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Title: Targeting antigen heterogeneous B cell malignancies with multi-specific CAR molecules

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Abstract:

B-cell malignancies represent a major clinical challenge due to high relapse rates and limited efficacy of existing therapies. While CAR-T cell therapy has transformed the treatment landscape, long-term outcomes remain limited by antigen-loss-mediated escape and inadequate CAR-T cell persistence. Consequently, addressing these limitations becomes critical. The study focuses on the rational design and optimization of bispecific third-generation CAR-T cell constructs to effectively target antigen-heterogeneous B-cell malignancies. Bispecific CAR constructs targeting combinations of key B-cell antigens like CD19, CD20, and CD22 were designed and systematically evaluated to mitigate antigen escape. In silico structural analyses were integrated to guide scFv orientation with optimal structural stability. Because access to patient-derived antigen-loss samples is limited, clinically relevant antigen-loss model systems were generated using CRISPR-Cas9 engineered B-cell lymphoma cell lines with defined antigen knockouts to facilitate screening of bispecific CAR constructs. Functional evaluation in these models revealed significant differences among different bispecific combinations, demonstrating that not all bispecific CAR constructs perform equivalently under antigen-loss conditions. The study also aimed to enhance CAR-T cell persistence and functional efficacy using a comprehensive, multi-parametric screening of dual costimulatory domain combinations. Unlike conventional approaches emphasizing primarily on cytotoxicity or proliferation, this screening evaluated multiple functional parameters under repeated antigen exposure. This integrated analysis revealed that certain costimulatory combinations excelled in one parameter but underperformed in others, emphasizing the necessity of multi-parameter evaluation for rational CAR design. Some costimulatory domains, however, demonstrated balanced performance across all functional parameters examined. Overall, this study underscores the importance of scFv pairing and rational costimulatory selection, providing a framework for developing effective and persistent CAR-T cells with translational potential in antigen-heterogeneous B-cell malignancies.

The CD19 CAR T hinge region influences avidity and cytotoxicity in CD19 SNP variants in B-cell Lymphoma

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Abstract

Outcomes after CD19-directed chimeric antigen receptor (CAR) T-cell therapy in relapsed/refractory diffuse large B-cell lymphoma (DLBCL) are heterogeneous, and biomarkers to guide product selection are lacking. The germline CD19 SNP rs2904880, encoding Val174 (V174) or Leu174 (L174), has been linked to differential responses to FMC63-based CAR T cells. We investigated how CAR hinge architecture modulates interactions with CD19 variants and relates to clinical outcomes.

In silico modeling of the FMC63–CD19 complex (PDB 7URV) showed that L174 induces distinct conformational changes and additional hydrophobic contacts. In B-cell lymphoma lines, CD19 surface expression was higher in V174L (VAL/LEU) than in V174 (VAL/VAL) cells. Two FMC63-based CARs differing only in the hinge (CD8 vs CD28) displayed similar proliferation. Cytotoxicity against V174 targets was comparable, whereas the CD28-hinge CAR showed superior killing of V174L-expressing cells. Avidity measurements (z-Movi®) revealed higher overall avidity for CD8-hinge CARs and stronger binding to V174 than to V174L, despite lower CD19 expression, supporting an inverse relationship between very high avidity and optimal cytotoxicity.

In a single-center retrospective analysis of 91 r/r DLBCL patients performed at our department, V174 homozygotes had inferior overall survival (OS) compared with V174L carriers. Among V174 homozygotes, axi-cel (CD28 hinge/transmembrane) was associated with longer OS than tisa-cel, whereas V174L carriers tended to perform better with tisa-cel.

Mapping specificity of de novo designed pMHC-targeting minibinders

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Fully *in silico* designed proteins, termed minibinders (miBds) binding peptide major histocompatibility complexes (pMHCs) are emerging as immunotherapy tools. Unlike T cell receptors (TCRs), miBds bypass thymic selection and may therefore exhibit unforeseen cross-reactivity. High-throughput specificity mapping is therefore required to identify potential off-target pMHCs.

Using our *de novo* design and screening pipeline, we generated NY1-B04, a high-affinity miBd targeting the cancer-testis antigen NY-ESO-1 (SLLMWITQC) presented by HLA-A*02:01. NY1-B04 showed strong binding (KD=6.9 nM) and, when formatted as a chimeric antigen receptor (CAR) in primary T cells, drove potent killing of NY-ESO-1^{+} melanoma cells *in vitro*.

To quantify off-target risk, we applied a "one-pot" specificity screen for TCR cross-reactivity termed TCR fingerprinting. We measured binding of NY1-B04 to a barcoded pMHC library containing 180 single-position variants (9 peptide positions × 20 amino acids). The resulting "miBd fingerprint" defined tolerated versus disruptive residues and enabled comparison with TCR specificity profiles.

Additionally, we diversified NY1-B04 in silico using partial diffusion and sequence redesign. Fingerprinting revealed distinct specificity profiles, with lower-avidity variants showing increased selectivity, resembling natural TCR behaviour.

In situ tumor-to-cDC1 reprogramming improves expansion and anti-tumor activity of TILs for adoptive cell therapy

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Adoptive transfer of tumor-infiltrating lymphocytes (TILs) can induce durable clinical responses, yet standard manufacturing often expands bystander or non-tumor-reactive clones. Neoantigen-focused selection strategies can enrich tumor specificity, but require extensive sequencing, antigen prediction and functional screening, prolonging manufacturing timelines, while restricting TIL products to selected neoantigens. Here, we present a strategy to amplify tumor-reactive TILs by reprograming tumor cells in situ into conventional dendritic cells type 1 (cDC1)-like antigen-presenting cells during early TIL manufacturing.

Fresh tumor biopsies from 9 patients with metastatic melanoma were fragmented and transduced with an adenoviral vector encoding PU.1, IRF8, and BATF3 (PIB) to reprogram tumor cells in situ into cDC1-like cells. Non-transduced (NT) and GFP-transduced fragments were used as controls. After 9 days, fragments were enzymatically digested for phenotypic analysis. For each condition, young TILs were isolated and expanded through the rapid expansion protocol (REP TILs). TIL reactivity and cytotoxicity were evaluated against autologous tumor cell lines, either before or after interferon gamma (IFNγ) stimulation, using intracellular cytokine staining and real-time tumor killing assays.

In situ reprogramming induced a cDC1-like phenotype in tumor fragments, reflected by increased expression of CD45 (2.8-fold vs NT; and 2.2-fold vs GFP, $p<0.05$) and HLA-DR (2.7-fold vs NT; and 1.9-fold vs GFP, $p<0.05$). Young TILs were successfully generated from 8/9 biopsies and expanded to REP TILs in 7/9. PIB-treated fragments yielded higher REP TIL expansion compared to GFP (9.4-fold, $p<0.05$) and NT fragments (1.6-fold, $p=0.059$), and were enriched in $CD8^+$ T cells (CD8/CD4 ratio: 22.3 ± 41.9) compared to NT (2.9 ± 7.7, $p<0.05$) and GFP controls (1.3 ± 3.5, p= $p<0.05$). Phenotypic analysis revealed enrichment of $CD8^+$ $TIM3^{low}$ REP TILs in PIB-treated cultures ($p<0.05$), consistent with enhanced activation. Functionally, PIB-derived CD8+ REP TILs showed higher reactivity against autologous tumor cells compared to NT (10.1-fold, $p<0.05$) and GFP controls (4.9-fold, $p<0.05$). This effect was further enhanced by IFNγ treatment (15.3-fold vs NT; and 9.5-fold vs GFP, $p<0.05$). Finaly, PIB-derived REP TILs demonstrated increased tumor cell killing compared to NT (2.6-fold, $p<0.005$) and GFP controls (1.9-fold, $p<0.005$).

Together, these findings demonstrate that incorporating in situ tumor-to-cDC1 reprogramming into TIL manufacturing enhances the expansion and functional potency of tumor-reactive T cells. This approach provides a strategy to improve TIL therapy, particularly in tumor with low frequencies of tumor-reactive lymphocytes.

Dissecting the Impact of RAS Pathway Inhibition on TIL Expansion and Therapeutic Efficacy

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BRAF inhibitors (BRAFi) and immune checkpoint blockade (ICB) have improved outcomes for patients with solid tumors, yet durable responses remain limited to a subset of patients. Adoptive cell transfer (ACT) therapies have emerged as a promising alternative. Trials of tumor-infiltrating lymphocyte (TIL) therapy show durable benefits in cutaneous melanoma. These results led to the FDA approval of Lifileucel for BRAF-mutant and wild-type melanoma patients who progressed on BRAFi (± MEKi) or ICB, though response rates remain modest. **Thus, there is a need to find new strategies to improve the efficacy of TIL therapy in BRAF-V600-mutated and wild-type melanoma**. BRAFi enhance anti-tumor immunity on tumor cells and, in BRAF–wild-type cells, boosting T cell activation through paradoxical ERK activation within a specific dose range. BRAFi also enhance the efficacy of ACT in BRAF-V600-mutated melanoma models. Our preliminary data support the use of YUMMER1.7 progressor melanomas to study how MAPK inhibition impacts TILs. Based on these findings, **we hypothesize that RAS pathway inhibition can improve TIL expansion and therapeutic efficacy when optimally timed and dosed.** To test this, we will **assess the effect of BRAF inhibitors on TIL expansion *ex vivo* and on TIL efficacy *in vivo*.** Alongside, clinical observations from Drs. Pavlick and Ma suggest that BRAFi during the bridging phase –between tumor resection and TIL infusion– can delay metastasis, allowing sufficient time for TIL administration. Based on this, **we will Investigate the effect of BRAF inhibitors as bridging therapy on TIL efficacy in vivo.** This study will inform strategies to improve the therapeutic efficacy of TIL therapy against BRAF-V600-mutated melanoma.

Neoantigen Identification using Dendritic Cell Reprogramming

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Genetic mutations in cancer generate tumour-specific neoantigens that are ideal targets for therapeutic vaccination. However, current methods for identifying immunogenic neoantigens remain inefficient. Type 1 conventional dendritic cells (cDC1s) play a key role in presenting peptides to T cells and driving anti-tumour immunity. We have shown that overexpression of three transcription factors, PU.1, IRF8, and BATF3 (PIB), can reprogram cancer cells into a cDC1-like phenotype. We hypothesize that this dendritic cell reprogramming strategy can be leveraged to identify highly immunogenic tumour neoantigens.

First, we examined the relative composition of the proteosome and immunoproteasome upon reprogramming, which induced upregulation of major histocompatibility complexes (MHC) I and II. Transcriptional and proteomic profiling of reprogrammed cancer cell lines showed upregulation of immunoproteasome components (PSME2, PSMB8, PSMB9, PSMB10) and downregulation of the proteosome, suggesting differential antigen processing. To define the peptide repertoire, we established protocols for MHC peptide extraction followed by high-sensitivity TimsTOF mass spectrometry, optimised for low-input samples. In the melanoma B16 mouse cell line, unique cDC1-derived peptides exhibited a distinctive H2.Kb presentation pattern, which was not observed in IFNγ-treated reference samples (despite IFNγ also leads to increased MHC expression), suggesting a cDC1-specific antigen presentation mechanism. From a panel of well-characterised mutated melanoma genes and proteins, we detected two uniquely present in reprogrammed cells, highlighting the enhanced capacity of cDC1-reprogrammed cells to present novel neoantigens.

These findings establish cDC1 reprogramming as a promising platform to sensitively identify tumour-specific, immunogenic peptides, enabling the development of more precise and effective cancer vaccines.

CRISPR-PD1 modified tumor infiltrating lymphocytes for adoptive therapy for patients with metastatic melanoma.

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Background:
Immune checkpoint inhibitors and adoptive cell therapy using tumor-infiltrating lymphocytes (TILs) have improved outcomes in metastatic melanoma (MM), but durable responses remain limited. Gene engineering strategies, such as PD-1 knockout, may enhance TIL efficacy by overcoming tumor-induced immunosuppression. We previously demonstrated that non-viral CRISPR-Cas9 delivery can efficiently disrupt PD-1 in TILs (Chamberlain et al, 2022). Here, we report early feasibility data from the first-in-human trial testing non-viral CRISPR-Cas9 PD-1 knockout in TILs (CRISPR-TILs) in advanced MM.

Methods:
Patients with metastatic or inoperable cutaneous melanoma progressing after first-line checkpoint inhibitors were enrolled (NCT06783270). PD-1 knockout was integrated into the in-house GMP TIL manufacturing process using non-viral CRISPR-Cas9. Following lymphodepletion (cyclophosphamide, fludarabine), patients received $\geq 5 \times 10^9$ CRISPR-TILs and up to six doses of high-dose IL-2. Infusion products were assessed for PD-1 knockout efficiency, cellular composition, memory phenotype, and tumor-reactivity by flow cytometry. Clinical responses were evaluated per RECIST 1.1, and safety was monitored throughout.

Results:
Seven patients have received CRISPR-TILs. Infusion products showed efficient PD-1 disruption, preserved cellular composition and effector memory phenotype. Tumor recognition assays confirmed antigen-specific reactivity and preserved cytokine production post-editing. No unexpected adverse events were observed. One patient experienced a prolonged infusion reaction with an increase in severity compared to traditional, unedited TILs. At six weeks post-infusion, two patients achieved objective responses per RECIST 1.1.

Conclusion:
These initial feasibility data from a first-in-human clinical trial demonstrate that non-viral CRISPR-Cas9–mediated PD-1 knockout can be efficiently integrated into the TIL manufacturing workflow and safely administered to patients with metastatic melanoma, with a preserved ability to induce deep clinical responses. Recruitment is ongoing, and continued follow-up will provide further insights into the therapeutic potential and durability of CRISPR-engineered TIL therapy.

Title:

Enhancing anti-cancer immunity with combinatorial CRISPR perturbations in tumour-infiltrating lymphocytes

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Tumour-infiltrating lymphocytes (TILs) play a central role in anti-tumour immunity and have demonstrated durable clinical benefit in metastatic melanoma. However, their efficacy is frequently limited by functional exhaustion and suppressive signalling within the tumour microenvironment. Genetic engineering using CRISPR/Cas9 offers a powerful strategy to overcome these barriers. At our centre, this approach is already being translated clinically through an ongoing first-in-human trial of PD-1 knockout (KO) TILs, demonstrating the feasibility of CRISPR-edited TIL products for patient treatment.

Building on this clinical experience, we investigated whether simultaneous disruption of multiple inhibitory pathways could further enhance TIL function. Specifically, we examined the effects of double knockout (DKO) of PD-1 and CISH (Cytokine-Inducible SH2-containing protein), a negative regulator of cytokine signalling, hypothesising that targeting checkpoint inhibition and cytokine-signalling release would potentiate TIL effector activity.

Using CRISPR/Cas9-mediated genome editing, we generated PD-1/CISH DKO TILs from previously treated melanoma and ovarian cancer TIL patients. Edited TILs were assessed for viability, phenotype, cytokine production, and metabolic fitness. DKO TILs were efficiently generated without evidence of toxicity or impaired expansion. Notably, dual editing resulted in enhanced pro-inflammatory cytokine production and improved metabolic profiles relative to single-KO or unedited TILs, supporting additive or synergistic functional benefits.

These findings extend our ongoing clinical translation of PD-1-edited TILs and provide a strong preclinical rationale for multiplex CRISPR editing to further improve TIL potency. Ongoing studies will evaluate tumour-killing capacity and inform the design of next-generation clinical trials incorporating multi-gene-edited TIL products.

Modeling CAR T Cell Dysfunction in Solid Tumors Using 3D Heterospheroids

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Background: CAR T cells show strong clinical responses in hematologic cancers but are less effective against solid tumors. Solid tumors create physical and immunological barriers that limit CAR T cell infiltration, persistence, and cytotoxic activity, which are not adequately represented in traditional in vitro systems. Developing preclinical models that more accurately mimic these constraints is critical for improving CAR T cell therapy in solid tumors.

Methods: We established an advanced 3D heterospheroid model using either MDA-MB-231 or HT-29 cancer cells, incorporating fibroblasts and healthy donor-derived macrophages, to study CAR T cell infiltration, activation, and cytotoxic function. To generate exhausted CAR T cells, we repeatedly stimulated them through co-culture with cancer cells. Non-exhausted and exhausted CAR T cells were evaluated in both 2D monolayer and 3D heterospheroid cultures, including analyses of killing kinetics, phenotype characterization, and cytokine production.

Results: CAR T cells demonstrated faster and more robust killing, higher activation, and increased proliferation in 2D cultures compared to 3D heterospheroids. Exhaustion led to reduced cytokine secretion and cytotoxicity, with the effect most pronounced in 3D heterospheroids. Furthermore, in HT-29 heterospheroids, the presence of macrophages decreased CAR T cell cytotoxicity, activation, and proliferation, indicating myeloid-driven suppression. By contrast, macrophages did not influence cancer cell killing in MDA-MB-231 heterospheroids.

Conclusions: These 3D heterospheroid models capture critical barriers to CAR T cell function in solid tumors, including spatial restrictions, exhaustion, and immunosuppressive interactions. They provide a versatile platform for assessing CAR T cell constructs and for investigating how tumor, fibroblast, and myeloid interactions shape CAR T cell efficacy, helping to better predict and optimize therapeutic responses in solid tumors.

Optimizing tumor-infiltrating lymphocytes through nonviral CRISPR/Cas-engineering for enhanced adoptive T cell therapy

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Cancer immunotherapy relying on tumor-infiltrating lymphocytes (TILs) has provided durable responses in metastatic melanoma, but treatment resistance due to T cell exhaustion and tumor immune evasion remain major challenges. CRISPR/Cas9 genetic engineering of TILs has emerged as a promising strategy to enhance their persistence and antitumor activity, for example, by delivering immunostimulatory cytokine payloads or by disruption of immune checkpoints. While high CRISPR knock-in (KI) efficiencies have been accomplished using non-integrating viral vectors, their production and purification pose significant challenges for their clinical application. Nonviral delivery of CRISPR/Cas elements could reduce these issues; therefore, we aimed to optimize these methods to increase nonviral KI efficiency in TILs. We nucleofected PBMCs and TILs with Cas9/sgRNA RNP complexes and donor DNA templates to achieve targeted integration through homology directed repair (HDR) of an inducible IL-7 cassette into the PD1 gene locus, thereby disrupting its expression (gene knock-out, KO) and preventing tumor-mediated immunosuppression. Gene KO and KI efficiency were assessed by INDEL analysis, flow cytometry and ELISA. Preliminary results demonstrate efficient PD1 disruption (~90% KO) and a 6-fold increase of IL-7 secretion upon cell activation using linear dsDNA as the donor template. Future experiments will compare different donor template formats (linear dsDNA vs. nanoplasmids) to identify the most efficient CRISPR/Cas KI strategy in TILs. In addition, *in vitro* functional assays will be performed to evaluate reactivity, persistence and cytotoxicity of edited TILs to assess the effect these modifications have on T cell performance. These findings could provide valuable insight into strategies to optimize TIL therapy clinical protocols by targeting some major limitations such as TIL exhaustion and challenges in the clinical translation of gene therapy techniques.

Deciphering the molecular signature of induced pluripotent stem cell to T-cell differentiation using CRISPR activation screens

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Abstract

Induced pluripotent stem cells (iPSC) are a promising avenue for producing next-generation adoptive cell therapies (ACT). They serve as a potentially unlimited source of cells to differentiate any immune cell of interest. Specifically in T cell-based ACTs, this approach allows the customization and personalization of the final T cell product and the generation of an off-the-shelf, allogeneic product. The differentiation of iPSCs to T cells poses great challenges, mainly associated with the low efficiency of the process and protocol variability. Therefore, decoding the molecular signature of this process is of significant importance for advancing this type of therapy. In this regard, CRISPR activation (a) or interference (i) screens offer a comprehensive and systematic approach to unravel the complexities of gene function and regulate the differentiation process.

We have generated iPS cell lines that constitutively express dead Cas9 (dCas9) fused to either the VP64 transactivating domain. We have further optimized the constructs to avoid transgene silencing by incorporating an antisilencing element (UCOE), and made the system Doxycycline inducible to avoid library skewing. We have validated the CRISPRa technology in two iPS cell lines, where a robust gene upregulation of CD4, CD8 and CD14 has been achieved.

For CRISPRa screens, these iPS cell lines will be transduced with a whole-genome gRNA library. We will then follow the enrichment of gRNAs in differentiated populations to evaluate which genes regulate the differentiation at different stages.

We will use our in-house scaffold technology to improve the presentation of the current differentiation factors, as well as implementing the drugable hits from the screen, which allows for a more controlled and localized delivery and improvement of the T-cell yield.

We anticipate that the knowledge obtained from the screens will not only enhance the understanding of T cell development but will also provide opportunities for more precise manipulation of gene expression, which will lead to the generation of robust protocols for iPSC-derived T cell products. Moreover, hits from early stages of the differentiation (at HPSC for instance) can also be used to improve the yields of other immune populations of interest such as NK cells.

Keywords: CRISPR screens, iPS cell, T cell, CRISPR activation, differentiation protocol

Bridging T Cell Immunotherapy and Stem Cell Engineering

Advancing Allogeneic T Cell Manufacturing from Induced Pluripotent Stem Cells

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T cells have demonstrated enormous potential as cellular therapeutics for cancer immunotherapy. However, current autologous approaches face major limitations, including high manufacturing costs and significant lot-to-lot variability between patients. One promising alternative is the generation of allogeneic T cells from induced pluripotent stem cells (iPSCs), which could provide a renewable and standardized cell source for off-the-shelf therapies. Despite this promise, differentiation of iPSCs into functional T cells remains challenging, with several bottlenecks limiting clinical translation. Two of the most critical hurdles are effective Notch activation and proper positive and negative selection during T cell development. Notch signaling is the master regulator that drives T cell commitment from hematopoietic stem and progenitor cells (HSPCs). Replicating this pathway in vitro is difficult because physiological Notch activation depends on a biomechanical pulling force applied through juxtacrine interactions within the thymic microenvironment. Both positive and negative selection are processes that depend on antigen recognition by immature thymocytes. In the thymus, developing T cells are selected based on their ability to recognize peptides presented on major histocompatibility complex (MHC) molecules. However, iPSC-derived thymocytes typically express a highly diverse repertoire of T cell receptors (TCRs) due to recombination, making selection and downstream functionality difficult to achieve in vitro.

To generate a clonal iPSC cell line, cells were CRISPR engineered by inserting a NY-ESO-1-specific TCR into the TRAC locus while simultaneously knocking out the TRBC locus. This strategy bypasses the need for TCR recombination, while being highly attractive for immunotherapy applications. To generate mature T cells from iPSCs, CD34+ hematopoietic stem and progenitor cells (HSPCs) were first produced through embryoid body (EB) formation using a chemically defined protocol that guided differentiation through mesodermal, endothelial, and hematopoietic stages. The EBs subsequently released HSPCs as single cells, which were subsequently cultured under T cell differentiation conditions. T cell lineage commitment was induced through Notch activation using platebound DLL4 and VCAM1 in combination with a chemically defined medium. After 14 days, the HSPCs had differentiated into CD7+CD5+ pre-T cells, and by day 28 they further developed into CD4+CD8+ double-positive T cells. Although the NY-ESO-1-specific TCR was detectable from day 14 onward, its expression increased substantially by day 28. The immature double-positive thymocytes were then matured into CD8 single positive T cells using either irradiated NY-ESO-1-expressing tumor cells or anti-CD3/CD28 stimulation together with stage-specific cytokines. After one week of maturation, the iPSC-derived T cells demonstrated antigen-specific tumor cell killing activity in vitro, while expressing markers indicating a naïve phenotype.

Abstract for CATC

Title: Biomarkers of endothelial dysfunction for prediction of acute toxicities in patients treated with chimeric antigen receptor T-cells for hematological cancers

Authors: Sørine T. Neumann[1,2], Denise Horan[1,2], Monica D.M. Tran[1,2], Eva Kannik Haastrup[3], Claus H. Nielsen[2], Inge Marie Svane[4], Özcan Met[4], Marianne Ifversen[1], Søren L. Petersen[5], Klaus Müller[1,2,6], Katrine Kielsen[1,2,7]

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Background: CAR-T therapy has improved survival in hematological cancers but is limited by severe cytokine-release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS). Growing evidence suggests that endothelial dysfunction plays a central role in their pathogenesis.

We investigated the predictive value of 11 biomarkers reflecting complementary aspects of endothelial dysfunction and/or activation including vascular adhesion, glycocalyx damage, cellular integrity, and coagulation status.

Materials and methods: We included 37 patients treated with CAR-T cell therapy (Kymriah: n=13; Yescarta: n=12; DK-CLIC-1901: n=12) for B-ALL (n=14) and B-NHL (n=23) at Rigshospitalet from 2019-2025 with a median age of 45.6 years (range: 1-71).

Endothelial biomarkers were analyzed in plasma by Luminex or ELISA before the start of lymphodepleting chemotherapy (LDT), on day of CAR-T infusion, and day +4, +7, +14, +21 and +90 after CAR-T infusion.

Results:
81% of patients developed CRS on median day +1 after CAR-T infusion (range: 0-12), and 35% developed ICANS on median day +6 (range: 1-14).

Following CAR-T infusion, nearly all endothelial markers temporarily decreased, except for VCAM-1, which increased, and syndecan-1, which remained stable.

Patients developing CRS ≥3 (n=6) had significantly elevated syndecan-1 levels prior to LDT (Figure 1A). Furthermore, VCAM-1, vWF, thrombomodulin, PAI-1 and sVEGFR-1 levels rose during the progression of CRS and were highest in patients with severe disease (all $p<0.05$).

Patients developing ICANS ≥3 (n=6) had significantly higher levels of VCAM-1 (Figure 1B) and vWF on day +4 after CAR-T infusion, while angiopoietin-2, syndecan-1, thrombomodulin and sVEGFR-1 levels increased during the progression of ICANS (all $p<0.05$).

There were no significant associations between ICAM-1, P-selectin, E-selectin or PECAM-1 and these toxicities.

Conclusion: Our findings support the emerging evidence of endothelial dysfunction in the pathogenesis of severe CRS and ICANS, and suggest syndecan-1, VCAM-1 and vWF as potential risk biomarkers, although confirmation in larger cohorts is needed.

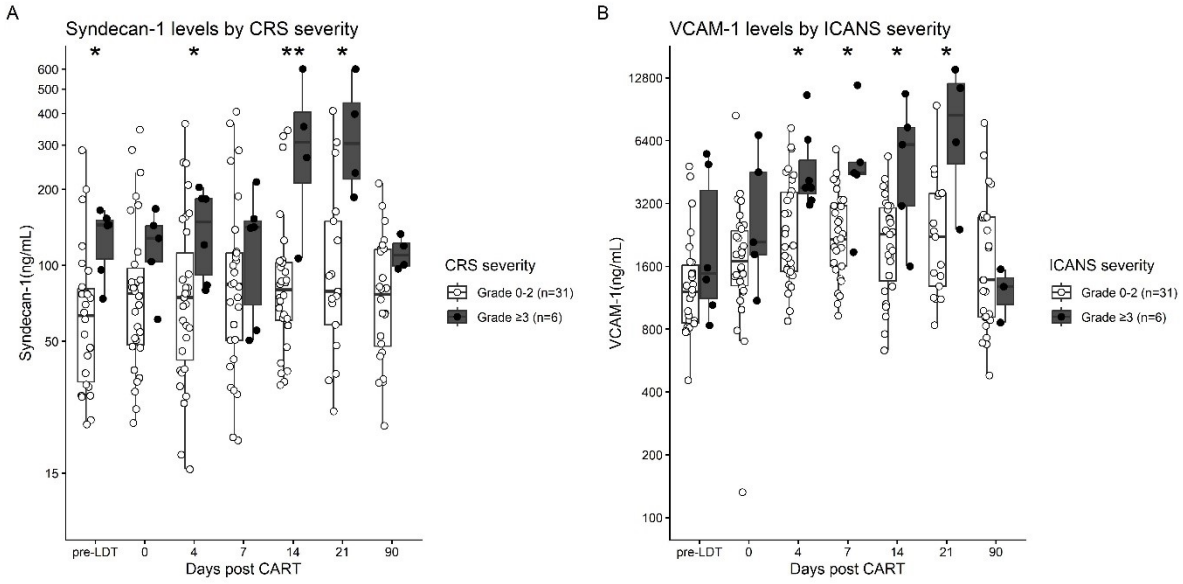
A
Syndecan-1 levels by CRS severity
Syndecan-1(ng/mL)
600
500
400
300
200
100
50
15
pre-LDT
0
4
7
14
21
90
Days post CART
CRS severity
Grade 0-2 (n=31)
Grade ≥3 (n=6)
B
VCAM-1 levels by ICANS severity
VCAM-1(ng/mL)
12800
6400
3200
1600
800
400
200
100
pre-LDT
0
4
7
14
21
90
Days post CART
ICANS severity
Grade 0-2 (n=31)
Grade ≥3 (n=6)

Figure 1

Single-cell based multiomics profiling of tumour infiltrating lymphocyte in adoptive cell therapy reveals signatures of relevance for clinical response

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Adoptive cell transfer (ACT) with tumour-infiltrating lymphocytes (TILs) can induce durable tumour regression, yet long-term clinical benefit remains inconsistent, and the determinants of anti-tumour T-cell persistence are poorly understood. To address this, we developed a multimodal platform, capturing pMHC-specificity and paired TCR sequences, transcriptomes and surface proteins in single cells, allowing us to link specificity, phenotype, and clonal dynamics. Across nine patients with diverse clinical outcome, we investigated a total of 67 longitudinal samples, including the infusion product, pre- and post-infusion PBMCs and pre-treatment tumour digest. From these patients we profiled T cells resognising neoepitopes, shared tumour associated antigens and viral epitopes. We often found several TCRs recognising the same tumour epitope and examples of TCRs with a flexible recognition profile, able to recognise different peptide targets. TCR specificity and functionality were experimentally validated by TCR CRISPR/Cas9 knock-in, confirming both the TCR-pMHC pairing and the direct tumour reactivity of both neoantigen and shared tumour antigen-specific TCRs. Across the longitudinal samples from the nine patients, we observed varying persistence of the tumour-specific T cell clones from the infusion product samples, with examples of clones detected up to 6 years after infusion. Infusion products and early post-infusion samples were consistently enriched for tumour-specific responses. However, in non-responders, we observed a rapid decline of tumour-reactive clonotypes. Additionally, these patients had fewer tumour-specific responses in blood prior to the TIL transfer. Based on the clonal dynamics and the phenotype of the T cell clones, we developed an “engraftment score” to compare the persistence of the anti-tumour T-cell clones and clinical outcomes. Interestingly, we observed that excessive expansion of T cell clones in the infusion product was linked to lower engraftment and expression of markers of exhaustion and terminal differentiation. These results highlight persistence of tumour-reactive clonotypes as a key determinant for ACT response and show potential for biomarker discovery.

Tracking antigen specific T cell responses in rapidly expanded TIL-ACT products of cervical cancer patients

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Persistent infection of human papillomavirus (HPV) causes the majority (>90%) cervical cancer (CC) cases. Tumor infiltrating lymphocyte based adoptive cell transfer (TIL-ACT) has previously shown an overall response rate of 44% in metastatic and recurrent CC. The manufacturing process of rapidly expanded TIL (REP TIL) product was further improved by the addition of monoclonal antibodies to the culture. However, there is still a lack of understanding with regards to the T-cell specificities in the TIL product and its correlation with patient outcome, especially in CC. In this study both standard and improved REP TILs along with their precursor cultures (pre-REP) from five CC patients were screened for T cell clones against HPV, tumor associated antigens (TAA) and other common viral antigens (CEF), using DNA barcoded peptide-major histocompatibility(pMHC) based multimer technology. Overall, 136 HPV responses were identified with 64 unique pMHC combinations, along with 23 TAA and 22 CEF responses. The improved REP process yielded a higher number of CD8 T-cell responses that were traceable and persistent from pre-REP to REP, with an increased expression of CD57, HLA-DR, and CD49d compared to standard REP. Top responses were validated using combinatorial tetramer staining. These findings reveal the antigenic landscape within CC TIL products, provide insight into the traceability of antigen-specific T-cell clones from culture initiation to infusion, and enable the discovery of novel TCRs for off-the-shelf therapies.

Title: Nonviral CRISPR/Cas-engineered tumor-infiltrating lymphocytes with inducible cytokine release and checkpoint resistance for adoptive T cell therapy

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Adoptive cell therapy (ACT) with tumor infiltrating lymphocytes (TILs) has achieved durable responses in patients with metastatic melanoma. However, insufficient expansion and persistence of tumor-reactive T cells remain a major cause of relapse. Cytokines such as IL-7 can drive T cells toward phenotypic subsets associated with enhanced persistence and antitumor activity, but toxicities are associated with systemic cytokine administration or constitutive expression of cytokines from TILs. Placing the cytokine transgene under control of a Nuclear Factor of Activated T cells (NFAT) inducible promoter restricts cytokine expression to antigen-dependent T cell activation, thereby minimizing such toxicities. CRISPR/Cas-mediated targeted integration into the PDCD1 gene locus further improves TILs by disrupting PD-1 to escape tumor-mediated immunosuppression. Here, we demonstrate efficient nonviral CRISPR/Cas-mediated targeted integration (>20%) of an inducible IL-7 cytokine transgene and simultaneous PD1 knockout (>81% decreased PD1 surface expression) in patient-derived TILs. Edited TILs demonstrated NFAT-inducible IL-7 expression only in response to anti-CD3/CD28 stimulation (13.64-fold increase over unstimulated controls) and co-culture with autologous tumor cells (5.59-fold increase over unstimulated controls), thereby demonstrating stimulus-dependent cytokine release. Furthermore, similar phenotypic and efficient cytotoxic profiles of edited and nonedited TILs were observed, confirming preserved antitumor function. This study establishes a clinically relevant, nonviral genome engineering strategy to generate TILs with inducible cytokine release and intrinsic checkpoint resistance. This approach is designed to enhance the persistence and antitumor activity, supporting translation into next-generation TIL therapies for patients with solid tumors.

Macrophage activation before lymphodepletion can predict CRS, ICANS, and late cytopenia after CD19 CAR-T cell therapy for hematological cancer

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Background: CAR-T cell therapy is challenged by immune-related toxicities, including cytokine release syndrome (CRS), immune effector cell-associated neurotoxicity (ICANS), and late cytopenia. Growing evidence suggests that macrophage activation plays a key role in the pathophysiology of these toxicities. We investigated whether circulating markers of macrophage activation (soluble mannose receptor, sMR and sCD163) could predict the severity of CRS, ICANS, and late cytopenia after CD19 CAR-T therapy.

Methods: We included 35 patients treated with CD19 CAR-T therapy for B-ALL (*n*=14) or B-NHL (*n*=21) at Rigshospitalet in Denmark from 2019-2025 (Table). Patients received tisagenlecleucel (*n*=13), axicabtagene ciloleucel (*n*=10), or an academic CAR-T product (DK-CLIC-1901, *n*=12). sMR and sCD163 levels were measured using in-house ELISA assays in plasma collected before lymphodepleting chemotherapy (pre-LD), on day of CAR-T infusion, daily from day +1 to +8, and on days +10, +12, +14, and +90 post-infusion. 100 healthy controls with a median age of 42 years (range: 19-69) were included for comparison.

Results: Pre-LD levels of sMR and sCD163 were significantly elevated in patients compared with healthy controls (median 0.35 vs. 0.22 mg/L, P<0.00054, and 2.32 vs. 1.58 mg/L, P<0.0001, respectively). After CAR-T infusion, both markers rose and peaked at day +6 for sMR (0.50 mg/L) and day +12 for sCD163 (4.1 mg/L). Pre-LD levels of sMR and sCD163 were not associated with age, diagnosis, prior lines of therapy, bridging therapy, or same-day CRP, IL-6 or LDH levels (all P≥0.05).
Patients developing grade ≥3 CRS (17%) and ICANS (14%) had higher levels of sMR and sCD163 before LD than those with grade 0-2 (Figure). In Cox analyses, the pre-LD levels of both markers were associated with increased risk of severe CRS (sMR: HR=2.7 per doubling, P=0.008; sCD163: HR=4.5 per doubling, P=0.034), and higher pre-LD sMR levels were associated with severe ICANS (HR=2.7 per doubling, P=0.027). Furthermore, high pre-LD LDH was associated with increased risk of severe CRS (HR=2.1 per doubling, P=0.011), while age, diagnosis, CAR-T product, and CAR-T cell dose were not associated with severe CRS or ICANS in our cohort.
Patients with grade ≥3 neutropenia (55% by ICAHT), thrombocytopenia (36%), and anemia (19%) beyond day +30 after CAR-T infusion had higher pre-LD sMR levels compared with grade 0-2 (P=0.006, P=0.002, and P=0.02, respectively), and higher pre-LD sCD163 levels were observed in patients with late grade ≥3 thrombocytopenia (P=0.04). In logistic regression analyses, higher pre-LD sMR was associated with increased risk of late grade ≥3 ICAHT (OR=3.6 per doubling, P=0.028), thrombocytopenia (OR=13.6 per doubling, P=0.016), and anemia (OR=8.2 per doubling, P=0.057).

Conclusions: Elevated levels of sMR and sCD163 before the start of lymphodepletion were associated with the development of severe CRS, ICANS, and late cytopenia in patients treated with CD19 CAR-T therapy, supporting the emerging evidence of a role of activated macrophages in the pathogenesis of CAR-T-related toxicities. These findings further suggest a clinical role of macrophage activation markers in guiding anti-inflammatory prophylaxis in high-risk patients.

Table:

Patient and treatment characteristics	**N=35**
Age at infusion (years), median (range)	40 (1.7-71.8)
Male sex, n (%)	29 (82.9%)
Diagnosis, n (%) B-ALL B-NHL	 14 (40.0%) 21 (60.0%)
No. of prior lines of therapy, median (range)	2 (1-5)
Prior HSCT, n (%) Allogeneic HSCT Autologous HSCT	 7 (20.0%) 2 (5.7%)
Refractory to previous treatment line, n (%)	20 (57.1%)
Pre-LD LDH (U/L), median (range)	228 (142-2230)
Elevated pre-LD LDH, n (%)	19 (54.3%)
Bridging therapy, n (%) Chemotherapy-based regimens IT therapy only Radiotherapy ± steroids Targeted therapy Steroids only None	 9 (25.7%) 5 (14.3%) 3 (8.6%) 4 (11.4%) 5 (14.3%) 9 (25.7%)
CAR-T product, n (%) Tisa-cel (Kymriah®) Axi-cel (Yescarta®) DK-CLIC-1901	 13 (37.1%) 10 (28.6%) 12 (34.3%)
CAR-T dose (cells x 10^6/kg), median (range)	2.0 (0.23-4.8)
Days from leukapheresis to infusion, median (range)	33 (13-58)

Figure:

Fig. 1

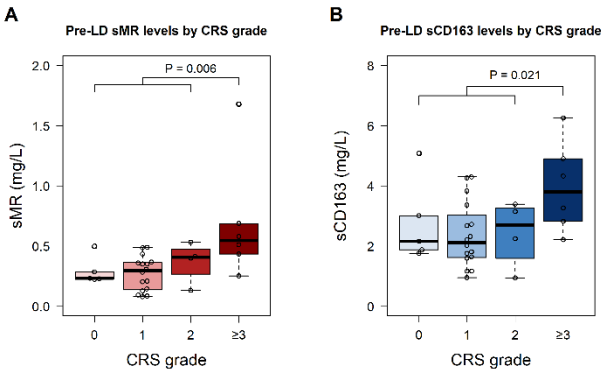
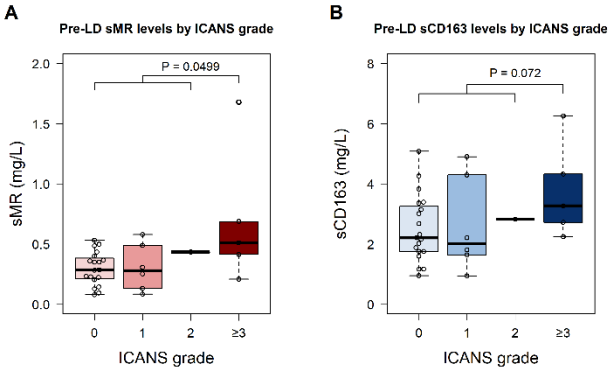


Fig. 2



Title: DAN-CART 1901: Preliminary results of safe and feasible administration of the fresh autologous anti-CD19 CAR-T product DK-CLIC-1901

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Background: Treatment with chimeric antigen receptor T-cells (CAR T) has demonstrated efficacy in patients with CD19-positive hematological malignancies and lower toxicity than other treatment options. We established an academic CAR T-cell program to broaden access for patients without other curative treatment options and to enable faster, more flexible local production.

We report preliminary results from the initial phase 1b/2 trial using the academic autologous 4 1BB anti-CD19 CAR-T product DK-CLIC-1901 (CTIS # 2024-515174-27-00). The primary objective was to evaluate the safety of DK-CLIC-1901. The secondary objectives were to assess feasibility, clinical efficacy, and immunological response after DK-CLIC-1901 infusion.

Methods: The main inclusion criteria were relapsed or refractory B-cell acute lymphoblastic leukemia (ALL) or non-Hodgkin lymphoma (NHL), age between 1-70 years, adequate performance status and organ function (lung, heart, liver, and kidney), life expectancy ≥ 12 weeks, and written informed consent.

Patients received a single infusion of fresh-to-fresh autologous, in vitro-expanded DK-CLIC-1901 CAR T-cells at a minimum dose of $1x10^6$ CAR T-cells/kg, with a dose reduction to $2x10^7$ CAR T-cells in total in case of high tumor burden. The DK-CLIC-1901 product was manufactured on the CliniMACS Prodigy platform (Miltenyi) using a lentiviral vector carrying a 4-1BB anti-CD19 CAR transgene [N Kekre et al, Front Immunol 2022].

Results: Thirteen patients passed the initial screening for participation. One patient was excluded before apheresis due to rapid progression of hematological malignancy; 12 patients underwent apheresis and received CAR-T infusion (Table).

Cytokine release syndrome (CRS) occurred in 9 patients (grade ≥3: n=3) and immune effector cell-associated neurotoxicity syndrome (ICANS) in 3 patients (grade ≥3: n=2), with a higher frequency in patients with high tumor burden (CRS grade ≥3: 67% vs. 11%; ICANS: 67% vs. 11%). One patient with a high tumor load died at day +26 from intracerebral hemorrhage following severe CRS/ICANS. Another patient developed HHV-6 encephalitis after CAR-T infusion with persistent neurological sequelae [NM Birk et al, Front Hematol 2025]. Late cytopenia was manageable, with grade ≥3 neutropenia (ICAHT), thrombocytopenia, and anemia occurring in 11%, 33%, and 0% of 9 evaluable patients at day 90 following CAR-T infusion.

Median follow-up time was 12 months (26 days to 2.0 years). Nine of 11 evaluable patients (82%) responded to CAR-T infusion, of which 5 remain in complete remission more than 6 months from CAR-T infusion (Figure). Two patients had loss of B-cell aplasia within the first year after CAR-T infusion (day +83 and +125),

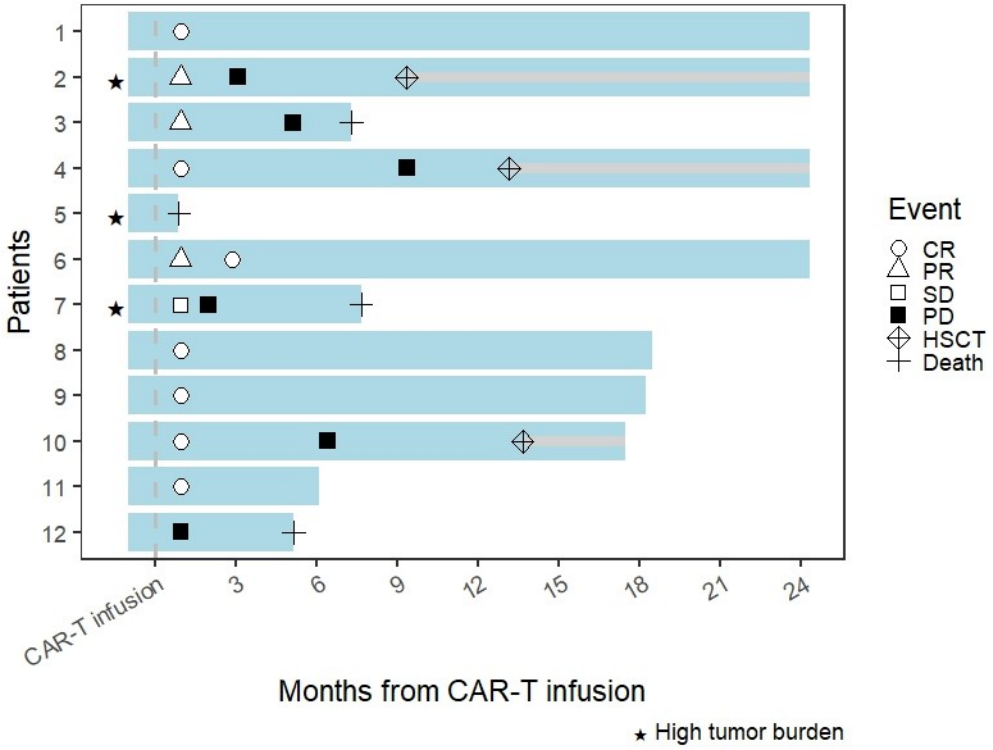
which was followed by a CD19-positive relapse in one patient. The estimated 1-year overall and progression-free survival were 65% (95%CI: 42-100) and 39% (95%CI: 19-82), and the 1-year cumulative incidence of non-relapse mortality was 8%. Three of the 6 patients with relapse or progressive disease were confirmed CD19 positive, and three patients (25%) were subsequently rescued with allogeneic hematopoietic stem-cell transplantation.

Conclusion: This is the first trial of point-of-care manufactured CAR-T cells in Denmark and demonstrates that administering fresh CLIC-1901 product is safe, feasible and efficacious, especially in patients with low tumor burden. Our platform provides a feasible and rapid manufacturing procedure for future CAR-T products, which may also include targets outside commercial interests.

Table:

Clinical characteristics	**N=12**
Age (years), median (range)	54 (16-67)
Male sex, *n*. (%)	11 (92%)
Diagnosis, *n*. (%)	
B-cell ALL	2 (17%)
B-cell NHL	10 (83%)
Diffuse large B-cell lymphoma	3 (25%)
Transformed follicular lymphoma	4 (33%)
High-grade B-cell lymphoma	1 (8%)
Burkitt lymphoma	1 (8%)
Mantle cell lymphoma	1 (8%)
High tumor load, *n*. (%)	
ALL: >20% blasts in BM	0 (0%)
NHL: ≥10 cm tumor mass or BM involvement	3 (25%)
LDH at the start of LDT (U/L), median (range)	229 (130-2200)
Prior lines of treatment, median (range)	3 (1-4)
CAR-T indication, *n*. (%)	
Primary refractory	5 (42%)
≥2 relapse	4 (33%)
Relapse after autologous/allogeneic HSCT	3 (25%)
LDT with fludarabine (30 mg/m^2 x4) and cyclophosphamide (500 mg/m^2 x2), *n*. (%)	12 (100%)
Time from enrollment to apheresis (days), median (range)	6 (2-16)
DK-CLIC-1901 production time (days from apheresis to CAR-T infusion), median (range)	13 (13-19)
Cellular composition of DK-CLIC-1901 product, median (range)	
CAR-T cell count (x10^6 CAR T-cells / kg bodyweight)	2 (0.23-3.1)
CAR-T cell count (x10^8 CAR T-cells in total)	200 (20-200)
CD4/CD8 T cell ratio	0.63 (0.29-3.69)

Figure:


Patients
1
2
3
4
5
6
7
8
9
10
11
12
CAR-T infusion
3
6
9
12
15
18
21
24
Months from CAR-T infusion
Event
CR
PR
SD
PD
HSCT
Death
★ High tumor burden

GD2-targeting Tumor Infiltrating Lymphocytes for Pediatric Patients with CNS Tumors

Cecilie Ølvang Madsen[1*], Thomas Morgan Hulen[1], Maria Ormhøj[2], René Mathiasen[3], Marianne Ifversen[3], Jane Skjøth-Rasmussen[4], Sine Reker Hadrup[5], Inge Marie Svane[1], Özcan Met[1,5]

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Introduction: Central nervous system (CNS) tumors are among the most common pediatric cancers, but prognosis for several subtypes remains poor, particularly at relapse where curative options are lacking. Adoptive cell therapy with chimeric antigen receptor (CAR) T cells has demonstrated success in hematological malignancies, while tumor-infiltrating lymphocyte (TIL) therapy has mediated durable responses in solid tumors. Combining these approaches may generate poly-specific TILs with improved tumor recognition and clearance.

Method: TILs were expanded from tumor biopsies of pediatric patients with CNS tumors and transduced with a GD2-specific CAR using a lentiviral vector, followed by rapid expansion. TILs engineered with GD2-CAR were tested *in vitro* for activation, effector function, cytotoxicity, and reactivity against autologous $GD2^+$ tumor cells when available.

Results: TILs could successfully be expanded from 6 out of 12 pediatric CNS tumor biopsies (50%), yielding a mean of 16.2×10^6 $CD3^+$ cells (range: 0.57×10^6-34×10^6), with up 8×10^6 cells/fragment. All patient-derived TIL cultures were successfully transduced and expanded under clinically adaptable conditions. TILs modified with GD2-CAR showed enhanced recognition and cytotoxicity against $GD2^+$ tumor cells, including autologous CNS tumor cell lines.

Conclusion: TILs engineered to express a GD2-targeting CAR demonstrated feasibility and enhanced anti-tumor activity in pediatric CNS tumor models. These findings provide proof-of-concept for combining TIL-therapy with CAR engineering and support further preclinical development toward clinical translation for children with CNS tumors.

Impaired function of CLL-derived CD19 CAR T cells in a 3D tumor microenvironment reveals CXCR4 and IL-10 as potential targets

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Introduction: Although targeted therapies have improved outcomes, CLL remains largely incurable, underscoring the need for novel therapeutic strategies. While CAR T-cell therapy has significantly advanced the treatment of acute lymphoblastic leukemia (ALL), its efficacy in relapsed/refractory CLL remains limited, suggesting disease-specific constraints on anti-tumor immunity. Increasing evidence indicates that malignant B cells shape the tumor microenvironment (TME) into protective niches, promoting immune dysfunction and therapy resistance. Understanding the mechanisms underlying reduced therapeutic responses, particularly the contribution of the TME to treatment failure, may reveal novel targets to enhance CAR T-cell function in CLL.

Methods: To dissect disease-specific differences in CAR T-cell functionality, we established a three-dimensional (3D) scaffold-based co-culture system with primary CLL or ALL cells, autologous CAR T cells, and bone marrow-derived stromal cells (BMSCs). CAR T-cell function was assessed by flow cytometry for activation, cytotoxicity, proliferation, exhaustion, and differentiation in core and peripheral regions of the scaffold. Spatial distribution and target cell co-localization were analyzed by confocal immunofluorescence imaging. The soluble milieu was examined by multiplex cytokine assays and ELISA. Targeted interventions were tested in combination with CAR T cells in the CLL 3D model to evaluate functional improvements.

Results: Comparative analyses of autologous CAR T cells in the 3D co-culture system revealed marked differences between CLL- and ALL-derived CAR T cells. Although exhibiting strong activation, CLL CAR T cells showed limited proliferation and reduced cytotoxicity, with decreased co-localization with malignant B cells and a more differentiated, exhausted phenotype. Functional impairments in cytotoxicity and exhaustion were particularly pronounced in core regions, suggesting that TME-mediated factors exacerbate intrinsic defects. IL-10 secretion was elevated in CLL co-cultures, pointing to disease-specific immunosuppression. Furthermore, $CXCR4^+$ CLL cells and $CXCL12^+$ BMSCs were enriched in core regions, indicating spatially regulated stromal support via the CXCR4/CXCL12 axis. Targeting these TME-pathways partially improved CAR T-cell function, with both IL-10 and CXCR4 blockade enhancing cytotoxicity and reducing exhaustion, while IL-10 inhibition reduced CLL viability in a patient-dependent manner.

Conclusion: This study highlights key functional impairments of CLL-derived CAR T cells within the 3D model. These findings underscore the importance of elucidating mechanisms of CAR T-cell dysfunction and support the potential of TME-targeted combination strategies, such as IL-10 and CXCR4 blockade, to partially restore effector function. The autologous 3D co-culture system offers a translational platform to investigate CAR T-cell function and evaluate novel therapeutic approaches to improve CAR T-cell efficacy in CLL.

Generation and Validation of HER2-targeting CAR-TILs for Ovarian Cancer: Investigating the potential as an adoptive cell therapy

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Introduction: Ovarian cancer (OC), the second most deadly gynecological cancer globally, remains challenging for immunotherapy due to low tumor mutational burden and low numbers of tumor-reactive tumor-infiltrating lymphocytes (TILs). Current immunotherapy approaches have shown limited success. CAR-TILs, TILs engineered to express a chimeric antigen receptor (CAR), represent a novel OC immunotherapy and remain poorly explored. This study investigates whether engineering OC-derived young TILs (yTILs) with a HER2-targeting CAR can improve adoptive cell therapy for OC.

Materials and Methods: To generate CAR-TILs, a low affinity HER2-targeting CAR was introduced to TILs via lentiviral transduction. Functional validation of CAR-TILs was performed using intracellular cytokine staining (ICS), flow cytometry, and ^{51}Cr release cytotoxicity assays. Autologous patient-derived tumor cell lines (TCLs) and young TILs were used to investigate CAR-TIL potential and function.

Results: CAR-TILs were generated from three OC patients. Transduction efficiencies ranged from 1-13%. When co-cultured with autologous TCL, $CD3^+$ CAR-TILs showed an increase in reactivity to 5-6%, compared to 1-3% reactivity from non-transduced (NTD) TILs, measured by CD107a, CD137, IFN-γ, and TNF co-expression. Upon co-culture with HER2 target-bearing SKOV-3 cell line, $CD3^+$ CAR-TILs were 4-20% reactive, compared to <1% reactivity in $CD3^+$ NTD TILs. In short-term cytotoxicity assays, CAR-TILs produced 10-15% lysis of autologous TCL at an effector:target ratio of 90:1, compared to 1-5% lysis by NTD. Overall, HER2-targeting CAR-TILs showed increased reactivity and cytotoxicity towards high HER2-expressing and autologous TCLs compared to NTD controls. Increased HER2 expression was associated with stronger CAR-TIL responses.

Conclusions: HER2-targeting CAR-TIL production is feasible and may improve the limited baseline activity typical of OC-derived TILs. CAR-mediated effector functions appeared to be antigen density- and dose-dependent. Together, these results support further investigation of low-affinity HER2-targeting CAR-TILs as a potentially safer and more effective OC immunotherapy.

T cell-targeted gene delivery for *in vivo* CAR-T cell therapy

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Chimeric antigen receptor (CAR)-T cell therapy has revolutionized the management of haematological malignancies, yet its application is limited by individualized, labor-intensive, and costly *ex vivo* manufacturing processes. *In vivo* genetic reprogramming of T cells offers a compelling alternative but relies on gene delivery systems that achieve high specificity, efficiency, and safety. In this study, we explored the use of rationally engineered, T cell–targeted adeno-associated virus (AAV) vectors as a modular platform for selective gene delivery and stable genome editing in human T lymphocytes, employing CAR-T cell generation as a translational model.

DARPin-retargeted AAVs (DART-AAVs) demonstrated highly selective binding and transduction of human $CD4^+$ and $CD8^+$ T cells within complex cellular environments. To support long-term CAR expression, AAV-mediated delivery of donor template CAR constructs was combined with lipid nanoparticle (LNP)-mediated CRISPR/Cas9 editing, enabling targeted integration of the CAR transgene into the TRAC locus. This combined AAV–LNP strategy achieved cell-specific gene transfer, stable CAR expression, and sustained cytotoxic activity *in vitro.*

In addition, leveraging our expertise in viral vector retargeting, we developed RNA-LNPs directed toward $CD8^+$ T lymphocytes through the incorporation of DARPins with high affinity for murine or human CD8. For surface display, DARPins were genetically fused to apolipoprotein E2 (ApoE2), produced in bacteria, and subsequently associated with RNA-LNPs. These engineered particles effectively distinguished between $CD8^+$ and $CD8^-$ T cells in terms of both binding and reporter gene expression across murine and human systems. Importantly, when introduced into human donor blood or administered systemically in humanized mice, CD8-LNPs labeled a significant proportion of $CD8^+$ T cells, while showing minimal binding to $CD4^+$ T cells. These findings indicate that ApoE2-DARPin constructs remain stably associated with RNA-LNPs even in serum-containing conditions, highlighting this strategy as a novel platform technology based on biological engineering rather than chemical conjugation for precise targeting of RNA-LNPs to therapeutically relevant cell populations.

Overall, this study establishes DART-AAVs and RNA-LNPs as a promising platform for CAR-T cell engineering and provides mechanistic insights with broad relevance for cell type–specific and durable genetic modification beyond CAR-T cell therapy.

Functional Dissection of Cytotoxic and Non-Cytotoxic Cellular States of a CD19 CAR T Infusion Product from the DAN-CART 1901 Trial Using Xdrop® Co-Encapsulation

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Background

Adoptive cell therapies can induce durable tumor regression, yet functional heterogeneity within infused T cell populations limits consistent efficacy. While single-cell transcriptomics has defined diverse T cell states, direct links between these states and cytotoxic function remain unclear. Emerging evidence suggests that cellular state, rather than receptor specificity or abundance, is a primary determinant of T cell function[1,2]. We previously demonstrated that Xdrop®-based microfluidic co-encapsulation enables high-throughput functional interrogation of CAR T cell–tumor interactions[3]. Here, we analyze Denmark's first domestically produced CAR T product (DK-CLIC-1901) using Xdrop DE50 technology, integrating functional sorting with single-cell transcriptomics mapping cytotoxic activity onto transcriptional state and clonal architecture.

Methods

CAR T cells from a single DK-CLIC-1901 infusion product were enriched using CD19 CAR specific magnetic selection and co-encapsulated with $CD19^+$ Daudi cells in Xdrop DE50 droplets as single-cell pairs. Droplets were sorted by FACS according to functional signals indicating whether the encapsulated CAR T cell exhibited a "killer" (caspase3/7^+, granzyme B^+) or "nonkiller" (caspase3/7^-, granzyme B^-) phenotype. Droplets were then broken, and the recovered cells processed for 10x Genomics single-cell RNA sequencing. Analyses included unsupervised clustering, differential expression, gene ontology, functional enrichment across states, and assessment of CAR expression and TCRβ-defined clonotypes.

Results

Of the co-encapsulated CAR T cells, 11.7% were classified as killer and 13.2% as nonkiller, with the remaining droplets being negative or positive for only one of the cytotoxic markers. Upon single cell sequencing analysis, detectable CAR transcript expression comprised 77% of killer and 74% of nonkiller populations. Both populations exhibited significant transcriptional heterogeneity. Gene ontology analysis revealed that both killer and nonkiller populations segregate into several distinct biological programs. Nonkillers were notably in stem-like/memory-like and heat-shock/stress-associated states, indicating that lack of cytotoxic function arises from divergent cellular contexts. In contrast, killer cells were enriched in more functionally aligned effector programs, including proliferative interferon-primed, terminal cytotoxic and oxidative stress-associated states. TCR clonotype analysis revealed a broadly polyclonal repertoire with limited overlap between killer and nonkiller clonotypes.

Discussion

These findings directly link cytotoxic function to transcriptional state and support a model in which functional capacity is primarily governed by cellular state rather than receptor abundance. The coexistence of killer and nonkiller cells within shared states highlights functional heterogeneity not captured by transcriptional clustering alone, consistent with emerging state-based models of T cell function. Ongoing analysis of additional infusion products will extend these findings. Together, this work provides a framework for resolving functional heterogeneity in adoptive cell therapy and identifying states associated with effective tumor killing.

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FLIC - Flow Cytometry and Imaging Core:

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The FLIC facility at DTU provides comprehensive flow cytometry, cell sorting, and imaging services to support academic and industrial research. Established to deliver cutting-edge analytical capabilities, FLIC houses state-of-the-art instrumentation including spectral analyzers with imaging (BD FACSDiscover A8), spectral cell sorters (BD FACSDiscover S8), high-parameter conventional analyzers (BD LSRFortessa SORP), and biosafety-equipped sorters (BD FACSAria Fusion). Additional platforms include confocal microscopy with AiryScan 2 super-resolution detection (Zeiss LSM 900), live-cell imaging (IncuCyte S3), and single-cell genomics systems (10x Chromium X, BD Rhapsody). FLIC operates as an open-access facility, providing hands-on training and technical support to enable independent user operation across all platforms. Our services span experimental design consultation, multi-parameter panel optimization, rare cell sorting, high-throughput screening, and data analysis support. The facility is accessible to all DTU personnel and external partners from academia and industry. With expertise in spectral cytometry, high-parameter immunophenotyping, and advanced sorting techniques, FLIC serves as a central resource for researchers requiring advanced single-cell analysis and imaging solutions.

Title: Adenoviral vectored full-length antigens for expansion of polyclonal CD4+ and CD8+ T cell responses in human dendritic cell and T cell co-culture.

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Targeting large full-length antigens for polyclonal T cell expansions *ex vivo* is difficult using peptides but is attractive due to the possibility to expand broad responses in patients with different HLA alleles. We evaluated adenoviral vectored (AdV) full-length antigens in human monocyte derived dendritic cells as stimulators for human T cell expansions with viral antigens from human papillomavirus 16 (HPV16) and the human endogenous retrovirus K/HML-2 (HERV-K). Initially, both the AdV strategy and a strategy based on confirmed single peptides in HLA-A2 donors expanded T cell responses with a majority of responses toward HPV16 E1. Expanded cells were sorted for polyclonal rapid expansion using either peptides or AdV transduced DCs. Here the peptide-based strategy resulted in higher frequencies of specific T cells whereas the AdV strategy resulted in T cells that performed functionally better against cancer cells in a chromium release lysis assay. However, AdV vectors introduced substantial challenges for both HPV and HERV-K antigens including high background cytokine production (IFNγ, TNFα). Prime-boost strategies using heterologous AdV serotypes further exacerbated non-specific activation. This was partially mitigated by IFNγ-based magnetic enrichment followed by rapid expansion, where the final expanded cell exhibited low background and good tumor recognition. During repeated assays aiming at expanding HERV-K self-antigen specific responses we observed a considerable difference in the obtained T cell responses between assays and between donors and even within assays using the same donors with different expansion protocols. Overall, we were able to expand anti-viral and anti-HERV-K specific CD4+ and CD8+ T cell responses towards most donors with adequate quality of their stored antigen presenting cells. Further experiments are required to understand the sources of the pronounced variability, and to further increase the yield for possible therapeutic purposes, it seems possible to systematically expand functional, antigen specific CD4+ and CD8+ T cells, with relevant antigen recognition towards cancer cells, without prior consideration of donor HLA types.

Title: Harnessing Endogenous miRNAs for Targeted Activation of CRISPR/Cas Systems

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The therapeutic potential of CRISPR/Cas-based genome editing for treating genetic disorders is immense, yet its clinical translation remains hindered by challenges related to specificity, safety, and in vivo delivery. Current strategies to restrict CRISPR/Cas activity to target tissues are based on tissue-specific promoters or ex vivo manipulation, both of which can be either labor-intensive or suffer from lack of specificity and customizability. In this work, we present a novel approach to achieving tissue specificity through integration of endogenous microRNA (miRNA) biology with CRISPR-based technologies. Over 2,000 miRNAs have been annotated in the human genome; while many are broadly expressed, others are specific to particular cell types, tissues, or developmental stages. By making the correct processing of CRISPR RNA (crRNA) dependent on the presence of a specific miRNA, we confer cell-type specificity to CRISPR/Cas activity, ensuring that editing occurs only in the intended target cells. This system enables efficient genome editing while minimizing off-target effects and our approach represents a significant step toward safe, precise, and cell-specific genome editing for therapeutic applications.

Novel T-Cell Epitope Mapping and In-Depth Characterization of T-Cells in Respiratory Syncytial Virus Infection

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Lower respiratory tract infections from seasonal and pandemic respiratory viruses remain a leading cause of morbidity and mortality worldwide. Despite their global impact, therapeutic options remain limited and clinical management is predominantly supportive. This highlights the need for novel immunological targets and biomarkers to improve prophylactic/treatment interventions, and predictive models for personalized care. Our focus is to identify and characterize T-cells specific to Respiratory syncytial virus (RSV) antigens.

To achieve this, we employed a large-scale, high-throughput screening platform utilizing DNA-barcoded peptide–MHC (pMHC) multimer (MULT) libraries. We screened T-cell responses against a comprehensive RSV peptidome based on 2023–2024 Danish strains. The peptide library consisted of 1,791 unique peptides presented across 12 HLA alleles, yielding 2,642 distinct pMHC complexes.

Our initial screen was conducted in a cohort of 53 healthy individuals to define the baseline RSV-specific epitope landscape in a non-acute immunological setting. This enabled optimization of MULT libraries and insights into RSV-specific T-cell immunity in healthy individuals. Notably, we identified 278 unique pMHCs recognized by circulating T-cells, of which only 2 epitopes were previously reported in the Immune Epitope Database (IEDB), highlighting the substantial novelty and depth of the dataset. The screening results were validated using a combinatorial tetramer staining approach, using the ten most frequently recognized pMHCs in an HLA-matched subset of the donor cohort.

Then, we investigated 15 acutely RSV-infected patients with the established pMHC library, now in a single cell approach (10X platform) allowing us to identify intrinsic determinants in their gene expression, TCR clonality, and epitope-specificity, all at ones. This, to further characterize RSV-specific T-cells in a disease setting. In parallel, we sorted RSV-specific B cells using barcoded RSV-antigen-tetramers, to enable a multi-faceted investigation of the adaptive immune system. Next, we will screen 16 lung-resident lymphocyte (LRL) samples from acutely RSV-infected infants with the same approach. The single cell data is being collected and analyzed, and preliminary results will be available for the conference.

Collectively, the initial healthy donor screen demonstrated the robustness of our epitope discovery platform and provided a novel reference map of RSV epitopes recognized by T-cells. The Single cell work provides an in-depth characterization on RSV specific T- and B-cells, where we can dissect the phenotypical and functional properties of antigen-specific T- and B-cell populations during active RSV infection. As part of the REACT consortium group, our T- and B-cell discoveries will be considered as one part out of many approaches across the REACT consortium group and collectively can be applied to improve on prophylactic/treatment interventions in the future.

Title: Bispecific immunotherapy of Multiple Myeloma: understanding the clonal diversity of treatment response

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Bispecific antibodies that engage T cells represent a revolution in immunotherapy. By recognizing CD3 on one arm and a cancer-specific surface protein on the other, they bridge the recognition gap between T cells and poorly immunogenic cancer cells. Because hematologic malignancies often invade T cell-rich environments like the bone marrow, T cell-engaging bispecific antibodies (BsAbs) have proven effective against advanced blood cancers.

In multiple myeloma, three BsAbs are currently approved for multirefractory disease, including teclistamab, which targets CD3 and the plasma cell-specific protein BCMA. While more than two-thirds of patients present deep and durable responses, the median duration of response is around 14 months, indicating that both primary and acquired resistance occur[1]. Understanding these phenomena requires analysis of drug-tolerant cancer cells, antigen escape mechanisms, and the immune microenvironment. Accumulating evidence suggests that the baseline immune landscape could predict response, yet conflicting results have been reported on naive versus effector T cell populations[2,3] and the role of T cell exhaustion[4]. Moreover, the impact of BsAb stimulation on T cell trafficking remains largely underexplored.

The ResisTec study addresses these questions through a design focused on the first months of teclistamab treatment in a real-world population. This single-arm, multicentric French study (NCT05945524) enrolled 100 multi-refractory myeloma patients treated with weekly teclistamab injections for three months, then bi-weekly. We collected bone marrow aspirates at baseline, 3 months, and recurrence; blood draws at baseline, monthly for 3 months, then at 6, 12, and 18 months. Single-cell RNA and TcR sequencing on purified CD45 immune cells revealed an enrichment of myeloid-derived populations in primary resistance patients, at the detriment of T cell and NK subsets. In blood, early changes at one month were more predictive of response than baseline frequencies — notably, a failure of cytotoxic CD8 T cells to expand in non-responders alongside regulatory T cell expansion. Canonical T cell exhaustion was absent from circulating T cells over six months regardless of clinical response. Finally, T cell clones expanded in the bone marrow at baseline accumulated in the blood during treatment, particularly in responders.

Collectively, these results suggest that: 1) early immune changes in the circulation are important predictors of treatment response; 2) T cell responsiveness may not always be captured by baseline transcriptional phenotype, possibly implicating epigenetic mechanisms; and 3) treatment response may partly rely on enhanced T cell patrolling of disseminated lesions. Further work is needed to understand how these changes affect NK cells and to contextualize findings within the transcriptional and genomic landscape of malignant plasma cells.

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CHARACTERIZING CD8$^+$ KIR$^+$ REGULATORY T CELL-MEDIATED IMMUNOSUPPRESSION IN CANCER

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Cancer immunotherapy relies on cytotoxic CD8$^+$ T cells to eliminate tumor cells and can induce durable responses in some patients; however, many individuals either fail to respond or initially respond and later develop resistance. These outcomes highlight the role of immunosuppressive mechanisms within the tumor microenvironment. While CD4$^+$ regulatory T cells have been extensively studied, CD8$^+$ T cells expressing killer immunoglobulin-like receptors (KIRs) have recently emerged as a regulatory population capable of suppressing effector T cell responses, yet their role in cancer remains poorly understood. In my PhD project, I investigate CD8$^+$ KIR$^+$ regulatory T cells in solid tumors. Using peripheral blood and tumor-infiltrating lymphocytes from cancer patients, I analyze their frequency, phenotype, and KIR receptor expression using high-dimensional flow cytometry. Antigen specificity is investigated using DNA-barcoded peptide–MHC multimers, while single-cell approaches integrate transcriptional and TCR clonotype information. Functional assays evaluate their suppressive effects on effector T cell responses. Through this work, I aim to better understand the contribution of CD8$^+$ KIR$^+$ regulatory T cells to immune suppression in solid tumors and identify potential strategies to enhance anti-tumor immunity and improve immunotherapy outcomes.

IDENTIFICATION AND CHARACTERIZATION OF SPLICE-DERIVED T-CELL ANTIGENS IN BLOOD CANCERS

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Identification of tumor-specific antigens lays at the foundation of novel immunotherapy approaches, such as adoptive cell transfer and vaccine development. Although most research has studied somatic mutations as neoantigen sources, other cancer-specific phenomena, such as transcriptional aberrations, may have undervalued potential of generating targetable neoantigens. In this study, we are focused in understanding how somatic mutations in spliceosome genes can influence the transcriptional landscape of cancer cells and give rise to a pool of novel, splice-derived neoantigens that can be leveraged for T-cell based therapies.

We will perform long-read RNA sequencing of splice-mutation bearing MDS and CLL patient samples to develop a computational pipeline for the generation of a cancer-specific, splice-derived neoantigen library. By employing a DNA-barcode-labelled, peptide-MHC-I dextramer system, we will perform large-scale screenings of T cell reactivities against predicted peptides across multiple HLAs, in mutation-bearing patients and healthy donors to identify immunogenic targets. We will then validate these epitopes by performing a comprehensive characterization of epitope-specific CD8+ T cells, through the implementation of single-cell RNA sequencing, TCR sequencing and immunophenotyping assays to understand the actionability of splice-antigen reactive T cell clones.

Develop and validate DNA-barcode labelled MHCII multimers for detection of antigen-specific CD4 T cells displayed towards large libraries of epitopes

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Methods for broad-scale detection of antigen-specific CD4 T cells are lacking, while such methods have demonstrated great value in exploring CD8 T cell response in health and disease. Here we use peptide-loaded major histocompatibility complex II (MHCII) proteins multimerized on a barcode- and fluorophore-labelled dextran backbone to provide a method for the detection of CD4 T cell responses to a large display of MHCII-associated peptides. We have established a protocol for MHCII production and peptide exchange suitable for generating large libraries of peptide-MHCII complexes. We validate the use of such pMHCII complexes in the form of barcode-labelled MHCII multimers to detect CD4 T cells. We demonstrate that antigen-specific T cell populations can be identified by amplifying the co-attached barcode by sorting the MHCII-multimer binding CD4 T cell population and that such signals correlate with the CD4 T cell population identified based on fluorescence-labeled MHC II multimers.

We demonstrate that individual CD4 T cell populations can be identified, with a peptide-MHC II panel consisting of 151 peptides derived from Flu, EBV, HCMV, and HCV loaded on DRB1*01:01 and DRB1*01:04. We could detect T cell populations at frequencies down to 0.001% of all CD4 T cells. Low-frequency responses were validated using peptide-driven expansion of antigen-specific CD4 T cells. This methodology was applied to identify MHC-II epitopes in HCV, recognized by CD4 T cells. Using PBMC samples from a patient cohort previously tested for CD4 functional reactivity to HCV-derived 20mer peptides, we identified 15 minimal epitopes within such sequences, presented in DRB1*01:01 and DRB1*04:01, being recognized by CD4 T cells in these donors. This method would allow for in-depth analyses of the specificity of immune interactions driven by CD4 T cells, and provide a better understanding of the antigen-driven association between CD4 and CD8 T cell responses in infection and cancer. Large-scale screening would also facilitate the identification of neoepitopes targeting CD4 T cells providing more insight into immunotherapies and cancer vaccines.

In vivo Expansion of genetically engineered CAR T cells using antigen presenting polymer particles(T-Boost)

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Abstract

The engineering of autologous T cells for the expression of chimeric antigen receptors (CAR) can induce profound clinical responses in haematological malignancies. However, the clinical production protocol for the engineered T cells is exhaustive and often leads to highly differentiated and exhausted effector T cells. To circumvent this, we have adapted an alternative approach in which we inject freshly CRISPR-engineered T cells followed by the injection of polymer nanoparticles (T-Boost) that present antigen and co-stimulation in vivo to support the expansion and survival of antigen-specific T cells.

Here we expand CRISPR/Cas9-engineered CAR-T cells with CD19 antigen for anti-CD19 CAR-T cells, together with nanoparticle-bound IL2 and IL21. One stimulation with the T-Boost nanoparticles in vitro achieved more than 100-fold expansion of antigen-specific cells after 5-6 days of culture. The expanded CAR-T cells effectively killed antigen-presenting cancer cells in vitro.

Previous technologies were either non-biocompatible or inactivated by serum upon injection. However, we have shown in vitro that pre-incubation with serum does not affect T-Boost activity. We also have preliminary data on in vivo maintenance of OT-1 T cells in pre-immunized BalbC mice, and we hypothesize that we can inject freshly engineered CAR T cells followed by T-Boost for stimulation, expansion, and maintenance of the engineered antigen-specific T cells. This approach will preserve T cell phenotypes and circumvent exhaustion due to ex vivo expansion.

Human Endogenous Retroviruses as novel targets for Cancer Immunotherapy

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Myelodysplastic syndromes (MDS) are haematological malignancies with limited curative treatment options outside allogeneic stem cell transplantation. Because mutational neoantigens in MDS are often scarce and highly patient-specific, alternative tumour-associated antigen sources are needed. Human endogenous retroviruses (HERVs) are remnants of ancient retroviral infections that make up approximately 8% of the human genome and are normally epigenetically silenced but can become re-expressed in malignant cells and following hypomethylating agent treatment, making them attractive candidates for immunotherapy. Here, we investigate the immunogenicity and therapeutic potential of HERV-derived antigens in an MDS cohort treated with anti-PD-L1 and guadecitabine.

To identify candidate targets, we established an in-house HERV discovery pipeline based on Telescope and applied it to RNA-sequencing data from the MDS cohort. This identified 442 candidate HERVs, from which the most significantly upregulated HERVs were selected for downstream antigen prediction. Using patient-specific HLA profiles (n = 16), we predicted 2558 unique peptides corresponding to 3887 peptide-MHC complexes, which were screened in 17 patients at two timepoints, at screening and after treatment, using DNA-barcoded peptide-MHC multimer technology. Ongoing analyses assess the frequency and activation state of HERV-reactive T cells to determine whether HERV-derived antigens elicit naturally occurring T-cell responses in MDS, whether these responses are enhanced by treatment, and whether shared immunogenic HERV targets exist across patients.

Title: CREATIC – Building a European Excellence Centre in Central Europe for ATMPs. From Capacity Building to Therapy Development to Patient Treatment

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Abstract:

Advanced Therapy Medicinal Products (ATMPs) offer major opportunities for patients with rare diseases and paediatric cancers, yet their widespread use is constrained by fragmented development pathways, regulatory complexity, and limited as well as costly access. CREATIC – a Horizon Europe Teaming for Excellence project- establishes a sustainable Central European Centre of Excellence that connects ATMP therapy development, GMP manufacturing, regulation, and fair access within an integrated ecosystem based on a strong partnership and leadership. Equally, it manifests a strong partnership between the widening-country coordinator Masaryk University (Czech Republic) and the partners Fraunhofer Institute for Cell Therapy and Immunology IZI (Germany), Leipzig University (Germany) and the University of Copenhagen (Denmark).
CREATIC focuses on highly personalised cell and gene therapies for patient groups underserved by conventional pharmaceutical models. In parallel, the project embeds intellectual property management, regulatory science, biomedical law, and health economics to support regulatory readiness, responsible innovation, and sustainable pricing and reimbursement.
By linking scientific excellence with manufacturing capability, and fair access, CREATIC delivers an end-to-end ATMP ecosystem that accelerates translation to patients while strengthening European cohesion and competitiveness. In essence, the poster delivers:

- A snapshot of the CREATIC integrated ATMP ecosystem linking therapy development, GMP manufacturing, regulation, and fair access
- A demonstration for establishing a sustainable Centre of Excellence in Central Europe through strategic European partnerships and structured capacity transfer by looking at, CREATIC's scope, key challenges and impact
- An overview of existing project outputs and ecosystem, including webinars, ATMP newsletter, FAIR Medicine approach

Immune signatures associated with clinical response to the personalized neoantigen vaccine EVX-01 and pembrolizumab in advanced melanoma

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EVX-01, a personalized cancer vaccine (PCV) targeting neoantigens (neoAgs), is currently being evaluated in a phase 2 clinical trial with the Immune Checkpoint Inhibitor (ICI) pembrolizumab in stage III/IV unresectable melanoma patients with no prior therapy (NCT05309421). EVX-01 is a peptide-based PCV delivering up to 10 patient-specific neoAgs selected using Evaxion's proprietary AI-Immunology™ platform. At the two-year data cut-off, the objective response rate was 75% (12/16) in the overall cohort, with 54% (7/13) of the patients with stable disease or partial response after the 12-week pembrolizumab induction demonstrating additional tumour reduction following EVX-01 initiation. Additionally, 100% (15/15) harbored EVX-01 neoAg-specific, long-lasting T-cell responses. However, pharmacodynamic biomarkers associated with clinical response to PCV-ICI combination therapy, including the combination of EVX-01 with pembrolizumab, remain poorly defined.

To identify pharmacodynamic biomarkers of clinical response to EVX-01 and pembrolizumab, peripheral blood mononuclear cells (PBMCs) were collected from enrolled patients before, during and after study treatment and analyzed using immune assays. Strong and long-lasting immune responses specific to EVX-01 neoAgs were detected in all evaluable patients using IFNγ ELISpot. In-depth characterization of EVX-01 neoAg-specific immune responses directly *ex vivo* using spectral flow cytometry revealed that EVX-01 vaccination induced both EVX-01 neoAg-specific CD4+ and CD8+ T cells in the majority of analysed patients, with CD4+ T cells predominating. EVX-01 neoAg-reactive T cells harbored a functional and activated profile characterized by proinflammatory cytokine production, strong activation-induced marker and PD-1 expression indicative of antigen experience, and a predominant memory T-cell phenotype. In parallel, immune cell profiling using

a 25-color spectral flow cytometry panel revealed longitudinal, treatment-associated modulation of both myeloid and lymphoid compartments in peripheral blood.

Collectively, we report that EVX-01 induces strong, long-lasting IFNγ responses in all evaluable patients, driven predominantly by CD4+ T cells, and in a subset of patients, CD8+ T cells, with a proinflammatory, activated memory phenotype. Ongoing efforts to integrate the phenotypes of EVX-01 neoAg-specific T cells and treatment-induced peripheral immune changes with clinical outcomes will support the identification of biomarkers associated with clinical response.

Title: In-situ characterisation of T-cell reactivity

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Immunotherapies have revolutionized cancer-therapies through leveraging the tumor-targeting capabilities of the adaptive immune system. Immune-checkpoint inhibitors (ICI) and chimeric-antigen receptor (CAR) T-cells have been at the forefront of these advancements with both trying to use CD8+-T-cells to eliminate cancer cells. ICIs blocks the immunosuppressive signals from the tumor, reinvigorating T-cell anti-tumor activity and CAR T-cells have customized receptors for targeting neo-antigens. ICIs show great efficacies in melanoma and CAR T-cells have been effective in leukemia but the overall response of immunotherapies for most other cancers have been limited. The tumor microenvironment (TME) has been proposed as a reason for the variability in treatment outcomes. Through a hypoxic environment with high pH, and tumor suppressive signals it lowers T-cell viability and efficacy. Tertiary lymphoid structures (TLS) have also been associated with improved patient outcomes.

Peptide bound Major Histocompability complex (pMHC) multimers target T-cell receptors allowing for recognition of their specificities. pMHCs have been used extensively in flow-cytometry and other single-cell applications but have seen limited use in spatial settings. Spatial investigation of tissues is important for understanding cellular interactions. This project aims to optimize staining methods for allowing for the recognition of specific T-cells in tissue sections utilizing immunofluorescence techniques. After achieving optimization, neo-antigen libraries will be screened for investigation of patient tumor samples. This will allow for study of tumor infiltration of neo-antigen specific T-cells and how they interact with the TME to give a deeper understanding on their impact on patient outcomes and formation of TLSs.

Cell avidity: the next-gen binding assay to advance cell-based and cell-engaging therapeutic development

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While conventional assays such as affinity, cytokine secretion, and cytotoxicity provide valuable data at a molecular level, this information is insufficient to fully characterize and select the best cellular therapies. There is still a lack of understanding about the biophysical cell-cell interactions that drive functional processes.

Cell avidity, the integrated strength of multivalent interactions between an effector cell and its target, can help elucidate the mechanism of action for therapeutic candidates. Our Cell Avidity platform challenges these interacting pairs using contactless force and quantifies the strength of binding between effector and target cells to distinguish productive from unproductive cell binding in a physiological context. This biophysical metric provides a unique view into cell binding characteristics to interrogate binding potency, selectivity, sensitivity, and kinetics.

Here, we review recent publications highlighting how researchers have used Cell Avidity to:

- Fine-tune the affinity/cell avidity of CAR-T cells to mitigate on-target off-tumor toxicity in renal cell carcinoma.
- Assess the contribution of CD58:CD2 in CAR mediated cell avidity and how replacing that with synthetic PD-1:CD2 interactions reduces fratricide while maintaining potency.
- Format-tune bispecific T cell engagers to enhance efficacy against renal cell carcinoma.
- Validate glyco-bridge binding in the context of CAR-T cells to overcome immunosuppressive tumor microenvironment.
- Engineer CAR-T cells secreting a T-cell engaging molecule to overcome a challenging tumor microenvironment in pancreatic adenocarcinoma.
- Elucidate mechanism of action of tandem CAR-T targeting heterogenous solid tumors Phenotype tumor-primed NK cells for cell binding and function.

We developed a Cell Avidity platform that enables the characterization and screening of molecular binders and cellular products, including antibodies, small molecules, and cell therapies. Cell Avidity provides essential information revealing potency, selectivity, sensitivity, and kinetics, offering key biophysical insights into the mechanism of action for immune-based therapies.

Learning TCR–pMHC Binding from Designed TCRs

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T cell receptor (TCR) recognition of peptide–MHC complexes is central to adaptive immunity, yet predicting binding specificity remains a major challenge due to limited experimental data. We hypothesize that synthetic TCR repertoires generated using diffusion-based protein design [1] can effectively be used for training predictive models of TCR–peptide binding. To test this, we designed artificial TCR sequences targeting the influenza-derived GIL peptide and use them to train a NetTCR-based model [1]. We then evaluate its ability to predict binding of natural TCRs to the same peptide. Our preliminary results suggest that models trained on diffusion-designed TCRs can be a useful resource for predicting TCR–peptide binding. This approach highlights the potential of generative models to augment limited experimental data, enabling scalable and data-efficient learning of TCR specificity, with broad implications across disease areas.

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Improving CAR-T Cell Persistence and Efficacy Through Epigenetic Reprogramming

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Chimeric Antigen Receptor (CAR) T cell therapy has revolutionized the treatment of hematologic malignancies, leading to durable remissions in patients with B-cell acute lymphoblastic leukemia (B-ALL) and non-Hodgkin lymphoma (NHL). However, a substantial proportion of patients fail to achieve complete responses, and the efficacy of CAR-T cells against solid tumors remains limited. Key challenges include T cell exhaustion, expansion of regulatory CAR-T cells (CAR-Tregs), and the immunosuppressive tumor microenvironment. Although current strategies aim to generate less differentiated, stem-like CAR-T cells to improve persistence and reduce exhaustion, they do not fully prevent the emergence of dysfunctional or suppressive phenotypes. We hypothesized that epigenetic modulation could be a dual strategy by promoting a stem-like phenotype while limiting regulatory polarization in CAR-T cells. Here, we present an epigenetic therapy capable of enhancing stem-like features and reducing regulatory T cell frequency in healthy donor T cells, leading us to incorporate this epigenetic therapy during CAR-T cell manufacturing. In both anti-CD19 and anti-HER2 CAR-T cells, treatment with the epigenetic therapy enhanced and sustained a stem-like profile, reduced terminal differentiation and CAR-Treg development, increased IFNγ and TBET expression, and decreased inhibitory receptor markers. These effects persisted over time and under immunosuppressive conditions such as TGF-β exposure. Transcriptomics and proteomics analyses confirmed a shift toward persistence-associated programs while restraining regulatory gene signatures. Notably, treated CAR-T cells exhibited transcriptional profiles similar to those observed in B-ALL patients who respond to CAR-T therapy. Importantly, similar modulatory effects were observed in patient-derived CAR-T cells, where the epigenetic therapy also enhanced proliferation and antitumor activity in long-term killing and rechallenge assays against B-ALL and NHL cell lines. In vivo experiments using different tumor models ($CD19^+$ Nalm-6 and $HER2^+$ SKOV3) showed that epigenetically treated CAR-T cells achieved improved tumor control compared to control CAR-T cells. Mice receiving treated CAR-T cells also demonstrated improved overall survival across models. Consistent with enhanced long-term persistence, epigenetic therapy led to increased accumulation of adoptively transferred CAR-T cells in the bone marrow, spleen, and blood of tumor-bearing mice three weeks after infusion of patient-derived CAR-T products. Together, our results support the use of an epigenetic therapy as a promising strategy to improve CAR-T cell potency, persistence, and resistance to suppression, with translational potential for both hematologic and solid tumors.

Overcoming Tumor Apoptosis Resistance by Enhancing Chimeric Antigen Receptor T-cell Cytotoxicity by Delivery of Cathepsins through the Granzyme-perforin Pathway

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Chimeric antigen receptor (CAR) T cell therapy has demonstrated remarkable success in treating B-cell malignancies, providing sustained clinical responses in patients with relapsed or refractory disease. However, tumor intrinsic resistance remains a significant barrier, with one contributing mechanism being the upregulation of anti-apoptotic proteins that interfere with programmed cell death. Proteins such as serine protease inhibitor B9 (SerpinB9), B-cell lymphoma 2 (BCL-2), and X-linked inhibitor of apoptosis protein (XIAP) disrupt distinct stages of the apoptotic cascade. They are highly associated with resistance to T-cell-mediated cytotoxicity.

To address these resistance mechanisms, we developed a strategy that enables targeted intracellular delivery of therapeutic proteins using the T cell's native perforin-granzyme pathway. CAR T cells were engineered to co-express granzyme B fused to cathepsin D, a lysosomal protease with cytosolic activity. These engineered CAR T cells were evaluated for fusion protein localization, effects on T cell phenotype and activation, and their capacity to enhance cytotoxicity against wild-type and apoptosis-resistant tumor cells.

The fusion proteins localized efficiently to cytotoxic granules and were selectively delivered into tumor cells during CAR T cell engagement, without impairing granule function, T cell phenotype, or activation. Cathepsin D CAR T cells showed significantly enhanced cytotoxicity against wild-type NALM-6 cells and restored killing activity against apoptosis-resistant NALM-6 cells overexpressing SerpinB9, BCL-2, or XIAP.

These findings establish a modular protein-delivery platform that enables CAR T cells to bypass intrinsic resistance mechanisms and execute potent cytotoxic responses. This approach represents a promising way to enhance the therapeutic efficacy of CAR T cell therapies.

Vaccine-induced PD-L1-specific T cells indirectly enhance antitumor immunity through myeloid cell modulation

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Background

The immune modulatory vaccine (IMV) targeting IDO and PD-L1 is designed to enhance T cell responses against both cancer cells and immunosuppressive populations in the tumor microenvironment (TME). When combined with anti-PD-1 therapy, it showed clinical activity in patients with advanced metastatic melanoma in a phase I/II trial[1]. Here we investigate how vaccine-activated T cells modulate target-expressing cancer and immune cells and contribute to tumor elimination.

Methods

PD-L1-specific $CD4^+$ T cell clones expanded from peripheral blood mononuclear cells (PBMCs) of vaccinated patients from the phase I/II trial were co-cultured with autologous cancer cells and PBMC-derived $CD14^+$ myeloid cells. T cell reactivity toward cancer and $CD14^+$ cells was assessed using IFN-γ ELISPOT. Phenotypic profiles of myeloid cells were analyzed with flow cytometry, and cancer cell killing was assessed using xCELLigence system, in the presence and absence of autologous tumor-infiltrating lymphocytes (TILs).

Results

Vaccine-induced PD-L1-specific $CD4^+$ T cells showed reactivity against $PD\text{-}L1^+$ myeloid cells co-cultured with $PD\text{-}L1^+$ cancer cells in ELISPOT assays. Phenotypic analysis of myeloid cells revealed a shift toward an immunosuppressive profile induced by cancer cells, which was reversed by the addition of PD-L1-specific $CD4^+$ T cells. In xCELLigence assays, targeting of $CD14^+$ cells by PD-L1-specific $CD4^+$ T cells promoted cancer cell killing and simultaneously enhanced the cytotoxic activity of tumor-reactive TILs.

Conclusions

Our data demonstrate that vaccine-activated PD-L1-specific T cells can directly target and reprogram $PD\text{-}L1^+$ myeloid cells, leading to cancer cell elimination both through myeloid cell modulation and by improving TIL cytotoxicity. These results highlight the potential of IMVs to promote an immunopermissive TME by reprogramming immunosuppressive myeloid cells, supporting their combination with checkpoint therapies.

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TR-TIL Platform: Antigen-agnostic pan-cancer identification of polyclonal tumor-reactive CD4$^+$ and CD8$^+$ TILs for TCR discovery

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Background: Adoptive cell therapy (ACT) with tumor-infiltrating lymphocytes (TILs) has shown promising results in melanoma and other immunogenic solid tumors. For poorly immunogenic tumors, TCR T-cell ACT using genetically engineered T cells targeting tumor antigens may be more effective. Existing TCR-discovery approaches typically focus on MHC I–presented tumor antigens and thus depend on prior antigen knowledge. We introduce a high-performance, antigen-agnostic platform — the TR-TIL Platform — to identify polyclonal tumor-reactive CD4$^+$ and CD8$^+$ TILs for TCR discovery.

Methods: We developed an *in vitro* co-culture assay recapitulating TIL–tumor interactions to capture the transcriptomic landscape of tumor-reactive CD4$^+$ and CD8$^+$ TILs (TR-TILs). PreREP TILs from 10 patients across five tumor histologies were incubated with autologous or control tumor cells. Following tumor recognition, cells underwent single-cell RNA sequencing (scRNA-seq). We derived CD4$^+$ and CD8$^+$ transcriptional signatures (TR-TIL$_{sigs}$) by comparing autologous and control conditions. We then assessed TR-TIL sigs on an independent validation cohort of co-culture scRNA-seq data.

Results: Recognition of autologous tumor antigens induced transcriptomic changes in TR-TILs, enabling the generation of TR-TIL$_{sigs}$ that delineate distinct subclusters of CD4$^+$ and CD8$^+$ TR-TILs. To validate signature predictive accuracy, a total of 35 (to date) top-scoring TCRs from six patients (4 melanoma, 1 renal cell carcinoma, 1 colorectal cancer) were screened for tumor reactivity in co-culture assays. Among CD8$^+$ TCRs, 21/23 (91%) exhibited reactivity; among CD4$^+$ TCRs, 10/12 (83%) exhibited reactivity. In melanoma, over 95% of selected top-scoring TCRs showed reactivity; in non-melanoma samples, 70% did so. The tumor-reactive TCRs were detectable in longitudinal peripheral blood samples from two long-term responders up to seven years after TIL-ACT.

Conclusions: We report a pan-tumor, antigen-agnostic TR-TIL Platform for discovery of CD4$^+$ and CD8$^+$ tumor-reactive TCRs. These TR-TIL signatures inform enrichment strategies and may enhance the therapeutic efficacy of adoptive TIL and TCR T-cell approaches.

Broad TGFβ-specific T cell immunity is associated with improved outcomes following radiotherapy and checkpoint blockade: rationale for multi-epitope TGFβ immune-modulatory vaccination in pancreatic cancer

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Background: Pancreatic ductal adenocarcinoma (PDAC) is characterized by a profoundly immunosuppressive tumor microenvironment (TME), in which transforming growth factor beta (TGFβ) plays a central role by promoting immune suppression, fibrosis, and, thus, resistance to immunotherapy. Immune-modulatory vaccines (IMVs) targeting TGFβ-expressing immunosuppressive cells represent a promising strategy to reprogram the TME and enhance T cell-mediated anti-tumor immunity.

Methods: In this study, we evaluated the immunogenicity and functional relevance of multiple TGFβ-derived epitopes in peripheral blood mononuclear cells from healthy donors and patients with PDAC. T cell responses were assessed by IFNγ ELISPOT and intracellular cytokine staining. Correlations between the TGFβ-specific responses and clinical outcomes were analyzed in patients treated with combined immune checkpoint inhibitors and radiotherapy. Functional recognition of TGFβ-expressing target cells was investigated using peptide-pulsed dendritic cells and TGFβ-expressing malignant cells. Aside from peptide stimulation, a multi-epitope mRNA construct encoding several TGFβ-derived epitopes was developed to assess simultaneous activation of epitope-specific T cells.

Results: Robust T cell responses against multiple TGFβ-derived epitopes were detected in both healthy donors and PDAC patients. These responses were predominantly mediated by $CD4^+$ T cells exhibiting pro-inflammatory and cytotoxic features, including granzyme B and TNFα expression. The TGFβ-specific T cells recognized target cells in a TGFβ-dependent manner, confirming their ability to target relevant immunosuppressive cells. Importantly, higher baseline frequencies of TGFβ-specific T cells correlated with improved overall and progression-free survival following combined checkpoint blockade and radiotherapy. Notably, patients with responses to multiple TGFβ-derived epitopes demonstrated better clinical outcomes compared to those with baseline responses to only a single epitope or none. Lastly, a single mRNA construct encoding multiple TGFβ-derived epitopes enabled efficient activation of T cells with distinct specificities, supporting the feasibility of multi-epitope vaccination strategies.

Conclusions: Our findings demonstrate that TGFβ-specific T cells are naturally occurring, functionally active, and clinically relevant in patients with PDAC. Moreover, the presence of T cell responses targeting multiple TGFβ-derived epitopes was associated with improved clinical outcomes. This suggests that a broader TGFβ-specific T cell repertoire may contribute to more effective anti-tumor immunity. These data provide a strong rationale for developing multi-epitope TGFβ-based IMVs, including mRNA or peptide-based platforms, as a strategy to reprogram the immunosuppressive TME and enhance responses to other immunotherapeutic interventions.

MelTMG: A Tandem Minigene Platform Encoding Melanoma Minimal Epitopes for Detection of Tumor Antigen–Specific T Cell Reactivity in Melanoma

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Background: Robust functional assessment of tumor antigen-specific T cell responses remains a key limitation in the development and benchmarking of T cell-based immunotherapies. Current approaches rely on autologous tumor material, which is not consistently available and limits standardization across patients and studies. This represents a major bottleneck for both translational research and clinical implementation. To address this, we developed a melanoma-specific tandem minigene (MelTMG) encoding shared tumor-associated antigen (TAA) epitopes as a standardized system for assessing T cell reactivity.

Methods: Feasibility was first established using a viral CEF-TMG construct encoding CMV, EBV, and influenza minimal epitopes, demonstrating efficient antigen processing and presentation in transfected antigen-presenting cells (APCs) and robust recognition of minimal epitopes by autologous T cells. Epitope positioning did not affect T cell recognition, as demonstrated using MART-1- and gp100-specific T cells and HLA-matched K562 cells transfected with TMG constructs encoding MART-1- and gp100-epitopes in different positions. Based on this, two melanoma-specific constructs were generated: an HLA-A2–restricted MelTMG2 and a multi-HLA panMelTMG. Functional validation was performed using antigen-specific and bulk T cells. APCs included HLA-matched K562 cells and autologous immortalized B cells transfected with TMG mRNA. T cell reactivity was assessed by ex vivo IFN-γ ELISpot.

Results: MelTMG constructs induced robust IFN-γ responses in both defined and bulk T cell populations. Antigen-specific responses were observed across experimental systems, including in 3 out of 6 bulk samples using K562-based assays and in a more physiological autologous B-cell setting in 1 out of 3 patient samples. Ongoing validation in expanded cohorts is expected to further establish reproducibility of MelTMG-mediated detection of tumor antigen–specific T cell responses.

Conclusions: MelTMG constructs encoding minimal epitopes represent a feasible and flexible tool for assessing T cell reactivity against shared melanoma antigens. The system supports antigen processing across multiple APCs, including K562 cells and autologous B cells, and enables detection of antigen-specific responses in bulk TIL populations. MelTMG represents a promising standardized positive control for functional T cell assays, with ongoing validation to support broader application.

Identification and Characterization of MCP-1 (CCL2) Specific T cell Immunity

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Monocyte Chemoattractant Protein-1 (MCP-1/CCL2) is a chemokine expressed by tumor- and stromal cells in the tumor microenvironment (TME), where it recruits immunosuppressive myeloid cells and thereby promotes immune evasion. Elevated MCP-1 expression associates with poor clinical outcome across multiple malignancies. Other immunosuppressive molecules in the TME have been shown to harbor immunogenic epitopes and thereby act as attractive targets for immune modulatory vaccines (IMVs). We therefore investigated whether MCP-1-derived epitopes are naturally recognized by T cells, and if MCP-1 may serve as a target for immunomodulatory vaccination.

Peripheral blood mononuclear cells from healthy donors and from patients with cholangiocarcinoma and pancreatic cancer were stimulated in vitro with MCP-1-derived peptides. The resulting cell cultures were characterized by IFNγ ELISPOT and intracellular cytokine staining (ICS) upon restimulation with MCP-1-derived peptide. Recognition of endogenously processed MCP-1 was assessed against HLA-matched tumor cells and autologous monocytes. MCP-1 expression was quantified by flow cytometry and ELISA, and antigen dependency was probed through siRNA-mediated MCP-1 knockdown and cytokine-driven MCP-1 induction.

Pro-inflammatory $CD4^+$ and $CD8^+$ T cell responses specific for MCP-1 were detected at high frequencies in both healthy donor and patient PBMC. MCP-1-specific T cell cultures recognized HLA-matched, MCP-1-expressing tumor cells. Silencing MCP-1 in these targets by siRNA significantly reduced T cell activation both in ELISPOT and ICS, confirming antigen-specific recognition. Conversely, T cell recognition of autologous $CD14^+$ cells was markedly enhanced when target cells were stimulated with GM-CSF which enhances the expression of MCP-1.

MCP-1 contains immunogenic epitopes, and T cells specific to these epitopes recognize target cells in an MCP-1-dependent manner. Together, these findings identify MCP-1 as an immunosuppressive TME molecule harboring naturally recognized T cell epitopes in both healthy individuals and cancer patients, with antigen dependency confirmed through complementary loss- and gain-of-expression approaches. This supports the further development of MCP-1-derived immune modulatory vaccines as a strategy to enhance antitumor immunity.

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