Addendum

Extended methodology and preliminary results by work package

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WP1: Centralized diagnostic review and sample collection.

Sample collection.

1. Creation of a National Cryopreserved TCL Sample Repository –– Lymphomoids, circulating PBMCs and serum/plasma.
    - Build a coordinated cryopreserved biobank linked to clinical and pathology data.
    - Harmonize standard operating procedures (SOPs) for sample processing and cryopreservation across all centers.
    - Collect both biopsy tissue from solid TCL lesions, serum/plasma, and multiple peripheral blood mononuclear cell (PBMC) samples from leukemic/disseminated presentations.

2. Standardized Cryopreservation and Biobanking for Functional Studies- Lymphomoids, circulating PBMCs and serum/plasma to provide samples/data for WP6 and WP8 purposes.
    - Develop cross-site SOPs for cryopreservation.
    - Generate a repository of viable samples for downstream experiments.

Prognosis.

Transcriptomic profiling

Fresh or viably cryopreserved TCL samples will be processed using 10x Genomics Chromium immune profiling workflows (5′ gene expression + V(D)J). This will allow simultaneous characterization of transcriptional programs and TCR clonotypes at single-cell resolution, distinguishing malignant from reactive T cells and defining activation, exhaustion, cytotoxic and other functional states. Sequencing will be performed on Illumina platforms at sufficient depth to enable robust clustering, differential expression and trajectory analyses (Seurat, Scanpy, scRepertoire). When single-cell workflows are not feasible (e.g. only FFPE samples available) bulk RNA-sequencing will be performed. Regulatory networks (including miRNA–lncRNA–mRNA axes) will be inferred computationally from single-cell or bulk transcriptomic data.

EC-NGS and ctDNA

T-cell clonality will be tested by the EuroClonality- NDC (Univ8Genomics) assay, a targeted capture NGS assay intended for the integrated detection of SVs (including immunoglobulin (IG) and T-cell receptor (TCR) rearrangements and translocations), single-nucleotide variations (SNVs) and copy number alterations (CNAs)[1,2]. Similarly, ctDNA monitoring will be performed with the EuroClonality-cfNDC panel[2] (Univ8Genomics) in samples obtained basal/pre-treatment (PET0), at the end of the induction treatment (PET6), 3 months after auto-stem cell transplantation, and at relapse, in all cases at the same time-points as PET/CT is performed.

Spatial analysis of the lymphoma microenvironment

The lymphoma microenvironment (LME) will be interrogated using spatial transcriptomics (Figure 1.1). A sub-cohort with paired diagnostic and relapse FFPE samples will be analyzed using high-resolution spatial transcriptomics (Visium HD, 10x Genomics). Spatial barcoding, alignment, and quantification will be performed with Space Ranger and integrated with Seurat. Cell-type deconvolution (SPOTlight, Giotto) will incorporate single-cell RNA-seq references. These analyses will identify immune–stromal interactions, cytokine networks, and microenvironmental niches associated with therapeutic failure and relapse.

Generation of monoclonal antibodies

Target selection for monoclonal antibody development will be guided by multi-omics analyses, prioritizing proteins selectively overexpressed in malignant T cells or in key LME subsets and associated with adverse prognosis or treatment resistance. The first step for monoclonal antibody production will consist of generating recombinant protein fragments (100–300 amino acids) corresponding to the target molecules for animal immunization. The selected fragments will be amplified and cloned using the Gateway® recombination system. Tagged variants (6×His, GST, or MBP) will be expressed in E. coli and tested for solubility. Soluble constructs will be scaled up and purified by affinity chromatography. BALB/c mice and Wistar rats will be immunized intraperitoneally with 100 µg of antigen per dose, following a standard schedule (days 0, 14, 28, blood check on day 39, boost on Day 42, and cell fusion on day 46). Antibody responses will be checked by ELISA and immunohistochemistry using HEK293T cells transiently overexpressing the protein of interest. Animals exhibiting robust antibody production

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will be sacrificed, and spleen cells will be collected for hybridoma generation. Hybridomas will be produced by fusing spleen-derived B lymphocytes with myeloma cells (NS-1), generating stable clones capable of secreting monoclonal antibodies specific to the target antigen. Culture supernatants from hybridoma colonies will be screened using ELISA and immunocytochemistry. The selected hybridomas will undergo multiple rounds of limiting-dilution cloning to ensure monoclonality and long-term stability, followed by cryopreservation. Final antibodies will be purified using an ÄKTA Prime system and subjected to deep validation by Western blotting, immunofluorescence, flow cytometry, and immunohistochemistry to confirm antibody specificity and reproducibility. Validation will include the generation of tissue microarrays incorporating well-characterized positive and negative controls (tumor cell lines and normal or tumoral tissues), as well as CRISPR/Cas9 knockout cell lines, to rigorously verify target specificity at both the cellular and tissue levels.

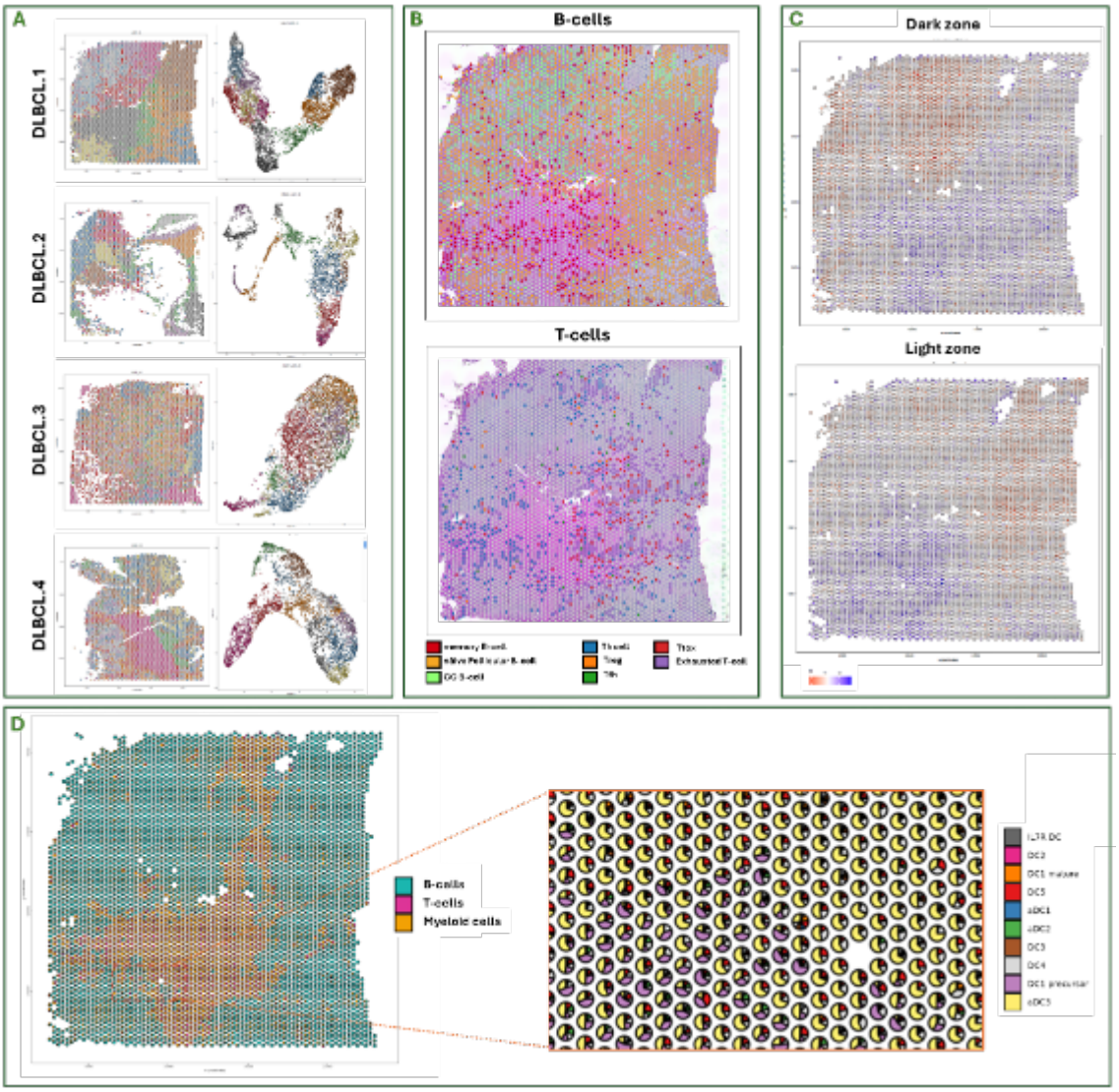

Figure 1.1: Spatial distribution and characterization of immune cells in DLBCL samples. A) Spatial map (left) and UMAP projection (right) showing major immune cell clusters identified in four DLBC cases within the tissue section**. B)** Detailed analyses of case DLBLC.1, with MYC and BCL2 translocations. Spatial localization of B-cell (top) and T-cell (bottom) subsets using Loupe Browser (10X Genomics), including memory B cells, naïve follicular B cells, germinal center (GC) B cells, T follicular helper (Tfh) cells, regulatory T cells (Tregs), Th1 cells, Tox, and exhausted T cells. **C)** Characterization of the DLBCL tissue microanatomy, highlighting dark zone (top) and light zone (bottom) regions based on specific gene expression signatures. **D)** Spatial mapping of major immune lineages, B cells, T cells, and myeloid cells, across the tissue section (left), with a magnified inset (right) detailing myeloid cell heterogeneity, including monocytes/macrophages, dendritic cells, granulocytes, mast cells, and cycling myeloid populations. RM, Head of the Bioinformatics Unit, performed the analyses in panels A, C, and D (unpublished results).

Immunophenotypic single-cell characterization by spectral flow cytometry

For retrospective TCL cases (diagnosed across Spain over the past 10 years), the immunophenotypic characterization and analysis will be centrally coordinated in Salamanca, where reports and expert reviews from

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the various participating centers will be compiled. For prospective cases, immunophenotyping by flow cytometry (FCM) will be centralized in Salamanca (Cancer Research Center and Service of Cytometry - https://nucleus.usal.es/es/citometria-, University of Salamanca), where samples will be processed ideally within 24 hours after collection. Fresh TCL tissue specimens (preferably dissociated at the site of origin and preserved in an appropriate buffer solution), bone marrow aspirates (when available) and peripheral blood samples will be stained following EuroFlow standard operating procedures (SOP; www.euroflow.org) with appropriate EuroFlow T/NK-panels1–4, and then immediately acquired on a 5-laser Cytek® Aurora spectral flow cytometer (Cytek Biosciences) or on a BD FACSDiscover™ A8 Cell Analyzer spectral flow cytometry (BD Biosciences). Data will be analyzed with the INFINICYT™ software (Cytognos/BD, Salamanca, Spain). FCM from tissues will be performed at diagnosis/recruitment and at relapse; peripheral blood samples will be also collected and analyzed at different time-points during follow-up, for treatment monitoring. The T/NK-cell panel will include a large number of T/NK-cell markers, aiming at the identification -within mature T/NK cell subsets (CD3, CD4, CD8, TCRgd, CD45, NKp80, CD56)- of aberrant/pathological cells, using T-cell clonality markers (TRBC1 and TRBC2) and other markers known to be aberrantly expressed (e.g., CD2, CD5, CD7, CD158k/KIRDL2, etc.), as well as markers for the classification into WHO/ICC-2022 categories (e.g., CD25, CD26, CD30, maturation markers such as CD27, CD28, CD45RA, CD45RO and CD197/CCR7, cytotoxic-related markers such as CD56, CD57 and cyGranzymes, among others, and follicular helper T-cell (TFH)-associated markers (CD10, CD185/CXCR5, CD278/ICOS, and CD279/PD-1), together with activation- (e.g., CD38, HLADR), and exhaustion/senescence-related markers (e.g., CD57, CD279, TIGIT) that would further allow for the identification of potential prognostic markers in each category. In parallel, to analyze the TCL-associated immune microenvironment, quantitation of both relative (tissue/blood) and absolute (blood) counts of a large number of immune T/NK-cell subsets will be performed (at diagnosis, during follow-up and at relapse), using the EuroFlow 36-color 43-antibody immune monitoring (IMM) T/NK-cell antibody panel (Almeida et al, ms. in preparation), which includes CD2 and CD7 for the detection of leukemia/lymphoma-associated immunophenotypes (LAIP) and anti-TRBC1 and anti-TRBC2 for the assessment of T-cell clonality (to distinguish clonal/aberrant from residual normal T cells). This panel allows the identification of an unprecedented number of T- and NK-cell subsets, including different functional Th (i.e., Th1, Th2, Th17, Th1/17, Th22), conventional cytotoxic, innate-like T- (e.g., TNK, MAIT) and maturation-associated T- and NK-cell subsets, together with innate lymphoid cells (ILC) other than NK cells, and also includes antibodies (against activation/inhibition-and senescence/exhaustion-related markers) for the precise characterization of each T/NK-cell subset. Samples will be processed using EuroFlow SOPs, and analyzed by spectral flow cytometry, as detailed above. Further, additional EuroFlow IMM panels will be applied (at least in EDTA-anticoagulated peripheral blood samples) for a deeper dissection of the non-T/NK/ILC residual immune system (myeloid and B-cell panels)1,5. Residual sample material (tumor and normal immune cells) will be stored (for further molecular assays) at the National/Spanish DNA Biobank (ISCIII-NUCLEUS, University of Salamanca, integrated in the Biobank HUB of the ISCIII Biobanks and Biomodels Platform, preferably following flow cytometry-based sorting, to ensure highly-purified tumor/normal residual cell fractions for future molecular analyses.

Overall, the different panels that will be used have been shown to precisely discriminate between normal/residual/polyclonal from clonal/tumor T cells, while at the same time a large number of maturational and functional subsets of normal residual T cells can be identified (Figure 1.2), which will help evaluate more precisely the tumor microenvironment and identify potential therapeutic targets.

Spectral flow data will be integrated with scRNA-seq and scTCR-seq using shared marker and clonotype information to align protein-defined subsets with transcriptional states and clonal architecture, thereby improving the definition of therapeutic targets and prognostic biomarkers.

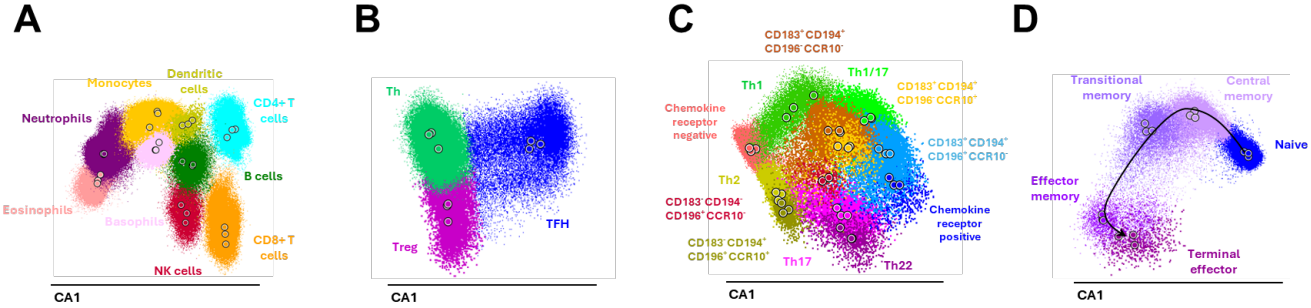

Figure 1.2. Major leukocyte and normal T-cell subsets that can be identified by flow cytometry with the EuroFlow immune monitoring (IMM) TCD4 tube. *In addition to the identification of the general leukocyte cell populations (A), the three major CD4+ T-cell subsets can be identified, i.e., Th, TFH and Treg (B) and within each of these, multiple functional-Th subsets (Th1, Th2, Th17, Th1/Th17 and Th22, as well as other subsets, defined by the expression pattern of chemokine receptors (C) at distinct maturation stages, e.g., naïve, central memory, effector memory and terminal effector (D) can be identified within the non-tumor compartment (Botafogo et al, Front Immunol 2020). Abbreviations (alphabetical order): CA, canonical analysis; Th, helper T cells; TFH, follicular helper T cells; Treg, regulatory*

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WP2: Multimodal data integration and building of AI algorithms for diagnosis, prognosis, stratification and prediction of therapy responses

The clinical and biological heterogeneity of TCL, together with the low incidence, hampers accurate diagnostic, prognostic and individualized treatment planning. On the one side, routine clinical practice increasingly generates high-dimensional information, which are difficult to synthesize using traditional statistical models. On the other side, treatment options have diversified, making optimal selection at the individual level challenging. For the creation of the AI models, techniques such as Machine learning (ML) and Deep learning (DL), structural causal models, ML-enhanced propensity score methods, and counterfactual inference methods will be used, integrated into multimodal pipelines. The combination of these approaches will allow for the construction of more robust, interpretable, and clinically actionable multimodal predictors for stratification, diagnosis, prognosis, and therapeutic selection in TCL. ML and DL methods provide powerful tools to analyze complex, multimodal datasets and to support precision medicine in TCL. ML is based on systems that are able to automatically learn knowledge and patterns from existing data (Pattern recognition and machine learning.CM Bishop, NM Nasrabadi, 2006). It helps to process large volumes of information and extract patterns from it that can inform decision-making. DL methods have been boosted lately to exploit the complexity of temporal data sequences such as data recorded during clinical visits as well as biomedical imaging for automatic biomarker computation and morphology to function relationship. DL has been shown to enhance ML performance and achieve state-of-the-art results in numerous clinical applications. When applied to rare diseases, these methods must explicitly address small-sample issues through careful model regularization, rigorous internal validation, and collaborative data sharing to expand training cohorts. ML models focused on survival can predict outcomes when combined with clinical, histopathological, and medical imaging data[3]. A recent study suggests that optimized models may outperform traditional indices and subtype-specific scores in predicting overall survival[4]. However, the application of ML/DL methods remains limited to relatively small, subtype-focused cohorts and there is still no broadly adopted ML tool in routine practice. There is a clear need for robust and interpretable prediction models that assist in risk stratification and diagnostic classification, estimate individualized risks of overall and progression-free survival, and forecast response to different therapeutic strategies in TCL. Developing and validating multivariable prediction models based on ML and/or DL algorithms, integrating routinely collected clinical, medical imaging, histopathological, immunohistochemical and multi-omics data from multiple centers, may help mitigate sample-size limitations, reduce overfitting and yield more generalizable tools to tailor treatment intensity, optimize transplantation decisions and assign patients for innovative therapies. Furthermore, by incorporating explainable AI techniques, such models can provide clinically interpretable insights into the relative contribution of specific prognostic factors, supporting shared decision-making and future refinement of prognostic indices.

Separate prediction models with different methods will be developed for several outcomes:

- Diagnosis: Probability of specific lymphoma subtype given integrated clinical, histopathological, immunohistochemical and multi-omics data, for patients with challenging diagnoses.
- Survival: Overall survival (OS) and progression-free survival (PFS).
- Treatment-related outcomes: Overall response Rate (ORR), complete and partial responses (CRs, PRs), Best response to first-line therapy according to standard criteria, primary refractory disease and relapse within predefined time windows (e.g. 1,2 and 5 years).

A graphical abstract of the proposed workflow is summarized in Figure 2.1.

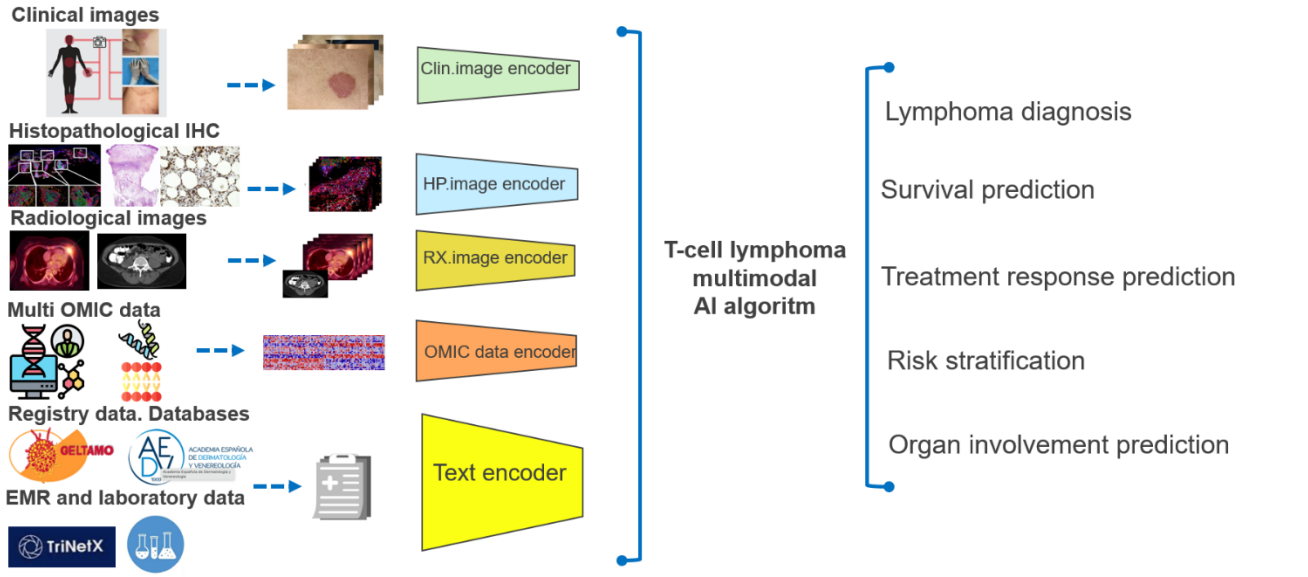
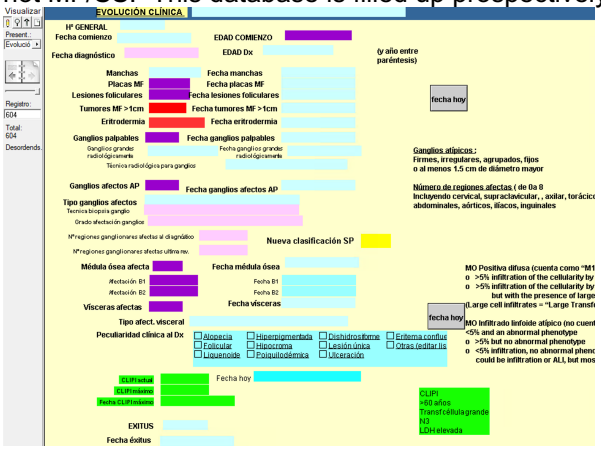
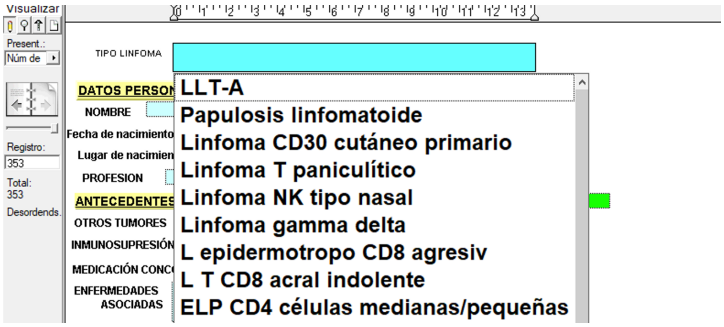


Figure 2.1. Graphical abstract of proposed multimodal TCL patient data integration, encoding and AI analysis for improved diagnosis, prognosis, stratification and prediction of therapy responses.

Task 2.1. Analysis, curation and characterisation of data sources

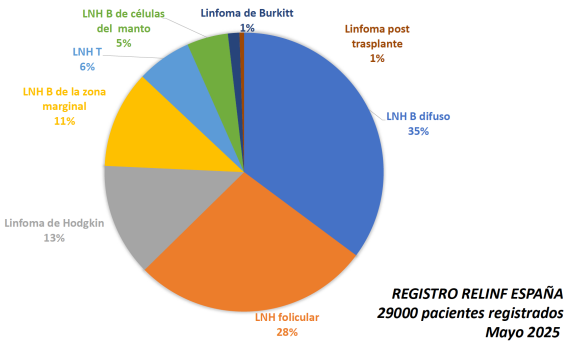
Database of primary cutaneous lymphomas from Hospital Universitario 12 de Octubre include data from 604 patients with mycosis fungoides or Sézary syndrome and 280 patients with primary cutaneous T-cell lymphomas not MF/SS. This database is filled up prospectively on a daily basis during the clinical interview of the patients.

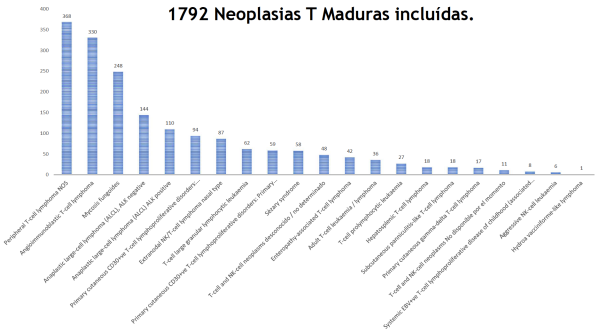




It includes a total of 240 fields covering personal data (sex, date of born, profession, exposition to ambient toxics, personal history, family history…); clinical evolution (date of appearance of cutaneous lesions, date of diagnosis, presence or not of patches, plaques, tumors, folliculotropic lesions, erythroderma, T-stage in skin (T0 to T4), palpable lymph nodes, histopathologically conformed lymph nodes (N0 to N3), bone marrow involvement, peripheral blood involvement, visceral involvement (M0 to M1), and their correspondent dates of occurrence); clinical evolution (stage at diagnosis, maximum stage, stage at last visits and their dates), last visit situation (death or alive, cause of exitus) CLIPI (age >60y.o. LDH, large cell transformation, N3 in lymph nodes and dates); histopathology (with all biopsies taken, dates, diagnosis, tissue analyzed, presence or not of large cell transformation or folliculotropism and their initial dates) immunohistochemistry (with up to 20 different markers) genetic rearrangement, FISH, mutations) laboratory studies (genetic rearrangement from different tissues, presence or not of the same clone, biochemistry, flow citometry with multiple time points), treatments administred (all lines dates of start, responses), one exploitable field of comments and one exploitable field of follow up.

GELTAMO includes data from 1797 mature T-cell neoplasias, including 593 primary cutaneous lymphomas.





Data are registered from different centers and updated on a yearly basis and include a limited number of fields and is updated every year.

RELCP (Spanish registry or primary cutaneous lymphomas) include data from more than 1500 patients with different cutaneous T-cell lymphomas. Includes epidemiological data (sex, age of born), diagnosis (type, subtype), treatments and follow up, including responses, time to responses, TTNT, PFS, OS. It is updated at least every year

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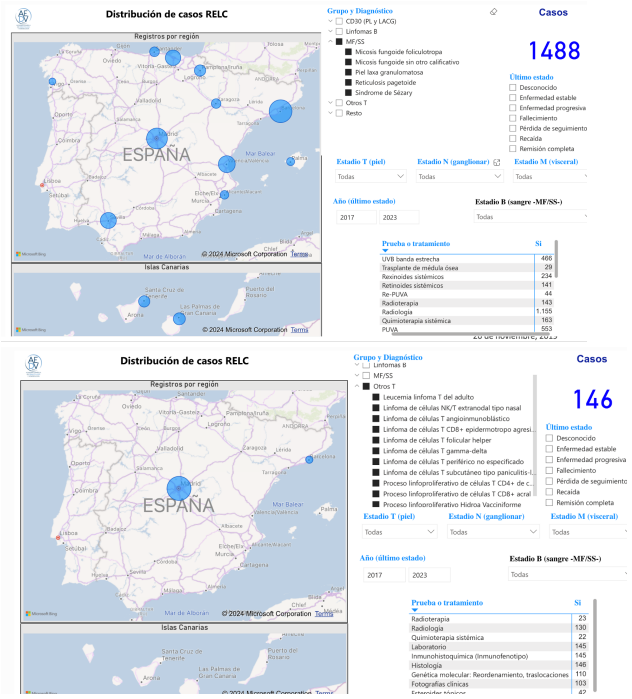

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Task 2.2. Establishment of a secure server. Design and creation of the standardised data model. Multimodal data loading and integration

A common data model will be defined to enable the consistent representation of all patient information within the platform. This model will support both structured data and the linking and management of unstructured data (imaging and -omics), enabling joint analysis and ensuring consistency of information throughout the project. This model will be mapped to standards such as OMOP to ensure semantic and technical interoperability with other national or international consortia. All necessary processes for data ingestion into the platform will be implemented, including automated ETL flows and manual procedures when necessary. Structured data, free text, digital medical and histopathological images, as well as multi-omic data will be integrated, ensuring anonymity, traceability and information quality Schlessinger D et al, JAAD, 2024, Dovigi E et al, JAAD 2024

To estimate the **sizing/capacity planning**, we will consider the different source of data:

-For **histopathology and immunohistochemistry** of slides of Cutaneous T-cell Lymphomas (CTCLs), we plan to scan at least 500 cases on early stage at diagnosis (25% of them will progress into advanced stage and will pass away), in order to predict which patients will progress and not losing the window of opportunity of a potentially curative treatment like bone marrow transplantation in a moment in which the disease is still not refractory and at least 300 cases on advanced stage at diagnosis or at follow up (30% are fast- progressors with survivals <3 years and 20% of them present an indolent behaviour and would not need aggressive treatments).

For Systemic TCLs, we plan to include at least 200 cases of every type of lymphoma

Every case has a mean of 14 slides to scan and every scan weights a mean of 1.5Gb

-For **clinical pictures of CTCLs**, at the present, we have 10.000 pictures from cases with CTCL and around 5000 pictures from cutaneous conditions similar to CTCL (psoriasis, eczema, atopic dermatitis, drug reactions…). All pictures come from historical cases treated at the 12 de Octubre Hospital. We plan to upload a minimum of 20000 pictures from other centers of the Consortium. Every picture weighs a mean of 5Mb

We plan to stablish a **VECTRA 360 set of pictures**. Using Vectra™ system, we plan to obtain prospectively full 3D skin images from 30 patients per month. At the end of the project, we will have images from at least 300 different patients at multiple time points with a median follow up of more than 3 years, enough to detect early progressors. Every full skin 3D image weighs around 2Gb. Due to the high price of this equipment, we plan to include only half of its price in the budget that will be completed with external sources.

-For **radiology images (CT scan) or nuclear medicine images, (PET) studies**, we plan to obtain images from at least 2000 patients. Every patient has a mean of 4 studies and every study weighs around 2 Gb. Besides, we are very interested in radiomics, segmentations and derived maps. All together include additional 200MB per study

-For **language models**, we will use the exploitable text included in the databases, the text included in the medical reports for pathology, immunohistochemistry, radiology or nuclear medicine, the text included in the Electronic Health Records and the data originated in the multi-omics studies that will be performed in other WPs of this proposal. We will ensure at least 1Gb per case (minimum 2000 cases).

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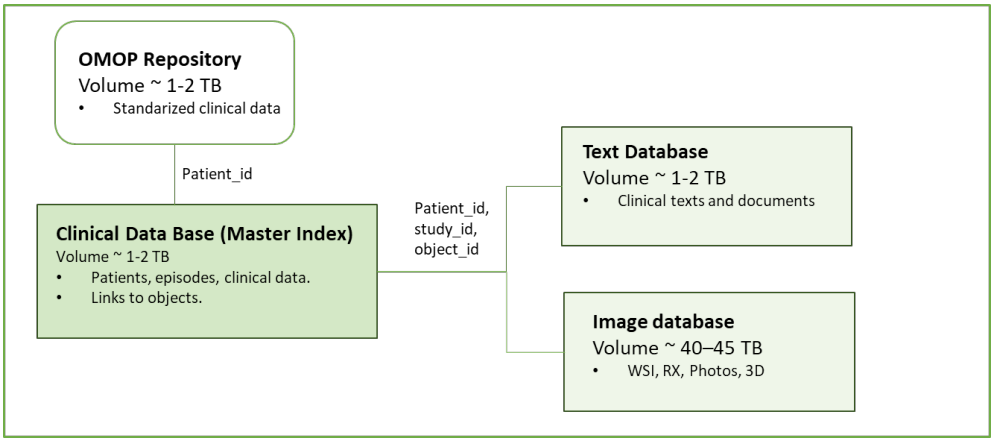

The figure summarizes the logical architecture of the data platform, organized as a set of specialized and interconnected databases. The Clinical Database centralizes pseudonymized identifiers, structured clinical variables, and study metadata, acting as an integration hub and enabling the longitudinal linkage of all patient information through persistent identifiers.
The Image Database stores large-volume data, including digital histopathology, radiology, and nuclear medicine images, clinical photography, and three-dimensional images, with an estimated aggregate volume of approximately 40–45 TB. The Clinical Text and Document Database manages reports and free text from electronic health records and other sources, enabling their exploitation using natural language processing techniques.
The Standardized Clinical Database, based on the OMOP Common Data Model (OMOP CDM), provides an interoperable semantic layer that facilitates secondary reuse of data, multicenter analysis, and integration with national and international initiatives.
All necessary processes for data ingestion into the platform will be implemented, including automated ETL flows and manual procedures when necessary. Structured data, free text, digital medical and histopathological images, as well as multi-omic data will be integrated, ensuring anonymity, traceability and information quality. **Standardized quality control and validation mechanisms will be defined and applied throughout the data ingestion process**. Natural language processing (NLP) techniques will be applied to extract relevant clinical information from free text (medical, pathology, radiology or nuclear medicine reports, lab-test results), allowing it to be transformed into structured variables that can be used in subsequent analyses. A common data split will be defined based on number of cases for each cancer subtype, data types and centers. Data from several centers will be held out as independent external data to validate generalizability.

Ethical considerations
Before uploading any data, we will ensure EC approval (we plan to design Ethics Committee of the 12 de Octubre Hospital as central EC). As we will use anonymized images and data, we plan to ask a waiver to obtain signed informed consent from every patient that will be included in the different studies.
In order to follow best practices in the development of our AI models, we will follow the CLEAR Derm Consensus Guidelines (Daneshjou R et al, JAMA Derm 2022)
(https://jamanetwork.com/journals/jamadermatology/fullarticle/2786912)
Besides that, we will made any effort to ensure fairness, equity, transparency, privacy and responsibility (as mentioned in the HHS TAI Playbook) (Adams L et al, NSM Perspect 2024, Schaemkerman M et al, EClinical.Med, 2024),

Task 2.3. Electronic Health Records (EHR) clinical, laboratory and oncogenomic data AI models
Real-world clinical, laboratory and main oncogenomic mutations data extracted from the health records will be used to define predictive models. AI and machine learning/deep learning models will be developed and validated. Causal machine learning (CML) techniques will also be incorporated for identification of patterns associated with diagnosis, prognosis, therapeutic response and inference of causal relationships between clinical variables, comorbidities, biomarkers, treatments administered, and outcomes. The generated models will estimate individual causal effects (ITE), predict which patients would benefit most from each intervention, and support personalised therapeutic decisions through counterfactual reasoning.
Variables considered have been specified above.
In order to secure transparency, we will document clearly data sources, preprocessing steps, model architectures, and training and validation methodologies. This will allow to confirm interpretability and explainability of the models. We will follow the 6 principles recommended by the US department of Health and Human Services (HHS) to promote AI trustworthiness (transparency and explainability, fairness/impartiality, responsibility, reliability, privacy, and security) (US Department of Health and Human Services. Trustworthy AI playbook, 2021. Accessed September 16, 2024. https://www.hhs.gov/sites/default/files/hhs-trustworthy-ai-playbook.pdf)
Prediction models will be built with data from multiple centers harmonized using the clinical database of the platform. Candidate predictors will be selected a priori based on clinical relevance and existing literature on prognostic indices and ML models in TCL. To address small sample sizes and reduce overfitting, the number of candidate predictors for each model will be tailored to the sample size (number of events) and, for high-dimensional

data (e.g. genomics, radiomics), dimensionality reduction will be applied. Statistical and ML/DL models based on survival analysis methods, such as Cox proportional hazard and accelerated failure time models will be used to process baseline information, while Bayesian Joint Models and transformers will be used for longitudinal data. Results from one of the consortium groups have demonstrate that models based on early treatment routine clinical and blood-test variables were better therapy response predictors than those obtained from baseline data only (RamosGuerra et al., 2025). In addition to ML/DL prognostic models, the project will estimate causal effects of alternative treatment strategies on overall and progression-free survival using causal machine learning (CML), which combines modern ML algorithms with causal inference principles to estimate treatment effects and counterfactual outcomes.

For ML/DL models, the following stages will be followed:

1. Pre-processing.

- Data cleaning and coding. Inconsistent values will be checked against source records where possible or set to missing. Continuous variables with extreme outliers will be inspected and, if appropriate, transformed.
- Handling of missing data. Patterns and mechanisms of missingness will be explored descriptively. For main analyses, multiple imputation by chained equations will be used to impute missing predictor values under a missing-at-random assumption. For variables with very high missingness or poor measurement quality, exclusion will be considered.
- Feature reduction: for high-dimensional molecular or imaging data, unsupervised methods (e.g. principal component analysis, autoencoders) or penalized models will be used to reduce dimensionality prior to model fitting.

All pre-processing steps will be performed within cross-validation or resampling loops to avoid information leakage where appropriate.

2. ML/DL models.

For diagnostic classification, supervised learning models such as XGBoost, random forests, Support Vector Machines and multilayer perceptrons will be used to predict specific TCL subtypes using clinical, histopathological and multi-omics features. On the other side, unsupervised learning or clustering algorithms will be considered to integrate clinical, histopathological, and multiomic data and identify novel patient subgroups with differential survival and treatment response, refine current classification scores, and generate hypotheses for subtype-adapted therapeutic strategies and future clinical trial design. Two stages are considered:

1. Clinical/histopathological clustering will be performed using a core set of routinely available variables at diagnosis, used as input to multiple unsupervised algorithms, including hierarchical and k-means clustering.
2. Multi-omics integrative clustering. Molecular features will be incorporated to refine subgroups and assess whether integrating omics data provides additional discriminatory power.

The final number of clusters will be determined using a combination of internal indices (e.g. average silhouette width, gap statistic) and stability metrics.

For survival, Random Survival Forests, which extend random forests (RF) to censored time-to-event data using survival-specific splitting rules and ensemble cumulative hazard estimates and gradient boosting (GBM) for survival, which adapts boosting to optimize survival losses will be developed to capture non-linear effects and interactions. Deep learning architectures for survival such as DeepSurv-type networks[5], will be explored when appropriate sample size permits.

For treatment response, multi-class or binary models (gradient boosting, random forests, penalized multinomial/logistic regression, neural networks) will be fitted, with appropriate handling of class imbalance using techniques such as class weighting or resampling.

For all model families, hyperparameters will be tuned using cross-validation or separate validation folds, with search spaces constrained to avoid overfitting given the limited sample size.

3. Models' performance and validation.

Clustering

Cluster stability and robustness will be assessed through resampling-based methods and consensus clustering. For each candidate algorithm and number of clusters, patients and/or features will be repeatedly subsampled (e.g. 80% of patients, 80% of features), clusters will be re-estimated, and a consensus co-membership matrix will be computed summarizing how often each pair of patients clusters together. Jaccard similarities between clusters obtained in the full dataset and those derived in resampled datasets will be calculated; clusters with Jaccard similarity >0.75 will be considered stable. Internal validity indices such as average silhouette width will be reported for each solution. Where sample size allows, external validation will be performed by applying the clustering model or a classifier trained on the discovered subtypes to an independent cohort and assessing concordance of subtype frequencies, molecular characteristics and outcome associations. Using the resulting clusters, their associations with clinical and biological characteristics and with patient outcomes will be investigated.

Prediction models

Performance of each model will be evaluated on held-out test data and through resampling. For discrimination of survival outcomes, Harrell's C-index and time-dependent area under the ROC curve (AUC) at clinically relevant time points (e.g. 1, 2 and 5 years) will be used. Also, calibration plots (predicted vs observed survival) and time-dependent Brier scores will be assessed.

For classification outcomes (diagnosis, treatment response), discriminability will be assessed with AUC, sensitivity, specificity, positive and negative predictive values, F1-score; for multiclass models, macro- and micro-averaged

metrics. And calibration will be assessed with calibration curves and Brier score.
Repeated k-fold cross-validation will be used to obtain estimates of model performance. For more complex ML/DL models, a train/validation/test split will be used, with cross-validation on the training set for hyperparameter tuning and a final locked test set for unbiased performance estimation.
When an independent external cohort is available for external validation, the best-performing models will be evaluated without re-training. Finally, clinical utility will be explored using decision curve analysis to quantify net benefit across risk thresholds.

4. Model presentation.

For models with acceptable performance, clinically oriented representations will be produced, including a risk calculator embedded in a web-based tool providing individualized survival probabilities or predicted risks of early mortality, or treatment response for defined time horizons, etc. Explainable AI techniques (e.g. SHAP values) will be used to quantify global and patient-level feature importance, highlighting which variables drive predicted risk and thereby enhancing clinical interpretability.
All modeling and reporting will follow current TRIPOD+AI[6] guidance for prediction model studies using ML methods. Data analyses and model building will be conducted using Python and version-controlled code will be documented for reproducibility.
For CML prognostic models, the following stages will be followed:

- **Causal Data Modelling and Construction of the Clinical Knowledge Graph.** The construction of a clinical causal graph is proposed, combining expert knowledge from the consortium, available retrospective and prospective information, and relationships derived from literature and medical databases. This graph will serve as a structural causal model (SCM) that will enable: modelling direct and indirect effects of clinical variables on survival and response; disambiguating spurious correlations; reducing the risk of shortcut learning to learn relationships that are consistent with underlying clinical mechanisms, thus improving their robustness and generalization.
- **Propensity Score Methods for Confounding Correction.** To address confounding bias in observational clinical data, a robust and complementary set of techniques based on Propensity Scores (PS) with ML will be implemented. These methodologies allow covariates to be balanced between treatment groups, approximating a quasi-experimental situation and improving the causal validity of the estimators. Propensity Score Matching (PSM), Inverse Probability of Treatment Weighting (IPTW), and doubly robust Augmented Inverse Probability Weighting (AIPW) techniques will be used. The balance will be verified using Love plots, which allow the Standardised Mean Differences to be visualised. Propensity curves/densities and overlap region analysis will also be included to detect areas of extrapolation. Sensitivity analyses (e.g., E-values or negative controls) will be incorporated to assess robustness to potential unmeasured confounding.
- **Estimation of treatment effect and identification of heterogeneity.** The estimation of individual causal effects (ITE) and treatment effect heterogeneity (HTE) will be addressed using ML-based meta-learners (including T-learner, S-learner, X-learner, R-learner, DR-learner). These allow the estimation of ITE per patient and HTE per subgroup, are flexible and combinable with non-linear base models (GBM, RF, networks), and useful for the selection of individual recommendation policies. A comparison will be made between methods to identify the stability and consistency of treatment effect estimates.
- **Policy evaluation and counterfactual decision-making support.** To assess the quality and reliability of treatment recommendation policies derived from these models, the following will be incorporated:
  - Policy value estimation: Assessment of expected clinical benefit, calculated as the estimated improvement in response with the learned policy compared to standard care allocation strategies.
  - Stability and equity analysis: Assessment of whether the policy behaves consistently across all clinical subgroups (e.g., age, disease stage, comorbidities) and does not amplify site-specific treatment biases.
  - Decision curve analysis (DCA): Quantify the net clinical benefit across all risk thresholds and verify that the policy recommendations would lead to clinically meaningful improvements.

Task 2.4. Image based analysis and AI models

This task will leverage a large, curated multimodal dataset comprising approximately 10,000 clinical images of cutaneous T-cell lymphomas, primarily mycosis fungoides, collected at multiple time points during patient follow-up. Additionally, more than 2000 PET/CT studies and 500 digital pathology studies from will be process to relate tumor evolution with tumor lesions micro and macroscopic clues. These image data will be integrated with longitudinal clinical records and molecular/genetic information generated in WP1. Clinical data will include demographic variables, disease stage, treatment history, laboratory parameters, response assessments and clinical outcomes, while genetic data will encompass relevant molecular alterations and TCR-related features. This multimodal and longitudinal dataset will enable the study of disease evolution and the identification of predictors of disease worsening and adverse prognosis.
Prior to model development, cutaneous images will undergo standardized pre-processing pipelines to minimize acquisition-related variability and ensure robust and reproducible analyses. Similarly harmonization algorithms on statistical and generative DL methodologies will be applied to radiological and pathology images to ensure homogeneity and proper model development. These pipelines will include image quality assessment, resolution and color normalization, illumination correction, artifact removal, automated hair-removal techniques and quantitative analysis independent of acquisition parameters such as manufacturer, reconstruction kernel, Xray

energy, active radioisotope concentration, etc… When appropriate, lesion localization and region-of-interest extraction will be performed using manual, semi-automatic and DL based approaches. Images not meeting predefined quality criteria will be excluded from downstream analyses.
For image-based analyses, convolutional neural network (CNN) architectures will be explored to automatically learn discriminative visual patterns associated with disease severity, progression and prognosis in mycosis fungoides and other TCL subtypes. CNN and transformer based models will be trained using supervised and self-supervised learning strategies, leveraging clinically relevant outcome labels such as disease progression, worsening of clinical stage, treatment failure or time-to-event outcomes. Transfer learning and fine tuning approaches based on pre-trained networks including foundational models will be considered to improve model performance and generalizability. Model development will incorporate appropriate validation strategies, including cross-validation and independent test sets when available.
In parallel with end-to-end CNN modelling, high-level imaging features extracted from intermediate layers of trained networks will be used as quantitative image descriptors (see also detailed methodology Task 2.5). These imaging-derived features will be integrated with structured clinical variables and molecular/genetic data to enable multimodal analyses. Feature selection and dimensionality reduction techniques will be applied to identify the most informative variables across modalities and to reduce model complexity. Multimodal machine learning models will be developed to combine imaging-derived features, clinical trajectories and genetic information with the objective of predicting clinically relevant outcomes, including disease progression, worsening of clinical stage, resistance to therapy and adverse prognosis.
Longitudinal and time-dependent modelling strategies will be explored to account for repeated measurements and disease evolution over time, enabling dynamic risk stratification of patients including CNN combined with recurrent neural networks as well as transformers based architecture to model the temporal behavior .
Model performance will be assessed using appropriate metrics depending on the prediction task, including discrimination, calibration and time-to-event performance measures. Model interpretability techniques will be applied to identify image regions, clinical variables or molecular features that contribute most strongly to predictions, supporting clinical trust, transparency and translational potential.
Computationally intensive image processing and model training will be performed using high-performance computing resources provided by the IT infrastructure of Comillas and Universidad Politécnica de Madrid ensuring sufficient capacity for large-scale image analysis and deep learning model development. All pipelines will be implemented using reproducible workflows to facilitate validation and future reuse.
AI models and computational pipelines showing clinical relevance and translational potential will be evaluated for intellectual property protection in coordination with the Technology Transfer Offices and the AECC Foundation, including potential co-ownership arrangements, to facilitate future clinical implementation and reuse.

Task 2.5. Multi-modal AI models and validation of AI models' performance. Based on the integrated multimodal information generated in previous tasks, comprehensive descriptive, visual, and exploratory analyses of the study cohorts will be conducted to characterize data distributions, inter-modality relationships, and clinically relevant patterns. These analyses will inform both feature engineering and model design choices. Multimodal artificial intelligence models will then be developed using early-fusion, late-fusion, and hybrid hierarchical integration schemes, enabling systematic comparison of different data-integration strategies.
In early-fusion approaches, features from all available modalities will be concatenated or jointly embedded prior to model training, allowing the learning process to capture cross-modal interactions from the earliest stages. Late-fusion strategies will combine modality-specific predictors at the decision level through weighted ensembling or meta-learning, enabling flexible integration when modalities differ in availability or predictive strength. Hybrid hierarchical models will integrate these paradigms by first learning modality-specific, task-aware latent representations, followed by higher-level fusion layers that capture inter-modal dependencies.
Each modality (e.g., clinical variables, imaging, molecular data, temporal biomarkers) will be encoded using dedicated neural architectures optimized for its data structure, as developed in Tasks 2.3 and 2.4. These latent representations will be integrated using attention mechanisms and/or adaptive gating modules, allowing the models to dynamically weight modalities and features according to their relevance for each prediction task and individual patient. Information sources demonstrating superior predictive performance in corresponding mono-modal models will be preferentially weighted within the multimodal architectures to enhance robustness and generalization.
Both mono-modal and multimodal models will be systematically benchmarked across multiple clinically relevant objectives, including enhanced diagnosis, patient stratification, identification of clinically meaningful subgroups, and prediction of prognosis and treatment outcomes. Model performance will be assessed using standard classification metrics—area under the receiver operating characteristic curve (AUC), sensitivity, specificity, and balanced accuracy—under cross-validation and held-out test scenarios. For time-to-event endpoints, survival predictions will be evaluated using Kaplan–Meier analyses, with statistical comparisons between risk groups performed using log-rank tests.
Strong emphasis will be placed on model interpretability and clinical transparency. Feature- and modality-level contributions will be analyzed using saliency and attention maps, while global and local explanations will be derived using Shapley value–based feature attribution methods. These analyses will support biological and clinical interpretability, facilitate hypothesis generation, and increase trust and usability of the proposed models in clinical decision-support settings.

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After development, we plan to test our models in trials that will be performed using the guidelines CONSORT-AI (https://www.equator-network.org/reporting-guidelines/consortartificial-intelligence/) and Spirit-AI (https://www.equatornetwork.org/reporting-guidelines/spirit-artificial-intelligence/) (Cruz Rivera S et al, Lancet Digit Health 2020, Liu X et al, Lancet Digit Health 2020)

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WP3: Generation and validation of an off-the-shelf library of CAR constructs targeting TCR Vβ and Cδ regions

***In vitro* cytotoxicity and cytokine secretion assays.** CAR-transduced T cells specific for each TCR Vβ and Cδ regions will be functionally validated using TCR-deficient TCL cell lines. Luciferase-expressing target cells, including STZ1 and Jurkat cells engineered to express defined TCR Vβ and Cδ regions, will be used to assess antigen-specific cytotoxicity.

Target cells (100,000–300,000 cells per well) will be labeled with eFluor™ 670 dye (Thermo Fisher Scientific) and co-cultured with either mock-transduced or CAR-transduced T cells in 96-well round-bottom plates at the indicated effector-to-target (E:T) ratios. Co-cultures will be maintained for 24 and 48 hours. Cytotoxic activity will be quantified by flow cytometry based on the percentage of residual live target cells, defined as eFluor 670^+ 7-AAD^- cells. Wells containing target cells alone will be included as negative controls in all experiments.

For experiments using primary TCL blasts, the absolute number of viable target cells will additionally be determined using BD Trucount™ tubes (BD Biosciences). To enable accurate comparison across different CAR constructs, transduction efficiencies will be normalized prior to functional analyses. Long-term cytotoxicity assays (3, 7 and 11 days) will also be performed using a range of E:T ratios to identify CAR constructs with the most sustained and potent antitumor activity. In parallel, cytokine release will be assessed by measuring levels of interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), and interleukin-2 (IL-2) in culture supernatants collected 24 hours after target cell exposure. Cytokine concentrations will be quantified using BD OptEIA™ Human ELISA kits (BD Biosciences), according to the manufacturer's instructions.

***In vivo* assessment of CAR-T cell efficacy in TCL models.** All animal experiments will be conducted under specific pathogen-free conditions. Seven- to twelve-week-old NSG mice (The Jackson Laboratory) will be systemically transplanted with 1×10^6 luciferase-expressing TCL cell lines (STZ1 or Jurkat cells engineered to express specific TCR Vβ and Cδ regions). For patient-derived xenograft (PDX) studies, NSG mice will be sublethally irradiated (2 Gy) prior to transplantation with 1×10^6 TCL PDX cells (Figure 3.1).

Following tumor engraftment, 3-4 days for cell line–based models or 3-4 weeks for PDX models, mice will receive intravenous infusion of either mock-transduced T cells or CAR-T cells ($3–5 \times 10^6$ cells per mouse). The dosing and timing of both target cell engraftment and CAR-T cell administration will be based on protocols previously optimized in a leading laboratory. Disease progression will be monitored weekly through flow cytometric analysis of peripheral blood samples. In mice bearing luciferase-expressing TCL cells, tumor burden will additionally be assessed by weekly bioluminescence imaging following intraperitoneal administration of D-luciferin (60 mg/kg; PerkinElmer). Bioluminescence signals will be quantified using Living Image software (PerkinElmer). Mice will be euthanized upon exceeding predefined humane endpoints, including greater than 20% body-weight loss, development of clinical symptoms, or tumor burden exceeding 20% blasts in peripheral blood. At study endpoint, peripheral blood, bone marrow, and spleen samples will be harvested for flow cytometric evaluation of tumor burden and CAR-T cell persistence. Plasma samples will also be collected from all treatment groups to assess systemic secretion of pro-inflammatory cytokines.

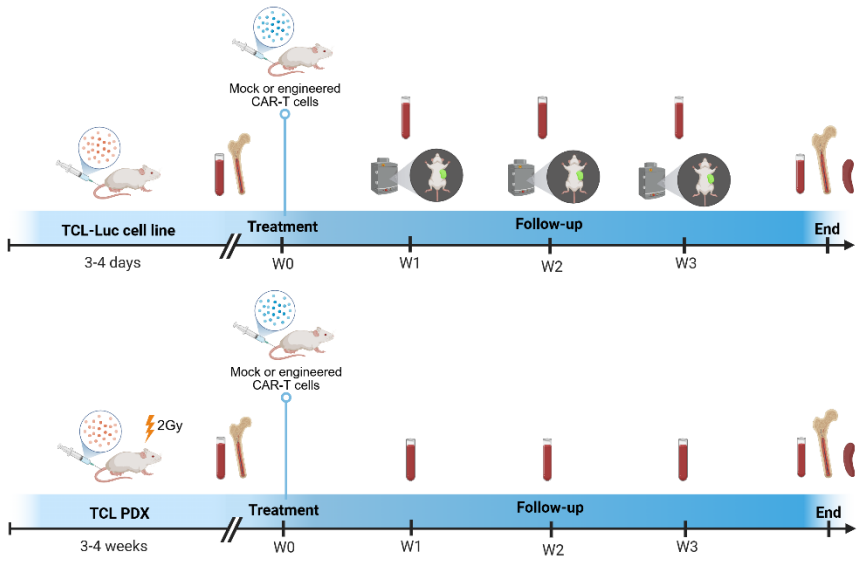

Figure 3.1. In vivo TCL xenograft and PDX models for CAR-T cell evaluation. NSG mice were transplanted with luciferase-expressing TCL cell lines or patient-derived TCL cells (PDX) and subsequently treated with mock or CAR-T cells. Tumor engraftment and progression were monitored by peripheral blood flow cytometry and, where applicable, bioluminescence imaging. At humane or experimental endpoints, tissues and plasma were collected to assess tumor burden, CAR-T cell persistence, and systemic cytokine levels.

WP4: Next-Generation TCR-Targeted Engager Platforms for TCL Immunotherapy

GMP Facilities for STAb-T cell production. BST offers outstanding GMP manufacturing facilities for CAR-

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T and STAb-T cell production, comprising nine GMP laboratories spanning more than 500 m²—the largest cell therapy manufacturing infrastructure in the country. In addition, BST hosts a dedicated process development laboratory and a highly experienced team of more than 25 professionals specialized in cell therapy manufacturing. Together, these capabilities ensure rapid scalability, robust GMP production, and efficient clinical translation of the advanced therapies to be developed within the TCRther4TCL program.

Design of TCR-specific biTCEs. The pCDNA3.1/ pCR3.1 expression vectors encoding the human kappa (κ) light chain signal peptide L1 followed by the anti-TCR scFv targeting Vβ and Cδ (VH–VL or VL–VH) (WP3), followed by a five-residue linker (Gly4Ser), the CD3LA scFv (VH–VL), the human c-Myc epitope tag, and a C-terminal polyHis tag, will be synthesized (Figure 4.1).

Cell transfection. HEK293 or HEK293T cells will be transfected with the expression plasmid using lipofectamine and selected in complete DMEM (DMEM supplemented with 10% v/v heat inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin) with 500 µg/mL geneticin (G-418) or 150 µg/mL hygromycin B to generate stable cell lines. Non-transfected cells will be used as negative controls. Supernatants from transiently and stably transfected cell populations will be analyzed by Western blotting, ELISA, and flow cytometry to test the functionality of the secreted biTCEs.

Production of the purified antibodies. We will perform small-scale production and purification of the anti-TCR antibodies in mammalian Expi293 cells using standard chromatography techniques. Following this, we will conduct structural and functional characterization, including evaluating the oligomeric state through SEC-MALS, assessing specificity and affinity using Biacore, and testing the stability of both scFv antibodies.

Generation of biTCE-encoding lentiviral transfer vectors. After functional validation of secreted biTCE, the antibody constructs will be cloned into a pCCL lentiviral transfer vectors (LV-TV) based on a polycistronic 2A (T2A, P2A, F2A) expression cassette, allowing the simultaneous expression of one or more TCE and/or a fluorescent protein (FP) such as EGFP, dTomato or mCherry, acting as a reporter gene. The biTCE sequence will contain tags for detection, such as c-Myc epitope tag or a poly-Histidine tag. The sequence of the LV vectors will be verified by Sanger sequencing using EF1α Fw, FP Rv and WPRE Rv primers.

T cell transduction. For T cell lines, Jurkat cells will be transduced with the indicated lentiviruses at a multiplicity of infection (MOI) of 5 and expanded in complete RPMI (RPMI-1640 supplemented with 10% v/v heat inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin). In the case of primary T cells, PBMC wil be plate-coated activated with 1 µg/mL anti-CD3 (OKT3) and 1 µg/mL anti-CD28 (CD28.2) mAbs for 2 days and transduced (MOI of 10) with the indicated lentiviruses in the presence of 10 ng/mL interleukin (IL)-7 and 10 ng/mL IL-15. T cells were expanded in RCM supplemented with IL-7 and IL-15 (10 ng/mL) for 5-10 days. Alternatively, after PBMC isolation, cells will be subsequently activated and expanded for 24 hours with Dynabeads™ Human T-Activator CD3/CD28 at a cell-to-bead ratio of 1:3 in complete RPMI (1 × 10^6 cells/mL) in the presence of 10 ng/ml IL-7 and IL-15. After 24 hours, cells will be transduced with lentiviruses (MOI of 5) and then cultured and expanded in complete RPMI supplemented with IL-7 and IL-15 (10 ng/mL) for up to 14 days. Transduction efficiency in both Jurkat and primary T cells will be determined based on FP expression by flow cytometry. Non-transduced Jurkat or primary T cells will be used as negative controls.

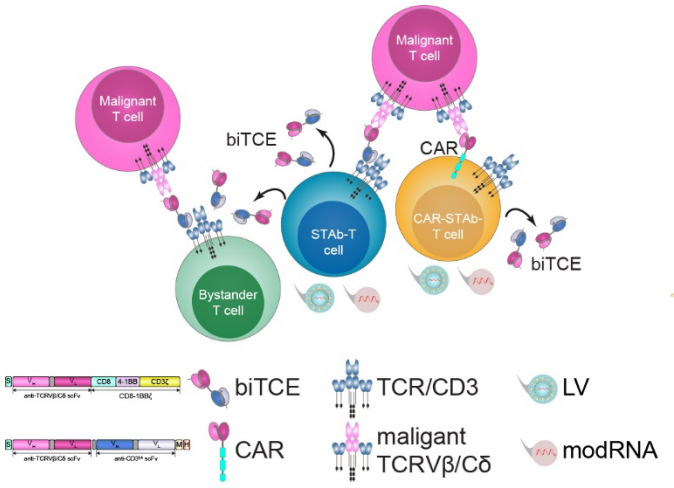

***Figure 4.1**. Next-Generation TCR-Targeted Engager Platforms for TCL Immunotherapy. STAb-T cells (blue cells) or CAR-STAb-T cells (yellow cells) are T lymphocytes engineered to secrete the TCR-biTCE or to simultaneously express a CAR and secrete a TCR-biTCE redirected to specifically kill malignant clones of TCL. Autologous peripheral blood T cells will be permanently engineered to express CARs (WP3) or TCR-biTCEs (stable by LV or transient by modRNA), enabling simultaneous targeting of multiple TCR regions to prevent tumor escape. The soluble biTCEs secreted by STAb-T cells and by CAR-STAb-T cell can recruit the entire T cell repertoire (bystander non-engineered T cells) to attack the malignant TCL.*

mRNA construct design and transfection. A capped and polyadenylated modRNA encoding the anti TCR-biTCEs will be synthesized, using wild type bases, by TriLink BioTechnologies or GenScript Biotech Corporation. The mRNA construct will comprised the L1 signal peptide, followed by the anti-TCR scFv targeting Vβ and Cδ (VH–VL or VL–VH), followed by a five-residue linker (Gly4Ser), the CD3LA scFv (VH–VL) and the C-terminal human c-Myc and polyHis tags. For transfection of HEK 293T cells, TransIT®-mRNA Transfection Kit will be used following manufacturer's instructions; After 48 hours transfection efficacy will be analyzed by flow cytometry. Non-transfected (MOCK) HEK 293T cells will be used as controls. For transfection of primary T cells, isolated PBMC will be activated with plate-bound 0.5 µg/mL anti-CD3 (OKT3) and 2µg/mL soluble anti-CD28 (CD28.2) antibodies for 2 days. T cells will be further expanded for 3–4 days in complete RPMI supplemented with 100 IU/mL recombinant human IL-2 (rhIL-2, Proleukin®). Then, T cells will be washed and resuspended in OPTI-MEM (final concentration of 100x10^6 cells/mL). Subsequently, the cells will be mixed with 10 µg of TCR-biTCEs encoding mRNA per 10^6 cells and electroporated (4mm cuvettes, single 10 ms square-wave pulse of 250V) using the Gene

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Pulser Xcell Electroporation System (BioRad). As controls, T cells will be left non-electroporated or will be electroporated without modRNA (mock-electroporated, MOCKe). Cells will be maintained in complete RPMI supplemented with 50 IU/mL of rhIL-2. Viability and electroporation efficiency will be analyzed at 3, 7, 24, 48, 72 and 96 hours post-electroporation by flow cytometry.

***In vitro* functional evaluation of TCR-biTCEs.** The bioactivity exerted by the anti-TCR-biTCEs will be assessed *in vitro* using primary T cells (and, when pertinent, Jurkat T cells) through binding assays; cell activation (CD69 and CD25 expression by flow cytometry); proliferation (CFSE, CellTrace™ Violet, and CellTrace™ Far Red); viability (BD Trucount™ tubes); cytokine secretion (Luminex platform); and cytotoxicity (bioluminescence and flow cytometry, after 24 or 48 hours). These assays will be performed in the presence of antigen-negative or antigen-positive target cells.

***In vivo* studies**. NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ female mice (NSG, The Jackson Laboratory) mice bearing TCL-derived cell line xenografts (CDX) or patient-derived xenografts (PDX) (WP6) will be used to evaluate the preclinical outcome of the TCR-Targeted Engager Platform. Tumor progression will be monitored by bioluminescence imaging (CDX) or flow cytometry in peripheral blood or bone marrow samples (PDX).

WP5: Development of patient-tailored vaccines targeting CDRs and neoantigens

Design of peptide and mRNA vaccines. The team has extensive experience in the design and preclinical evaluation of antigen- peptide- and mRNA-based nanovaccines. As examples, protein-based vaccines have been previously developed and tested for hepatitis B and HIV, achieving robust humoral and cellular immune responses (Figure 5.1 A,B)[7,8]. In addition, an intranasal HIV peptide vaccine prototype was advanced up to non-human primate studies, where it conferred protection in macaques and was successfully scaled up to an industrially compatible manufacturing process, illustrating the feasibility of translating peptide nanovaccines towards clinical-grade production.

In parallel, the group has engineered and optimized lipid–polymer hybrid nanocapsules and nanoemulsions for mRNA vaccination, generating broad nanocarrier libraries that support efficient encapsulation, protection and delivery of mRNA cargoes. These platforms have been applied to SARS-CoV-2 vaccine research, where selected formulations achieved potent antigen expression in vivo, elicited strong spike-specific antibody responses and Th1-biased T-cell immunity, and showed excellent stability (Figure 5.1C)[9–11]. Together, these data provide a robust technological and translational foundation to design and characterize the peptide and mRNA nanovaccines proposed in WP5.2.

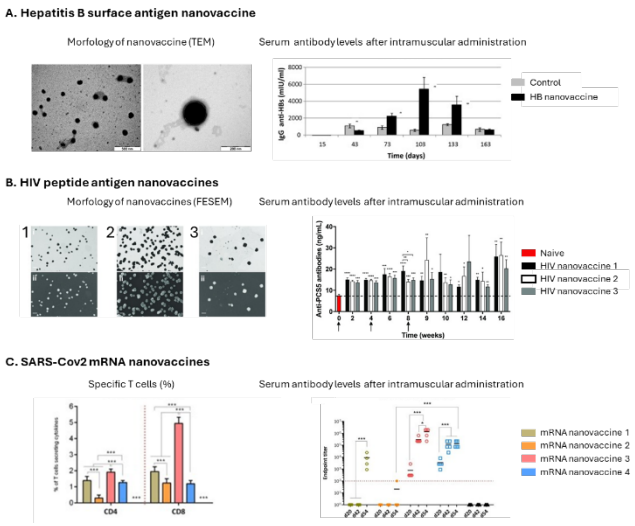

***Figure 5.1**. Representative examples of our prior work on nanovaccine platforms, including recombinant protein surface antigen nanovaccines (A), peptide-based nanovaccines (B), and mRNA nanovaccines (C). The figure shows electron microscopy images of nanovaccine platforms, and quantitative analyses of antigen-specific serum antibody titers over time and. For the SARS-CoV-2 mRNA nanovaccines, frequencies of antigen-specific CD4$^+$ and CD8$^+$ T cells, demonstrating robust humoral and cellular immunity induced by the different nanoparticle formulations.*

Characterization of mRNA vaccines. For the delivery of RNA constructs, suitable nano-formulations will be prepared from safe, biocompatible and regulatory accepted polymers and lipids (including ionizable, PEG-lipids and oils). Compositions will be adapted from prototype families previously developed by USC[7–9,11], characterized with satisfactory stability and high loading of biomacromolecules. These carriers will be prepared by simple and mild methods (e.g. solvent displacement, self-emulsification) well established at Alonso's lab and by microfluidics mode, resulting in nanocarriers wit adjustable size and surface charge. Two main types of nanostructures will be developed: (i) core-shell type nanocapsules based on a lipid core, surrounded by a polymer shell and (ii) matrix-type polymeric and/or lipid nanoparticles. Particle size, PDI, and zeta potential will be measured (Zetasizer Nano-ZS, Malvern). Selected prototypes will be characterized by nanoparticle tracking analysis (NanoSight NS300, Malvern) and by transmission electronic microscopy (TEM Jeol JEM 2010). Gel electrophoresis retardation and Quant-it™ RiboGreen RNA Assay (ThermoFisher) or UPLC/SPS-PAGE will be performed to evaluate mRNA or peptide encapsulation efficiency, respectively. To study the stability in relevant biological media (human plasma, 37ºC, 0-24h) and under storage conditions (4ºC, 0-5 weeks) size, zeta potential and mRNA encapsulation will be monitored.

Functional characterization of peptide and mRNA nanovaccines will be performed in human monocyte-derived dendritic cells, macrophages and T cells obtained from PBMCs of healthy donors. Antigen uptake and cell activation will be assessed by flow cytometry and confocal microscopy using fluorescently labelled cargo and antibodies against MHC–peptide complexes and co-stimulatory markers. Transfection efficiency of

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mRNA nanovaccines will be quantified by reporter expression (eGFP), while viability and activation markers will define non-toxic effective doses. Antigen-specific $CD4^+$ and $CD8^+$ T-cell responses will be measured in autologous co-cultures by ELISpot, intracellular cytokine staining, proliferation assays and cytotoxicity readouts to prioritize the most immunogenic formulations for in vivo testing (WP6).

Clinical translation: stability and scale-up studies. To support a future First-in-Human Phase I trial, long-term stability of the freeze-dried nanocarrier (NC) formulation will be evaluated after lyophilization in the presence of trehalose, monitoring residual moisture, reconstitution behavior, particle size, PDI, zeta potential and cargo integrity under ICH-compatible storage conditions. Stability-indicating assays will be performed at predefined time points to confirm preservation of physicochemical properties and encapsulated peptide/mRNA. Process scale-up will be addressed using microfluidic-based manufacturing, optimizing total flow rate, phase ratio and lipid concentration, and benchmarking these runs against a stirred-tank batch process in terms of size, PDI, encapsulation efficiency and run-to-run reproducibility.

T cell epitope identification. There is a large difference between MHCI and MHCII epitope prediction tools being the performance of class II epitope predictors considerably poor. Provided the relevance of $CD4^+$ T cells for effective immune responses, we consider critical to target this compartment for improved vaccine designs. It is therefore essential to identify peptides selected for presentation and triggering immunodominant responses. We will prioritize epitope predictions and validation of peptide binding for MHCI. However, we consider essential to use orthogonal and complementary methods for MHCII besides predictions. A reconstituted antigen processing system along with predictions[12,13] and/or immunopeptidomics will be therefore used.

Peptide affinity measurements. Peptide affinity measurements involve Fluorescence Polarization (FP) or Time Resolved Florometry (TRF). We will determine IC50 for each candidate using reporter labelled reported peptides (FITC or Europium) whose binding will be blocked by titrated concentrations of the peptide under evaluation[13–15] .

MHC-II Reconstituted antigen processing system. Antigens or peptide mixtures are incubated with recombinant MHC molecules, DM and cathepsins. Processing reactions are quenched (pH and chemicals) and MHCII-peptide complexes are pulldown, and reactants in excess are removed away by washing. Peptides bound to MHCs are eluted by acidic treatment and cleaned up via Stage-tips. Resulting peptide mixtures are measured by MS informing on the preferential selection of specific peptides by individual MHC-II allotypes.

Peptidome analysis. Patient MoDCs are attained upon differentiation from monocytes (GM-CSF and IL4 treatment) isolated from PBMCs. MoDCs in cell culture are fed with the corresponding antigens (proteins, long polypeptides, and/or 15-mer peptides). Likewise, mRNA constructs can be fed to the cultures mimicking the proposed vaccination strategy. After the corresponding incubation times with the desired antigenic source, MoDCs are harvested, snap-frozen and subject cell lysis. After lysis, MHC molecules are isolated via pulldown using available antibodies crosslinked to protein G Sepharose. Available antibodies include W6/32 for MHC-I, L243 for HLA-DR, SPV-L3 for -DQ and B7/21 for HLA-DP. Isolated MHC-peptide complexes undergo acidic treatment for eluting the bound peptides. Samples are then fractionated and cleaned up via Stage-tips prior to be measured via MS. Analysis pipelines have been extensively described in the literature and by our own[16].

WP6: Preclinical models for therapy testing

TCL-Lymphomoids: Tumor fragments will be minced with a into 0.75–1.5 mm3 fragments and cryopreserved in FBS +10% DMSO for future use. Four to ten explants will be embedded in Collagen I and layered on top of a pre- solidified collagen bed in a 0.4 mm inner transwell to form the double dish air-liquid interface (ALI) system .The insert will be placed in a 12-well plate and left in a 5% CO2 incubator at 37 °C for 20 min for the matrix to gelify, followed by addition of 200 µL of the medium mentioned above supplemented with 10 ng/mL IL2/IL7. Media will be replaced every 2-3 days.

TCL-Tumoroids: PBMCs will be thawed in sterile conditions and resuspended in supplemented medium (RPMI 1640, 1% Glutamax, 10% FBS, 1% Penicillin-Streptomycin) and counted using trypan blue in a Neubauer chamber to assess viability before culture. To assess proliferation, cells will be labeled with carboxyfluorescein succinimidyl ester (CFSE, 0.5 µM). CFSE-labeled TCL samples will be then mixed with irradiated YK6 cells (provided by Daniel J. Hodson-https://pubmed.ncbi.nlm.nih.gov/33837304/)) at a ratio of 2:1 (TCL:YK6). Cells will be then resuspended in a 1.35 mg/mL dilution of cold rat tail collagen I, seeding 100,000 TCLcells/well and 50,000 YK6 cells/well in a final volume of 100 µL/well in Nunclon™ Sphera 96-wells Ultra-Low Attachment (ULA) microplates. Tumoroids will be then incubated at 37ºC for 45 minutes to allow jellification and followed by addition of 200 µL of the medium mentioned above supplemented with 10 ng/mL IL2/IL7. Media will be replaced every 2-3 days

TCL-on chip: Lymphomoids/tumoroids will be integrated in a microfluidic platform (https://beonchip.com/) to better mimic in vivo conditions allowing therapy delivery studies in a dynamic system and under cytokines gradients. if necessary, (PPG and JPV).

TCL on Chicken Embryo CAM models to study tumorigenesis, tumoral angiogenesis and TCL dissemination (JPV). In vivo CAM assay: In vivo assays: CAM assay: Fertilized hen eggs (Granja Gibert, Spain) are incubated at 60% humidity and 38ºC in a rotating incubator. Briefly, on day 10 of chick development (without developed immune system), eggs are windowed and subject experimental TCL-Lymphomoids resuspended in 20% Matrigel (Corning, VA, USA) and 80% serum-free media, placed onto the chorioallantoic membrane (CAM). Cells are allowed to expand for up to 7 days in a stationary incubator at 60% humidity and 38ºC. This model enables

quantification and visualization of tumor growth, angiogenesis and cell dissemination (human Alu or specific viral sequences vs. chicken gapdh). The CAM does not require obtaining ethics committee approval for animal experimentation according to European law (Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes). It constitutes an attractive alternative to other animal experiments.

Adapting TCL-Lymphomoids in humanized pre-clinical settings. In vivo: NSG or NOG (IL2Rγnull) mice will be conditioned (irradiation or busulfan) and transplanted with human CD34+ hematopoietic stem cells + Lymphomoids containing at least 2 million malignant cells. TCL_Lymphomoids will be marked using fluorescent TCL_DNA-aptamers or using a vital colorant and transplantated by intradermal injection in 20 µL of phosphate-buffered DMSO/Tween vehicle in the ear (for Cutaneous CTLs) or in XXX. Specific and rationally selected therapies amongst those selected above as well as CART and RNA-vaccines will be administrated and the biological and molecular effects will be addressed in samples (FG, JPV, BA).

Quantification of treatment response in the in vivo murine model

Mice will be bled weekly and presence of aberrant human T lymphoid populations in the murine blood will be quantified by flow cytometry using the appropriate markers (hCD45+, hCD4+, hCD26-, hCD7-). We will determine the efficacy of the treatment by comparing the evolution of the tumor populations in the different experimental groups (control vs treatment). In addition, we will monitor the presence of aberrant circulating malignant SS cells using the binomial flow cytometry markers TRBC1/TRBC2, previously tested in its clinical counterparts. Flow cytometry analysis of TRBC1/2 is rapid and convenient, as it allows assessing T-cell clonality and tumor quantity simultaneously. This analysis is commonly used for initial blood staging and monitoring tumor burden during therapy in patients with MF or SS. Assessment of TRBC1 expression within a comprehensive single-tube FC assay has been proven to overcome interpretative uncertainties in the identification of SS cells, without the need for a separate T-cell clonality assay (Horna et al. 2020). Those SS-PDX presenting tumor mass or visceral infiltration will be additionally evaluated to measure treatment responses according to CTCL tumor PDX protocol.

WP7: Identification and repurposing of inhibitors targeting central nodes in the TCR signaling pathway as possible anti-TCL drugs

Flow-cytometry-based in vitro screening assay. Lymphocyte-rich preparations from TCL patients obtained from apheresis or blood will be labeled with 1 µM CellTrace™ Violet (ThermoFisher) and incubated for 4 days on 96-well plates (Costar) coated with 10 µg/mL of the anti-CD3 antibody OKT3 Co-stimulation will be provided with soluble anti-CD28 (0.3 µg/mL). Inhibitors will be added from stock DMSO solutions to reach final concentrations of 1 µM, 100 nM and 10 nM. After 4 days cells will be resuspended and incubated with a panel of antibodies to distinguish αβ T cells from γδ T cells and CD4+ from CD8+ T cells. Malignant cells will be distinguished from normal T cells according to the expression of CD3, CD26, PD1, CD5, CD7, CD9, CD10 and CD30 to search for the cells with diminished or loss CD3, CD26, CD5, CD7 and/or gain of PD1, CD9, CD10 or CD30. The multiparametric analysis will be carried out using a spectral flow cytometer (Aurora 5L ESP, Cytek) available at the CBMSO (partner BA). The effect of the inhibitors on the spontaneous and CD3-induced proliferation of malignant versus normal T cells will be measured according to CTV dilution. An example is shown in Figure 7.1. In this case, the CD26 marker was used to distinguish healthy (CD26+) from malignant (CD26-) Sezary Syndrome CD4+ T cells. The PI3K and mTOR inhibitor Dactolisib inhibited the proliferation of Sezary cells but also, and very strongly, the proliferation of healthy normal T cells.

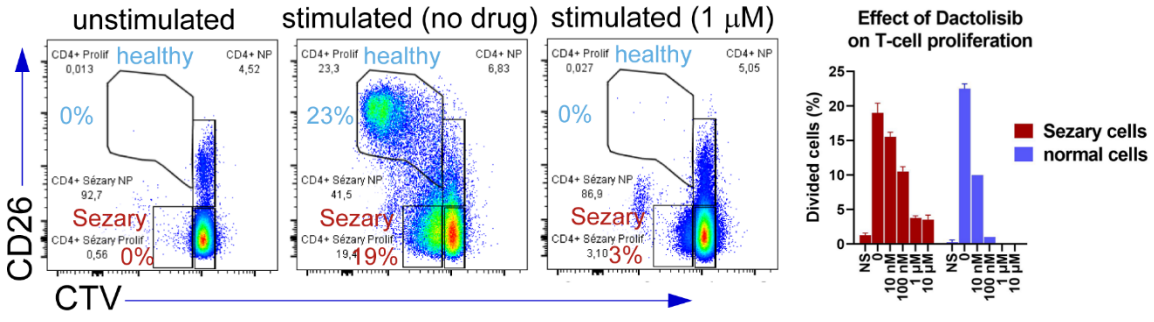


Figure 7.1. Effect of the PI3K/mTOR dual inhibitor dactolisib on the proliferation of both normal and malignant CD4+ T cells from Sezary Syndrome patient SS1. Lymphoid cells obtained from apheresis were labeled with CTV and stimulated on anti-CD3-coated plates for 4 days in the presence of soluble anti-CD28. Cell proliferation was assessed according to CTV dilution on CD4+CD26+ (normal) and CD4+CD26-negative malignant T cells. Dactolisib inhibited the proliferation of Sezary cells but also of the normal T cells (bar plot).

WP8: First in Human Prospective clinical Trial.

Once the treatment to be tested on patients has been decided, [TCRther], a phase 1 trial will be initiated.

Protocol Title:

First-in-Human, open-label, adaptive, translationally driven study to evaluate the safety, tolerability, pharmacology, and preliminary efficacy of TCRther in adult patients with relapsed/refractory T-cell lymphoma without available therapeutic options.

Study code: [XXX-FIH-RR_PTCL]**

Phase: I (First-in-Human)

Design: Adaptive, to assess feasibility, toxicity and clinical activity

Rationale

Patients with relapsed or refractory peripheral T-cell lymphomas (RR PTCL) represent an unmet clinical need in hematologic oncology. Conventional chemotherapies and autologous or allogeneic stem cell transplantation provide limited efficacy in this population, with median overall survival often measured in months for high-risk or multiply relapsed patients. The heterogeneous biology of T-cell lymphomas, including subtypes such as PTCL and cutaneous T-cell lymphoma (CTCL), is associated with intrinsic chemoresistance and poor prognosis, highlighting the necessity for novel, mechanism-driven therapies that target malignant T cells while sparing normal hematopoietic cells as much as possible.

Immunotherapeutic approaches offer a compelling rationale for intervention in this setting. Bispecific monoclonal antibodies can simultaneously engage T-cell effectors and lymphoma-specific antigens, thereby redirecting the immune system to selectively kill malignant T cells. Similarly, CAR T-cell therapies can be engineered to recognize tumor-associated antigens on T-cell lymphoma cells, providing potent and sustained cytotoxic activity. Preclinical studies and early clinical reports have demonstrated promising anti-tumor activity with acceptable safety profiles for both modalities, justifying the translation into first-in-human (FIH) trials. The design of such FIH trials must carefully balance therapeutic potential against safety, given the risks of cytokine release syndrome, T-cell aplasia, and other immune-mediated toxicities.

A prospective FIH clinical trial allows for systematic evaluation of safety, tolerability, pharmacokinetics/ pharmacodynamics, and preliminary efficacy in a population with no remaining standard treatment options. Using rational dose-escalation strategies informed by MABEL (Minimum Anticipated Biological Effect Level) and NOAEL (No Observed Adverse Effect Level) calculations from preclinical data ensures patient safety while enabling early assessment of biologically active doses. Additionally, embedded biomarker analyses and immune monitoring can guide dose selection, identify predictive response markers, and inform future combination strategies. Such trials are essential to establish a foundation for safe, effective, and scalable immunotherapy approaches for patients with RR PTCL, ultimately advancing therapeutic options in this high-risk patient population.

Type of Study

First-in-Human, open-label, adaptive, translationally driven study to evaluate the safety, tolerability, pharmacology, and preliminary efficacy of [TCRther] selected via a pre-specified translational algorithm in adult patients with RR PTCL without available therapeutic options.

Study Duration

Recruitment period: 18 months (from month #42 until month #60 in the global planning). Follow up period: 1 year after the last dose / treatment

Patient Numbers

In Part 1, it is anticipated that 9-18 participants will be enrolled, and the total number of participants will depend on the number of dose levels explored to identify the RDE

In Part 2, up to approximately 15 participants will be enrolled.

The total number of participants enrolled in the clinical trial is anticipated to be up to approximately 30 participants.

Study Sites

Number of centres: 5 centres for the escalating dose phase (part 1), increasing to 10 centres for the expansion phase (part 2). The number of patients to be recruited per centre: 4-5.

Objectives

General objective

To evaluate the safety, tolerability, clinical pharmacology, and preliminary antitumour activity of [Product X], a novel immunotherapeutic strategy targeting [TARGET], prospectively selected using a translational algorithm, in adult patients with RR PTCL without available therapeutic options.

Primary

- Safety: incidence of adverse events (AEs) /serious adverse events (SAEs) and dose-limiting toxicity (DLT).
- Determination of maximum tolerated dose (MTD) and recommended dose for expansion (RDE)

Secondary

- Preliminary antitumour activity: overall response rate (ORR), complete remission rate (CRR), disease control rate (DCR), duration of response (DOR), duration of complete remission (DCR), progression free survival (PFS)
- Pharmacokinetics (PK) / pharmacodynamics (PD) (species-specific) and expansion/persistence (CAR-T).
- Biology: cytokines, T activation/exhaustion, subpopulations of normal T-cells

Exploratory

- Predictive biomarkers of response/toxicity.
- Correlations with histological subtypes and target expression.

Characteristics of the study population

Patients enrolled will be >= 18 years old. It is expected a slightly higher prevalence of male patients considering the prevalence of the disease between the males and females (1.3:1 ratio).

Inclusion and Exclusion Criteria

Inclusion criteria

- Age >= 18 years.
- Karnofsky Performance status > 70% (ECOG < 2) and a life expectancy >= 12 weeks.
- Patients must have a histologically confirmed diagnosis of RR PTCL according to the WHO classification, confirmed by central pathology review.
- Measurable disease according to Lugano 2014.
- Patients must have failed at least one prior line of therapy and should not have other standard treatment option available
- Adequate hepatic function (AST/ALT < 3 x upper limit of normal values, total serum bilirubin < 2 x upper limit of normal value), adequate renal function < 2 mg/dL, adequate cardiac function (FE > 40%), estimated glomerular filtration rate >30 mL/min per 1.73 m2 (to be calculated using the Modification of Diet in Renal Disease (MDRD) formula)
- Absence of other clinical comorbidities which can represent an exclusion to treatment.
- Participants with childbearing potential will have to practice effective birth control while on study.
- Must sign an informed consent form (ICF) (or their legally acceptable representative must sign) indicating that he or she understands the purpose of, and procedures required for, the study and is willing to participate in the study. Subjects must be willing and able to adhere to the prohibitions and restrictions specified in this protocol, as referenced in the ICF.

Exclusion criteria

- Enrollment in another interventional clinical trial within 1 month from study enrollment
- Active malignancies (ie, progressing or requiring treatment change in the last 24 months) other than the disease being treated under study. The only allowed exceptions are:
    1. non-muscle invasive bladder cancer treated within the last 24 months that is considered completely cured.
    2. skin cancer (non-melanoma or melanoma) treated within the last 24 months that is considered completely cured.
    3. non-invasive cervical cancer treated within the last 24 months that is considered completely cured.
    4. localized prostate cancer (N0M0): with a Gleason score of ≤6, treated within the last 24 months or untreated and under surveillance, with a Gleason score of 3+4 that has been treated more than 6 months prior to full study screening and considered to have a very low risk of recurrence, or history of localized prostate cancer and receiving androgen deprivation therapy and considered to have a very low risk of recurrence.
    5. Breast cancer: adequately treated lobular carcinoma in situ or ductal carcinoma in situ, or history of localized breast cancer and receiving antihormonal agents and considered to have a very low risk of recurrence.
    6. Malignancy that is considered cured with minimal risk of recurrence
- Positive pregnancy test for female patients.
- Active breastfeeding
- Previous allogeneic hematopoietic cell transplantation within the previous 6 months and/or presence of active graft-versus-host-disease and/or active immunosuppression
- Received a cumulative dose of corticosteroids equivalent to ≥70 mg of prednisone within the 7 days prior to study enrollment (if the CART cell construct is the product to be used)
- Contraindications or life-threatening allergies, hypersensitivity, or intolerance to the experimental drug or its excipients, including dimethyl sulfoxide, or to fludarabine, cyclophosphamide, tocilizumab, anakinra, dexamethasone.
- Active CNS involvement
- Seizures or stroke during the last 6 months
- Active and non-controlled infection
- Any other medical condition considered significant by the principal investigator which could contraindicate the clinical trial participation.

Endpoints (primary and secondary endpoints / outcome measures)

Primary outcome:

- Defining the maximum tolerated dose of the experimental drug. Dose limiting toxicities will be considered:
* grade > 3 cytokine release syndrome (CRS) and/or grade > 3 immune cell associated neurotoxicity syndrome (ICANS) or any grade > 3 non-hematological toxicity (including non-ICANS neurotoxicity) non existing before infusion and possibly related to the experimental drug, according to the criteria and grading defined in the international consensus document (ASTCT criteria for CRS and ICANS, CTCAE for other toxicities)
- Defining the recommended dose for the expansion phase

- Safety outcomes:
  - Non-relapse mortality (NRM) at 12 months, defined as any death not directly caused by disease progression
  - Incidence of all grade >3 adverse events (AEs) as per CTCAE version 5.0. b.
  - Incidence of all the following AEs of special interest (AESI), any grade:
    - Cytokine release syndrome
    - Immune effector cell-associated neurotoxicity syndrome (ICANS)
    - Immune effector cell hemophagocytic lymphohistiocytosis-like syndrome (IECHS)
    - Tumour lysis syndrome (TLS)
    - Prolonged cytopenia (beyond 6 months)
    - Infections
    - Hypogammaglobulinemia
    - Second primary malignancies

Secondary outcomes:

- Overall Response Rate (ORR), defined as the proportion of patients who achieve either a complete remission (CR) or a partial remission (PR) as their best overall response during the study treatment period, as assessed by the investigator according to standardized lymphoma response criteria. ORR is calculated as the number of patients with CR or PR divided by the total number of evaluable patients.
- Complete Remission Rate (CRR), defined as the proportion of patients who achieve a complete remission (CR) at any time during the study, based on the best overall response, as determined by standardized response criteria. A complete remission requires the disappearance of all clinical, radiologic, and metabolic evidence of active disease, with normalization of disease-related laboratory abnormalities when applicable.
- Disease Control Rate (DCR), defined as the proportion of patients who achieve complete remission (CR), partial remission (PR), or stable disease (SD) as their best overall response during the study. DCR reflects the ability of the investigational therapy to control disease progression, particularly relevant in early-phase trials where prolonged disease stabilization may represent clinical benefit.
- Duration of Response (DoR), defined for responding patients (CR or PR) as the time from the first documented evidence of objective response (CR or PR) until the earliest occurrence of disease progression or death from any cause, whichever occurs first. Patients without an event at the time of analysis are censored at the date of last adequate disease assessment.
- Duration of Complete Remission (DoCR), defined for patients who achieve a complete remission as the time from the first documentation of CR until disease progression or death from any cause, whichever occurs first. Patients who remain in complete remission at the time of analysis are censored at the date of their last disease assessment confirming CR.
- Progression-Free Survival (PFS), defined as the time from the date of first administration of the investigational product to the first documentation of disease progression or death from any cause, whichever occurs first. Patients who have neither progressed nor died at the time of analysis are censored at the date of their last adequate disease assessment.

Statistical plan. General Considerations

This section is a summary of the planned statistical analyses of the most important endpoints including primary and key secondary endpoints.

Data will be presented by dose regimen and cohort.

Descriptive statistics will be used for all variables.

Continuous variables will be summarized by the number of observations, mean, standard deviation, median, minimum and maximum.

Categorical variables will be summarized by frequency counts and percentages for each category. Unless otherwise stated, percentages will be calculated based on the population total (all participants who receive at least 1 dose of any study intervention) by dose regiment and by timepoint as appropriate.

Time-to-event variables will be summarized by the number of events, number of censored participants and the censoring reasons, rates al specific timepoints and median time to the event using de Kaplan-Meier methodology.

R version 4.5.1 (or later) will be used for analyses presented.

Overall Design

The aim of the dose escalation is to determine the safety, tolerability and preliminary efficacy of TCRther.

Evaluation of data from dose escalation, including appropriate safety, tolerability, PK, pharmacodynamics, and efficacy, will inform the dose and schedule to be evaluated in dose expansion.

Dose escalation will start with participants receiving TCRther:

- Bispecific: a cycle duration 21-28 days. The study treatment period is finite with a maximum of 3-6 cycles for all participants, extendable until progression/toxicity. DLT evaluation period will be done at first cycle of treatment.
- CAR-T: a single infusion per patient per dose level. DLT evaluation period 28 days post infusion.

Target dose per cohort defined by Bayesian optimal interval (BOIN) design:

<u>BOIN design in case the winning treatment represents the use of bispecific monoclonal antibodies:</u>

We will employ the Bayesian optimal interval (BOIN) design[17,18], with the 3+3 design run-in, to find the MTD. The

Addendum

BOIN design is implemented in a simple way like the traditional 3+3 design but is more flexible and possesses superior operating characteristics that are comparable to those of the more complex model-based designs, such as the continual reassessment method (CRM)[19].

The target dose-limiting toxicity (DLT) rate for the MTD is $\phi = 0.25$ and the maximum sample size is 30. We will enrol and treat patients in cohorts of size 3. DLTs have already been defined and only those DLTs that occur within the first cycle will be used for dose finding. As shown in Figure 8.1, the BOIN design uses the following rule, optimized to minimize the probability of incorrect dose assignment, to guide dose escalation/de-escalation:

- if the observed DLT rate at the current dose is ≤ 0.197, escalate the dose to the next higher dose level;
- if it is > 0.298, de-escalate the dose to the next lower dose level;
- otherwise, stay at the current dose.

The 3+3 design run-in will be applied to override the above decision rule when the number of patients treated at the current dose is 3. That is, we will escalate the dose if 0/3 DLT, stay at the current dose if 1/3 DLT, and de-escalate the dose if >= 2/3 DLTs. For the purpose of overdose control, doses j and higher levels will be eliminated from further consideration if $\Pr(p_j > 0.25 \mid \text{data}) > 0.95$ and at least 3 evaluable patients have been treated at dose level j, where p_j is the true DLT rate of dose level j, $j = 1, \ldots, 3$. This posterior probability is evaluated based on the beta-binomial model $y_j \mid p_j \sim \text{binomial}(p_j)$ with $p_j \sim \text{uniform}(0, 1)$, where y_j is the number of patients experienced DLT at dose level j. When the lowest dose is eliminated, stop the trial for safety. The probability cutoff 0.95 is chosen to be consistent with the common practice that when the target DLT rate $\phi \leq 1/6$, a dose with 2/3 patients experiencing DLT is eliminated. The above dose escalation/de-escalation and elimination rule can be equivalently presented in Table 8.1, which will be used to conduct the trial.

The steps to implement the BOIN design are described as follows:

1. Patients in the first cohort are treated at dose level 1.
2. To assign a dose to the next cohort of patients, conduct dose escalation/de-escalation according to the rule displayed in Table 8.1. When using Table 8.1, please note the following:
    a. "Eliminate" means eliminate the current and higher doses from the trial to prevent treating any future patients at these doses because they are overly toxic.
    b. When we eliminate a dose, automatically de-escalate the dose to the next lower level. When the lowest dose is eliminated, stop the trial for safety. In this case, no dose should be selected as the MTD.
    c. If none of the actions (i.e., escalation, de-escalation or elimination) is triggered, treat the new patients at the current dose.
    d. If the current dose is the lowest dose and the rule indicates dose de-escalation, treat the new patients at the lowest dose unless the number of DLTs reaches the elimination boundary, at which point terminate the trial for safety.
    e. If the current dose is the highest dose and the rule indicates dose escalation, treat the new patients at the highest dose.
3. Repeat step 2 until the maximum sample size of 30 is reached, or stop the trial early if the number of evaluable patients treated at the current dose ≥ 12 and the decision according to Table 8.1 is to stay at the current dose.

Table 8.1. Dose escalation/de-escalation rule for the BOIN design.

Actions*	The number of evaluable patients **at the current dose**								
	1	2	3	4	5	6	7	8	9
Escalate if # of DLT ≤	0	0	0	0	0	1	1	1	1
De-escalate if # of DLT ≥	1	1	2†	2	2	2	3	3	3
Eliminate if # of DLT ≥	NA	NA	3	3	3	4	4	4	5

* When none of the actions (i.e., escalate, de-escalate or eliminate) is triggered, stay at the current dose for treating the next cohort of patients. Note that "# of DLT" is the number of patients with at least 1 DLT, and "NA" means that a dose cannot be eliminated before treating 3 evaluable patients. "†" indicates the 3+3 design run-in.

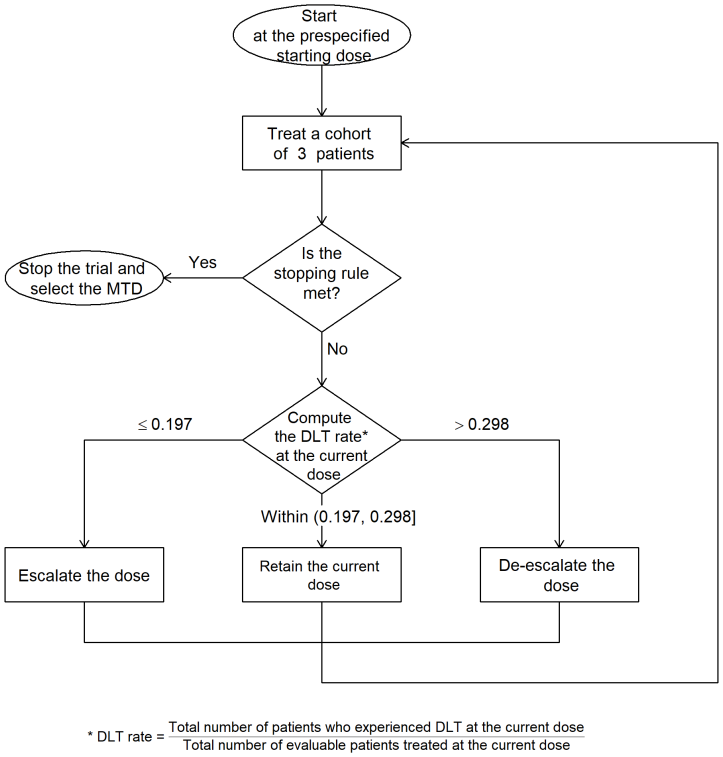


Figure 8.1. Flowchart for trial conduct using the BOIN design.

After the trial is completed, select the MTD based on isotonic regression as specified in[18]. This computation is implemented by the "Estimate MTD" tab of the BOIN Design Desktop Program[20]. Specifically, select as the MTD the dose for which the isotonic estimate of the DLT rate is closest to the target DLT rate. If there are ties, select the higher dose level when the isotonic estimate is lower than the target DLT rate and select the lower dose level when the isotonic estimate is greater than or equal to the target DLT rate.

Optional: [In case there is cohort expansion after identifying the MTD] Once we determine the MTD, an additional *n* patients will be enrolled for additional experience with safety and efficacy. We will use the elimination boundaries in Table 8.1 for DLT monitoring.

Operating characteristics

Table 8.2 shows the operating characteristics of the trial design based on 1000 simulations of the trial using the BOIN Design Desktop Program[20]. The operating characteristics show that the design selects the true MTD, if any, with high probability and allocates more patients to the dose levels with the DLT rate closest to the target of 0.25.

Table 8.2. Operating Characteristics of the BOIN design.

	Dose Level			Number of Patients	% Early Stopping
	1	2	3		
Scenario 1					
True DLT Rate	0.25	0.43	0.62		
Selection %	76.7	14.8	0.3		8.2
% Pts Treated	62.6	32.4	5.0	18.5	
Scenario 2					
True DLT Rate	0.11	0.25	0.39		
Selection %	19.7	61.2	18.5		0.6
% Pts Treated	33.1	43.4	23.5	24.3	
Scenario 3					
True DLT Rate	0.05	0.11	0.25		
Selection %	1.1	20.3	78.6		0.0
% Pts Treated	18.0	34.7	47.2	23.4	

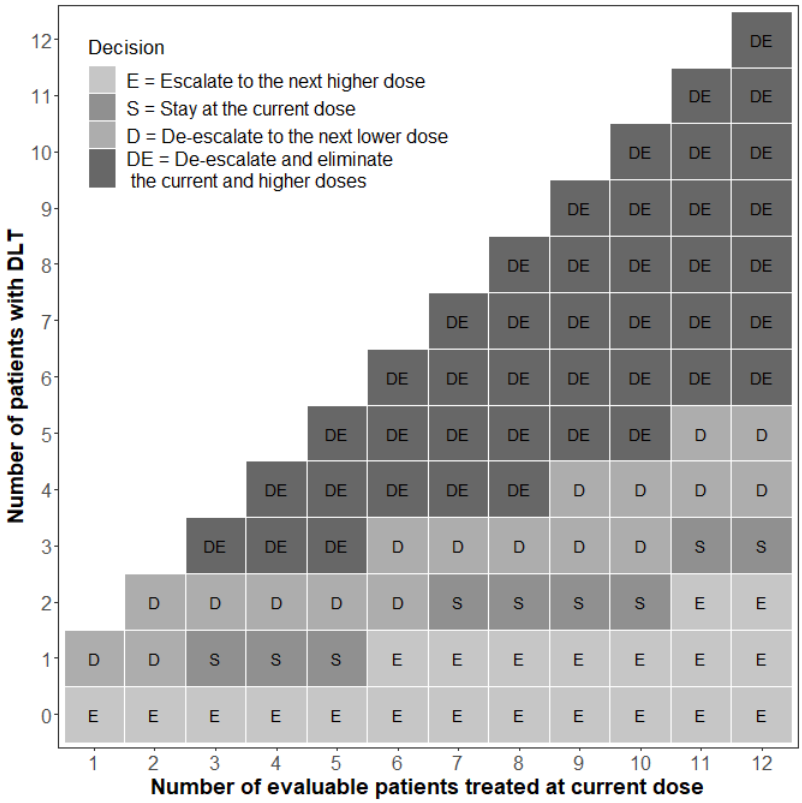
Note: "**% Early Stopping**" refers to early stopping due to excessive DLT.

Appendix 8.1: Trial and Design Specifications

Parameter	Value
Number of doses	3
Starting dose	1
Max sample size	30
Cohort size	3

Stop trial if # patients assigned to single dose reaches	12
Apply the 3+3 design run-in	True
Use accelerated titration	False
Target toxicity probability	0.25
Use the default alternatives to minimize decision errors	True
Alternative (unacceptable high toxicity) for optimization	Default
Alternative (unacceptable low toxicity) for optimization	Default
Eliminate dose threshold (pE)	0.95
Impose a more stringent safety stopping rule	False
Number of repetitions per scenario	1000
Random number generator seed	6

Appendix 8.2: Alternative BOIN decision table



BOIN design in case the winning treatment represents the use of an autologous CAR-T cell construct:

We will employ the Bayesian optimal interval (BOIN) design[17,18] to find the MTD. The BOIN design is implemented in a simple way similar to the traditional 3+3 design but is more flexible and possesses superior operating characteristics that are comparable to those of the more complex model-based designs, such as the continual reassessment method (CRM)[19].

The target dose-limiting toxicity (DLT) rate for the MTD is $\phi = 0.25$ and the maximum sample size is 30. We will enrol and treat patients in cohorts of size 2. DLTs have already been defined and only those DLTs that occur within the first cycle will be used for dose finding. As shown in Figure 8.1, the BOIN design uses the following rule, optimized to minimize the probability of incorrect dose assignment, to guide dose escalation/de-escalation:

- if the observed DLT rate at the current dose is ≤ 0.197, escalate the dose to the next higher dose level;
- if it is > 0.298, de-escalate the dose to the next lower dose level;
- otherwise, stay at the current dose.

For the purpose of overdose control, doses j and higher levels will be eliminated from further consideration if $\Pr(p_j > 0.25 \mid \text{data}) > 0.95$ and at least 3 evaluable patients have been treated at dose level j, where p_j is the true DLT rate of dose level j, $j = 1, \ldots, 3$. This posterior probability is evaluated based on the beta-binomial model $y_j \mid p_j \sim \text{binomial}(p_j)$ with $p_j \sim \text{uniform}(0, 1)$, where y_j is the number of patients experienced DLT at dose level j. When the lowest dose is eliminated, stop the trial for safety. The probability cutoff 0.95 is chosen to be consistent with the common practice that when the target DLT rate $\phi \leq 1/6$, a dose with 2/3 patients experiencing DLT is eliminated. The above dose escalation/de-escalation and elimination rule can be equivalently presented in Table 8.1, which will be used to conduct the trial.

The steps to implement the BOIN design are described as follows:

Addendum

1. Patients in the first cohort are treated at dose level 1.
2. To assign a dose to the next cohort of patients, conduct dose escalation/de-escalation according to the rule displayed in Table 8.1. When using Table 8.1, please note the following:
    a. "Eliminate" means eliminate the current and higher doses from the trial to prevent treating any future patients at these doses because they are overly toxic.
    b. When we eliminate a dose, automatically de-escalate the dose to the next lower level. When the lowest dose is eliminated, stop the trial for safety. In this case, no dose should be selected as the MTD.
    c. If none of the actions (i.e., escalation, de-escalation or elimination) is triggered, treat the new patients at the current dose.
    d. If the current dose is the lowest dose and the rule indicates dose de-escalation, treat the new patients at the lowest dose unless the number of DLTs reaches the elimination boundary, at which point terminate the trial for safety.
    e. If the current dose is the highest dose and the rule indicates dose escalation, treat the new patients at the highest dose.
3. Repeat step 2 until the maximum sample size of 30 is reached or stop the trial early if the number of evaluable patients treated at the current dose ≥ 12 and the decision according to Table 8.1 is to stay at the current dose.

* When none of the actions (i.e., escalate, de-escalate or eliminate) is triggered, stay at the current dose for treating the next cohort of patients. Note that "# of DLT" is the number of patients with at least 1 DLT, and "NA" means that a dose cannot be eliminated before treating 3 evaluable patients.

After the trial is completed, select the MTD based on isotonic regression as specified in[18]. This computation is implemented by the "Estimate MTD" tab of the BOIN Design Desktop Program[20]. Specifically, select as the MTD the dose for which the isotonic estimate of the DLT rate is closest to the target DLT rate. If there are ties, select the higher dose level when the isotonic estimate is lower than the target DLT rate and select the lower dose level when the isotonic estimate is greater than or equal to the target DLT rate.
Optional: [In case there is cohort expansion after identifying the MTD] Once we determine the MTD, an additional *n* patients will be enrolled for additional experience with safety and efficacy. We will use the elimination boundaries in Table 8.1 for DLT monitoring.
Operating characteristics
Table 8.2 shows the operating characteristics of the trial design based on 1000 simulations of the trial using the BOIN Design Desktop Program[20]. The operating characteristics show that the design selects the true MTD, if any, with high probability and allocates more patients to the dose levels with the DLT rate closest to the target of 0.25.

Table 8.2. Operating Characteristics of the BOIN design.

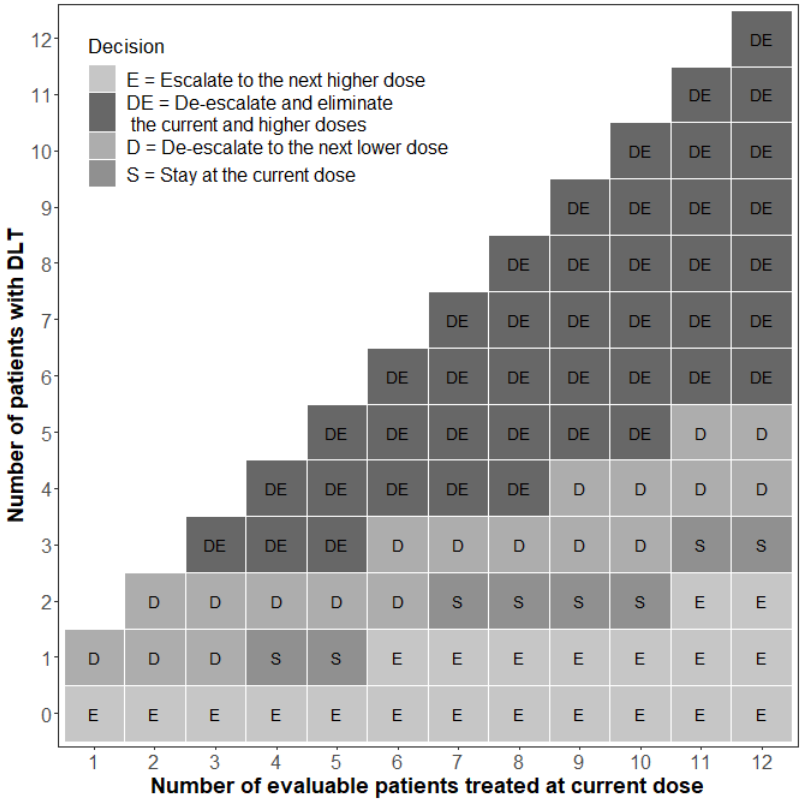
	Dose Level			Number of Patients	% Early Stopping
	1	2	3		
Scenario 1					
True DLT Rate	0.25	0.43	0.62		
Selection %	72.8	13.7	0.2		13.3
% Pts Treated	62.2	31.6	6.2	17.8	
Scenario 2					
True DLT Rate	0.11	0.25	0.39		
Selection %	22.7	59.7	17.0		0.6
% Pts Treated	32.5	44.0	23.5	23.2	
Scenario 3					
True DLT Rate	0.05	0.11	0.25		
Selection %	0.7	25.2	74.0		0.1
% Pts Treated	16.2	35.1	48.7	21.9	

Note: "**% Early Stopping**" refers to early stopping due to excessive DLT.

Appendix 8.3: Trial and Design Specifications

Parameter	Value
Number of doses	3
Starting dose	1
Max sample size	30
Cohort size	2
Stop trial if # patients assigned to single dose reaches	12
Apply the 3+3 design run-in	False
Use accelerated titration	False
Target toxicity probability	0.25
Use the default alternatives to minimize decision errors	True
Alternative (unacceptable high toxicity) for optimization	Default
Alternative (unacceptable low toxicity) for optimization	Default
Eliminate dose threshold (pE)	0.95
Impose a more stringent safety stopping rule	False
Number of repetitions per scenario	1000
Random number generator seed	6

Appendix 8.4: Alternative BOIN decision table



PRODUCT	PHASE	OBJECTIVES	VARIABLES	STATISTICAL ANALYSIS
TCRther	DOSE ESCALATION PHASE (N = 18, *9-18 patients will be enrolled, and the total number of participants will depend on the number of dose levels explored to identify the RDE*)	SAFETY (primary endpoint)	DLT (Dose-limiting toxicity) Incidence of AE Incidence of SAE NRM (non-relapse mortality) at 12 months	BOIN design Descriptive statistics Descriptive statistics Estimation, 95% CI
		TOLERABILITY (primary endpoint)	MTD (Maximum tolerated dose)	BOIN design
		PRELIMINARY EFFICACY (primary endpoint)	RDE (Recommended dose for expansion)	BOIN design
		PHARMACOLOGY (secondary endpoint)	PK (Pharmacokinetics) PD (Pharmacodynamics)	Trapezoidal method estimation Descriptive statistics
		ANTI-TUMOR ACTIVITY (secondary endpoint)	ORR (Overall Response Rate) CRR (Complete Remission Rate) DCR (Disease Control Rate) DOR (Duration of Response) DCR (Duration of Complete Remission) PFS (Progression-Free Survival)	Estimation, 95% CI Estimation, 95% CI Estimation, 95% CI Estimation, 95% CI Estimation, 95% CI Kaplan-Meier methodology
	EXPANSION PHASE (N = 15)	TO STABLISH PREDICTIVE MARKERS OF RESPONSE AND TOXICITY (secondary exploratory endpoint)	Predictive biomarkers of response Expansion and persistence of CAR-T cells Expansion and persistence of STAb-T cells Expansion and persistence of antigen-specific T cells Phenotypic characteristics Expression and production of cytokines Expression of markers of T-cell activation and exhaustion Ex vivo test of anti-tumoral activity T-cell clonality Subpopulations of normal T-cells Correlations with histological subtypes and target expression	For each time point, the mean cell count will be estimated with its 95% CI.

If the winning treatment represents the use of an autologous CART cell construct

Key considerations

- CAR-T therapy is single infusion per patient per dose level.
- Toxicity is acute (CRS, ICANS) and may require hospitalization.
- DLT evaluation period: 28 days post-infusion.

Key principles

- Single IV infusion over 30–60 minutes
- Step-up dosing optional if high tumour burden or risk of severe CRS
- Target dose per cohort defined by BOIN method

Tentative dose level	CAR-T cells infused
Level 1	1×10^6 CAR+ T cells/kg
Level 2	3×10^6 CAR+ T cells/kg
Level 3	5×10^6 CAR+ T cells/kg

- Cohort size: 2 patients (FIH)
- Escalation follows BOIN based on DLT occurrence

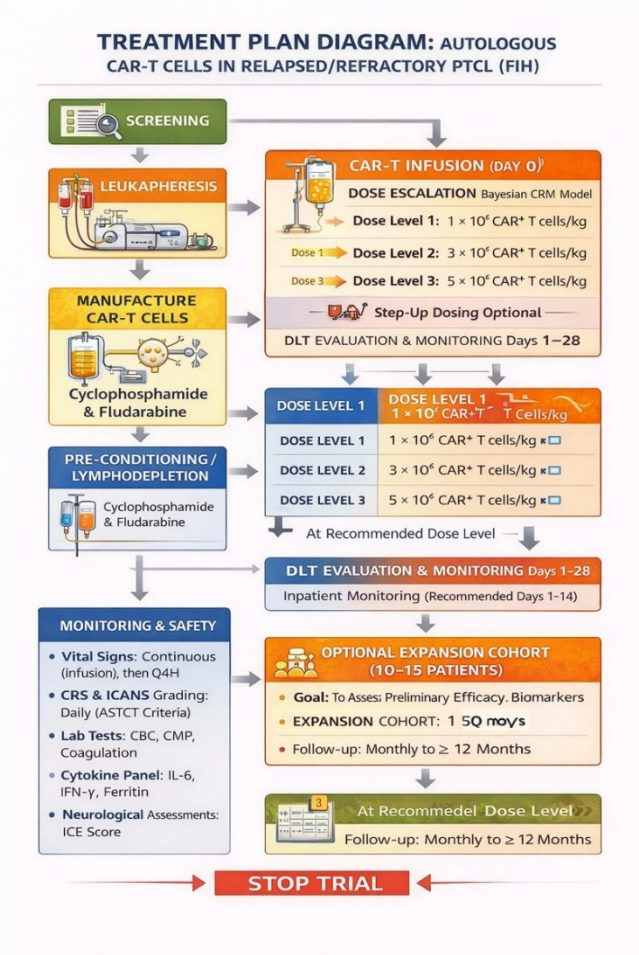
Summary treatment flow

1. **Screening:** confirm eligibility
2. **Leukapheresis:** collect autologous T cells
3. **Manufacture CAR-T cells:** 2–3 weeks. Bridging therapy allowed according to treating physician, with confirmation of disease persistence prior to lymphodepletion.
4. **Pre-conditioning:** lymphodepletion with cyclophosphamide (300 mg/m2 iv x 3 days) + fludarabine 30 mg/m2 iv x 3 days) (from day – 5 to day -3.
5. **Infusion:** single IV CAR-T dose
6. **Inpatient monitoring:** 7–14 days
7. **DLT observation:** 28 days
8. **Dose escalation:** BOIN method per cohort
9. **Expansion cohort:** RDE, monitor efficacy and biomarkers
10. **Long-term follow-up:** ≥12 months

Image elaborated with AI

Clinical Relevance and Ethical Considerations

RR PTCL are a heterogeneous group of aggressive hematologic malignancies associated with poor prognosis and limited effective treatment options after failure of conventional chemotherapy, autologous or allogeneic stem cell transplantation. Median overall survival in these patients is typically short, emphasizing an urgent need for novel therapeutic approaches.



Immunotherapeutic strategies, including bispecific monoclonal antibodies and CAR T-cell therapies, offer a rational mechanism-driven approach. Bispecific antibodies can redirect T-cell effectors toward tumor cells, whereas CAR T cells provide highly specific, potent, and potentially long-lasting cytotoxic activity. Preclinical and early clinical data suggest these therapies may induce meaningful anti-tumor responses even in heavily pretreated populations. Conducting a FIH Phase I trial allows for the systematic assessment of safety, tolerability, pharmacokinetics/pharmacodynamics, and preliminary efficacy, while also generating essential biomarker and mechanistic insights that can guide further development and dosing strategies.

FIH trials must carefully balance potential clinical benefit against known and unknown risks. Key ethical considerations include:

1. Patient Safety: Given the novelty of the intervention, the trial design has incorporated robust dose-escalation strategies and dose-limiting toxicity monitoring to minimize harm. Continuous monitoring for cytokine release syndrome, neurotoxicity, and T-cell aplasia is mandatory.
2. Informed Consent: Patients will be fully informed about the experimental nature of the therapy, potential risks and benefits, available alternative therapies, and the uncertainty inherent in first-in-human studies. Consent will be obtained prior to any study-specific procedures.
3. Scientific Justification: Enrolment is ethically justified only if there is a reasonable scientific rationale and preclinical evidence supporting potential efficacy and manageable safety. The trial has been designed to maximize knowledge gain while minimizing patient exposure to risk.
4. Independent Oversight: The trial will include Data Safety Monitoring Boards (DSMBs) or independent safety committees to review adverse events and stop or modify the study if risks exceed acceptable thresholds.
5. Fair Patient Selection: Inclusion and exclusion criteria ensure that only patients for whom standard therapies have failed or are unavailable are enrolled, avoiding exploitation of vulnerable populations.

Although this study is not designed to provide definitive conclusions on sex or gender-related differences, it will contribute valuable exploratory data to a largely understudied area. Ensuring that sex and gender are considered in patient documentation and data analysis aligns with ethical principles of inclusivity in clinical research.

Regulatory Framework

Competent Authority: The Agencia Española de Medicamentos y Productos Sanitarios (AEMPS) is the national authority responsible for the authorization and oversight of clinical trials involving investigational medicinal products (IMPs), including advanced therapy medicinal products (ATMPs) such as CAR T cells. We will submit a clinical trial application (CTA) to AEMPS including preclinical pharmacology, toxicology, and manufacturing data, as well as the trial protocol, investigator brochure, and informed consent forms.

1. Ethics Committee Approval: We will also obtain approval from an independent Research Ethics Committee (Comité de Ética de la Investigación, CEI) at the institution where the study is conducted.
2. Compliance with European and International Regulations: Spanish FIH trials follow EU Clinical Trials Regulation (EU No 536/2014), EMA guidance on first-in-human studies, and ICH-GCP (International Council for Harmonisation – Good Clinical Practice). CAR T-cell and other ATMP trials are also subject to

Addendum

Directive 2001/83/EC and Regulation (EC) No 1394/2007 on ATMPs. Compliance with Lugano criteria, EMA FIH guidelines, and pharmacovigilance standards is expected.

3. Risk Mitigation and Dose Escalation Design: Dose-escalation strategies have already been integrated to minimize patient exposure to toxic doses. Detailed stopping rules, dose-limiting toxicity criteria, and safety monitoring plans are already existing in the protocol.

Ethical Considerations

1. Patient Safety and Minimization of Risk: FIH trials involve patients with high unmet medical need, but safety must remain paramount. Intensive monitoring for acute toxicities (e.g., cytokine release syndrome, neurotoxicity) is mandatory and already included in the protocol.
2. Informed Consent: Patients will receive comprehensive oral and written information about the investigational nature of the therapy, potential benefits and risks, alternative treatments, and the voluntary nature of participation. Consent will comply with Spanish Law 14/2007 on Biomedical Research and EU General Data Protection Regulation (GDPR) for data privacy.
3. Scientific and Social Value: Enrolment is ethically justified only when the trial has a sound scientific rationale, preclinical evidence supports safety and biological activity, and knowledge gained could benefit future patients. Vulnerable populations will not be exposed unless there is direct clinical benefit, and patient selection must be fair and transparent.
4. Transparency and Reporting: The trial will be registered in EU Clinical Trials Register and published according to ICMJE guidelines. Adverse events will be reported to AEMPS, CEI, and EMA (if applicable) within prescribed timelines.

Multimeric reagents for T cell staining. We will prioritize transient co-transfections (MHC- and BirA Ligase-encoding plasmids) in Expi293 for attaining biotinylated MHC molecules, and we will use the insect cell baculovirus system for the production of required accessory molecules (e.g., the peptide exchange catalyst for MHCII molecules as a soluble molecule sDM) (Figure 8.2). We have at hand more than 20 constructs (16 for MHCII and 6 for MHCI) to express MHC molecules and we will expand the portfolio of MHCI molecules present in TCL patients using the same construct design. Purification, protein modification and/or cleanup methods have been described by us[14]. MHCI molecules are covalently linked via G_4S linker to beta 2 microglobulin and include a double mutant in conserved positions (Y84C-A138C) yielding “empty and loadable molecules”. In case of MHCII molecules, the beta chain encodes the MHCII-placeholder peptide CLIP spaced by a G_4S linker and a Thrombin cleavage site. These molecules require thrombin cleavage prior to any downstream usage. Both MHCI and II include a Biotin Acceptor Site and His-Tag allowing their purification and site-specific biotinylation.
MHC is conjugated to commercial Fluorophore-labelled Streptavidin (Fluo-SA). Tetramerized MHCI molecules are efficiently loaded using a relatively mild peptide excess whereas tetramerized MHCII require the MHC-II peptide loading catalyst (expressed here as sDM). Size exclusion chromatography ensures the homogeneity of the tetramers and remove undesired molecules. Adequate peptide loading is assessed by eluting the loaded peptides and MS measurement as referred for immunopeptidomics studies. Note as well that: 1) the peptide can be incorporated at the cDNA level of both MHCI and MHCII constructs, and 2) the array of different dyes conjugated to SA enable the building up of personalized tetramer panels for each TCL patient.

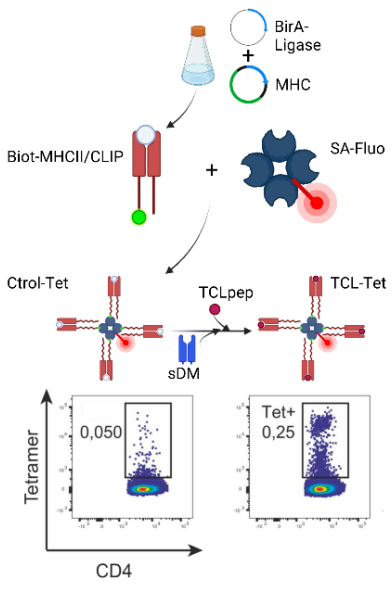


Figure 8.2. *Tetramer preparation. Experimental workflow for attaining MHCII tetramers. Expi293 cells grown in suspension are transiently co-transfected with a plasmid encoding a given MHC and the BirA-Ligase. Recombinant MHC is biotinylated at the cellular level and then secreted and accumulated in the medium, from where it is purified. The construct design implies that the protein is pre-loaded with the place-holder peptide CLIP tethered via G4S linker to the beta chain and spaced by a Thrombin cleavage site. Pretreatment with Thrombin (not shown) and conjugation to Fluorescently labelled Streptavidin (SA-Fluo) yields MHCII tetramers pre-loaded with CLIP (control Ctrol-Tet). CLIP-loaded tetramers incubated with TCL-relevant peptides and soluble HLA-DM (sDM, the peptide loading catalyst for MHCII presentation) results in MHCII-tetramers loaded with TCL-relevant peptides. Representative dot-plots for Tetramer positive cells for a specific T cell epitope upon ex vivo expansion. For MHCI tetramers we use a similar workflow. In this case cDNA constructs encode MHCI molecules tethered to beta 2 microglobulin and mutated in the conserved Y84C-A138C positions. The result of protein expression is a biotinylated “empty” MHCI that can be tetramerized. Tetramerized MHCI reagents need to be loaded with control and TCL-relevant peptides to yield Ctrol-Tet and TCL-Tet respectively. The final result is the specific stain of antigen-specific T cells that can be further isolated and/or immunophenotyped. Tetramer stains provide direct evidence for the expansion of specific T cells at a given time or during the course of a treatment. Thus, when applied in the context of TCRther4TCL we will be able to monitor the performance of the peptide and mRNA vaccines.*

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