection immediately prior to imaging.

To further investigate T-cell persistence, blood samples were collected from both treatment and control groups on days 9 and 21 after ACT; an additional sample was collected from the treatment group on day 29. Flow cytometric analysis using an anti-human CD3 antibody confirmed the presence of circulating human T cells in all mice shown in [Figure 18](#).

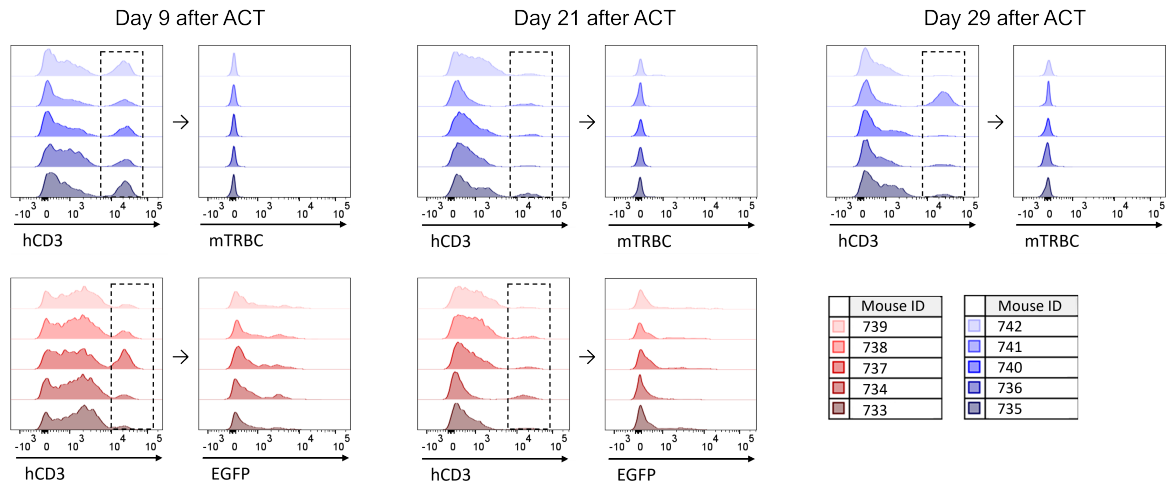


Figure 18: Flow cytometric detection of transferred human T cells in peripheral blood.

Blood samples collected at the indicated time points were stained for hCD3 expression to detect transferred human T cells. Top row: Treatment group (shades of blue); hCD3⁺ cells (dashed gate) were assessed for mTRBC expression. Bottom row: Control group (shades of red); hCD3⁺ cells (dashed gate) were assessed for EGFP expression. Each histogram represents an individual mouse.

In the treatment group, all five mice exhibited a prominent population of hCD3⁺ T cells in peripheral blood on day 9 after ACT. However, mTRBC was not detectable on these circulating hCD3⁺, suggesting loss or downregulation of the transgenic cTCR. By day 21 after ACT, the number of circulating hCD3⁺ T cells had declined in all animals, with only trace amounts detectable and again no evidence of mTRBC expression. On day 29, an increase in hCD3⁺ T cells was observed exclusively in mouse 741, while levels remained low in the other treatment mice; nevertheless, mTRBC expression was absent across all animals.

In the control group, hCD3⁺ T cells were detected on both days 9 and 21 after ACT, albeit at reduced levels on the latter. Notably, a subset of these cells retained EGFP expression at both time points, indicating sustained transgene expression in control T cells.

At the end of the experiment, organs were harvested, fixed in 4% paraformaldehyde, and processed into formalin-fixed paraffin-embedded (FFPE) sections for immunohistochemical (IHC) analysis at the Institute of Pathology. Histological evaluation was performed by [REDACTED], and representative images are presented in [Figure 19](#).

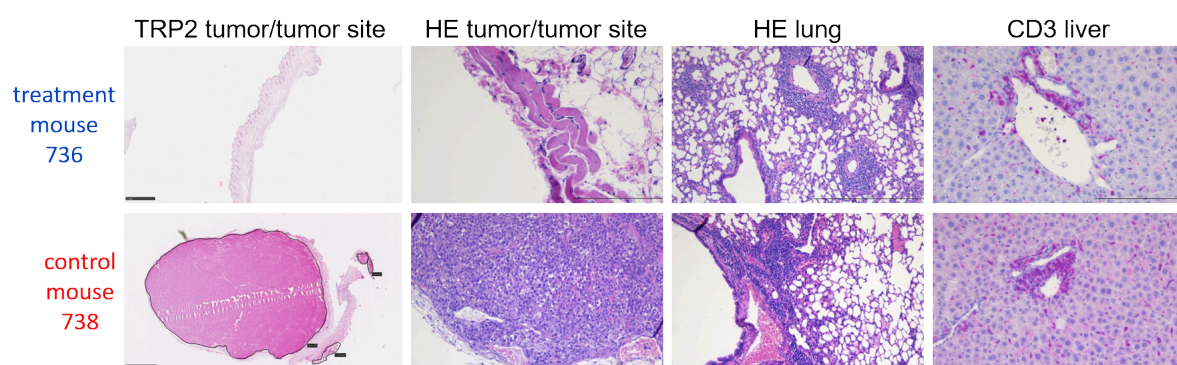


Figure 19: Immunohistochemical analysis of tumor site, lung, and liver.

Representative hematoxylin and eosin (H&E) and IHC stainings from a treatment mouse (ID 736, top row) and a control mouse (ID 738, bottom row). Sections include tumor site (H&E and hTRP2 staining), lung (H&E staining), and liver (H&E and hCD3 staining). TRP2 staining (pink) highlights tumor tissue in the control mouse. CD3 staining (pink) shows infiltration of human T cells in liver sections.

In the control group, HE staining and IHC confirmed the presence of viable tumor tissue exhibiting strong TRP2 expression. In contrast, IHC analysis of primary tumor sites in the treatment group revealed no detectable tumor cells in the examined sections, indicating complete tumor clearance. Further histopathological analysis showed infiltration of human T cells in both groups, as evidenced by HE staining of

the lungs and IHC for human CD3 in the liver. These infiltrates were primarily localized to the peribronchial and perivascular regions of the lung and the portal triads of the liver. Importantly, no histopathological signs of T-cell-mediated tissue damage or off-tumor toxicity were observed in any of the analyzed organs.

In summary, adoptive transfer of anti-TRP2-cTCR-transduced human T cells into NSG mice bearing TRP2-overexpressing melanoma tumors led to complete and durable tumor regression, as demonstrated by both caliper-based measurements and BLI. The transferred T cells exhibited antigen specificity *in vitro* and preferentially localized to tumor sites *in vivo*, with persistence detectable over several weeks. Although expression of the chimeric TCR declined over time in peripheral blood, tumor control was maintained throughout the study. Histopathological analysis confirmed the complete absence of tumor cells in treated mice and revealed no evidence of off-target toxicity, supporting the apparent safety of this approach.

4.3.3 Evaluation of HLA-independent anti-TRP2-cTCR T cells in NSG mice bearing tumors with natural TRP2 expression

Following the initial experiment using HLA class I- and class II-deficient TRP2-overexpressing 86b_KO.hTRP2.NLUC.EGFP tumor cells, a second xenograft experiment was performed using HLA class I- and class II-deficient tumor cells expressing TRP2 endogenously (86b_KO.NLUC.EGFP). In this experiment, control T cells were transduced with a cTCR construct targeting the HLA-A2-restricted NY-ESO-1 peptide SLLMWITQC, which was expected to be non-functional in this HLA-deficient setting. This modification was implemented to achieve more comparable FLUC expression between control and treatment T cells, as the previous control construct resulted in disproportionately high FLUC signals.

As in the first experiment, PBMCs from three buffy coats were screened, and the buffy coat with the highest proportion of CD8⁺ T cells and the lowest proportion of CD16⁺CD56⁺ NK cells was selected. Transduction efficiency was assessed by flow cytometry on days 5, 6, and 7 after transduction and results are shown in [Figure 20 A and B](#). The transfer was originally planned for day 6; however, due to low expression of the control TCR (21.7%), the culture was extended by one day. This did not improve, but rather reduced, TCR expression in control cells. In both groups, the percentage of CD3⁺CD4⁺ T cells increased over time, while CD3⁺CD8⁺ and CD16⁺CD56⁺ cells declined. In treatment T cells, cTCR expression peaked on day 7, with 43.5% of

CD3⁺mTRBC⁺ double-positive cells. In control T cells, cTCR expression peaked on day 6 at 21.7% CD3⁺mTRBC⁺ cells and declined to 16.1% by day 7. Based on these dynamics, T-cell transfer was performed on day 7 after transduction, as further culture was unlikely to improve cell numbers or TCR expression.

A fraction of transduced cells was retained in culture for IFN- γ ELISpot analysis performed on day 13 after transduction. As shown in Figure 20 C and D, treatment T cells specifically recognized the target tumor cell line used for *in vivo* inoculation (86b_KO.NLUC.EGFP), but not the TRP2-negative control cell line 86a_KO. Control T cells reacted exclusively to the HLA-A2⁺ NY-ESO-1⁺ melanoma cell line D05-Mel and did not respond to the HLA-deficient 86b_KO cells. No background IFN- γ release was observed in the absence of target cells.

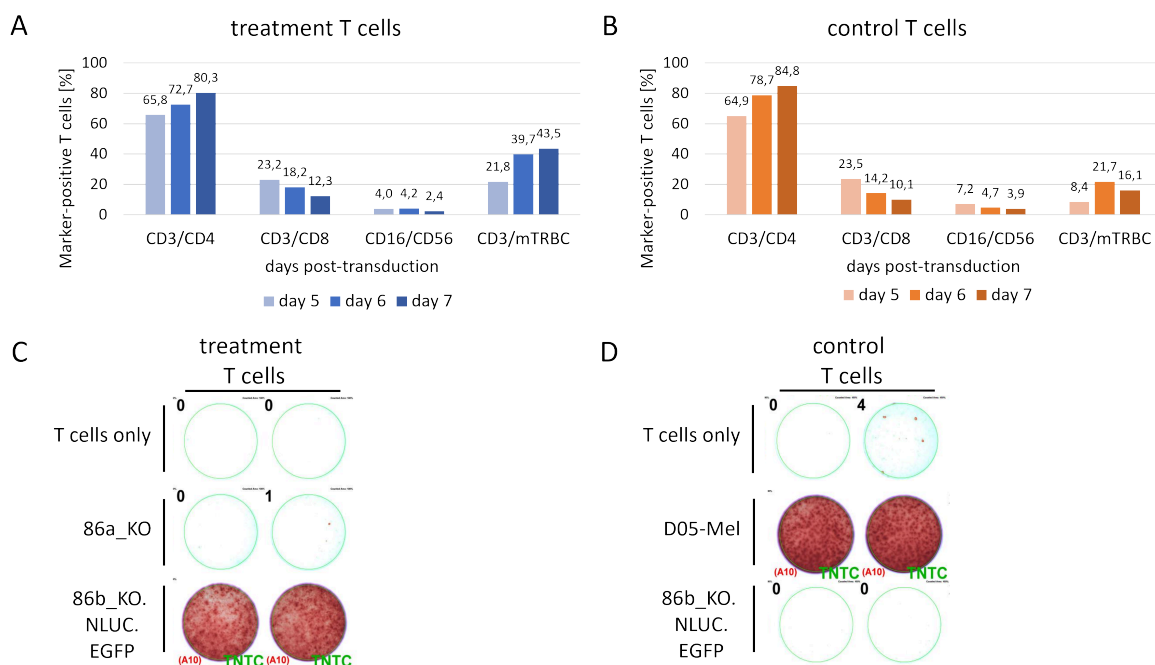


Figure 20: Flow cytometric characterization and IFN- γ ELISpot assay of cTCR-transduced T cells used in the second xenograft experiment.

(A) Treatment T cells were analyzed by flow cytometry on days 5, 6, and 7 after transduction for CD3, CD4, CD8, and cTCR (mTRBC) expression. (B) Control T cells were analyzed by flow cytometry on days 5, 6, and 7 after transduction for CD3, CD4, CD8, and cTCR (mTRBC) expression. (C) IFN- γ ELISpot assay performed on day 13 after transduction using 20,000 cTCR⁺ T cells and 50,000 indicated target cells per well; no IL-2 was added during co-culture. (D) NY-ESO-1-specific control T cells were tested under the same conditions with HLA-A2⁺/NY-ESO-1⁺ D05-Mel and the HLA-deficient inoculated tumor cell line 86b_KO.NLUC.EGFP. T cells alone were included as a control. TNTC ('too numerous to count') indicates wells in which spot density exceeded the software's capacity to accurately identify individual spots.

NSG mice were subcutaneously inoculated on day 0 with 1×10^6 86b_KO.NLUC.EGFP tumor cells suspended in 100 μ L PBS. Tumor BLI on day 6 after inoculation confirmed successful engraftment in all but one treatment mouse, as shown in [Figure 21 D](#). The experimental timeline is shown in [Figure 21 A](#). On day 8, mice received an adoptive transfer of 1×10^7 total T cells via tail vein injection. T-cell preparations contained 43.5% CD3⁺mTRBC⁺ double-positive cells in the treatment group and 16.1% in the control group, corresponding to approximately 4.35×10^6 cTCR⁺ T cells per mouse in the treatment group and 1.61×10^6 cTCR⁺ T cells per mouse in the control group. As in the previous experiment, each mouse received 7.2×10^5 IU of IL-2 intraperitoneally. The experiment concluded 55 days after tumor inoculation (47 days after ACT).

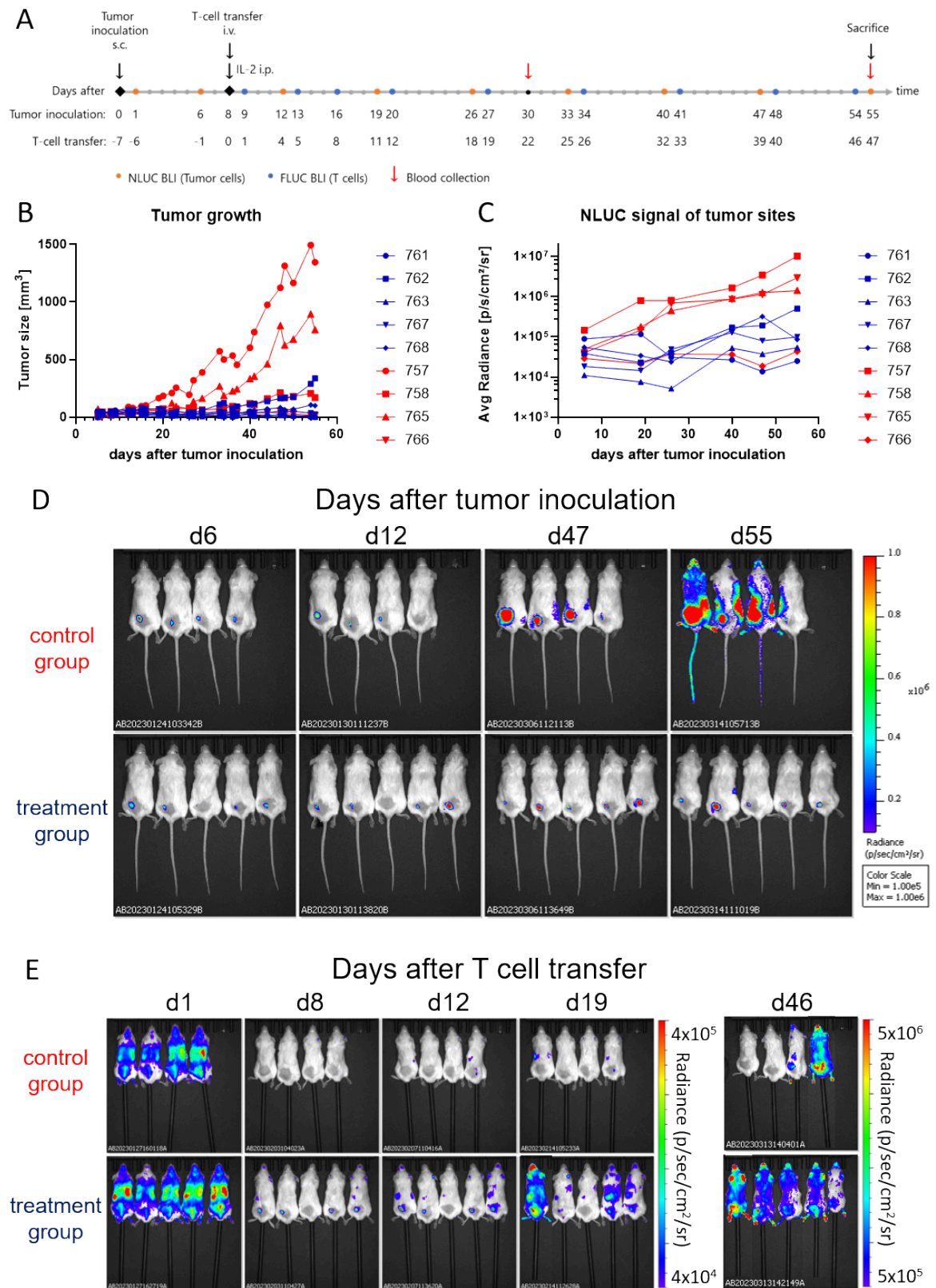


Figure 21: Experimental timeline, tumor size and bioluminescence imaging of NLUC and FLUC of NSG mice bearing endogenously TRP2-expressing tumors.

(A) Experimental timeline indicating days of tumor inoculation, BLI, T-cell transfer, and IL-2 administration. (B) Tumor size measured by digital caliper and plotted over time (mm^3). Treated mice are shown in blue; control mice in red. Each line represents a single mouse. (C) Quantification of NLUC-derived tumor bioluminescence signal over time (average radiance [photons/s/cm²/sr]). Each curve represents one mouse. (D) Representative NLUC-based tumor BLI images at selected time points. Control mice (top row; IDs 757, 758, 765, 766) and treatment mice (bottom row; IDs 761, 762, 763, 767, 768). Mice received 100 μL furimazine (1:40 in PBS) intraperitoneally immediately before imaging. (E) Representative FLUC-based T-cell BLI images at selected time points. Control mice (top row; IDs 757, 758, 765, 766) and treatment mice (bottom row; IDs 761, 762, 763, 767, 768). Mice received 2.5 mg D-luciferin in 100 μL dH₂O intraperitoneally immediately before imaging.

BLI of T cells, shown in [Figure 21 E](#), confirmed successful T-cell transfer and localization of transduced T cells to the tumor site. However, based on caliper measurements and tumor BLI ([Figure 21 B and D](#)), complete tumor regression was not achieved. Nevertheless, tumor growth was noticeably delayed in the treatment group compared to controls, indicating a moderate but measurable therapeutic effect.

Peripheral blood samples collected during the experiment at time points indicated in [Figure 21A](#) confirmed the presence of hCD3⁺ T cells in both treatment and control groups. However, as previously observed in the first xenograft experiment, mTRBC expression could not be detected on these circulating T cells, suggesting loss or down-regulation of transgene expression *in vivo* (data not shown).

At experimental endpoints, organs were again harvested, fixed in 4% paraformaldehyde, and processed into formalin-fixed paraffin-embedded (FFPE) sections for immunohistochemical (IHC) analysis at the Institute of Pathology. Histological evaluation was performed by [REDACTED], and representative images are presented in [Figure 22](#). Importantly, no histopathological signs of T-cell-mediated toxicity were observed in any of the analyzed organs.

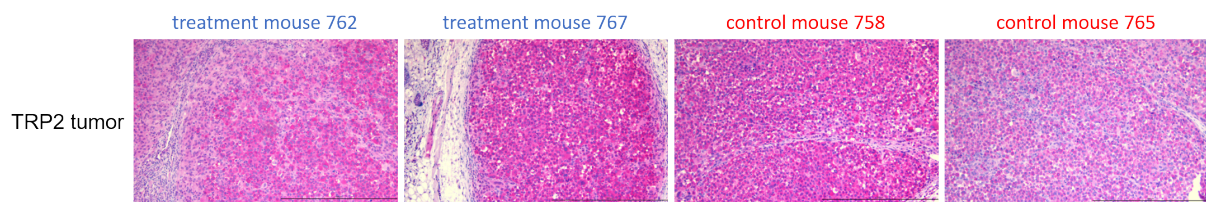


Figure 22: Immunohistochemical staining of TRP2 in tumor tissue at the endpoint of the second xenograft experiment.

Representative immunohistochemical staining for TRP2 on tumor sections from four mice (two from the treatment group and two from the control group) collected at the experimental endpoint. Staining patterns include both uniform and mosaic TRP2 expression within tumors.

TRP2 staining was detectable in all tumors at endpoint, with both uniform and mosaic patterns observed, indicating that TRP2 expression was still present at the time of analysis; however, expression dynamics at earlier timepoints during tumor development could not be assessed.

In this xenograft experiment using tumor cells with endogenous TRP2 expression, adoptive transfer of anti-TRP2 TCR-T cells into NSG mice led to delayed tumor growth but did not induce complete regression. Transferred T cells localized to the tumor site, as confirmed by *in vivo* BLI, and specifically recognized TRP2⁺ target cells *in vitro*. Despite effective tumor localisation, caliper and tumor BLI measurements indicated only moderate therapeutic efficacy. TRP2 expression was retained in all tumors at endpoint, as shown by IHC staining, though expression during earlier phases remains unknown. No signs of off-target toxicity were detected histologically in any of the analyzed organs. These results confirm antigen recognition and tumor homing by transduced T cells, but also highlight limitations in achieving full tumor control in this model.

4.3.4 Repeat evaluation of HLA-independent anti-TRP2-cTCR T cells in NSG mice bearing tumors with natural TRP2 expression

To evaluate reproducibility, the *in vivo* experiment using 86b_KO.NLUC.EGFP tumor cells with natural TRP2 expression was repeated. Again, PBMCs from three buffy coats were screened by flow cytometry to identify a donor with a high percentage of CD3⁺CD8⁺ T cells and low CD16⁺CD56⁺ NK cell content. The selected donor's PBMCs were used for transduction and downstream transfer. Treatment T cells were transduced with pMX-puro_anti-TRP2-cTCR-FLUC #1.1.G. and received antigen-specific stimulation after transduction with irradiated TRP2-overexpressing 86b_KO.hTRP2 cells. Control T cells were transduced with pMX-puro_anti-NY-ESO-1-cTCR-FLUC #4D and received no antigen-specific stimulation, consistent with the previous experiment.

On day 6 after transduction (the day of adoptive transfer), flow cytometric analysis showed that 55.3% of treatment T cells were CD3⁺mTRBC⁺, including 18.9% CD8⁺mTRBC⁺ cells. The proportion of CD16⁺CD56⁺ NK cells in this group was 2.25%. In the control group, 23.6% of T cells were CD3⁺mTRBC⁺, with 4.61% CD8⁺mTRBC⁺ cells, while NK cell frequency remained at 5.24%, as shown in [Figure 23 A](#). T-cell functionality was evaluated using an IFN- γ ELISpot assay on the day of transfer. As shown in [Figure 23 B](#), anti-TRP2 T cells produced IFN- γ in response to naturally TRP2-expressing 86b_KO cells, their NLUC-transduced variant, and

TRP2-overexpressing 86b_KO stimulator cells. No cytokine release was observed in response to the TRP2-negative 86a_KO cell line or in the absence of target cells. In contrast, control T cells reacted specifically to the HLA-A2⁺/NY-ESO-1⁺ melanoma cell line D05-Mel, but not to any 86b_KO-derived targets. No background IFN- γ secretion was detected in wells containing only T cells.

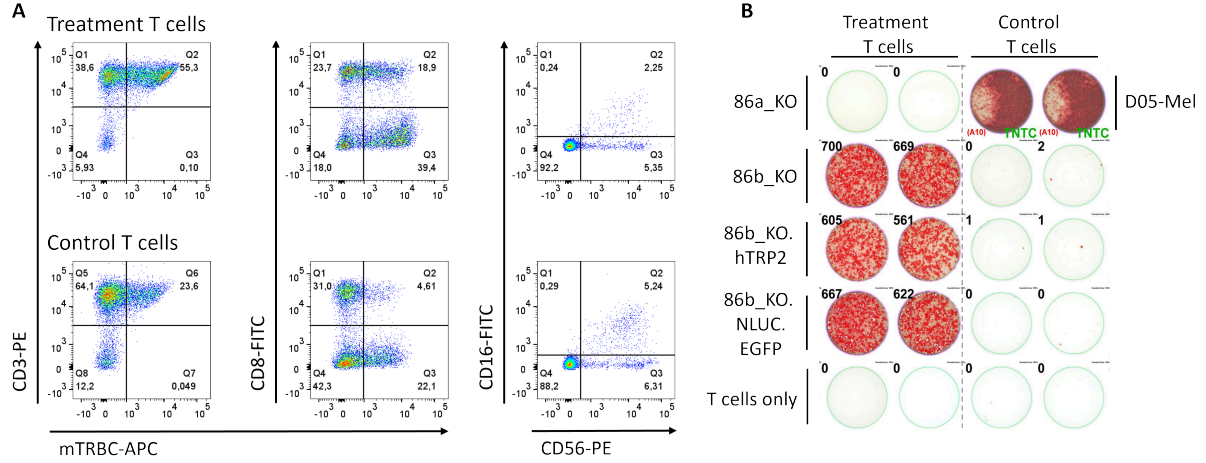


Figure 23: Flow cytometric analysis and IFN- γ ELISpot assay for transduced T cells used in the third xenograft experiment.

(A) Flow cytometric analysis of transduced T cells on day 6 after transduction, immediately prior to ACT, assessing CD3, CD4, CD8, and cTCR (mTRBC) expression. (B) IFN- γ ELISpot assay setup performed on the same day using 20,000 cTCR⁺ T cells and 50,000 target cells per well. Experimental conditions included TRP2-positive tumor targets (86b_KO, 86b_KO.hTRP2, 86b_KO.NLUC.EGFP), TRP2-negative tumor targets (86a_KO), HLA-A2⁺/NY-ESO-1⁺ targets (D05-Mel), and T-cell only controls. TNTC ('too numerous to count') indicates wells in which spot density exceeded the software's capacity to accurately identify individual spots.

In this repeated xenograft experiment, NSG mice were subcutaneously inoculated on day 0 with 1×10^6 86b_KO.NLUC.EGFP tumor cells. The experimental timeline is shown in [Figure 24 A](#). On day 6 after inoculation, tumor burden was assessed by BLI, and mice were randomly assigned to treatment and control groups to ensure balanced NLUC signal intensities across cohorts, consistent with the previous xenograft experiments. On day 7, all mice received 1×10^7 total T cells intravenously, together with 7.2×10^5 IU IL-2 intraperitoneally. Based on flow-cytometric cTCR expression, this corresponded to approximately 5.5×10^6 cTCR⁺ T cells per mouse in the treatment group and 2.3×10^6 cTCR⁺ T cells in the control group. The experiment ended 51 days after tumor inoculation (44 days after ACT).

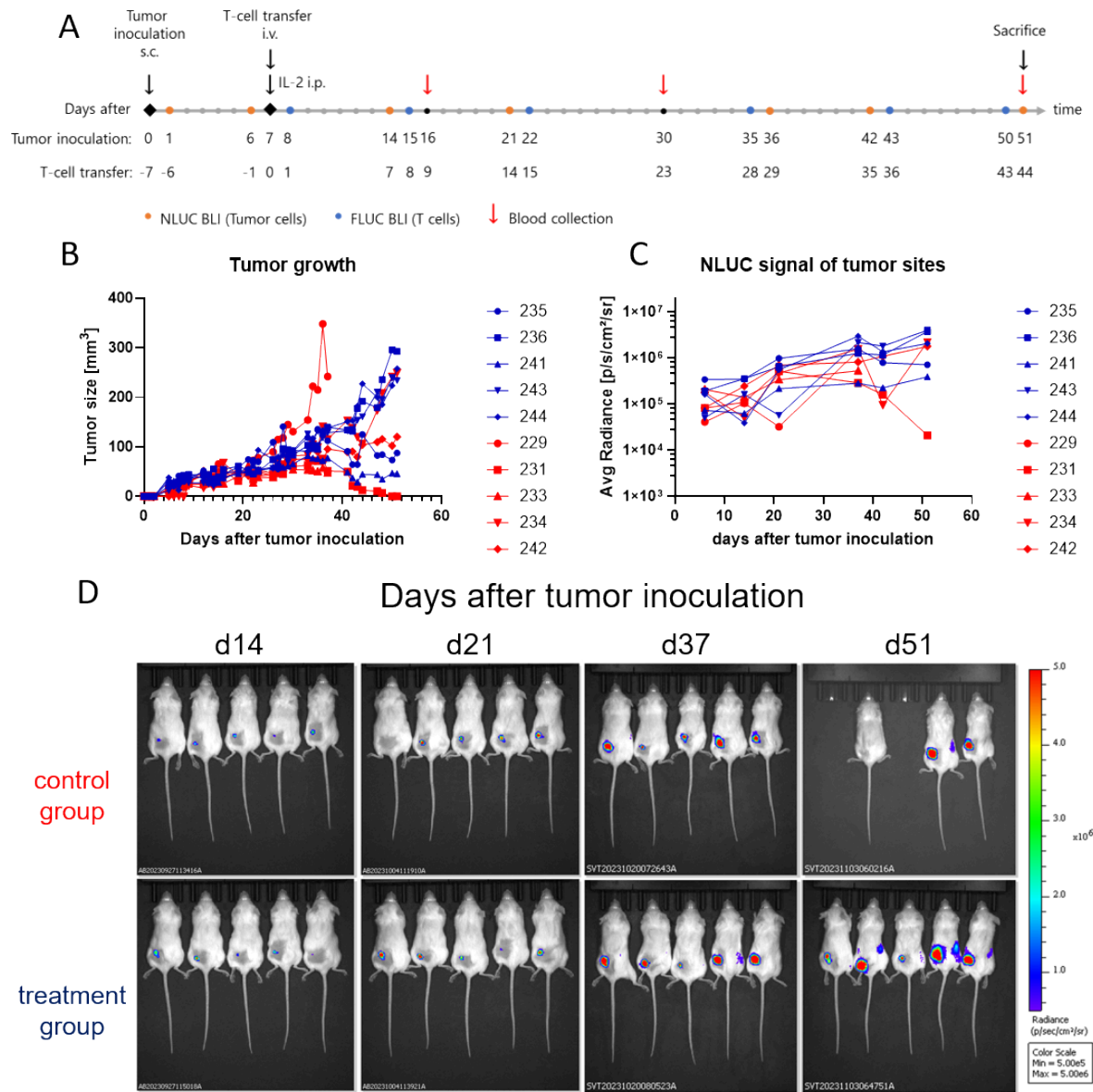


Figure 24: Experimental timeline, tumor size and bioluminescence imaging of NSG mice bearing endogenously TRP2-expressing tumors of the third xenograft experiment.

(A) Experimental timeline indicating tumor inoculation, BLI assessments, adoptive T-cell transfer, and IL-2 administration. (B) Tumor growth curves measured by digital caliper; each line represents an individual mouse (blue: treatment group, red: control group). (C) Quantification of NLUC-derived bioluminescence signal from the primary tumor site over time, expressed as average radiance [p/s/cm²/sr]; each line corresponds to a single mouse (blue: treatment group, red: control group). (D) Representative NLUC BLI images of tumor signals at selected time points. Control mice (top row; IDs 229, 231, 233, 234, 242) and treatment mice (bottom row; IDs 235, 236, 241, 243, 244) were imaged following intraperitoneal injection of 100 μ L furimazine (1:40 dilution in PBS).

Throughout the experiment, neither quantification of NLUC bioluminescence signals nor caliper-based tumor measurements indicated any therapeutic benefit in treated animals compared to controls. Tumor growth progressed at a similar rate in both groups, with no observable signs of suppression or delay. These results suggest a complete lack of antitumor efficacy under the tested conditions, despite successful TCR transduction and confirmed antigen-specific functionality of the T cells *in vitro*. A summary of cTCR expression profiles and the number of transferred cTCR⁺ T cells for all three xenograft experiments is provided in Appendix [Figure 39](#).

In vivo BLI of FLUC-transduced T cells revealed the accumulation of treatment T cells at the primary tumor site and in the spleen, as shown in [Figure 25 A](#). Compared to controls, treated mice showed more pronounced T-cell accumulation at the tumor site, as demonstrated by T-cell BLI on day 15. In both groups, BLI signal intensity increased over time in three of five control mice and two of five treatment mice, suggesting *in vivo* proliferation of the transferred T cells. The presence of circulating T cells was confirmed by flow cytometric analysis of peripheral blood using anti-human CD3 antibody and counting beads for quantification ([Figure 25 B and C](#)). Quantification of hCD3⁺ T cells in peripheral blood revealed a strong increase only in treatment mouse 241, correlating with the high BLI signal, whereas treatment mouse 244 exhibited a strong BLI signal without a corresponding increase in circulating hCD3⁺ T cells. Among the control mice, mice 231, 233, and 242 showed increasing FLUC signals. Mouse 233, which had to be removed early from the experiment, exhibited the highest absolute T-cell counts at day 23 after ACT. In mouse 231, T-cell counts peaked at day 23 and subsequently declined by day 44, while mouse 242 showed the highest T-cell counts in the control group at day 44 after ACT.

Despite rising FLUC signal and peripheral hCD3⁺ T-cell levels, mTRBC staining remained undetectable on circulating T cells (data not shown). This observation, consistent with previous xenograft experiments, indicates *in vivo* loss or downregulation of cTCR expression. It should also be noted that endogenous TCR expression was not knocked out in these human T-cell products; therefore, potential GvHD-related effects cannot be excluded and may stem either from the transduced cTCR or from reactivity mediated by residual endogenous TCRs.

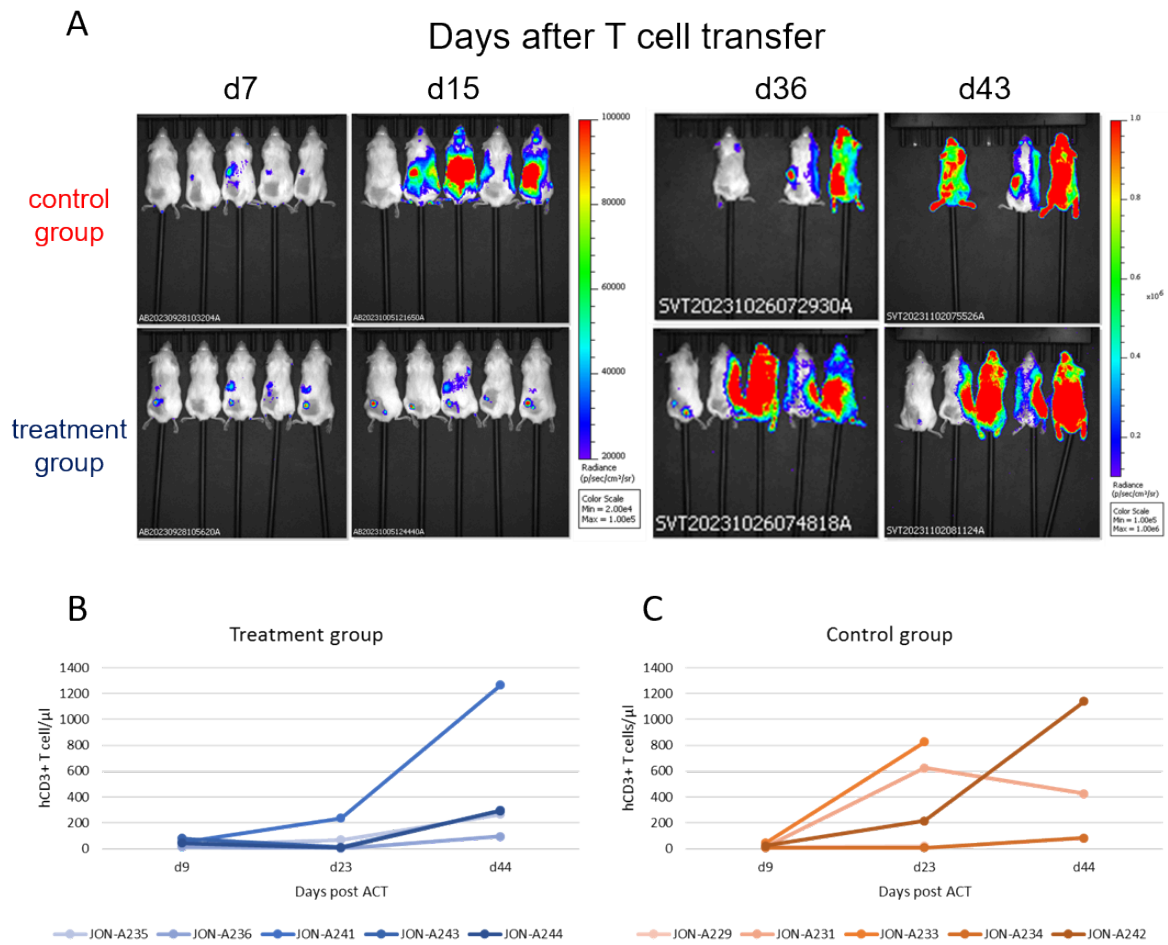


Figure 25: *In vivo* bioluminescence and peripheral blood analysis of transferred T cells in the third xenograft experiment.

(A) Representative *in vivo* BLI images of FLUC-expressing T cells at indicated time points. Control mice (top row; IDs 229, 231, 233, 234, 242) and treatment mice (bottom row; IDs 235, 236, 241, 243, 244) were imaged following intraperitoneal injection of D-luciferin (2.5 mg/100 μ L in dH₂O). (B–C) Flow cytometric quantification of hCD3⁺ T cells in peripheral blood using counting beads, shown as absolute T-cell numbers per μ L. Each line represents an individual mouse (blue: treatment group; orange: control group).

Immunohistochemical analysis of tumor sections once again revealed strong TRP2 expression in tumor cells, consistent with findings from the previous xenograft experiment using tumors with natural TRP2 expression. However, T-cell infiltration was sparse, as determined by the pathologist. No histopathological evidence of T-cell-mediated cytotoxicity was observed in any of the other examined organs in either group (data not shown).

In conclusion, anti-TRP2 TCR-T-cell therapy exhibited limited efficacy in xenograft models bearing tumors with natural TRP2 expression. While modest tumor growth delay was observed in the second experiment, the third experiment failed to show any therapeutic benefit despite *in vitro* antigen-specific activity. Transferred T cells localized to the tumor sites and expanded systemically, as shown by T-cell BLI. Nevertheless, mTRBC expression could not be detected on circulating hCD3⁺ T cells, a finding consistently observed in all three xenograft experiments. Histological analyses confirmed continued TRP2 expression at the experimental endpoint and revealed limited tumor infiltration, with no evidence of off-tumor/on-target or off-tumor/off-target toxicity, as assessed by the pathologist (data not shown). These findings suggest that although transferred T cells persist *in vivo*, the loss of transgenic cTCR expression on circulating cells along with potential antigen downregulation may limit therapeutic efficacy in settings with physiological TRP2 expression.

4.3.5 Analysis of TRP2-specific T-cell responses to melanoma cells after cytokine and tivozanib treatment

Previous studies have demonstrated that inflammatory cytokines such as TNF- α can induce reversible dedifferentiation of melanoma cells, resulting in downregulation of melanocytic differentiation antigens including TRP2 [130]. This process, referred to as inflammation-induced reversible dedifferentiation, contributes to immune escape and therapy resistance. Consistent with this, anti-TRP2-cTCR therapy *in vivo* showed limited efficacy against tumors with natural TRP2 expression, suggesting a transient cytokine-driven antigen loss as a potential resistance mechanism. Recent work by Ma et al. (2025) identified the receptor tyrosine kinase inhibitor tivozanib as a pharmacologic activator of the MITF-TRP2 axis, capable of restoring TRP2 expression under inflammatory conditions [131].

To explore whether tivozanib could reverse cytokine-induced dedifferentiation and thereby re-sensitize tumor cells to TRP2-specific T-cell recognition, melanoma cell lines 86a_KO and 86b_KO cells were seeded at 1×10^6 cells per T75 flask and treated for 72 hours with either DMSO (control), 10 ng/mL TNF- α , or 300 U/mL IFN- γ , each with or without 10 μ M tivozanib. After treatment, cells were used as target cells in an IFN- γ ELISpot assay with anti-TRP2-TCR-transduced T cells to assess antigen recognition.

As shown in [Figure 26](#), exposure to TNF- α or IFN- γ reduced TRP2-specific recognition by anti-TRP2 T cells, consistent with cytokine-induced dedifferentiation and antigen loss. Co-treatment with tivozanib mitigated this effect in 86b_KO cells, maintaining TRP2-dependent recognition, indicating that tivozanib can preserve or restore

MITF activity and TRP2 expression under inflammatory conditions. In contrast, 86a_KO cells, representing a fully dedifferentiated, MITF-inactive phenotype, did not respond to tivozanib, confirming that its activity depends on residual MITF functionality.

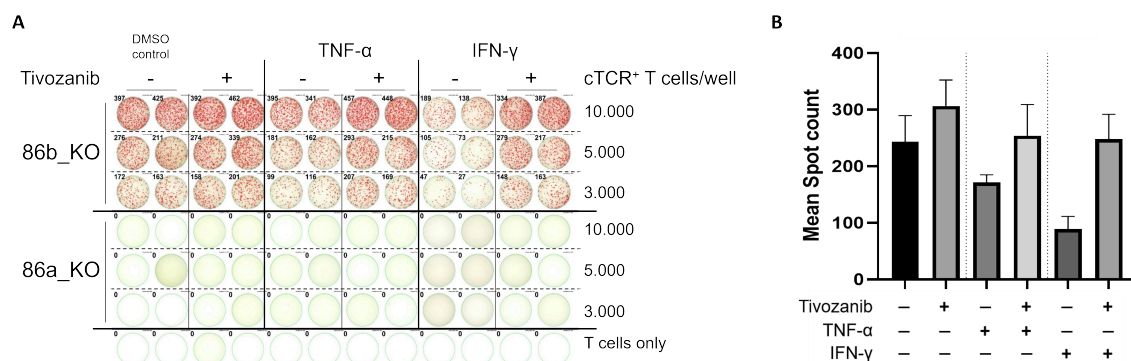


Figure 26: IFN- γ ELISpot assay of anti-TRP2-cTCR⁺ T cells co-cultured with cytokine- and tivozanib-treated melanoma cells.

Anti-TRP2-cTCR⁺ T cells (10,000, 5,000, or 3,000 cells per well) were co-cultured with 50,000 86a_KO or 86b_KO target cells in the presence of 250 IU/mL IL-2. Tumor cells were pretreated for 72 h with TNF- α (10 ng/mL) or IFN- γ (300 U/mL), with or without 10 μ M tivozanib. (A) IFN- γ spot patterns from co-cultures with the indicated target cells. (B) Mean spot counts for wells containing 5,000 cTCR⁺ T cells co-cultured with pretreated 86b_KO target cells, as indicated, shown as bar graphs.

These findings suggest that pharmacologic modulation of differentiation pathways may enhance the susceptibility of melanoma cells to TRP2-specific T-cell therapy by counteracting inflammation-induced antigen loss.

4.4 Syngeneic experiments

4.4.1 Generation and functional validation of transgenic murine T cells

To generate transgenic T cells for syngeneic adoptive transfer, primary splenocytes were isolated from C57BL/6 mice and activated for 24 hours with 2 µg/mL Concanavalin A (ConA) and 10% T-cell growth factor (TCGF). The following day, activated cells were electroporated with CRISPR/Cas9 ribonucleoprotein complexes targeting the murine TCR alpha (mTRAC) and beta (mTRBC) chains to disrupt endogenous TCR genes, as described in chapter [3.1.7.3](#). Immediately after electroporation, cells were transduced with retroviral particles encoding cTCR constructs using retronectin-coated plates. A second transduction was performed approximately 24 hours later. A detailed description of the procedure can be found in chapter [3.1.7.3](#).

Treatment T cells were transduced with anti-TRP2-cTCR constructs lacking a puromycin resistance cassette, either with or without the FLUC reporter gene. Control T cells were transduced with anti-NY-ESO-1 cTCR constructs, also without a selection marker and $\pm$ FLUC. On day 2 after transduction, treatment T cells received antigen-specific stimulation using irradiated B78.H1 tumor cells overexpressing human TRP2, whereas control T cells were not stimulated. Flow cytometry, IFN- γ ELISpot, and bioluminescence-based killing assays were performed on day 4 after editing and transduction to assess cTCR surface expression and antigen-specific activity; the corresponding results are presented in [Figure 27](#).

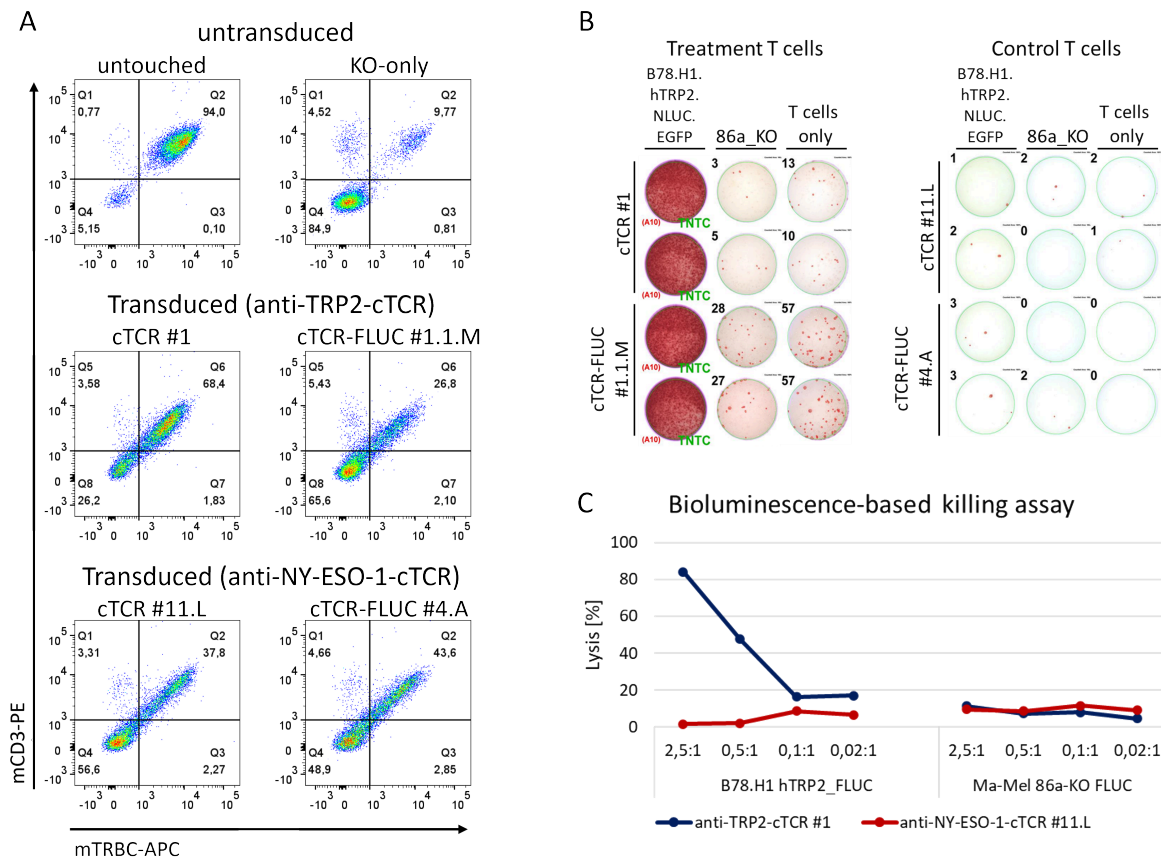


Figure 27: Characterization of cTCR-engineered murine T cells on day 4 after electroporation and retroviral transduction.

(A) Flow cytometric analysis on day 4 after electroporation showing endogenous TCR knockout efficiency ($mCD3^+mTRBC^+$ frequencies) in untouched control splenocytes and CRISPR/Cas9-edited cells (KO-only), as well as surface expression of transduced cTCR constructs (mTRBC staining) for anti-TRP2-cTCR (#1), anti-TRP2-cTCR-FLUC (#1.1.M), anti-NY-ESO-1-cTCR (#11.L), and anti-NY-ESO-1-cTCR-FLUC (#4.A). (B) IFN- γ ELISpot performed on day 4 using 10,000 cTCR $^+$ T cells and 50,000 target cells per well; indicated co-cultures include B78.H1.hTRP2.NLUC.EGFP cells, 86a_KO (negative control), and T-cell-only wells. TNTC ('too numerous to count') indicates wells in which spot density exceeded the software's capacity to accurately identify individual spots. (C) Bioluminescence-based cytotoxicity assays using FLUC-expressing hTRP2 $^+$ B78.H1 tumor cells co-cultured with engineered T cells to assess cytolytic capacity in vitro.

To precisely assess cTCR expression on the day of adoptive transfer, flow cytometric analysis was performed on day 6 after transduction/knockout. This included staining for CD3 and the specific TCR variable beta chains hV β 3 (anti-TRP2 cTCR) and hV β 1 (anti-NY-ESO-1 cTCR). The results are presented in [Figure 28](#). The approx-

imately 10% difference between mTRBC⁺ and hVβ3⁺ populations may reflect the presence of T cells that were not successfully knocked out for the endogenous TCR. Notably, a comparable discrepancy was not observed in the control T-cell population.

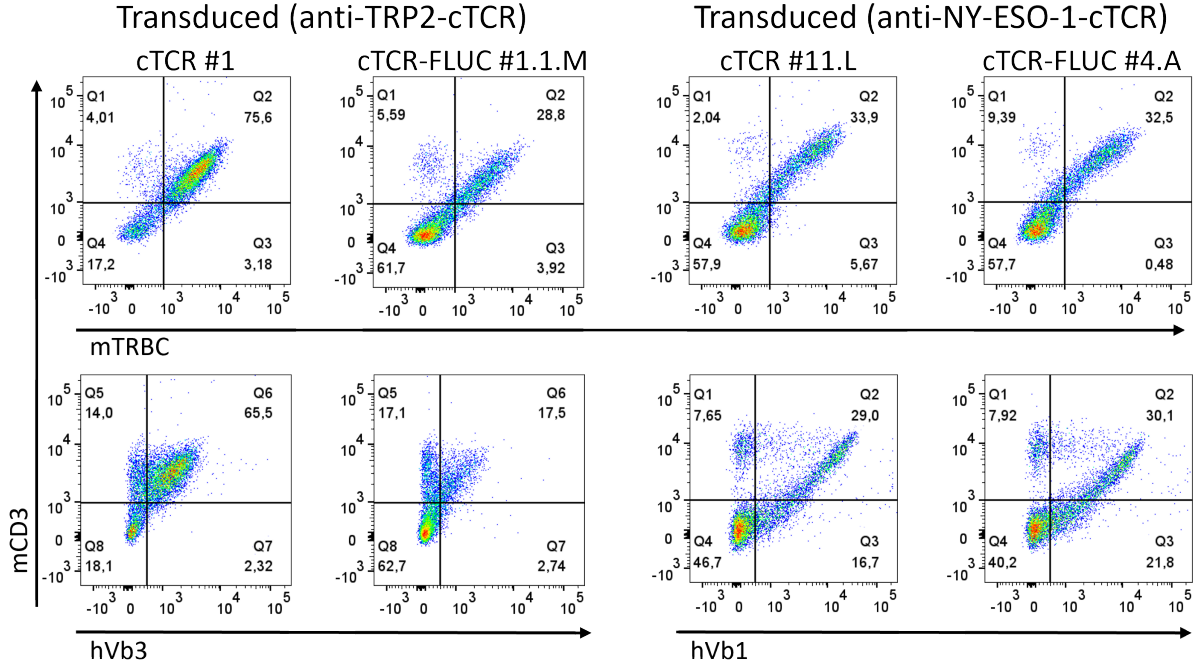


Figure 28: cTCR surface expression of engineered murine T cells on the day of adoptive transfer.

Flow cytometric analysis on day 6 after transduction (day of transfer). Transduction efficiencies based on mCD3⁺mTRBC⁺ frequencies were 75.6% (anti-TRP2-cTCR #1), 28.8% (anti-TRP2-cTCR-FLUC #1.1.M), 33.9% (anti-NY-ESO-1-cTCR #11.L), and 32.5% (FLUC-expressing control #4.A). Specific staining of variable TCR β-chain regions (anti-TRP2-cTCR monitored via hVβ3; anti-NY-ESO-1-cTCR via hVβ1) showed mCD3⁺hVβ3⁺ frequencies of 65.5% (anti-TRP2-cTCR #1) and 17.5% (anti-TRP2-cTCR-FLUC #1.1.M), and mCD3⁺hVβ1⁺ frequencies of 29.0% (anti-NY-ESO-1-cTCR #11.L) and 30.1% (anti-NY-ESO-1-cTCR-FLUC #4.A).

To further characterize the phenotype of transduced T cells at the time of adoptive transfer, flow cytometry analysis was performed on cryopreserved cells collected and frozen on day 6 after transduction. Following thawing, cells were stained with a panel of twelve monoclonal antibodies targeting murine CD3, CD8, CD25 (IL-2Rα), CD69, CTLA-4, PD-1 (CD279), TIGIT, IL7Rα (CD127), CD44, and CD62L, as well as human TCR variable regions Vβ1 and Vβ3. A live/dead viability dye was included to exclude non-viable events. The gating strategy involved sequential gating on lymphocytes, single cells, and live cells, followed by the identification of CD3⁺CD8⁺ and CD3⁺CD8⁻ subsets. An exemplary gating strategy is shown in the Appendix [Figure 42](#) and was

applied consistently for all samples, including T cells prior to transfer and those analyzed at the experimental endpoint. Within these, cTCR-transduced cells were identified based on hV β expression, and activation or exhaustion markers were subsequently quantified as frequencies within the respective hV β ⁺ T-cell populations.

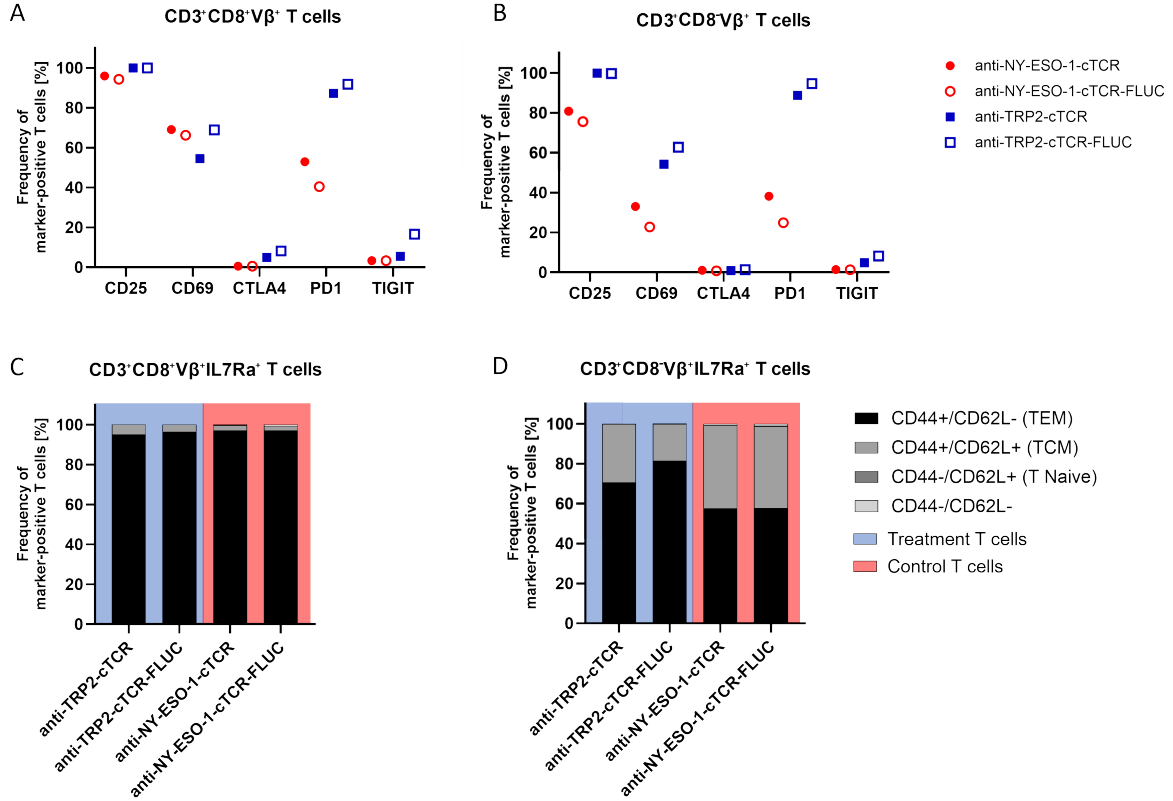


Figure 29: Phenotypic characterization of activation, exhaustion, and differentiation markers in transduced T cells on the day of adoptive transfer.

Cryopreserved T cells collected on day 6 after electroporation/transduction were thawed and analyzed by flow cytometry. Cells were stained with an antibody panel to assess expression of activation, exhaustion, and differentiation markers. Transduced T cells were first gated on CD3 and CD8 expression to distinguish CD8⁺ and CD8⁻ (putative CD4⁺) subsets, and hV β 1 or hV β 3 staining was used within these subsets to identify cells expressing the transduced TCRs. (A) Frequencies of CD25⁺, CD69⁺, CTLA-4⁺, PD-1⁺, and TIGIT⁺ cells gated on CD3⁺CD8⁺hV β ⁺ T cells. (B) Frequencies of CD25⁺, CD69⁺, CTLA-4⁺, PD-1⁺, and TIGIT⁺ cells gated on CD3⁺CD8⁻hV β ⁺ (putative CD4⁺) T cells. (C) Differentiation phenotypes of CD3⁺CD8⁺hV β ⁺ T cells based on CD44 and CD62L expression within IL-7R α ⁺ cells (TN: CD44⁻CD62L⁺, TCM: CD44⁺CD62L⁺, TEM: CD44⁺CD62L⁻, DN: CD44⁻CD62L⁻). (D) Differentiation phenotypes of CD3⁺CD8⁻hV β ⁺ (putative CD4⁺) T cells within IL-7R α ⁺ cells, categorized as in (C).

Among CD8⁺hVβ⁺ T cells, expression of CD25, CD69, CTLA-4, PD-1, and TIGIT was detected at comparable frequencies in anti-TRP2- and anti-NY-ESO-1-transduced cells, except for PD-1. PD-1 expression was higher in anti-TRP2 T cells (82.2% and 91.8% for non-reporter and FLUC-expressing constructs, respectively) compared to anti-NY-ESO-1 T cells (52.9% and 40.5%). More pronounced differences were observed within the CD3⁺CD8⁺hVβ⁺ subset, corresponding to putative CD4⁺ T cells. Here, PD-1 expression reached 88.8% and 94.6% in the anti-TRP2 group, strongly exceeding the 38.2% and 24.9% observed in anti-NY-ESO-1-transduced cells. Notably, CD25 and CD69 also displayed a more pronounced divergence between treatment and control groups within this subset: CD25 expression was 99.9% and 99.7% in anti-TRP2 cells versus 80.8% and 75.6% in controls, while CD69 expression reached 54.2% and 62.7% compared to 33.0% and 22.8%, respectively.

To assess the differentiation status of transduced T cells, expression of CD44 and CD62L was analyzed within the IL7Rα⁺ population. Among CD8⁺hVβ⁺ T cells, the vast majority displayed a T effector memory (TEM; CD44⁺CD62L⁻) phenotype, comprising 94.9% (anti-TRP2), 96.4% (anti-TRP2-FLUC), 97.0% (anti-NY-ESO-1), and 96.9% (anti-NY-ESO-1-FLUC) of cells, respectively. Central memory (TCM; CD44⁺CD62L⁺) cells accounted for the remainder, while naïve (CD44⁻CD62L⁺) and double-negative (CD44⁻CD62L⁻) phenotypes were each <1% across all samples. In the CD8⁺hVβ⁺ (CD4⁺) subset, TEM frequencies were lower overall: 70.5% and 81.4% for anti-TRP2 constructs, and 57.5% and 57.8% for anti-NY-ESO-1 constructs, respectively. The remaining cells largely displayed a TCM phenotype, with naïve and double-negative populations consistently below 1.13%.

Collectively, these data suggest that the transferred T-cell product consisted predominantly of effector T cells that were activated, as indicated by moderate to high expression of CD25, CD69, and PD-1, but not yet exhausted, as reflected by low expression of CTLA-4 and TIGIT.

4.4.2 Assessment of anti-TRP2-cTCR T-cell activity in syngeneic melanoma model

To assess *in vivo* efficacy in an immunocompetent model, C57BL/6 mice were subcutaneously injected with 0.5 × 10⁶ B78.H1 hTRP2-NLUC tumor cells on day 0. The experimental timeline is presented in [Figure 30 A](#). On day 5 after tumor inoculation, mice received sublethal total body irradiation (5 Gray), followed by BLI on day 6 to confirm successful tumor engraftment and allow for controlled randomization into treatment and control groups. Adoptive transfer of 1 × 10⁷ total T cells and systemic IL-2 administration were performed on day 7. Based on cTCR expression levels, each mouse in the

treatment group received approximately 2.6×10^6 anti-TRP2-cTCR⁺ T cells and 0.9×10^6 anti-TRP2-cTCR-FLUC⁺ T cells, corresponding to a total of about 3.5×10^6 cTCR⁺ T cells transferred. Control mice received approximately 1.1×10^6 anti-NY-ESO-1-cTCR⁺ and 0.9×10^6 anti-NY-ESO-1-cTCR-FLUC⁺ T cells, corresponding to a total of about 2×10^6 cTCR⁺ T cells transferred. Serial BLI revealed a rapid and robust anti-tumor response in the treatment group, with strong signal reduction by day 12 and complete loss of detectable tumor bioluminescence by day 19, as shown in Figure 30 B-D.

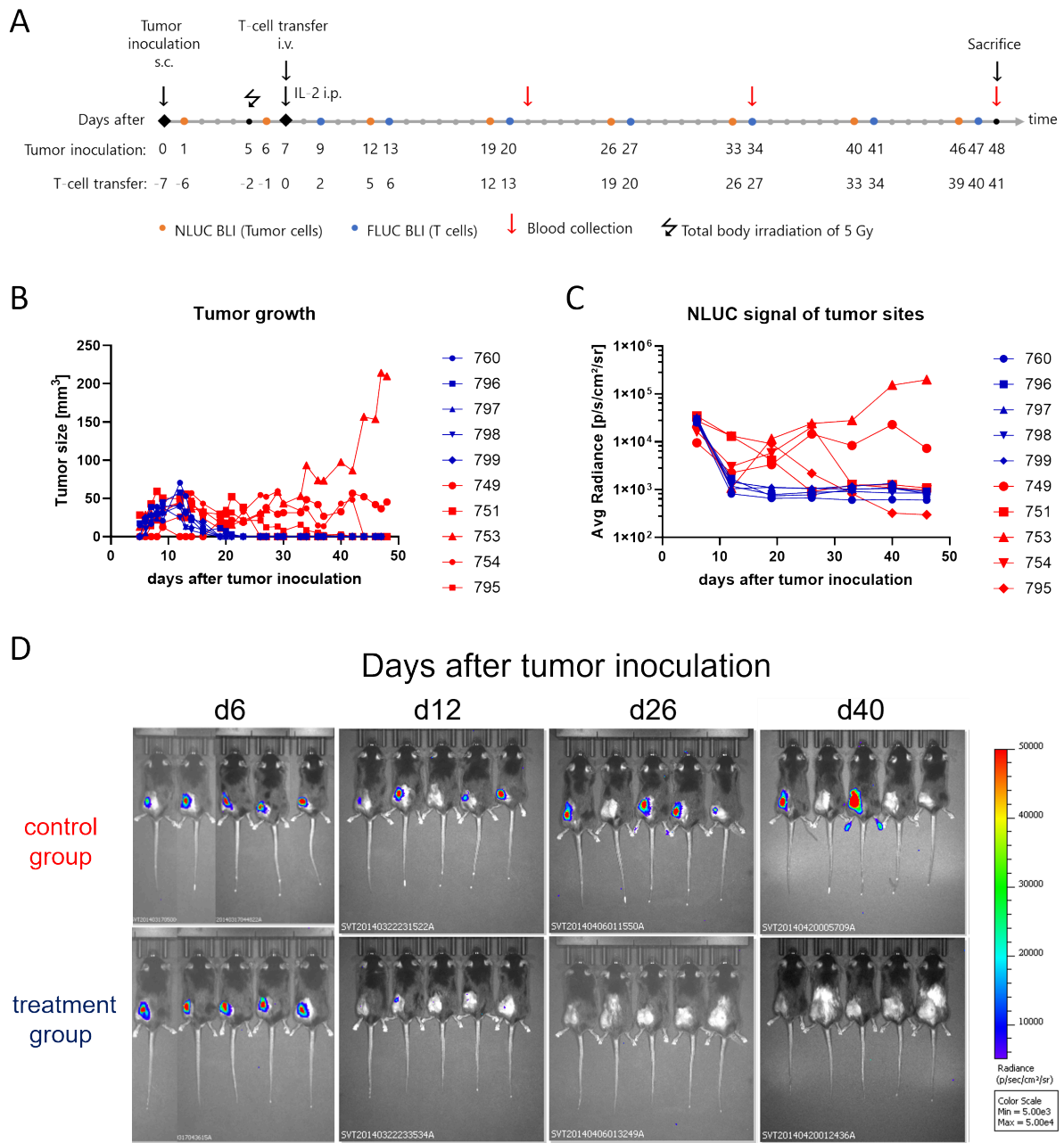


Figure 30: *In vivo* evaluation of TRP2-specific T cells in a syngeneic subcutaneous melanoma model.

(A) *Experimental timeline.* C57BL/6 mice were injected subcutaneously with 0.5×10^6 B78.H1.hTRP2.NLUC.EGFP cells on day 0. On day 5, mice received sublethal total-body irradiation (5 Gy). Tumor engraftment was confirmed on day 6 by bioluminescence imaging (BLI), followed by controlled randomization into treatment and control groups. On day 7, mice received adoptive transfer of 1×10^7 total T cells and systemic IL-2. The study ended on day 48 after tumor inoculation. (B) Tumor growth curves based on digital caliper measurements. Each line represents an individual mouse (blue: treatment group; red: control group). (C) Quantification of NLUC-based bioluminescence signal at the tumor site (average radiance

[photons/s/cm²/sr]) over time. Each line represents an individual mouse (blue: treatment group; red: control group). (D) Representative BLI images of tumors in the control group (upper row; mouse IDs 749, 751, 753, 754, 795) and treatment group (lower row; mouse IDs 760, 796, 797, 798, 799) at indicated time points. Mice received 100 μ L of furimazine (1:40 in PBS) intraperitoneally immediately prior to imaging.

After tumor clearance, treated mice remained tumor-free for the duration of the study, which ended on day 48 after tumor inoculation (d41 after ACT). In the control group, tumor progression was observed in two mice, while three of five animals exhibited spontaneous tumor regression. However, tumor clearance in control mice occurred at a markedly slower rate compared to the treatment group, indicating a potent and accelerated therapeutic effect mediated by the TRP2-specific T cells.

4.4.3 Characterization of vitiligo-like depigmentation and T-cell localization patterns

BLI on day 2 after ACT revealed specific localization of FLUC⁺ T cells to the tumor sites in both treatment and control groups, as shown in [Figure 31](#). In control mice, T cells remained localized to the tumor region even in cases of spontaneous tumor regression. In contrast, T cells in the treatment group initially localized strongly to the tumor, as evidenced by BLI on days 2 and 6 after ACT, but began to migrate beyond the tumor site into adjacent skin tissue as the tumors regressed. It is important to note that only a subset of the transferred T cells expressed FLUC and were therefore trackable by BLI; however, the localization of FLUC⁺ cells is assumed to represent the overall distribution of the transgenic T-cell populations. Equal numbers of FLUC⁺ cells were used in both groups, enabling valid comparison of migration patterns.

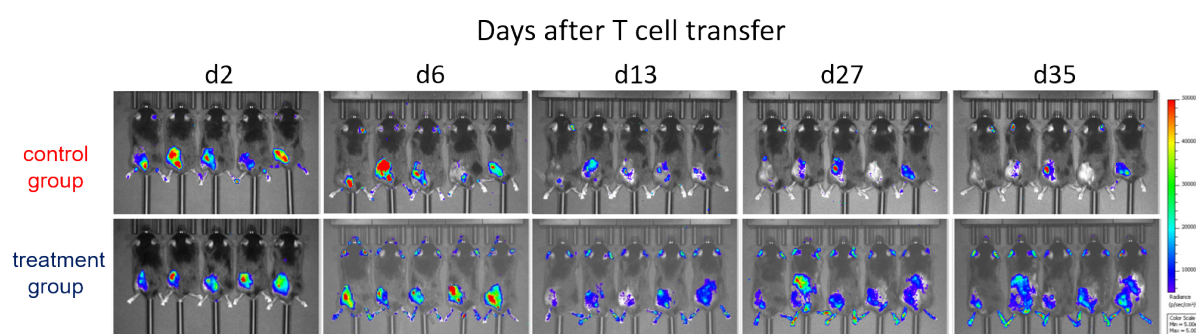


Figure 31: *In vivo* BLI of transduced T cells in the syngeneic melanoma model.

Representative BLI images showing FLUC signal from transduced T cells at indicated time points after adoptive cell transfer. The upper row shows control mice receiving anti-NY-ESO-1-cTCR T cells (mouse IDs 749, 751, 753, 754, 795). The lower row shows treatment mice receiving anti-TRP2-cTCR T cells (mouse IDs 760, 796, 797, 798, 799). T-cell bioluminescence was recorded following intraperitoneal injection of D-luciferin (2.5 mg/100 μ L in dH₂O). FLUC⁺ T-cell signals are visible at tumor sites and other anatomical locations over time in both groups.

Strikingly, all mice receiving anti-TRP2-cTCR T cells developed a progressive fur depigmentation phenotype, varying in extent and anatomical distribution. Depigmentation became apparent around day 13 after ACT and continued to increase thereafter. In all cases, the extent of depigmentation remained stable or further progressed over time, with no evidence of spontaneous repigmentation observed during the experimental period. In contrast, no such changes occurred in control animals, supporting a TRP2-specific, cTCR-mediated on-target/off-tumor effect. Notably, the anatomical distribution of depigmented fur closely mirrored the post-tumor migration pattern of FLUC⁺ T cells, exemplified by mouse 796 in [Figure 32](#), that displayed the most extensive depigmentation. This spatial correlation suggests a direct association between T-cell infiltration and melanocyte loss or dysfunction within the skin or hair follicles.

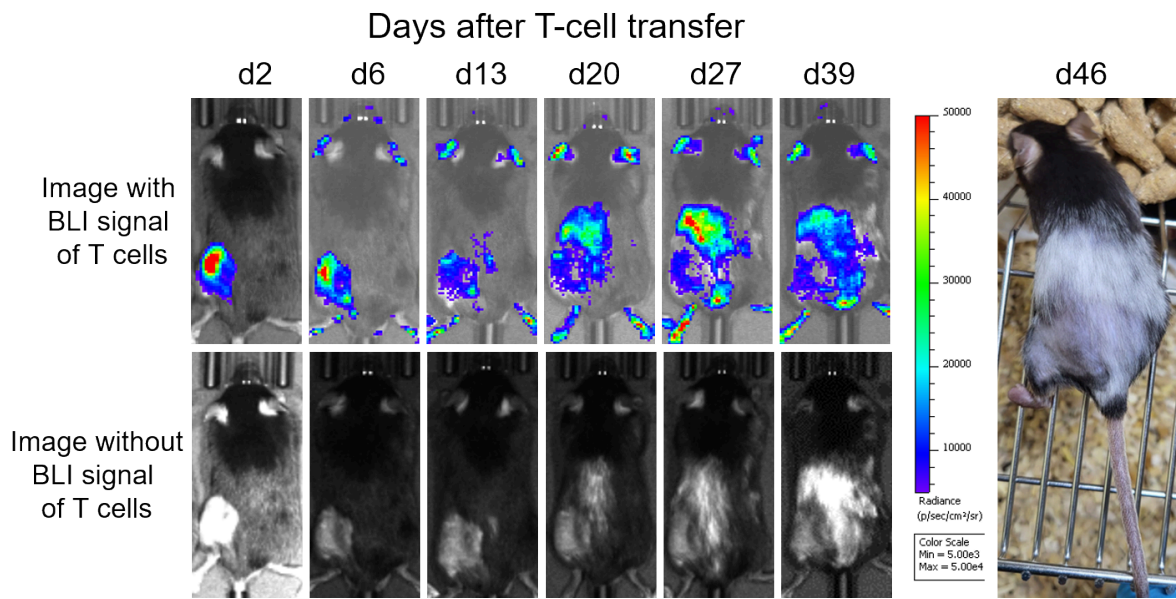


Figure 32: *In vivo* BLI of transduced T cells and fur pigmentation patterns in an anti-TRP2-cTCR-treated mouse over time.

Representative images of mouse 796 at the indicated time points after adoptive T-cell transfer. Top row: FLUC⁺ T-cell bioluminescence signal following intraperitoneal administration of D-luciferin (2.5 mg/100 μ L in dH₂O). Bottom row: corresponding images without BLI overlay, showing coat pigmentation changes in the same anatomical regions over time. Day 46 image illustrates depigmented fur areas at the experimental endpoint.

Peripheral blood was collected on days 14 and 27 after ACT to assess the persistence of transferred T cells *in vivo*. Blood samples were stained with anti-mouse CD3 antibody in combination with specific antibodies against the TCR β chain: anti-hV β 3 for anti-TRP2-cTCR-transduced T cells (treatment group) and anti-hV β 1 for anti-NY-ESO-1-cTCR-transduced T cells (control group). Quantification was performed by flow cytometry using counting beads to determine absolute cell numbers per μ L of blood and results are shown in [Figure 33](#).

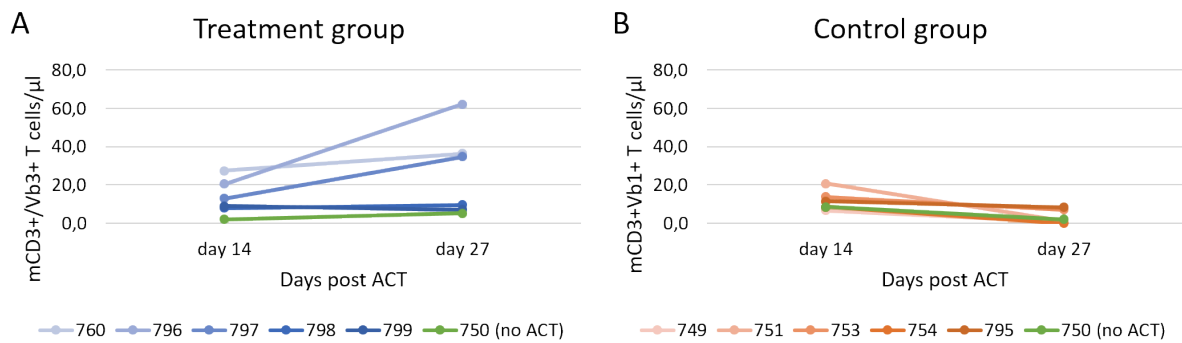


Figure 33: Quantification of transferred T cells in peripheral blood by flow cytometry.

Absolute T-cell counts per μL blood were determined using anti-CD3 and TCR $\text{hV}\beta$ -specific antibodies together with counting beads. Each line represents an individual mouse. (A) Treatment group (anti-TRP2-cTCR): $\text{CD3}^+\text{hV}\beta3^+$ T-cell counts shown from day 14 to day 27 after adoptive transfer. (B) Control group (anti-NY-ESO-1-cTCR): $\text{CD3}^+\text{hV}\beta1^+$ T-cell counts shown over the same time frame. Mouse 750, which did not receive adoptive T-cell transfer, served as a negative control in both panels.

In the treatment group, the number of $\text{CD3}^+\text{hV}\beta3^+$ T cells per μL of blood remained stable or slightly increased from day 14 to day 27, suggesting sustained persistence or expansion of transferred anti-TRP2 T cells *in vivo*. In contrast, $\text{CD3}^+\text{hV}\beta1^+$ T-cell counts in the control group remained stable or slightly decreased over the same period. As a negative control, blood from mouse 750, which did not receive any T-cell transfer, was processed in parallel, confirming the specificity of the detection strategy.

To assess persistence and phenotypic characteristics of transduced T cells *in vivo*, peripheral blood, spleen, and lymph nodes were harvested at the experimental end-point. Single-cell suspensions were prepared, and flow cytometric analysis was performed following staining with a 12-marker panel targeting murine CD3, CD8, CD25 ($\text{IL-2R}\alpha$), CD69, CTLA-4, PD-1, TIGIT, $\text{IL7R}\alpha$ (CD127), CD44, and CD62L, as well as human TCR $\text{V}\beta1$ and $\text{V}\beta3$ to detect TCR-transduced cells. A viability dye was included to exclude non-viable events. T cells were first gated on lymphocytes, single cells, and live cells, followed by identification of $\text{CD3}^+\text{CD8}^+$ (putative cytotoxic) and $\text{CD3}^+\text{CD8}^-$ (putative helper) T-cell subsets. Within each subset, frequencies of $\text{hV}\beta^+$ cells were analyzed to quantify the proportion of transduced T cells present.

The corresponding results are presented in Figures 34-36, showing the distribution of $\text{hV}\beta^+$ transduced T cells (Figure 34), the expression of activation and exhaustion markers (Figure 35), and the differentiation status of transduced T cells (Figure 36) across peripheral blood, lymph nodes, and spleen.

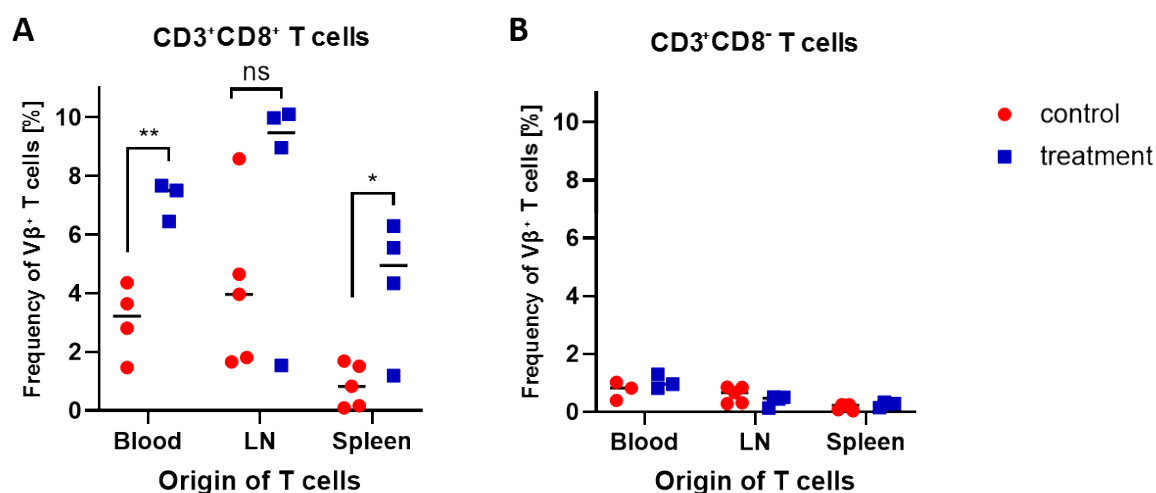


Figure 34: Frequency of hVβ⁺ transduced T cells in peripheral blood, lymph nodes, and spleen.

Flow cytometry was performed on cells from peripheral blood, lymph nodes (LN), and spleen at the experimental endpoint (day 48 after tumor inoculation; day 41 after adoptive T-cell transfer) to determine the proportion of T cells expressing the transduced TCR. Each data point represents an individual mouse. (A) Frequency of hVβ⁺ cells among CD3⁺CD8⁺ T cells. In peripheral blood, the frequency of hVβ⁺ CD3⁺CD8⁺ T cells was significantly higher in treatment compared to control mice (Welch's *t*-test, *p* = 0.0029), whereas no significant difference was observed in the LN (*p* = 0.2025). In the spleen, the frequency was moderately but significantly increased in treated mice (*p* = 0.0480). (B) Frequency of hVβ⁺ cells among CD3⁺CD8⁻ T cells.

Among CD3⁺CD8⁺ T cells, the frequency of hVβ⁺ cells was higher in mice treated with anti-TRP2 T cells than in mice treated with anti-NY-ESO-1 T cells, with tissue-specific significance patterns. Statistical significance was assessed using an unpaired two-sample Welch's *t*-test. The highest frequencies were observed in lymph node-derived T cells, with means of 7.6% in anti-TRP2-treated and 4.1% in anti-NY-ESO-1-treated mice (*p* = 0.2025). Blood samples showed a significant increase in the anti-TRP2 group, with 7.2% hVβ⁺ T cells compared to 3.1% in controls (*p* = 0.0029). The lowest frequencies were detected in spleen-derived T cells, with 4.3% in the anti-TRP2 group versus 0.9% in controls (*p* = 0.0480). In the CD3⁺CD8⁻ (putative CD4⁺) subset, the frequency of hVβ⁺ cells remained consistently below 1% across all tissues and treatment groups.

Expression of activation and exhaustion markers was assessed within CD3⁺CD8⁺hVβ⁺ and CD3⁺CD8⁻hVβ⁺ T cells isolated from peripheral blood, spleen, and lymph nodes at the experimental endpoint. Frequencies of CD25⁺, CD69⁺, CTLA-4⁺, PD-1⁺, and TIGIT⁺ cells were quantified separately for each tissue compartment. Overall, no striking differences in marker expression were observed between treatment and control groups or across tissue sources. However, an increased frequency of PD-1⁺ cells was detected within the CD3⁺CD8⁻hVβ⁺ subset in peripheral

blood of treated animals compared to the same T-cell subset in control mice, as well as compared to CD3⁺CD8⁺hVβ⁺ cells from either group. Furthermore, CD25 expression was consistently lower in both CD8⁺ and CD8⁻ transduced T-cell subsets derived from spleen samples relative to those from peripheral blood and lymph nodes.

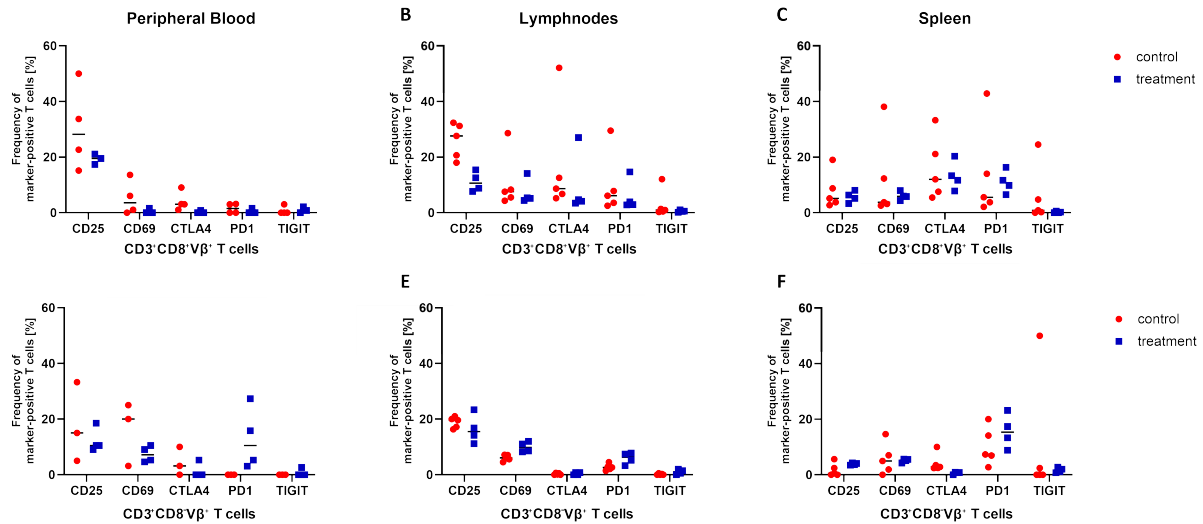


Figure 35: Expression of activation and exhaustion markers on transduced T cells in peripheral blood, lymph nodes, and spleen.

Flow cytometry was used to assess expression of CD25, CD69, CTLA-4, PD-1, and TIGIT on transduced (hVβ⁺) T cells at the experimental endpoint (day 48 after tumor inoculation; day 41 after adoptive T-cell transfer). Cells were stratified by tissue (peripheral blood, lymph nodes, spleen) and CD8 expression. Each data point represents an individual mouse. (A–C) Frequencies of marker-positive cells among CD3⁺CD8⁺hVβ⁺ T cells from (A) peripheral blood, (B) lymph nodes, and (C) spleen. (D–F) Frequencies of marker-positive cells among CD3⁺CD8⁻hVβ⁺ T cells from (D) peripheral blood, (E) lymph nodes, and (F) spleen.

To evaluate the differentiation status of transduced T cells, hVβ⁺ cells were gated on IL7Rα⁺ events (consistently >98% across all samples) and subsequently analyzed for CD44 and CD62L expression to define memory subsets. T effector memory (TEM; CD44⁺CD62L⁻) and central memory (TCM; CD44⁺CD62L⁺) populations were compared between anti-TRP2-cTCR-treated and anti-NY-ESO-1-cTCR-treated control animals. No consistent or striking differences in memory phenotype distribution were observed between treatment groups, although notable variability was seen across individual mice and tissues, as shown in [Figure 36](#).

In peripheral blood, CD8⁺hVβ⁺ T cells displayed variable phenotypes, with some animals exhibiting a dominant effector memory (TEM; CD44⁺CD62L⁻) population, while others had a predominance of central memory (TCM; CD44⁺CD62L⁺) cells. In the CD8⁻hVβ⁺ subset, TCM cells were dominant in all animals except control mouse 753, where 55% of cells exhibited a TEM phenotype. In lymph nodes, CD8⁺hVβ⁺ T cells

were mostly TCM across all mice, with the exception of treatment mouse 760, in which TEM cells exceeded 50%. CD8⁺hVβ⁺ cells in lymph nodes consistently showed TEM frequencies below 40%. In spleen-derived CD8⁺hVβ⁺ T cells, the highest overall TEM frequencies were observed, with only control mouse 754 showing a TEM proportion below 50%. In contrast, CD8⁺hVβ⁺ cells in the spleen displayed a more balanced distribution of TEM and TCM subsets. Naïve (CD44⁺CD62L⁺) and double-negative (CD44⁺CD62L⁻) phenotypes were generally rare across all tissues and groups; however, control mouse 751 showed a prominent double-negative population in the spleen within both CD8⁺hVβ⁺ and CD8⁺hVβ⁻ compartments.

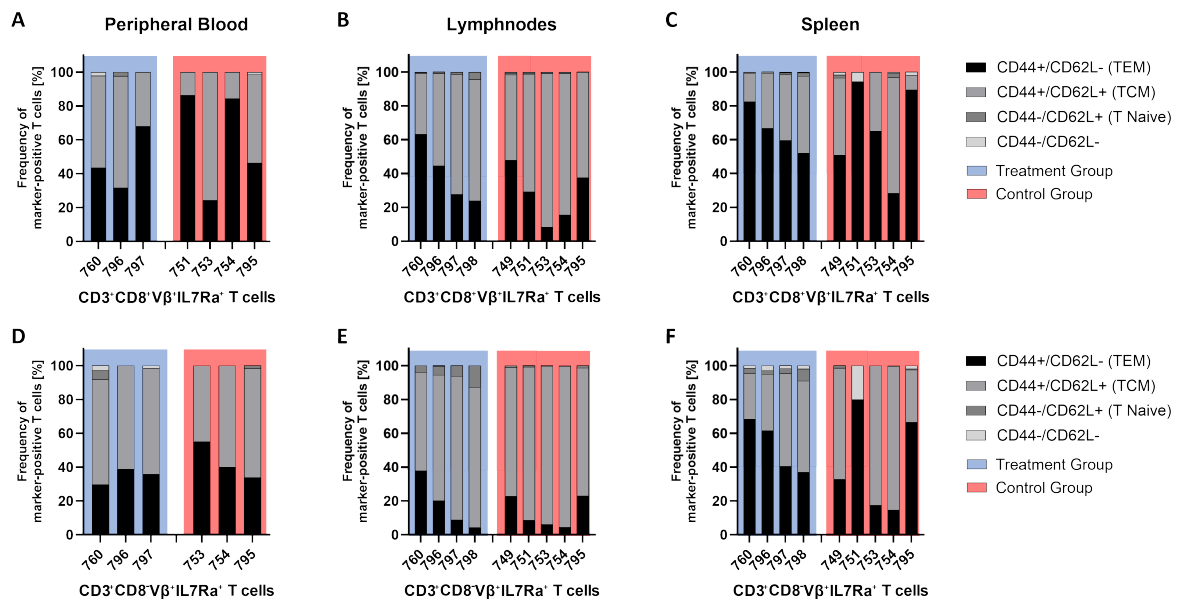


Figure 36: Differentiation status of transduced T cells in peripheral blood, lymph nodes, and spleen.

Flow cytometry analysis of CD3⁺hVβ⁺ T cells gated on IL7Ra⁺ events. CD44 and CD62L expression was used to define differentiation subsets: T effector memory (TEM: CD44⁺CD62L⁻), central memory (TCM: CD44⁺CD62L⁺), naïve (T naïve: CD44⁻CD62L⁺), and double-negative (DN: CD44⁺CD62L⁻). Each stacked bar represents an individual mouse. (A–C) Differentiation subset distribution among CD3⁺CD8⁺hVβ⁺ T cells from (A) peripheral blood, (B) lymph nodes, and (C) spleen. (D–F) Differentiation subset distribution among CD3⁺CD8⁺hVβ⁻ T cells from (D) peripheral blood, (E) lymph nodes, and (F) spleen.

Together, these results indicate that transduced T cells predominantly exhibit memory phenotypes *in vivo* after ACT, with substantial variability across mice and tissues but without consistent or treatment-specific differences in differentiation status.

4.4.4 Histopathological evaluation of tissues

To assess potential therapy-associated tissue alterations and evaluate the safety profile of the treatment, histological and immunohistochemical analyses were performed on skin, ocular tissue, liver, brain, lungs, intestines, and spleen. All samples were evaluated by pathologist [REDACTED]. In skin sections, CD3 immunostaining revealed no pathological accumulation or clustering of T cells in either treatment or control groups. CD3⁺ T cells were sparsely distributed, primarily localized intraepithelially or as isolated cells within the subepithelial connective tissue. Preliminary quantification of CD3⁺ cells per 100 epithelial cells revealed no significant differences between groups, suggesting a lack of localized T-cell-mediated immune activation at the experimental endpoints.

H&E staining of skin and internal organs further supported these findings, as shown in [Figure 37](#). No histopathological signs of hepatitis, cholangitis, or inflammatory lesions were detected in the liver or bile ducts. Similarly, ocular histology revealed no evidence of retinal degeneration, sensory epithelial damage, or inflammatory infiltrates (data not shown). Minor preparation artifacts, such as retinal detachment or vitreous body shrinkage, were observed but deemed non-specific and unrelated to T-cell treatment.

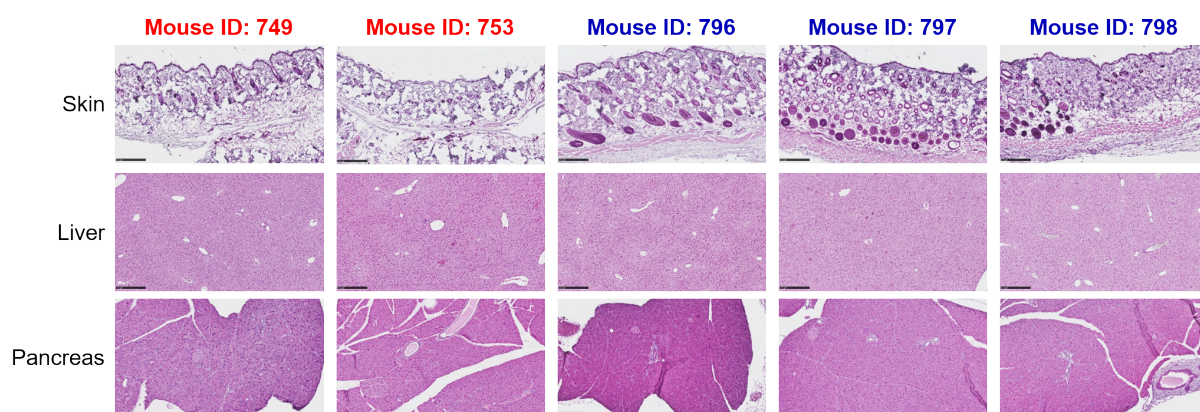


Figure 37: Histopathological analysis of skin, liver, and pancreatic tissues from control (red) and treated mice (blue).

Representative H&E stainings of skin (top), liver (middle), and pancreatic tissue (bottom) collected at the experimental endpoint from control (red mouse IDs: 749, 753) and treatment (blue mouse IDs: 796, 797, 798) groups. No histopathological alterations or inflammatory lesions were detected in any examined tissues.

Collectively, histological analyses revealed no evidence of treatment-associated inflammation or tissue damage across examined organs, supporting a favorable safety profile. The observed depigmentation occurred in the absence of detectable tissue pathology within the affected areas.

4.4.5 Serum cytokine profiling following TRP2-specific T-cell therapy

To investigate whether systemic immune activation contributed to the vitiligo-like depigmentation observed in treated mice, serum cytokine levels were analyzed via multiplex assay at the experimental endpoint (day 41 after T-cell transfer). Multiplex cytokine analysis was conducted in collaboration with PD Dr. Matthias Bros. As shown in [Figure 38](#), concentrations of 15 cytokines were measured, including key pro-inflammatory markers such as IFN- γ , TNF- α , IL-1 β , IL-6, IL-17, and chemokines like CXCL1, CXCL10, CCL2, and CCL5 (RANTES). Mice treated with anti-TRP2 TCR-T cells (blue) showed comparable cytokine profiles to those receiving control anti-NY-ESO-1 TCR-T cells (red), with no consistent group-wide elevations indicative of systemic immune activation.

Notably, while most cytokine levels remained low or undetectable across both groups, sporadic elevations were observed in individual mice. For instance, elevated levels of IFN- α , IL-6, and CXCL1 were detected in one anti-TRP2-treated mouse (ID: 760), while other treatment animals showed increased concentrations of IL-1 β , IL-12, and CCL2. In the control group, isolated increases in IFN- γ , IFN- α , IL-10, CXCL10, GM-CSF, and CCL5 were observed in different mice. These isolated elevations did not follow a treatment-specific pattern and may reflect inter-individual variability rather than a therapy-associated effect. Importantly, the “no ACT” control mouse, which did not receive adoptive cell therapy, exhibited baseline cytokine levels similar to both treatment groups.

Overall, these findings do not support the presence of sustained systemic immune activation in anti-TRP2 TCR-T-cell-treated mice. However, given that serum samples were collected only at the experimental endpoint, transient cytokine elevations occurring earlier in the course of therapy cannot be excluded.

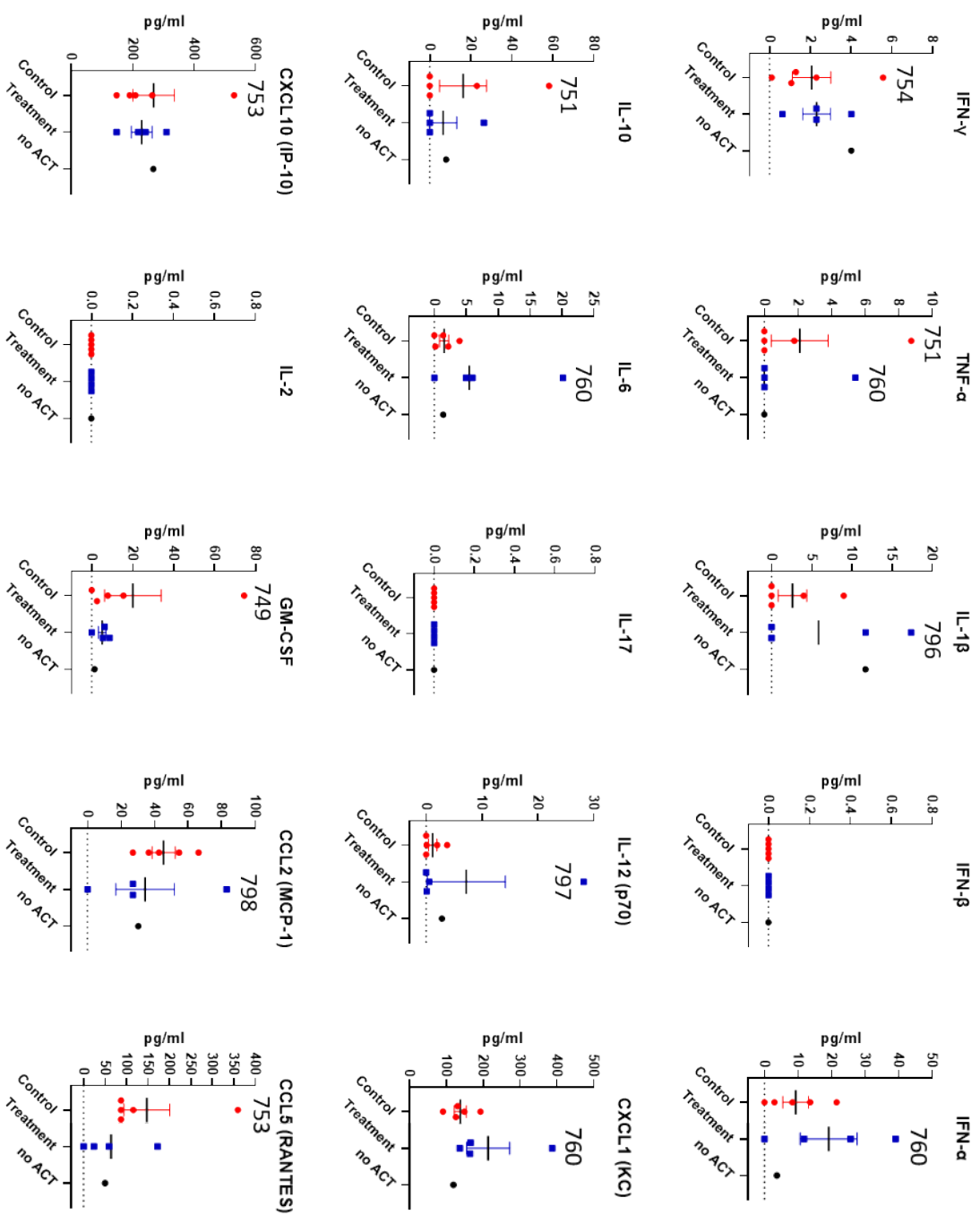


Figure 38: Endpoint serum cytokine profiling in cTCR-T-cell-treated mice.

Multiple serum cytokine analysis was performed at the experimental endpoint (day 40 after ACT for control mice, day 41 after ACT for treatment mice) in syngeneic C57BL/6 tumor-bearing mice treated with anti-TRP2 TCR-T cells (blue) or control anti-NY-ESO-1 TCR-T cells (red). One tumor-bearing mouse that did not receive adoptive cell therapy ("no ACT", black) served as an additional reference. Mouse 799 was excluded due to insufficient serum volume. Cytokine concentrations [pg/mL] are shown for individual mice for IFN- γ , TNF- α , IL-1 β , IFN- β , IFN- α , IL-10, IL-6, IL-17, IL-12, CXCL1, CXCL10, IL-2, GM-CSF, CCL2, and CCL5.

Taken together, engineered murine T cells exhibited high transduction and knockout efficiencies and retained antigen-specific functionality *in vitro*, as demonstrated by IFN- γ ELISpot and bioluminescence-based cytotoxicity assays. Phenotypically, the T cells predominantly displayed an effector memory (TEM) profile prior to adoptive transfer. Upon administration into C57BL/6 mice bearing TRP2-overexpressing B78.H1 melanoma tumors, these T cells induced rapid tumor regression and complete elimination of detectable disease, as monitored by serial BLI. In contrast, control animals displayed either delayed spontaneous regression or progressive tumor growth, underscoring the antigen-specific and accelerated therapeutic effect mediated by TRP2-targeted T cells.

Overall, the syngeneic mouse model reinforced the robust antitumor efficacy of TRP2-specific TCR-T cell therapy in an immunocompetent setting, validating the therapeutic potential of an HLA-independent TCR targeting a melanoma-associated antigen. Furthermore, the absence of histopathological signs of T-cell-mediated organ toxicity, despite the occurrence of vitiligo-like depigmentation, suggests a favorable safety profile.

5 Discussion

Antigenic heterogeneity and immune evasion through MHC class I loss remain significant barriers to effective immunotherapy in solid tumors such as melanoma. In this study, a naturally occurring, HLA-independent T-cell receptor targeting TRP2 was evaluated with the aim of bypassing classical antigen presentation constraints in adoptive T-cell therapy. Using both xenograft and syngeneic melanoma models, it was demonstrated that T cells transduced with this TRP2-specific TCR effectively localized to tumor sites. Robust antitumor activity was observed only in models with TRP2 overexpression, whereas in tumors with endogenous TRP2 expression, responses were limited to delayed growth or absent altogether. Notably, the therapy was well tolerated without systemic toxicity, although a vitiligo-like depigmentation phenotype emerged. These findings provide preclinical evidence supporting the feasibility and apparent safety of HLA-independent TCR-T-cell therapy targeting TRP2, offering a promising strategy to overcome immune evasion in melanoma and potentially other solid tumors.

5.1 Direct antigen recognition by HLA-independent $\alpha\beta$ T-cell receptors

Direct recognition by MHC-independent $\alpha\beta$ T-cell receptors offers a promising pathway to overcome key limitations in T-cell-based immunotherapy, particularly in the context of tumors that evade immune detection through MHC downregulation. Unlike conventional $\alpha\beta$ TCRs that depend on peptide-MHC complexes and CD4/CD8 co-receptors for activation, HLA-independent TCRs can directly recognize conformational epitopes on native cell-surface proteins, thereby bypassing the requirement for antigen processing and MHC-mediated presentation. This expanded versatility enhances our understanding of immune surveillance and opens new possibilities for antigen targeting in solid tumors.

The Quad-KO mouse model, developed by Alfred Singer's group at the NIH (Bethesda, USA), which lacks MHC class I and II molecules as well as CD4 and CD8 co-receptor expression, exemplifies this concept. It demonstrates that TCRs can undergo thymic selection and mediate immune responses by directly recognizing self-proteins such as CD155, even in the absence of MHC molecules and co-receptor engagement. These MHC-independent TCRs display high-affinity interactions during thymic selection while maintaining canonical TCR architecture, thereby revealing a non-classical mode of antigen recognition [132], [133], [134].

Early work by Olivera J. Finn and colleagues showed that $\alpha\beta$ TCRs can directly recognize tumor antigens without the requirement for peptide-MHC presentation. Barnd et al. (1989) first described a human cytotoxic T-cell line (W.D.) derived from a pancreatic cancer patient that specifically recognized and lysed pancreatic and breast tumor cells in an MHC-unrestricted, yet TCR-dependent, manner through interaction with the highly glycosylated tumor-associated mucin MUC1. Blocking experiments confirmed antigen specificity and excluded MHC involvement [135]. Later, Alajez et al. (2005) demonstrated the translational potential of this approach by engineering a MUC1-specific, MHC-independent single-chain TCR fused to a CD3 ζ signaling domain, which could be stably expressed across multiple immune lineages and mediated selective tumor control *in vivo* without inducing autoimmunity [136].

Additional studies in murine models have further characterized MHC-independent $\alpha\beta$ TCRs capable of recognizing a range of native molecules, including chemical haptens and intact foreign proteins. For example, Rao et al. described arsonate-reactive TCRs that bind haptens conjugated to carrier proteins [137], while Almeida et al. reported a TCR capable of directly recognizing the fluorescent protein phycoerythrin (PE) [138], both independently of MHC presentation. Also, Hanada et al. identified a murine $\alpha\beta$ TCR that directly recognizes a soluble TRAIL/DR4 complex [139].

Beyond murine models, further evidence for HLA-independent TCRs in humans is beginning to emerge, highlighting the translational potential of this approach for immunotherapy. Notably, Hiscox et al. identified a fully human, HLA-independent TCR that directly recognizes mesothelin without the need for peptide-HLA presentation, demonstrating robust, antigen-specific cytotoxicity and potent anti-tumor activity in preclinical models [140]. This receptor was identified using phage display libraries, and it remains unclear whether such $\alpha\beta$ TCR pairing would naturally occur in the human repertoire. In contrast, the HLA-independent TRP2-specific TCR characterized in this study was isolated directly from a melanoma patient with HLA class I-deficient disease, demonstrating that such receptors can arise naturally.

Building on this, the present study demonstrates that a naturally occurring, HLA-independent TCR targeting TRP2, a melanocyte differentiation antigen frequently expressed in melanoma, can mediate effective tumor control *in vivo* when TRP2 is overexpressed. Engaging the native TCR signaling pathway may offer advantages in achieving more physiological T-cell activation and improved safety, presenting a promising alternative to synthetic CAR-based strategies in solid tumors. Supporting this, Baeuerle et al. showed that TRuC-T cells, which integrate antibody domains into the native TCR complex, achieve potent cytotoxicity with lower cytokine release and avoid the tonic signaling seen in CAR-T cells [141]. Similarly, Mansilla-Soto et al. demonstrated that HIT receptors, combining CAR-derived antigen recognition

domain with native TCR-CD3 signaling, mediate faster, more sensitive cytotoxic responses at lower antigen densities, resulting in superior tumor control in xenograft models [63]. Together, these findings suggest that engaging the full native TCR signaling pathway can improve the efficacy and safety of engineered T-cell therapies, underscoring the potential of HLA-independent TCR-T cells to address the limitations of current CAR-T approaches in solid tumors.

An open question is whether HLA-independent antigen recognition by $\alpha\beta$ TCRs represents a physiologically intended mechanism of immune surveillance or is merely a consequence of random cross-reactivity [142], [143] or molecular mimicry [144], [145]. The fairly limited number of reports describing MHC-independent TCRs, together with the diversity of their identified targets, ranging from native proteins to haptens and intact foreign antigens, suggests that such receptors may arise stochastically within the highly diverse TCR repertoire. However, these receptors may not be purely coincidental but could reflect a conserved immune mechanism that enables T-cell recognition of cells that have lost MHC expression as a means of immune evasion. Supporting this notion, the Wölfel group identified HLA-independent anti-TRP2 TCRs in three other melanoma patients and one healthy donor [34], [35], indicating that these receptors may indeed represent a physiological mechanism of immune surveillance that can be harnessed therapeutically. Clarifying whether these TCRs arise through selective pressures during thymic development or peripheral immune responses, or whether they exploit structural features enabling direct antigen engagement, will be essential for understanding their physiological relevance and for guiding their safe and effective therapeutic application.

While the limited number of characterized HLA-independent TCRs prevents definitive conclusions regarding their affinity spectrum, current data, including unpublished preliminary cDNA titration studies from the Wölfel group (personal communication, [REDACTED]), suggest that these receptors exhibit a range of affinities comparable to conventional TCRs. This is critical given the established relationship between TCR affinity, therapeutic efficacy, and the risk of off-target toxicity [146]. Moving forward, the therapeutic implementation of MHC-independent TCRs will require careful selection, engineering, and safety validation to fully realize their potential in overcoming antigen escape and enhancing the effectiveness of T-cell therapy in solid tumors.

5.2 TRP2 as a target antigen in adoptive cell therapy and potential adverse effects

TRP2 is a melanocyte differentiation antigen involved in melanin biosynthesis, possesses anti-apoptotic properties [36], and is considered a promising immunotherapy target due to its relatively restricted expression and functional relevance in melanoma cells. As shown in this study and supported by Avogadri et al., TRP2 is commonly expressed in primary melanomas, with immunohistochemical positivity in 84% of primary tumors but reduced to 58% in metastatic lesions, potentially limiting therapeutic efficacy in advanced disease stages [147].

A key concern when targeting TRP2 is its expression in normal melanocytes, which raises the risk of on-target, off-tumor toxicity. In this study, vitiligo-like depigmentation observed in mice treated with MHC-independent TRP2-specific TCR-T cells aligns with findings from other preclinical models. Steitz et al. demonstrated that gene gun vaccination with synthetic mRNA encoding murine TRP2 induced protective anti-melanoma cellular immunity, together with a humoral response, and induced vitiligo-like fur depigmentation in C57BL/6 mice, consistent with TRP2-specific immune targeting of melanocytes [148]. Similarly, Le Poole and Luiten reported that DNA vaccination with a plasmid encoding human TRP2 led to progressive depigmentation in mice, beginning at the vaccination site [149]. These consistent observations across studies support the notion that TRP2-directed immunotherapies can elicit antigen-specific autoimmune effects against melanocyte-lineage cells.

Clinical studies by Cohen et al. and Rosenberg and White suggest that melanoma-associated vitiligo reflects effective anti-tumor immunity and may serve as a surrogate marker of therapeutic efficacy [150], [151], [152]. Similarly, Jilani et al. reported vitiligo-like depigmentation as the sole off-tumor effect in TRP1-targeting CAR-T therapy, supporting the interpretation that localized autoimmune responses can occur without broader systemic toxicity [153]. In contrast, Palmer et al. demonstrated that adoptive transfer of gp100-specific TCR-transduced T cells in murine models led to severe ocular autoimmunity, characterized by CD8⁺ T-cell infiltration into immune-privileged ocular tissues and associated with the breakdown of immune privilege. Mechanistically, IFN- γ signaling induced MHC class I upregulation in ocular melanocytes, enabling antigen presentation in tissues typically shielded from immune surveillance. Notably, blocking IFN- γ or deleting its receptor significantly reduced ocular toxicity while preserving anti-tumor efficacy, revealing a pathway-specific mechanism of toxicity that could guide strategies to reduce adverse effects in TCR-T-cell therapy [154].

In this study, MHC-independent TRP2-specific TCR-T cells induced localized depigmentation without evidence of systemic or ocular toxicity. These findings support the feasibility of targeting TRP2 despite its expression on normal melanocytes. Consistent with this, Khong and Rosenberg demonstrated in a clinical setting that immunologic tolerance to TRP2 can be overcome without inducing severe autoimmunity. In their study, tumor-infiltrating lymphocytes from a patient with a strong clinical response recognized TRP2-derived epitopes, even in the absence of prior TRP2 vaccination, suggesting that TRP2-specific immune responses can naturally emerge as part of effective anti-tumor immunity [40].

Importantly, this study demonstrated complete tumor regression in syngeneic and xenograft models when tumors were engineered to overexpress TRP2, while tumors expressing only endogenous TRP2 showed limited responses, characterized by delayed growth or lack of control. Notably, immunohistochemistry revealed TRP2 expression at experimental endpoints even in cases with limited response, suggesting reversible antigen downregulation under immune pressure with subsequent re-expression. This hypothesis is based on the work by Landsberg et al., who showed that inflammatory cytokines, particularly TNF- α , can induce transient dedifferentiation and downregulation of melanocytic antigens, including TRP2 and gp100, facilitating immune evasion. Importantly, this dedifferentiation was reversible, and TNF- α blockade restored antigen expression and T-cell recognition, highlighting inflammation-induced tumor plasticity as a critical consideration in TRP2-targeted therapies [130]. Mehta et al. supported this with a case of a patient whose metastatic melanoma underwent inflammation-induced dedifferentiation as a resistance mechanism to adoptive cell therapy targeting the MART-1 antigen [155].

Overall, these findings highlight TRP2 as a viable target for TCR-T-cell therapy despite its expression on normal melanocytes, with depigmentation serving as a potential indicator of effective immune responses. Importantly, inflammation-induced tumor plasticity, including transient dedifferentiation and antigen loss under immune pressure, may limit the durability of TRP2-targeted therapies. Addressing this challenge will require strategies that stabilize melanocytic antigen expression and reduce antigen escape. One promising approach, supported by recent findings from Ma et al., is the use of the receptor tyrosine kinase inhibitor tivozanib, which can reverse inflammation-induced dedifferentiation in melanoma by restoring MITF activity and dependency, thereby re-sensitizing tumors to melanocytic antigen-targeting therapies [131].

In this study, it was observed that treatment of melanoma cells with the receptor tyrosine kinase inhibitor tivozanib restored TRP2-specific T-cell recognition following exposure to TNF- α or IFN- γ , consistent with reactivation of the MITF-TRP2 axis and partial reversal of cytokine-induced dedifferentiation. This effect was observed in melanoma cells retaining MITF activity (86b_KO), whereas fully dedifferentiated, MITF-inactive cells (86a_KO) remained unresponsive.

Future studies should evaluate whether combining tivozanib with TRP2-specific TCR-T-cell therapy *in vivo* can stabilize antigen expression and enhance therapeutic efficacy. In addition, the co-targeting of melanocytic and non-melanocytic antigens may further mitigate immune escape by limiting the capacity of tumors to downregulate a single lineage antigen under immune pressure. Future studies should focus on integrating such strategies to balance efficacy with safety, fully utilizing TRP2-directed T-cell therapies in melanoma while overcoming the barrier of inflammation-induced tumor plasticity.

5.3 Technical limitations and model-specific confounders

5.3.1 *In vivo* bioluminescence imaging

In vivo BLI provided a functional readout of both tumor burden and T-cell dynamics throughout treatment. Importantly, the ATP dependence of FLUC enabled the selective detection of metabolically active T cells, allowing assessment of T-cell viability and persistence following adoptive transfer. In contrast, the ATP-independent activity of NLUC ensured that a loss of signal directly reflected tumor elimination. Additionally, the shorter emission wavelength of NLUC was sufficient for reliable detection of subcutaneous tumors, while the longer wavelength emitted by FLUC facilitated the tracking of T cells in deeper anatomical sites. Combined, these features of the dual-luciferase system enabled accurate interpretation of treatment responses and supported the mechanistic evaluation of T-cell-mediated tumor control *in vivo*.

When applying this method in practice, however, technical aspects of substrate administration must also be considered. As noted by Keyaerts et al. (2012), intraperitoneal injection of luciferase substrates such as D-luciferin is widely used because it is simple and reliable, although occasional failed injections can lead to markedly reduced signal if the substrate is inadvertently delivered into the intestine [156]. This factor should be considered when interpreting tumor BLI results and likely explains the transient loss of tumor signal observed, for example, in [Figure 30D](#) in control mouse 753 on day 12 after tumor inoculation, given that clear signals were detected on days 6 and 26.

5.3.2 T-cell tracking and control vector design in BLI experiments

In the first NSG xenograft experiment, which demonstrated complete tumor eradication, anti-TRP2-TCR-FLUC transduced T cells were used for the treatment group. Untransduced T cells were initially planned as controls, however, to enable tracking by BLI using the FLUC reporter gene, the control T cells were transduced with a construct containing FLUC to allow visualization.

For this purpose, a bicistronic control vector encoding FLUC and EGFP linked via a P2A sequence was used, with EGFP serving as a flow cytometry reporter to verify successful transduction and indicate co-expression of FLUC. However, this approach resulted in differences in FLUC expression levels between treatment and control T-cell populations. Specifically, treatment T cells showed a transduction rate of 61% (CD3 and mTRBC co-expression), while 87.6% of control T cells expressed EGFP. To maintain consistency across groups, the same total number of T cells was transferred per mouse rather than adjusting for the proportion of successfully transduced cells, which would have resulted in unequal total T-cell numbers. This resulted in a higher number of FLUC-expressing cells in the control group and, consequently, stronger luminescence signals in BLI measurements.

Additionally, the lack of puromycin selection *in vivo* may have contributed to this discrepancy, as *in vitro* experiments showed a decline in surface TCR expression without selection, whereas intracellular EGFP expression in control T cells remained stable. It remains unclear whether TCR downregulation in the absence of puromycin similarly affected intracellular FLUC expression. Nevertheless, despite differences in luminescence signal strength between treatment and control groups, the approach allowed reliable tracking of T-cell localization within individual mice, although signal intensities were only comparable within each group, not across groups.

To improve comparability, the strategy was adjusted in subsequent experiments by transducing control T cells with an irrelevant TCR construct encoding FLUC in the same tricistronic format as the treatment vector. An HLA-A2-restricted TCR targeting NY-ESO-1 was selected, as the absence of human HLA in the murine models ensured that this irrelevant TCR would not mediate functional effects while maintaining equivalent vector design and expression levels. To prevent off-target activation, control T cells were not antigen-specifically stimulated, consistent with the approach used in the first experiment.

Despite this adjustment, some differences in TCR and FLUC expression levels remained. This likely reflects the experimental decision to apply antigen-specific stimulation exclusively to the treatment T cells, while control T cells were not stimulated, although such stimulation would have been possible. Antigen-specific stimulation may have preferentially promoted proliferation and survival of successfully transduced treatment T cells, thereby increasing the number of FLUC-expressing cells in this group and contributing to residual differences between treatment and control populations. However, these discrepancies were less pronounced than those observed with the previous FLUC-EGFP control construct, and FLUC expression in control cells was consistently maintained, enabling reliable tracking.

It is important to note that the FLUC-EGFP vector used in the initial experiment was smaller in size (approximately 7500 bp) than the tricistronic cTCR construct (approximately 9000 bp), which likely contributed to a higher transduction efficiency and elevated intracellular FLUC levels observed in the control group. This size-dependent effect on viral transduction efficiency has also been reported by others [157], [158]. Although FLUC expression is inferred from surface TCR expression, as both are encoded within the same tricistronic construct, it remains uncertain whether surface TCR levels directly correlate with intracellular FLUC protein expression. In addition, antigen-induced upregulation of surface TCR may not proportionally affect FLUC expression. To remain consistent with previous experiments, the same total number of T cells was transferred into each mouse, accepting group-specific differences in the proportion of cTCR⁺ and, consequently, FLUC⁺ T cells.

Overall, this strategy improved the comparability of BLI signals between treatment and control groups. However, the exact number of FLUC-expressing cells at the start of the experiment remains unknown, as surface TCR expression may not accurately reflect intracellular FLUC levels, which should be considered when interpreting T-cell BLI data.

5.3.3 Uncontrolled T-cell proliferation in NSG models

While absolute luminescence signals of T cells between groups are not directly comparable, the longitudinal assessment of FLUC signal within the same mouse can provide insights into T-cell dynamics, particularly regarding proliferation over time. In the first NSG experiment, BLI signals in the control group showed a distinct pattern: FLUC signal intensity was high on day 2 after ACT, decreased on days 7 and 14, and then increased again at a later time point (day 20) in all five animals ([Figure 17](#)). In contrast, this pattern of signal increase was not uniformly observed in the treatment group. Only one mouse (ID: 741) in the treatment group displayed a clear increase in

FLUC signal at later time points compared to earlier measurements. Further analysis of peripheral blood samples showed that mouse 741 exhibited a distinct hCD3⁺ T-cell population on day 29 after ACT that was not detectable on day 21 ([Figure 18](#)), consistent with the marked increase in FLUC signal observed by BLI and indicating *in vivo* expansion of human T cells. Notably, these circulating T cells lacked detectable mTRBC surface expression, despite robust intracellular FLUC signals, suggesting that surface cTCR expression and intracellular FLUC expression do not correlate.

Moreover, the absence of surface mTRBC indicates that the observed T-cell expansion was unlikely to be driven by signaling through the anti-TRP2 cTCR. Instead, proliferation may have been mediated by endogenous TCRs or other, currently undefined, stimuli independent of transgenic TCR expression. Consistent with this interpretation, control T cells, which were not transduced with a TCR in this experiment and retained their endogenous TCRs, exhibited similar proliferative patterns, as indicated by BLI signals in the control group. Unfortunately, peripheral blood samples were not collected on day 29 after ACT in the control group, limiting the ability to independently confirm the presence of hCD3⁺ T cells in circulation at this later time point.

Another important consideration when interpreting BLI-based T-cell tracking is that bioluminescence predominantly reflects T cells that have accumulated within tissues rather than cells circulating in the bloodstream. Consequently, BLI signals do not necessarily correlate with flow cytometric measurements of hCD3⁺ T cells in peripheral blood. FLUC imaging is particularly sensitive to localized tissue infiltration, where even relatively small numbers of T cells can generate a detectable signal. In contrast, circulating T cells typically produce diffuse or very weak signals that may remain below the detection threshold. In this study, no direct comparison of BLI-positive and BLI-negative tissue regions was performed using immunohistochemistry or flow cytometry to independently validate T-cell infiltration at corresponding sites.

In the second NSG experiment, in which tumor growth was merely controlled in treated animals, the control construct was replaced with one encoding an irrelevant TCR to achieve comparable FLUC expression profiles between treatment and control T cells. Flow cytometric analysis of mTRBC expression suggested a lower proportion of transduced, and consequently FLUC-expressing, cells in the control group. Despite this, FLUC signals were comparable between groups on day 1 after ACT and decreased over time in both groups at later time points ([Figure 21](#)). However, beginning on day 19 after ACT, the FLUC signal increased sharply in all five treatment mice. A similar rise was also detected in one of the four control mice, suggesting uncontrolled expansion of the transferred T cells in these animals. This increase

necessitated an adjustment of the BLI scale to a higher range to maintain visibility of localization patterns. Unfortunately, no peripheral blood samples were collected during this experiment, preventing additional confirmation of the BLI findings by flow cytometric analysis.

In the third NSG experiment, in which no tumor control was observed, BLI indicated pronounced T-cell expansion in three of five control mice and two of five treated mice. In contrast, flow cytometric analysis of peripheral blood revealed increased hCD3⁺ T-cell numbers in only two control mice and one treated mouse, demonstrating that elevated BLI signals do not necessarily reflect increased circulating T-cell levels ([Figure 25](#)). This finding is consistent with BLI preferentially detecting tissue-resident or locally accumulated T cells rather than cells in the peripheral blood compartment. Notably, despite rising FLUC signals and increasing hCD3⁺ T-cell levels in peripheral blood, again, staining for mTRBC showed no detectable expression of the transduced TCR in circulating T cells.

Importantly, these episodes of uncontrolled proliferation were observed exclusively in NSG mice, occurred in both treatment and control groups, and were independent of the specific control construct used. This suggests that the proliferation was not driven by the anti-TRP2 TCR but may instead reflect a xenogeneic graft-versus-host-like reaction mediated by endogenous TCRs still expressed on the transferred human T cells. Xenogeneic activation of human T cells in NSG mice is a well-described confounder in adoptive transfer studies, and published work has demonstrated that the onset of xenogeneic GvHD is dose dependent, with higher numbers of transferred human T cells leading to earlier disease onset [159].

5.3.4 Interpretation of BLI signals and tumor immunogenicity in the syngeneic model

In the syngeneic C57BL/6 melanoma model, murine B78.H1 tumor cells engineered to overexpress human TRP2 and NanoLuc were used to evaluate the efficacy and safety of anti-TRP2 TCR-transduced T cells in an immunocompetent setting. Treated mice showed robust tumor regression, while a notable fraction of control animals also exhibited spontaneous tumor rejection, raising questions about the immunogenicity of the modified tumor cells. Overexpression of human TRP2 rather than murine TRP2 likely caused the tumor cells to be more immunogenic, prompting endogenous immune responses even after total body irradiation (TBI). Although B78.H1 cells are

described as MHC class I- and II-deficient [122], [123], residual or inducible MHC expression under inflammatory conditions cannot be excluded, potentially allowing antigen presentation. Additionally, reporter genes such as NanoLuc and EGFP may have further increased tumor immunogenicity.

In this experiment, TBI was performed five days after tumor inoculation, potentially allowing innate or residual adaptive immune responses to clear tumor cells before immunosuppression was established. This timing could explain the lower tumor engraftment rate in control animals. These findings highlight the need to optimize tumor cell engineering and conditioning regimens to balance immune suppression with consistent tumor establishment. In future studies, adjusting irradiation timing or using less immunogenic constructs may improve tumor take in control animals.

Prior to adoptive transfer, endogenous TCRs were knocked out using CRISPR/Cas9, leaving approximately 10% residual mCD3⁺mTRBC⁺ cells. Each mouse received a total of 1×10^7 T cells. In the treatment group, this included 4 million anti-TRP2-cTCR-transduced cells, of which 65.5% (~2.6 million) were mCD3⁺hV β 3⁺, and 6 million anti-TRP2-cTCR-FLUC-transduced cells, of which 17.5% (~1 million) were mCD3⁺hV β 3⁺. Control mice received 5.5 million anti-NY-ESO-1-cTCR-transduced cells (20.1% or ~1.1 million mCD3⁺hV β 1⁺) and 4.5 million anti-NY-ESO-1-cTCR-FLUC-transduced cells (19.3% or ~0.9 million mCD3⁺hV β 1⁺).

Although the total number of transferred T cells was identical across groups, transduction efficiencies varied, resulting in higher numbers of cTCR⁺ T cells in the treatment group. BLI signal intensity at a given site reflects the abundance of FLUC⁺ T cells and enables comparative tracking across groups. While BLI visualizes only the FLUC⁺ subset, these signals are generally interpreted as representative of the overall distribution of the transgenic T-cell populations.

Overall, these technical limitations and model-specific confounders represent well-known challenges in adoptive T-cell therapy studies and must be considered when interpreting data.

5.4 Differences in TCR-T efficacy in NSG experiments

Data from the first xenograft experiment indicate that anti-TRP2 cTCR-transduced T cells mediate early antitumor activity, as treated mice exhibited a rapid decline in tumor-associated bioluminescence, whereas control mice showed continued signal increase. This divergence became apparent within the first five days after ACT. In contrast, the second experiment showed only a delay in tumor growth, while the

third experiment revealed no measurable antitumor effect over the course of the study, despite successful T-cell localization to the tumor. Collectively, these observations suggest that effective antitumor responses emerge early after transfer, whereas delayed onset of robust tumor control is unlikely in this model.

To assess whether differences in transduction efficiency or CD8⁺ T-cell numbers contributed to the varying outcomes, PBMC compositions and transduction data across experiments were examined. Although TRP2-specific TCR recognition is functionally independent of CD4/CD8 co-receptor expression, the proportion of CD8⁺ cells among transferred T cells was still considered as a potential modifier of overall effector function *in vivo*. The initial PBMC compositions were comparable, with CD8⁺ T-cell frequencies of 15.7%, 14.6%, and 13.4% in the first, second, and third experiments, respectively, and only minor variations in NK cell populations. On the day of transfer, the proportion of TCR-transduced T cells was 61% in the first experiment, 43.5% in the second, and 55.3% in the third. This corresponded to 6.1×10^6 , 4.35×10^6 , and 5.5×10^6 anti-TRP2 TCR-positive T cells transferred in the respective experiments, with the third experiment additionally confirming that approximately 20% of transferred cells were CD8⁺mTRBC⁺. A detailed summary of these PBMC compositions, transduction efficiencies, and T-cell transfer parameters across all three NSG experiments is provided in [Figure 39](#) in the appendix.

Although the second experiment involved the lowest number of TCR-transduced T cells (approximately 4.35×10^6 anti-TRP2-cTCR⁺ cells per mouse), it still resulted in a delay in tumor growth. In contrast, the first experiment, which achieved complete tumor regression, had the highest TCR expression levels and the largest number of transferred anti-TRP2-cTCR⁺ cells (approximately 6.1×10^6 per mouse). The third experiment featured an intermediate number of cTCR⁺ cells (approximately 5.5×10^6 per mouse) and included a substantial CD8⁺mTRBC⁺ subset, yet no tumor control was observed. Furthermore, IFN- γ ELISpot assays confirmed T-cell functionality in all experiments, and BLI demonstrated T-cell homing to the tumor sites in the second and third experiments. Together, these findings suggest that differences in transduction efficiency or the number of cTCR⁺ or CD8⁺ T cells transferred did not account for the variability in therapeutic efficacy observed.

Unfortunately, no comprehensive phenotyping analyses were performed on the transduced human T cells used in the xenograft experiments. As a result, differentiation subsets, including effector, memory, or exhausted T-cell states, were not determined. This lack of information limits the ability to assess whether differences in T-cell differentiation status may have influenced the observed antitumor efficacy. Another important variable that was not controlled for is the donor source of the PBMCs. Information regarding the age, sex, and health status of the buffy coat donors was unavailable,

and inter-donor variability in T-cell fitness may have influenced the therapeutic outcomes. It is plausible that intrinsic differences in donor T-cell quality contributed to the observed differences in efficacy, highlighting the need to consider donor-related factors in adoptive T-cell therapy studies and to perform thorough T-cell phenotypic characterization prior to transfer.

In conclusion, no clear mechanistic explanation was identified for the failure of anti-TRP2 T cells to control tumor growth in the second and third xenograft experiments, as key parameters such as transduction efficiency, CD8⁺ T-cell numbers, and T-cell functionality were comparable across experiments. The variability in therapeutic efficacy is therefore most likely explained by differences in TRP2 antigen expression on the tumor cells, rather than by differences in T-cell functionality. Tumor-dependent factors such as low baseline TRP2 levels or inflammation-induced reversible dedifferentiation as described in chapter 4.3.5 would directly limit antigen availability and thereby impair recognition by the anti-TRP2 TCR.

5.5 Potential causes of vitiligo-like depigmentation in the syngeneic model

Unlike in NSG xenograft models, BLI tracking in the syngeneic model showed no uncontrolled systemic T-cell expansion. As seen in [Figure 31](#), FLUC signals in the treatment group remained stable over time, while signals in the control group gradually declined, indicating limited *in vivo* proliferation. Flow cytometric analysis of cTCR-expressing T cells in peripheral blood at days 14 and 27 after ACT supported these observations, showing stable or slightly increasing T-cell numbers in the treatment group and stable to declining numbers in the control group ([Figure 33](#)).

BLI tracking revealed that FLUC⁺ treatment T cells localized early to tumor sites and subsequently migrated peripherally, a pattern that anatomically corresponded with the emergence of a vitiligo-like depigmentation phenotype in treated mice ([Figure 31](#)). This phenomenon, observed in all treated animals to varying degrees but absent in controls, suggests on-target/off-tumor targeting of TRP2-expressing melanocytes by the transgenic T cells. Notably, depigmentation progressed over time despite the absence of histological evidence for T-cell infiltration or inflammation in the skin at the experimental endpoint, indicating a non-destructive or transient immune-mediated mechanism.

Cytokines are known to play a central role in melanocyte biology and melanogenesis, providing a plausible mechanism for the observed depigmentation. As reviewed by Custurone et al. and Fu et al., cytokines such as IFN- γ , TNF- α , IL-6, IL-17, and IL-1 β can disrupt melanocyte function and inhibit melanin synthesis by downregulating MITF and melanogenic enzymes, contributing to vitiligo pathogenesis [160], [161].

Among treated mice, mouse 796 exhibited the most pronounced depigmentation, alongside the highest increase and total count of cTCR⁺ T cells in peripheral blood. However, histopathological analysis of skin with depigmented fur from this mouse showed no differences compared to skin with non-depigmented fur in control mice, with no evidence of increased T-cell infiltration or melanocyte destruction. These findings prompted us to investigate whether the depigmentation could result from transient, cytokine-mediated effects on melanocytes rather than direct T-cell-mediated cytotoxicity.

Serum cytokine analyses at experimental endpoints, presented in [Figure 38](#), did not reveal clear group-wide differences between treated and control mice, despite the progressive nature of depigmentation observed during the experiments. Levels of IFN- γ , TNF- α , and IL-17 were comparable between groups, while IL-6 was slightly elevated in some treated mice. Mouse 796 displayed the highest IL-1 β concentration among treated animals but did not exhibit elevated levels of other cytokines relative to group-mates.

Together, these findings suggest that localized inflammatory cytokine activity could have suppressed melanogenesis and could have contributed to melanocyte loss, leading to depigmentation even in the absence of systemic inflammation. This may explain why fur depigmentation developed at sites overlapping with T-cell presence without concurrent detection of elevated serum cytokine levels. It is plausible that depigmentation resulted from highly localized cytokine effects that were undetectable in serum or from transient systemic cytokine elevations that had resolved by the time of sampling.

5.6 T-cell persistence and exhaustion in the syngeneic model

Achieving durable responses with adoptive T-cell therapies remains a critical challenge in the treatment of solid tumors, where chronic antigen exposure and an immunosuppressive tumor microenvironment drive T-cell exhaustion [72]. This dysfunctional state, marked by reduced effector function, metabolic impairment, and upregulation of inhibitory receptors such as PD-1, CTLA-4, LAG-3, and TIGIT, is reinforced by transcriptional and epigenetic reprogramming and is not readily reversed by checkpoint blockade alone [71].

To assess the persistence and exhaustion of transferred T cells, flow cytometric analysis of activation and exhaustion markers, as well as markers distinguishing effector memory and central memory T-cell subsets, was performed both prior to adoptive transfer and at experimental endpoints. Flow cytometric analysis confirmed the persistence of cTCR⁺ T cells in peripheral blood, lymph nodes, and spleen in the syngeneic model, with higher frequencies observed within the CD8⁺ subset, particularly in blood and spleen ([Figure 34](#)). Analysis of hVβ⁺ T-cell frequencies in blood, spleen, and lymph nodes revealed detectable hVβ⁺CD8⁺ T cells in both treatment and control groups, with higher frequencies in the anti-TRP2 TCR treatment group. In contrast, hVβ⁺ cells were nearly absent within the CD4⁺ subset in both groups. These findings differ from the NSG experiments, where human CD3⁺ T cells were detected in peripheral blood, sometimes correlating with BLI signals, but hVβ expression was not observed, likely due to downregulation or loss of cell surface cTCR-expression in the xenograft setting. It would be of interest in future studies to assess whether the persisting T cells in the syngeneic model are capable of mediating protection upon tumor rechallenge.

To evaluate T-cell activation and exhaustion, marker expression was analyzed at the experimental endpoints ([Figure 35](#)). No pronounced differences were observed between treatment and control groups, with expression frequencies varying primarily by tissue type and T-cell subset. Pre-transfer analysis showed high CD25 expression across all T cells, likely due to IL-2 supplementation during expansion ([Figure 29](#)). CD69 expression was around 60% in CD8⁺ T cells in both groups, while CD4⁺ T cells in the treatment group showed similar levels but were lower (~30%) in the control group. CTLA-4 and TIGIT expression remained low across all samples, suggesting limited involvement of these checkpoint pathways in T-cell regulation at this timepoint.

PD-1 expression presents a complex indicator of T-cell status. While traditionally associated with exhaustion, PD-1 can also mark activated, high-avidity, tumor-reactive T cells, reflecting chronic antigen exposure rather than dysfunction alone [162]. Pre-transfer, PD-1 was expressed in >80% of CD8⁺ and CD4⁺ treatment T cells but was lower (<60%) in control T cells, consistent with antigen-specific stimulation performed only in the treatment group. At endpoint analysis, PD-1 expression decreased markedly in both CD8⁺ and CD4⁺ subsets, with CD8⁺ T cells showing near-zero expression in blood, ~5% in lymph nodes, and ~10% in the spleen. This reduction aligns with reduced antigen encounter at late time points, likely reflecting diminished tumor burden and consequently lower antigen-driven activation. A sim-

ilar pattern was observed for the activation markers CD25 and CD69, both of which showed substantially lower expression at endpoint compared with the day of transfer. Notably, PD-1 expression remained higher in CD4⁺ cells from treated mice compared with controls, particularly in peripheral blood.

According to Klebanoff et al., central memory T cells (TCM) exhibit superior antitumor efficacy in murine adoptive T-cell therapy models, largely due to their enhanced engraftment, persistence, and proliferative capacity compared to effector memory T cells (TEM) [73], [74]. In this study, differentiation profiling based on CD44 and CD62L expression showed that prior to transfer, CD8⁺ T cells in both treatment and control groups predominantly displayed a TEM phenotype, whereas CD4⁺ T cells consisted of a mixed population of approximately two-thirds TEM and one-third TCM. At the experimental endpoint, CD8⁺ T cells in both groups demonstrated a shift toward a more pronounced TCM phenotype, with a similar but less marked trend observed in the CD4⁺ compartment. However, no consistent differences in differentiation status were detectable between treatment and control groups. It is noteworthy that TCM proportions re-emerged and expanded as determined by endpoint analysis despite the predominantly TEM phenotype observed at the day of transfer. However, this shift was also observed in the control group and occurred independently of whether control mice exhibited spontaneous tumor regression, preventing any definitive conclusion as to whether the transition toward a memory-enriched phenotype was cTCR-specific. Based on these data, the shift is more likely attributable to the inherently superior engraftment, persistence, and proliferative capacity of TCM compared to TEM.

Recent single-cell multi-omics analyses of CAR T-cell therapies have highlighted functional phenotypes associated with long-term persistence. Bai et al. identified a type 2 functional phenotype, characterized by IL-4, IL-5, IL-13, and GATA3 expression, as a hallmark of ultra-persistent CAR T cells in patients with prolonged B-cell aplasia. This phenotype appeared to regulate dysfunctional subsets, preventing premature exhaustion and supporting persistence and efficacy. Enhancing type 2 signatures during CAR T-cell manufacturing improved outcomes in preclinical models, emphasizing the importance of balanced functionality over maximal initial cytotoxicity [163].

These insights are relevant for TCR-T-cell therapy in solid tumors, where persistence and functional stability are often limited. Although the role of type 2 functionality in TCR-T cells remains underexplored, strategies such as metabolic conditioning, IL-4-based priming, or modulation of key transcriptional programs (e.g., TOX, TCF1)

may enhance persistence and reduce exhaustion in TCR-T cells [72]. Ultimately, improving the long-term phenotype and persistence of TCR-T cells, rather than focusing solely on initial cytotoxicity, may be essential for achieving durable tumor control in the challenging environment of solid tumors.

6 Conclusion

This work demonstrates that a naturally occurring, HLA-independent, TRP2-specific T-cell receptor can effectively and safely target experimental melanoma tumors *in vivo*. Using xenograft and syngeneic mouse models, it was demonstrated that T cells engineered with this TCR exhibited robust tumor localization and persistence, as well as the ability to clear or delay tumor growth, even in the context of low or defective MHC expression. Importantly, treatment was well tolerated, with no systemic toxicity observed, and localized, vitiligo-like depigmentation representing the primary on-target/off-tumor effect, consistent with the TRP2-expression profile.

By bypassing classical antigen presentation pathways, HLA-independent TCR approaches offer a promising strategy to overcome immune evasion in solid tumors while maintaining antigen specificity and reducing the risk of therapy-induced toxicities. The findings presented in this dissertation provide preclinical proof-of-concept for the use of HLA-independent TCRs in adoptive cell therapy, highlighting their potential to expand the applicability and safety of TCR-based immunotherapies for patients with melanoma and other solid tumors even when exhibiting HLA loss.

Future work should focus on further investigating the mechanisms of antigen recognition by HLA-independent TCRs, optimizing T-cell engineering and manufacturing processes, and evaluating the long-term efficacy and safety of this approach in clinical settings. These efforts will be essential to translate HLA-independent TCR-based therapies into effective treatment options for patients with solid tumors.

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Appendix

Summary of PBMC composition, transduction efficiencies, and T-cell transfer parameters across all NSG experiments

NSG Experiment	Tumor Cell Line (1 mio per mouse)	Buffy Coat ID	CD3/CD8 (%) after isolation	CD3/CD4 (%) after isolation	CD16/CD56 (%) after isolation	CD3/mTRBC (%) at transfer	CD3/CD8 (%) at transfer	CD3/CD8/m TRBC (%) at transfer	CD16/CD56 (%) at transfer	Total T cells transferred per mouse (million)	cTCR ⁺ T cells transferred (million)
1st	Ma-Mel 86b-										
	KO TRP2- NLUC-EGFP- PAC #1.2	BC258	15.7	49.6	7.2	61.0	38.9	n/a	1.6	10	6.1
2nd	Ma-Mel 86b-										
	KO NLUC- EGFP #8	BC587	14.6	44.0	8	43.5	12.3	n/a	2.4	10	4.4
3rd	Ma-Mel 86b-										
	KO NLUC- EGFP #8	BC134	13.4	52.8	16.2	55.3	40.1	18.9	2.25	10	5.5

Figure 39: T-cell composition and transduction parameters across xenograft experiments.

Overview of PBMC composition, transduction efficiency, and T-cell transfer parameters for all three xenograph melanoma experiments. Buffy coat IDs and tumor cell lines used for each experiment are indicated. Cell populations were characterized by flow cytometry after PBMC isolation and on the day of adoptive T-cell transfer. Total numbers of transferred T cells and calculated cTCR⁺ T-cell equivalents per mouse are shown, providing context for interpreting differences in therapeutic efficacy across experiments.

Flow cytometry gating strategies

Exemplary analysis of PBMCs isolated from Buffy Coats

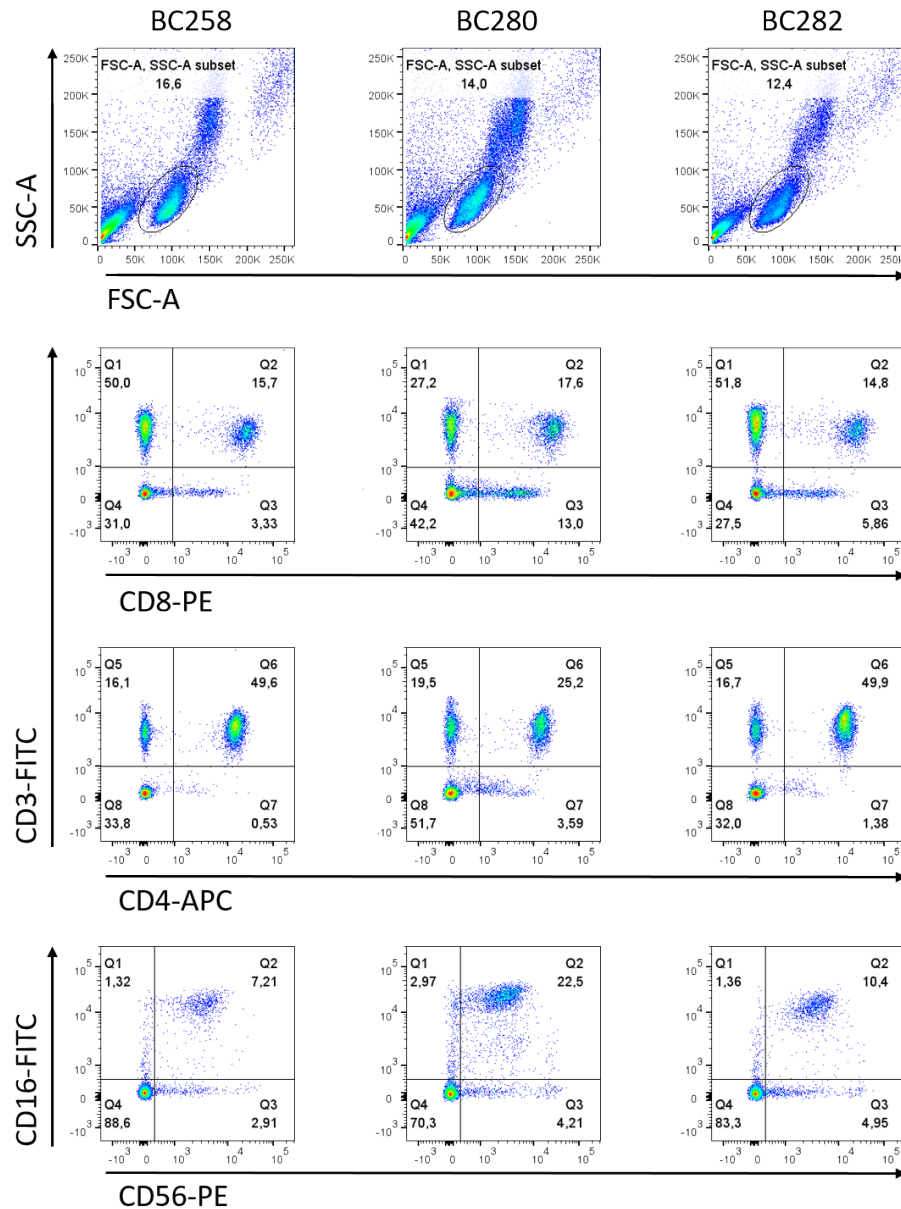


Figure 40: Flow cytometric analysis of freshly isolated PBMCs from three different Buffy Coats.

PBMCs were analyzed by multicolor flow cytometry to determine the proportions of CD4⁺ and CD8⁺ T cells, as well as CD16⁺CD56⁺ NK cells. Cells were first gated on lymphocytes based on FSC/SSC profiles, followed by marker-specific gating using CD3, CD4, CD8, CD16, and CD56 to quantify the respective lymphocyte subsets across the three donor samples.

Gating strategy for the quantification of T cells in peripheral blood samples

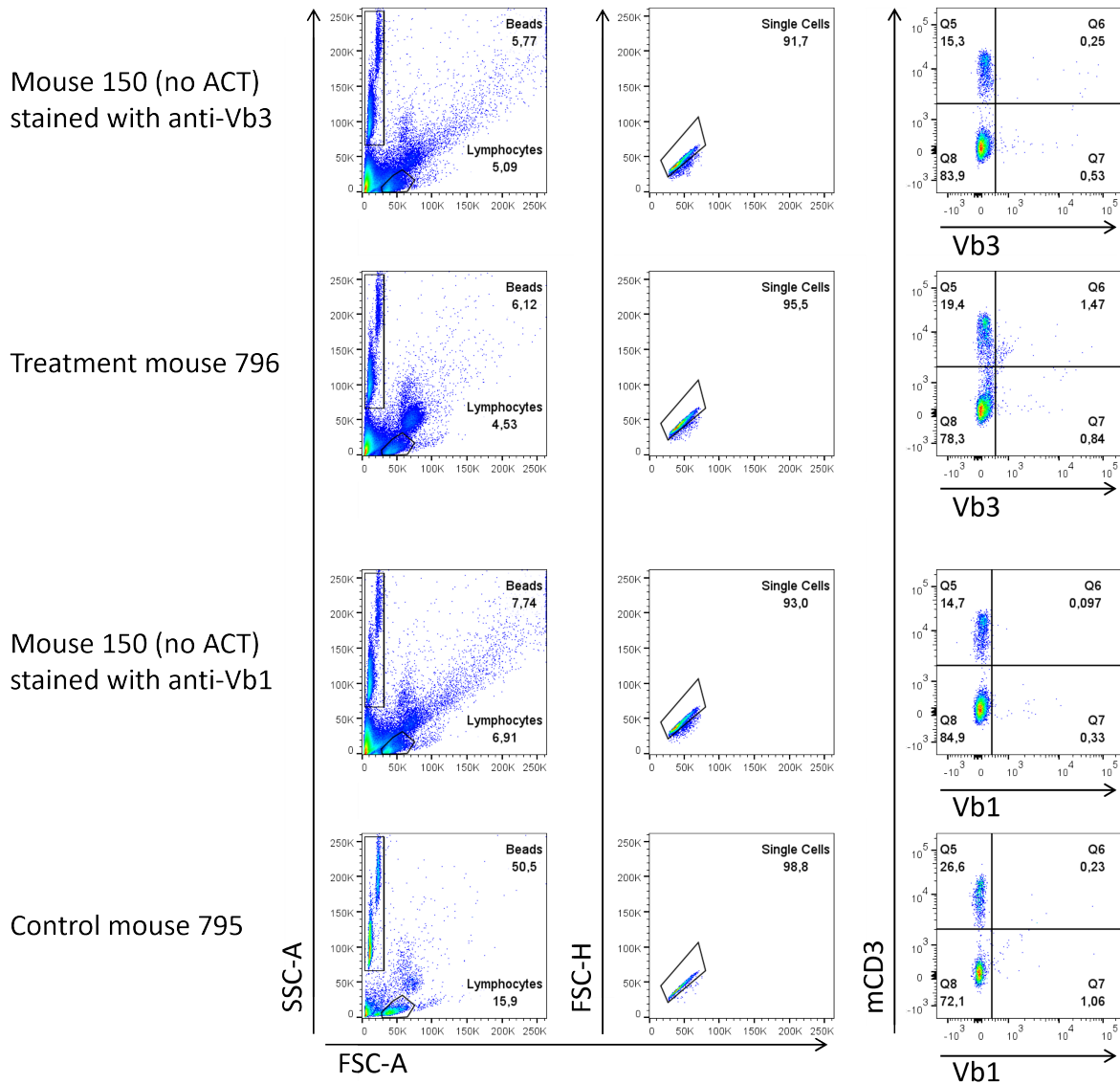


Figure 41: Exemplary gating strategy for quantification of peripheral T cells based on CD3 and V β expression.

Peripheral blood was subjected to red blood cell lysis prior to staining for mCD3 and specific TCR V β variants. Flow cytometry was performed until 10,000 counting beads were acquired. Cells were first gated on lymphocytes based on FSC/SSC, followed by exclusion of doublets (single-cell gating). CD3⁺V β ⁺ events were then identified, and their absolute number per μ l of blood was calculated based on bead counts. Shown are representative plots for a negative control mouse (150, no ACT) as well as for a treated (mouse 796, second row) and untreated control (mouse 795, bottom row). Mouse 150 served as a staining control for V β 3 and V β 1, respectively.

Gating strategy for murine T-cell phenotyping

Treatment mouse 798

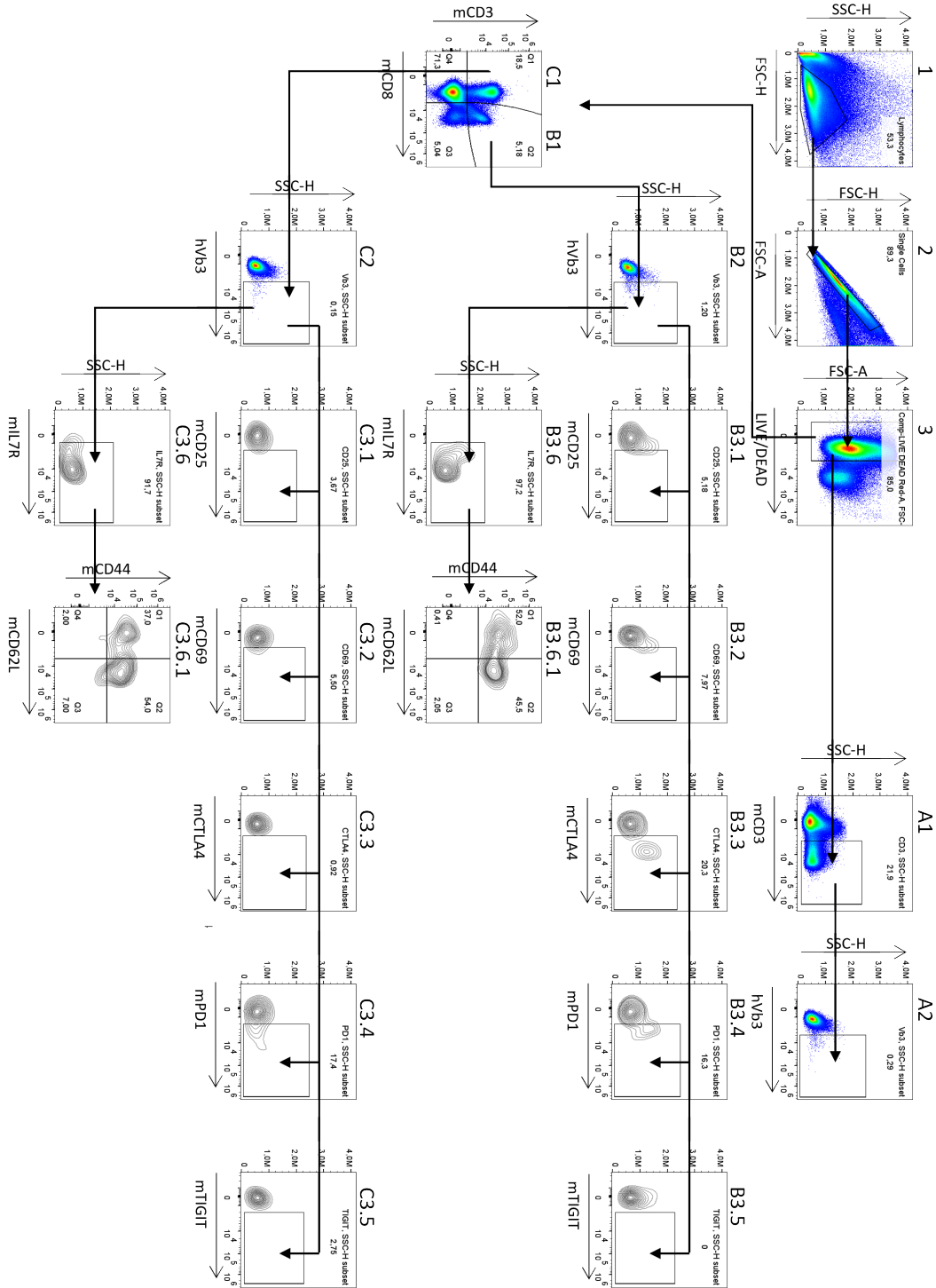


Figure 42: Exemplary gating strategy for multiparametric flow cytometry analysis of murine T cells isolated from spleens after adoptive T-cell transfer, here for treatment mouse 798.

Splenocytes were prepared by mechanical dissociation, followed by red blood cell (RBC) lysis. Antibody staining was then performed prior to flow cytometric analysis. Sequential gating was conducted as illustrated by the numbered plots and directional arrows. (1) Lymphocytes were first identified based on FSC-A vs. SSC-A. (2) Single cells were selected using FSC-A vs. FSC-H, followed by (3) exclusion of dead cells using LIVE/DEAD™ staining. From the live cell gate, three separate gating branches were analyzed in parallel:

For data presented in [Figure 34](#) (Frequency of $V\beta^+$ transduced T cells in peripheral blood, lymph nodes, and spleen) T cells were identified based on CD3 expression (A1), and $V\beta^+$ cells were gated as the transduced TCR $^+$ population (A2).

For the analysis of activation and exhaustion markers ([Figure 35](#)), and T-cell differentiation ([Figure 36](#)): CD3 $^+$ CD8 $^+$ T cells were gated based on CD3 vs. CD8 expression (B1), and from the $V\beta^+$ population (B2), the expression of CD25 (B3.1), CD69 (B3.2), CTLA-4 (B3.3), PD-1 (B3.4), and TIGIT (B3.5) was assessed.

Additionally, IL-7R α (CD127) expression (B3.6) and the expression pattern of CD62L vs. CD44 (B3.6.1) were evaluated to assess memory phenotypes.

In parallel, CD3 $^+$ CD8 $^-$ cells (putative CD4 $^+$ T cells) were analyzed: CD3 $^+$ CD8 $^-$ cells (C1) were gated for $V\beta^+$ expression (C2), followed by assessment of CD25 (C3.1), CD69 (C3.2), CTLA-4 (C3.3), PD-1 (C3.4), TIGIT (C3.5), IL-7R α (C3.6), and CD62L vs. CD44 expression (C3.6.1).

Percentages shown in the plots indicate the frequency of gated populations relative to the respective parent gate. This gating strategy was applied consistently across all analyzed samples derived from spleen, lymph nodes, and peripheral blood. For samples from control mice, $V\beta^+$ cells were used.

Vector maps

pDONR221

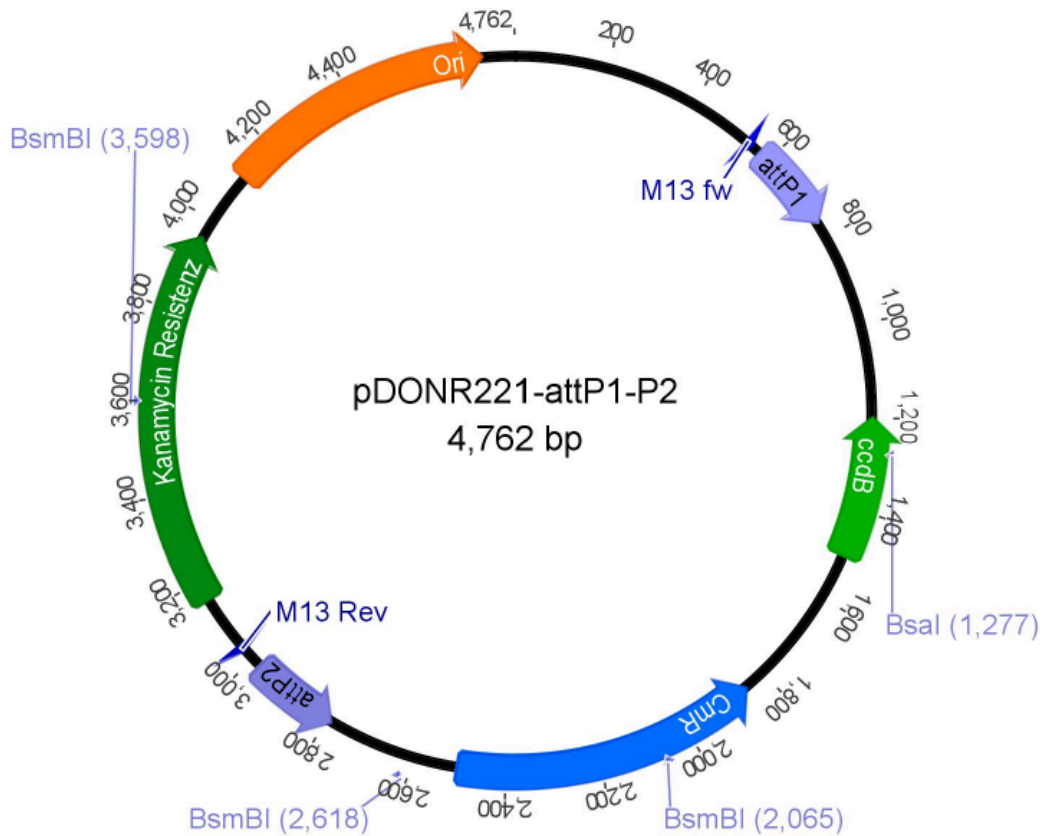


Figure 43: Plasmid map of pDONR221.

Entry vector used in the Gateway cloning system. It contains attP1 and attP2 recombination sites flanking the ccdB gene and a chloramphenicol resistance marker (CmR), enabling negative selection in *E. coli*. The plasmid also carries a kanamycin resistance gene for bacterial selection and an origin of replication (ori) for propagation. M13 forward and reverse primer sites facilitate sequencing. This vector is used to generate Gateway entry clones via BP recombination with a PCR product flanked by attB1 and attB2 sites.

pMX-IRES-puro-DEST#35

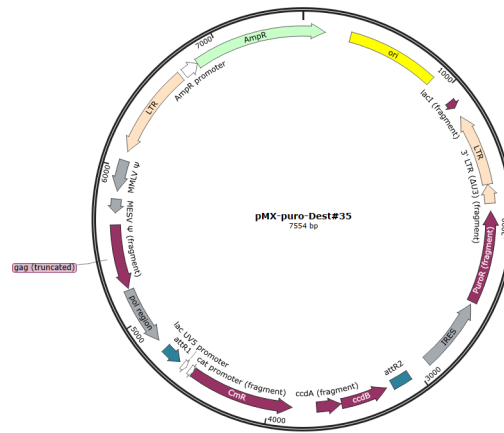


Figure 44: Map of the retroviral destination vector pMX-puro-DEST#35 (7,554 bp).

Gateway-compatible retroviral destination vector based on the pMX backbone. It contains an AmpR gene and origin of replication (ori) for bacterial propagation, and a Gateway cassette (attR1, attR2, ccdB, CmR) for recombination-based cloning. The mammalian expression cassette includes an IRES followed by a puromycin resistance gene (PuroR) for selection in transduced cells. Retroviral elements include truncated 5' and 3' LTRs, partial gag, and packaging sequences (ψ). Suitable for stable gene expression via retroviral transduction. Plasmid maps were created using SnapGene.

pMX-DEST#3



Figure 45: Map of the retroviral destination vector pMX-DEST#3 (6,282 bp).

Retroviral Gateway destination vector based on the pMX backbone. It includes an AmpR gene and origin of replication (ori) for propagation in E. coli, and a Gateway cassette (attR1, attR2, ccdB, CmR) for recombination-based cloning. The plasmid contains truncated 5' and 3' LTRs, partial gag, and packaging elements (ψ) required for retroviral particle generation. This version lacks a mammalian selection marker, making it suitable for applications where no antibiotic selection is needed after transduction. Plasmid maps were created using SnapGene.

pMX-puro_anti-TRP2 cTCR_FLUC #1.1.G

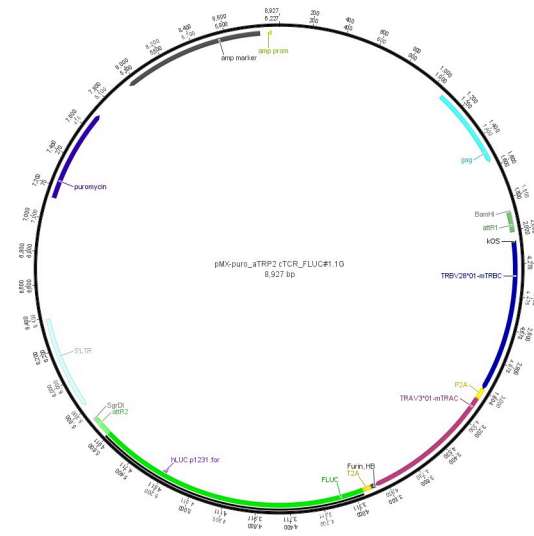


Figure 46: Plasmid map of pMX-puro_aTRP2 cTCR_FLUC#1.1G (8,927 bp).

*Retroviral vector for stable expression of a codon-optimized, murinized TRP2-specific TCR in mammalian cells. The construct encodes TRAV3*01–mTRAC and TRBV28*01–mTRBC chains, linked by P2A and furin-T2A sequences to ensure equimolar expression and proper cleavage. An in-frame firefly luciferase (FLUC) allows functional tracking of transduced T cells in vitro and in vivo. The vector includes a puromycin resistance cassette for mammalian selection and standard pMX retroviral elements (5' LTR, gag, 3' LTR) for packaging and transduction. Ampicillin resistance and ori enable propagation in E. coli. Designed for Gateway-mediated cloning and efficient retroviral gene transfer in functional T-cell assays. Plasmid map was created using Geneious.*

pMX-puro_FLUC-EGFP #3.6.C

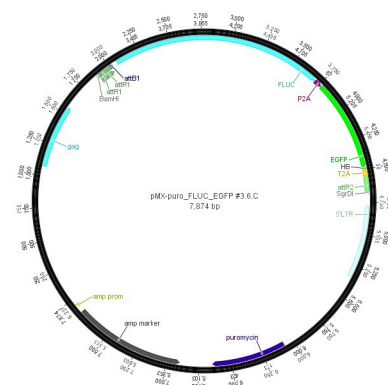


Figure 47: Plasmid map of pMX-puro_FLUC-EGFP #3.6.C.

Retroviral vector for co-expression of FLUC and EGFP linked by a P2A peptide. Includes a puromycin resistance gene for selection, attR1/attR2 sites for Gateway cloning, and standard pMX retroviral elements (LTRs, gag) for packaging. Ampicillin resistance allows propagation in E. coli. Plasmid map was created using Geneious.

pLenti

pLenti-EF1 α _NLUC_EGFP_PAC_WPRE #6.6.5

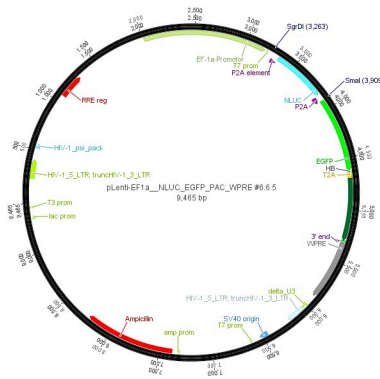


Figure 48: Plasmid map of pLenti-EF1 α _NLUC_EGFP_PAC_WPRE #6.6.5 (9,465 bp).

Third-generation lentiviral transfer vector enabling bicistronic expression of NLUC and EGFP linked by a P2A peptide under the control of the EF1 α promoter. A puromycin resistance gene (PAC) allows selection of transduced cells. The vector contains standard lentiviral elements (LTRs, RRE, psi, cPPT/CTS, and WPRE) for efficient packaging and expression. Restriction sites SgrDI and SmaI flank the insert region and can be used for cloning or validation. Plasmid map was created using Geneious.

pLenti-EF1 α _TRP2 fl_NLUC_EGFP_PAC #6.6.5.1

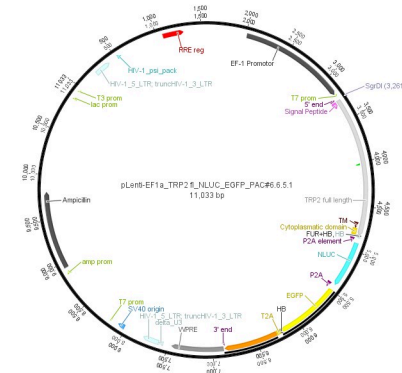


Figure 49: Plasmid map of pLenti-EF1 α _TRP2 fl_NLUC_EGFP_PAC #6.6.5.1 (11,033 bp).

Third-generation lentiviral vector for stable expression of full-length TRP2 fused to NanoLuc (NLUC) and EGFP via self-cleaving P2A/T2A elements. Expression is driven by the EF1 α promoter, allowing robust transgene expression in a wide range of mammalian cells. The construct includes a puromycin resistance gene (PAC) for selection and standard lentiviral elements (LTRs, psi packaging signal, RRE, cPPT, WPRE) for efficient viral packaging and integration. Ampicillin resistance and an origin of replication support bacterial propagation. This vector enables reporter-based monitoring of TRP2-expressing cells in vitro and in vivo. The indicated SgrDI restriction site was used for cloning. The plasmid map was created using Geneious.

Helper plasmids

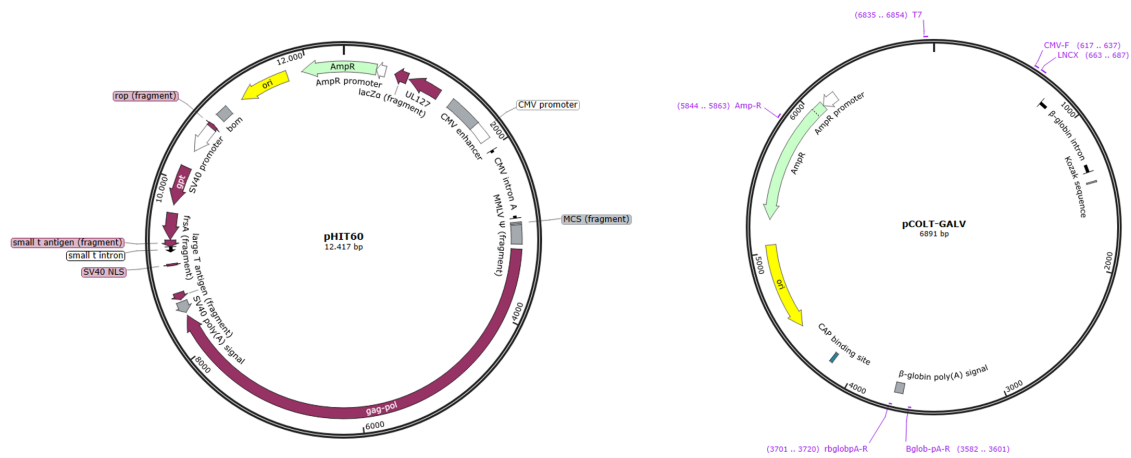


Figure 50: Plasmid maps of packaging and envelope plasmids used for retroviral particle production.

Left: *pHIT60* (12,417 bp) encodes the MMLV-derived *gag-pol* polyprotein under control of the CMV promoter and includes SV40 large and small T antigen fragments to support replication in packaging cells.

Right: *pCOLT-GALV* (6,891 bp) encodes the Gibbon Ape Leukemia Virus (GALV) envelope protein under a CMV promoter with a β-globin intron and Kozak sequence to enhance expression. Both plasmids contain Amp^R and bacterial origins of replication for propagation in *E. coli* and are co-transfected with a retroviral transfer vector for pseudotyped retroviral particle generation in HEK293T cells. Plasmid maps were created using SnapGene and obtained from the Addgene repository.

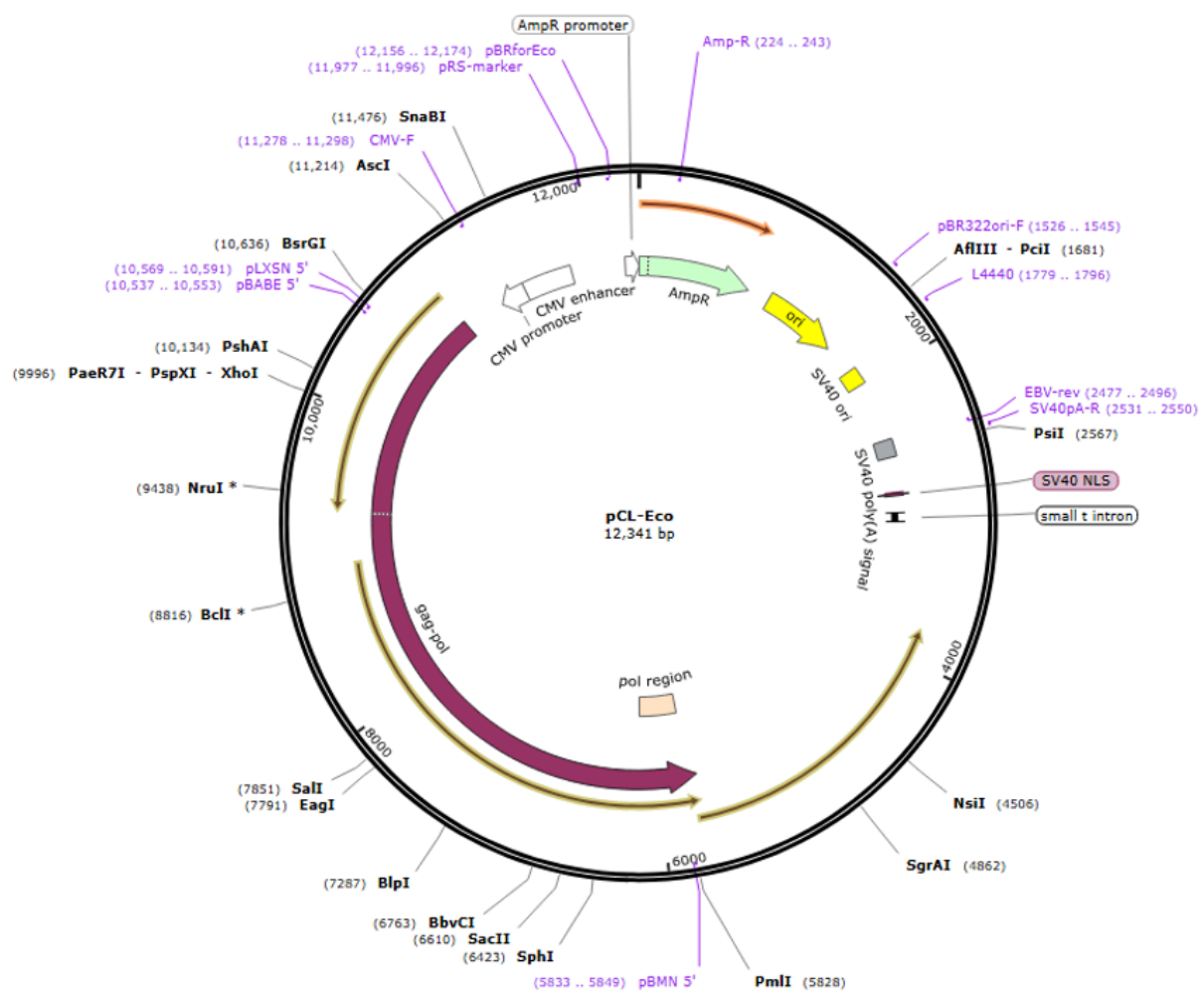


Figure 52: Plasmid map of pCL-Eco (12,341 bp).

Retroviral packaging plasmid encoding MMLV gag and pol genes, used for the production of ecotropic retroviral particles. Expression is driven by the CMV promoter, and the plasmid includes the SV40 origin of replication and polyadenylation signal. Ampicillin resistance (AmpR) and a bacterial origin of replication enable propagation in *E. coli*. pCL-Eco is typically used in combination with a retroviral transfer vector to generate retroviral particles for gene delivery to murine cells expressing the ecotropic receptor. Plasmid maps were created using SnapGene and obtained from the Addgene repository.

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Use of Generative AI Tools

During the writing process of this dissertation, generative AI tools (such as ChatGPT by OpenAI) were used exclusively for spell checking, grammar correction, and text editing. These tools assisted in refining the clarity, conciseness, and linguistic accuracy of the written text.

Importantly, AI tools were not used for generating scientific ideas, selecting methodologies, planning experiments, conducting or analyzing data, or interpreting results.

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