ESMO GOOD SCIENCE BETTER MEDICINE BEST PRACTICE

ESMO IMMUNO-ONCOLOGY AND TECHNOLOGY

ORIGINAL ARTICLE

Preclinical data and design of a phase I clinical trial of neoantigen-reactive TILs for advanced epithelial or ICB-resistant solid cancers

J. Palomero[1†], V. Galvao[2†], I. Creus[1], J. Lostes[2], M. Aylagas[3], A. Marín-Bayo[1], M. Rotxés[2], M. Sanz[2], M. Lozano-Rabella[1], A. Garcia-Garijo[1], A. Yuste-Estevanez[1], D. Grases[1], J. Díaz-Gómez[1], J. González[1], J. F. Navarro[1], J. J. Gartner[4], I. Braña[2], X. Villalobos[1], N. Bayó-Puxan[1], J. Jiménez[1], A. N. Palazón[1], S. Muñoz[1], G. Villacampa[1], A. Piris-Giménez[1], P. Barba[5], M. Codinach[3,6], L. Rodríguez[3], S. Querol[3], E. Muñoz-Couselo[2], J. Tabernero[1,2,7], S. Martín-Lluesma[1,8], A. Gros[1*] & E. Garralda[2*]

[1]Vall d'Hebron Institute of Oncology (VHIO), Vall d'Hebron Barcelona Hospital Campus, Barcelona; [2]Medical Oncology Department, Vall d'Hebron Barcelona Hospital Campus, Vall d'Hebron Institute of Oncology (VHIO), Barcelona; [3]Banc de Sang i Teixits (Blood and Tissue Bank, BST), Barcelona, Spain; [4]Surgery Branch, National Cancer Institute, Bethesda, USA; [5]Hematology Department, Vall d'Hebron Barcelona Hospital Campus, Vall d'Hebron Institute of Oncology (VHIO), Barcelona; [6]Vall d'Hebron Research Institute—Autonomous University of Barcelona (VHIR-UAB), Barcelona; [7]Institute of Oncology (IOB)-Quirón, Barcelona-Universitat de Vic-Universitat Central de Catalunya (UVic-UCC), Vic-CIBER en oncología (CIBERONC) ISCIII, Madrid; [8]Department of Basic Medical Sciences, Faculty of Medicine, University of Sant Pablo-CEU, CEU Universities, Madrid, Spain

Available online 2 December 2024

Background: Adoptive cell therapy (ACT) of *ex vivo* expanded tumor-infiltrating lymphocytes (TILs) can mediate objective tumor regression in 28%-49% of metastatic melanoma patients. However, the efficacy of TIL therapy in most epithelial cancers remains limited. We present the design of a phase I clinical study that aims to assess the safety and efficacy of NEXTGEN-TIL, a TIL product selected based on *ex vivo* neoantigen recognition, in patients with advanced epithelial tumors and immune checkpoint blockade (ICB)-resistant solid tumors.

Materials and methods: Pre-rapid expansion protocol (REP) TIL cultures expanded in high-dose interleukin 2 (HD-IL-2) from patients with metastatic solid tumors were screened for recognition of autologous tumor cell lines (TCLs) and/or neoantigens. Six good manufacturing practice (GMP)-grade validations of pre-REP TIL expansion were carried out and TIL cultures from these six intermediate products were selected to carry out the clinical-scale GMP validation of the REP.

Results: TILs expanded in 82% of patient-derived tumor biopsies across different cancer types and these frequently contained tumor- and neoantigen-reactive T cells. During GMP validations, a variable number of TIL cultures expanded, constituting the intermediate products (pre-REP). Three finished products were manufactured using a REP which reached cell doses ranging from 4.3e9 to 1.1e11 and met the established specifications. The NEXTGEN-TIL clinical trial entails a first expansion of TILs from tumor fragments in HD-IL-2 followed by TIL screening for neoantigen recognition and REP of selected neoantigen-reactive TIL cultures. Treatment involves a classical non-myeloablative lymphodepleting chemotherapy followed by NEXTGEN-TIL product administration together with HD-IL-2.

Conclusions: NEXTGEN-TIL exploits ex vivo expanded neoantigen-reactive TIL to potentially improve efficacy in patients with epithelial and ICB-resistant tumors, with a safety profile like traditional TILs.

Key words: tumor-infiltrating lymphocytes, adoptive cell transfer, neoantigen-reactive T cells, phase I clinical trial

INTRODUCTION

Immune checkpoint blockade (ICB) has shown unprecedented results in a variety of aggressive and hard-to-treat cancers.[1] However, transient responses and low response rates in many metastatic epithelial cancers remain significant challenges.[2] Thus, there is an unmet need to develop more effective therapies. Adoptive cell therapy (ACT) with autologous tumor-infiltrating lymphocytes (TILs), TIL-based ACT (TIL-ACT), aims to augment the immune system's ability to specifically recognize and kill cancer cells.[3,4] This therapeutic regimen consists of a non-myeloablative lymphodepleting (NMA-LD) chemotherapy followed by the infusion of *ex vivo* expanded autologous TILs and administration of high-dose interleukin 2 (HD-IL-2) to sustain lymphocyte activity.[5] TIL-ACT has been extensively explored for the treatment of metastatic melanoma and represents an interesting

*Correspondence to: Dr Alena Gros, C/Natzaret 115-117, Cellex Center Lab 4.04A, 08035 Barcelona, Spain. Tel: +34-93-254-34-50x8700
E-mail: agros@vhio.net (A. Gros).
Dr Elena Garralda, Pg. de la Vall d'Hebron, 119, Horta-Guinardó, 08035 Barcelona, Spain. Tel: +34-93-489-43-32x4332
E-mail: egarralda@vhio.net (E. Garralda).

†These authors contributed equally to this work.

therapeutic alternative.[6] In a meta-analysis combining data from 13 studies (published 1988-2016) using TIL-ACT, in 410 heavily pretreated patients (some with brain metastasis), the pooled objective response rate (ORR) estimate was 41%, and the complete response rate was 12%.[7] Subsequent studies including ICB and targeted therapy-refractory population have shown an ORR ranging between 28% and 49%,[8-13] leading to its accelerated approval for the treatment of adult patients with unresectable or metastatic melanoma refractory to standard therapy.[14] A recent meta-analysis found no difference in ORR or complete response rate between studies with and without prior anti-PD-1/PD-L1 treatment in TIL-ACT efficacy for melanoma patients. This suggests that previous anti-PD-1/PD-L1 treatment does not affect clinical response or survival benefit from TIL-ACT in advanced cutaneous melanoma, supporting its use as a second-line treatment option.[15] TILs can be successfully expanded from various types of tumors, and promising preliminary antitumor responses have been observed in non-small-cell lung cancer patients[16] and human papillomavirus-positive (HPV+) cancer patients.[17] Therefore, current evidence supports further testing of this therapeutic approach in solid tumors other than melanoma. Nevertheless, the adoptive transfer of 'unselected TIL' (without selection of anticancer T cells), commonly used to treat patients with metastatic melanoma, has shown limited activity in other epithelial cancers.[18,19]

Accumulating evidence supports that lymphocytes targeting neoantigens play an important role in the antitumor efficacy of cancer immunotherapy, including TIL therapy.[20-26] TIL infusion products from melanoma patients who experienced complete tumor regression following TIL therapy frequently contain neoantigen-reactive TILs.[25-27] The absolute number of infused tumor-reactive TILs,[28] tumor mutation burden, neoantigen burden[24] as well as the frequency of neoantigen-reactive lymphocytes[26] have been positively associated with TIL therapy efficacy in patients with advanced melanoma. This association between the clinical activity of TIL therapy and mutational burden and detection of neoantigen reactivity was also observed in patients refractory to ICB,[29] suggesting that delivering an enriched neoantigen-reactive TIL product or selecting patients for TIL therapy based on the detection of neoantigen reactivity could improve clinical outcome in this setting. In addition, adoptive transfer of TIL cultures selected for neoantigen recognition has been shown to induce antitumor responses in selected patients with cholangiocarcinoma, colorectal (CRC), breast (BrCa) and cervical cancers.[30-34] These findings suggest that enriching TILs for neoantigen recognition could prove critical to enhance the efficacy of TIL therapy in patients with epithelial cancers.

In this clinical study, we propose to manufacture a T-cell product composed of TIL cultures selected for their ability to recognize tumor-specific neoantigens and to use this product to individually treat patients with metastatic, refractory epithelial tumors as well as ICB-resistant solid tumors. Besides, we also propose screening tumors and T cells at baseline and after treatment to determine whether specific phenotypes and functional features may be related to clinical outcomes.

MATERIALS AND METHODS

Patient samples and TIL expansion for preclinical testing

For assessing the preclinical expansion of TILs from core tumor biopsies and for testing the clinical-scale good manufacturing practice (GMP) validation of TIL expansion, patients were enrolled on a project approved by the institutional review board of the Vall d'Hebron Hospital [PR(AG)252-2016, PR(AG)318-2018, respectively] and signed an informed consent. All patients had metastatic solid tumors with variable tumor burden and had received a wide range of prior therapies including, in some instances, immunotherapy. A fresh tumor biopsy and at least one blood sample were obtained for each patient. Peripheral blood mononuclear cells (PBMCs) were isolated from the blood samples or leukapheresis using a Ficoll gradient centrifugation and were cryopreserved for future use.

To expand TILs, core needle (trucut) biopsies from tumors (~2 mm × 12 mm) were cut into 6-18 2 mm × 2 mm fragments, which were used to expand independent TIL cultures in 24-well plates in 1 : 1 T-cell medium [RPMI-1640 with L-glutamine and AIM-V (Thermo Fisher Scientific, Paisley, UK) supplemented with penicillin 100 U/ml, streptomycin 100 μg/ml (Thermo Fisher Scientific, Paisley, UK), Hepes 12.5 mM (Biowest, Nuaillé, France), 10% human serum (prepared in-house) and IL-2 6000 IU (Novartis, Schiphol, Netherlands)]. TIL expansion was considered successful when the TIL culture reached confluency in 4 wells from a 24-well plate, time at which they were cryopreserved for future use. This phase of expansion constituted the pre-rapid expansion protocol (REP). TILs that did not reach this level of expansion were not considered for subsequent neoantigen screening. Additional tumor biopsies were frozen in optimal cutting temperature compound and were used as a source for DNA and RNA extraction.

Cell lines. Fresh tumor-derived fragments or 1 × 10e6 tumor-single suspension cells were cultured in RPMI-1640 with 20% Hyclone fetal bovine serum (FBS) (GE Healthcare), penicillin 100 U/ml, streptomycin 100 μg/ml and Hepes 25 mM (Thermo Fisher Scientific), at 37°C in 5% CO_2. Medium was replaced monthly until cell lines were established. Established tumor cell lines (TCLs) were sequenced and cryopreserved at early passage. NIH 3T3 CD40L cells were obtained from the National Cancer Institute by transduction of NIH 3T3 cells (American Type Culture Collection) with a retrovirus encoding CD40L. NIH 3T3 CD40L cells were maintained in Dulbecco's modified Eagle's medium with 10% Hyclone FBS, penicillin 100 U/ml, streptomycin 100 μg/ml and Hepes 25 mM, at 37°C in 5% CO_2.

Identification of non-synonymous mutations by tumor whole-exome sequencing and NA sequencing

Genomic DNA and total RNA were purified from optimal cutting temperature compound-embedded tumor sections

and normal DNA was extracted from PBMCs. The percentage of tumor was assessed by immunohistochemistry. DNA concentration was measured using Qubit™ Fluorometer (Thermo Fisher Scientific) and the quality and size of tumor and normal DNA were assessed using 4200 TapeStation (Agilent). Whole-exome sequencing (WES) libraries were generated by exome capture of ∼20 000 coding genes using SureSelect human. The All Exon V6 Kit (Agilent Technologies, Santa Clara, California, USA) and paired-end sequencing were carried out in the Illumina NovaSeq 6000 platform. The average sequencing depth ranged from 200× to 300× for each of the individual libraries generated. Alignment of WES to the reference human genome build hg19 was carried out using bwa-men[35] before quality trimming with TrimGalore.[36] Aligned reads were processed following the GATK4[37] best practices (MarkDuplicates and BaseRecalibration). Variant calling was carried out with VarScan2,[38] Strelka2,[39] SomaticSniper[40] and Mutect2.[41] All the somatic non-synonymous mutation (NSM) variants detected were filtered according to the following criteria: minimum coverage of 10 reads, minimum 4 variant reads, >7% variant allele frequency and called by two or more callers (single nucleotide variants) or one for insertions and deletions. Filtered variants were merged and annotated using VEP[42] and epitopes were generated from the variants using Varcode.[43] Affinity binding scores were assigned to the epitopes using MHCflurry[44] and the human leukocyte antigen (HLA) typing obtained from OptiType.[45] Epitopes were manually vetted using integrative genomics viewer and NSMs were selected as candidate neoantigens for generation of tandem minigenes (TMGs) based on the presence in the tumor. For VHIO-08, epitopes were prioritized for TMG generation using the affinity binding scores.

When possible, a messenger RNA (mRNA) sequencing library was generated from the respective samples using the Illumina TruSeq RNA Library Prep Kit. Alignment of RNA to the reference human genome build hg19 was carried out using STAR[46] before quality and adapter trimming with TrimGalore. Aligned reads were processed following the GATK4 best practices (MarkDuplicates, IndelRealignment and BaseRecalibration). Gene counts and fragments per million mapped reads values were calculated using featureCounts[47] and used to assess the expression of candidate mutations. The analysis pipeline (WES and RNA) is available at: https://github.com/jfnavarro/hla_pipeline.

Design and generation of TMGs

NSMs for immunological screening were selected based on their detection in the tumor exome. For each NSM identified by WES, one minigene construct was designed encoding the mutated amino acid (aa) flanked by 12 aa of the wild-type (wt) sequence; up to 25 minigenes were stringed together to generate TMG in a single open reading frame. TMG constructs were codon optimized and subcloned into pcDNA3.1+ modified to include two copies of the β-globin 5′ untranslated region to enhance RNA stability. *In vitro* transcribed RNA was then generated using the TMG constructs as a template using the HiScribe T7 RCA mRNA Kit with tailing (New England Biolabs) as instructed by the manufacturer and was subsequently used to transfect autologous B cells.

Transfection of autologous B cells with TMG RNA

Antigen-presenting B-cell lines were generated by CD19 microbead (Miltenyi Biotec) isolation from PBMCs. B cells were expanded using irradiated NIH 3T3 CD40L cells in B-cell medium, comprising Iscove's modified Dulbecco's medium (Quality Biological Inc.) with 10% human AB serum (processed in-house), penicillin 100 U/ml, streptomycin 100 µg/ml, L-glutamine 2 mM and IL-4 200 U/ml (PeproTech). On day 3, fresh B-cell medium was replenished. B cells were used fresh or cryopreserved at day 5-6. When used after cryopreservation, cells were thawed into B-cell medium the day before electroporation.

As a complementary neoantigen-reactive T-cell screening approach, 25-mer peptides encoding for one NSM and minimal epitopes were also purchased from JPT. Crude peptides were used for the initial *in vitro* screening of T cells. To validate reactivities observed in the initial screen, selected high-performance liquid chromatography (HPLC)-purified mutant peptides and their wt counterparts were purchased.

Peptide synthesis and pulsing. We purchased peptides from JPT. Crude peptides were used for the initial *in vitro* screening of T cells. To validate reactivities observed in the initial screen, we ordered selected HPLC-purified mutant peptides and their wt counterparts. B cells were harvested, washed and resuspended at 2e6-5e6 cells/ml in their corresponding media supplemented with the appropriate cytokines with 10 µg/ml or 1 µg/ml for 25-mer and minimal epitopes, respectively. Pulsing of peptide pools (PPs) was carried out at a final concentration of 10 µg/ml per peptide. After overnight pulsing, B cells were resuspended in T-cell medium, and immediately used in coculture assays.

Assessment of T-cell reactivity using interferon-γ ELISPOT assay and detection of 4-1BB

To test reactivity to neoantigens, 2e4-5e4 T cells were co-incubated with 1e5 or 3e5 peptide-pulsed or TMG RNA-electroporated B cells, respectively. Media alone were used as background for the assay, and irrelevant PPs or TMGs were used as negative controls. Alternatively, T cells were cocultured with 1e5 tumor cells to evaluate tumor reactivity. In this case, T cells were cultured with media and an irrelevant HLA mismatched TCL as negative controls. All cocultures were carried out in the absence of exogenously added cytokines. For all the assays, plate-bound anti-CD3 (OKT3) (1 µg/ml; Miltenyi Biotec) was used as a positive control.

Cell-surface T-cell activation-induced receptors OX40 and 4-1BB were assessed by flow cytometry at ∼20 h after co-incubation. Briefly, cocultured cells were pelleted, resuspended in staining buffer and incubated with anti-

CD3, -CD4, -CD8, -4-1BB and -OX40 antibodies for 30 min at 4°C. Cells were washed, resuspended in staining buffer containing PI (Sigma-Aldrich) and acquired on a BD LSRFortessa flow cytometer. T-cell activation was also measured using an interferon (IFN)-γ ELISPOT assay to detect secreted IFN-γ.

The criteria used to classify tested TIL cultures as tumor- or neoantigen-reactive were a frequency of expression of 4-1BB or OX40 on CD8+ or CD4+ T cells ≥0.5% and ≥2-fold the frequency of the corresponding negative control, and/or the presence of ≥40 IFN-γ spots and >2× the negative control. Moreover, the reactivity had to be detected in two consecutive coculture experiments.

Identification of the specific neoantigen recognized by reactive TILs in preclinical studies

To identify the specific mutations recognized within the reactive TMG/PP, expanded TIL cultures were cocultured with TMG-electroporated autologous B cells for 20 h. CD3+CD8+ cells expressing 4-1BB were sorted in BD FACS Aria and expanded using a REP. Briefly, 4-1BB+CD8+ cells were seeded in T25 flasks in T-cell medium containing anti-CD3, IL-2 3000 IU/ml and irradiated PBMCs pooled from three allogeneic donors. At day 14, cells were harvested and either used in coculture experiments or cryopreserved until further analysis. Crude 25-mer peptide preparations were used for neoantigen screening, and the reactivities were further confirmed with HPLC-grade peptides.

TIL expansion for GMP validations, pre-REP and REP

GMP-grade validations of TIL expansion were carried out for the pre-REP phase until cryopreservation of the intermediate TIL cultures and for the REP phase separately. Of note, the validation of the REP was carried out without screening the neoantigen reactivity of the intermediate TIL cultures. TIL expansion from tumor trucut biopsies was done following the same procedure as the TIL expansion for preclinical testing at the qualified GMP facility of the Banc de Sang i Texits (BST), Barcelona. Briefly, TIL cultures reaching confluency in 4 wells from a 24-well plate were analyzed by flow cytometry and when TIL cultures contained a minimum of 9e6 viable total cells, a viability of >70% and a frequency of >95% CD45+ cells, they were cryopreserved in at least three cryovials, constituting the intermediate cell products.

The validations of the REP phase were done following a very similar procedure to the one for the preclinical studies. However, some modifications were incorporated to obtain sufficient cell dose of the final product. Briefly, the intermediate TIL cultures were thawed and 1e6-3e6 TILs were seeded in G-REX vessels with irradiated PBMCs (pooled from 10-12 donors) in T-cell medium containing anti-CD3 and IL-2 3000 IU/ml. TIL cultures were split and REP was harvested on day 11.

RESULTS

Preclinical testing of TIL expansion from core tumor biopsies and tumor and neoantigen reactivity assessment

Given that surgical resection is not standard for patients with metastatic disease, and it considerably increases the costs of TIL therapy, we aimed to expand TILs from image-guided tumor core biopsies. To test expansion of TILs from tumor core biopsies, we expanded TILs from $n = 154$ trucut tumor biopsies from patients presenting different types of solid tumors all of which were refractory to standard therapies before inclusion in phase I clinical trial. TILs were successfully expanded from 82% of the biopsies (Figure 1). We observed a high variability in the percentage of tumor fragments giving rise to *ex vivo* expanded TILs, but TILs could be generated irrespective of the origin of the tumor and irrespective of whether the patient was immunotherapy experienced (Figure 2A and B). We generated 10 short-term culture TCLs from these biopsies, which enabled us to test whether TILs expanded at the pre-REP phase recognized the corresponding autologous TCL. As shown in Figure 2C, for patient VHIO-055, 4 out of 18 TIL cultures expanded *ex vivo* contained tumor-reactive TILs and were classified as tumor-reactive (TIL F6, F8, F14 and F18) since 4-1BB was up-regulated on CD8+ cells and IFN-γ was secreted when TIL cultures were cocultured against their autologous TCL, but not an irrelevant one. We carried out tumor reactivity assays by co-culturing the expanded TIL cultures against TCLs in 10 patients and 9 out of 10 independent cases of TIL cultures contained tumor-reactive TILs (Figure 2D). Importantly, the frequency of tumor-reactive TILs based on 4-1BB or OX40 up-regulation was very heterogeneous, ranging from 1.5% to 68% (Table 1).

Next, to test TILs derived from tumor core needle biopsies for neoantigen reactivity in preclinical studies, we carried out WES from tumor and normal DNA of four patients VHIO-008, VHIO-009, VHIO-029 and VHIO-055, and complemented it with RNA sequencing of the tumor samples, to identify all tumor-specific NSMs expressed and screened the *ex vivo* expanded TIL cultures for recognition of B cells electroporated with TMGs or pulsed with PPs encoding for the candidate neoantigens identified. This method to screen for neoantigen recognition was first reported in 2014,[27,33] and can be exploited to identify and select for TILs capable of recognizing neoantigens and autologous tumor.[48,49]

The results of a representative screening of VHIO-009 TILs for neoantigen recognition are shown in Figure 3. In this patient, TIL cultures 8, 9 and 10 recognized neoantigens encoded by TMG7, TMG2 or TMG6 and TMG8, respectively, as measured either by IFN-γ spots or by up-regulation of 4-1BB on the CD3+CD8+ TILs (Figure 3A and B). As shown in Figure 3C, TIL culture 9 recognized TP53RKp.S108C but not other mutations included in TMG2/PP2; moreover, it was HLA-I restricted (data not shown). TIL culture 10 recognized NSMCE1p.N156D (HLA-II restricted) and GBPp.E359K (HLA-I restricted) (data not shown). The neoantigen within TMG7

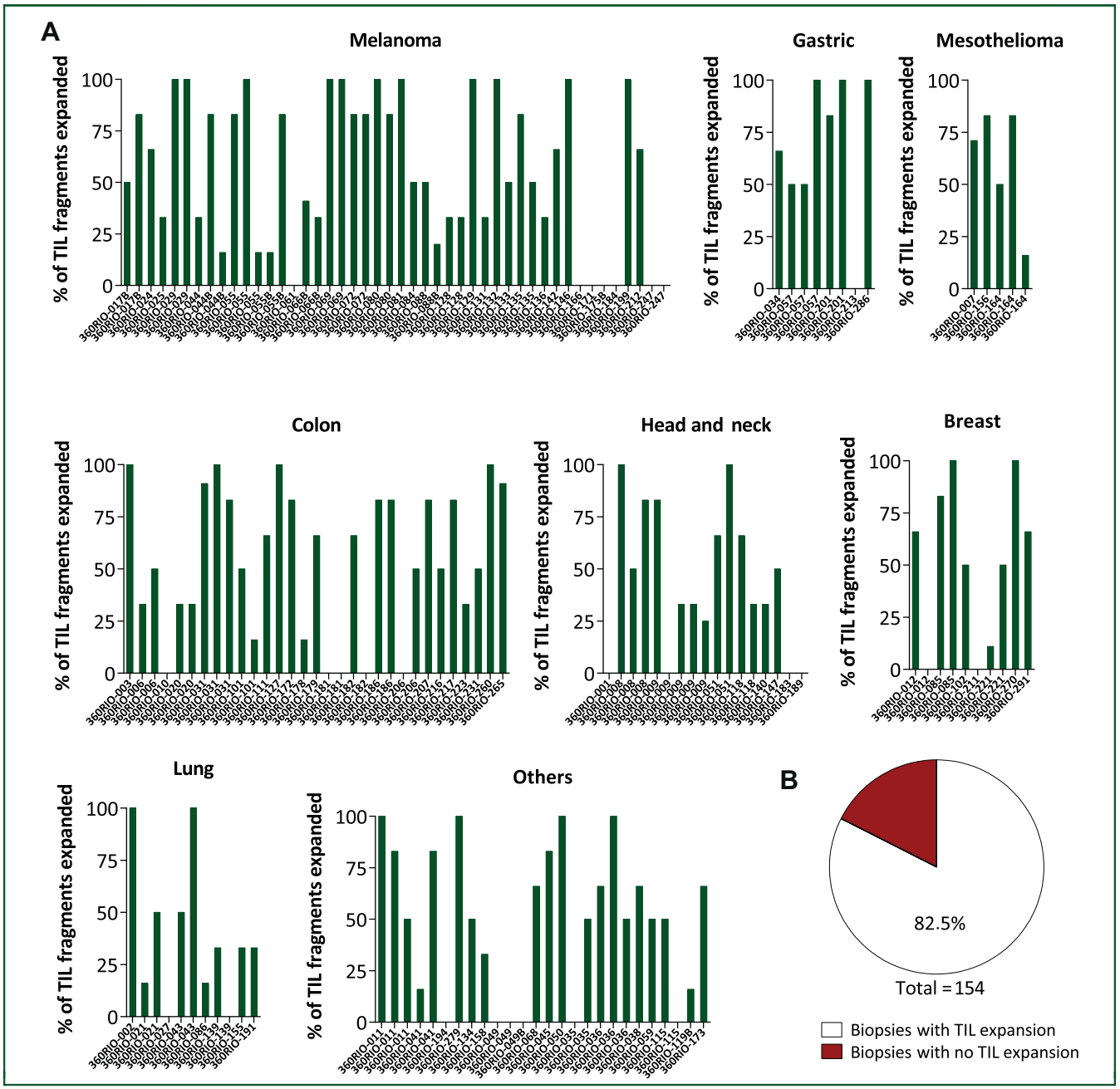

Figure 1. Expansion of TILs from core tumor biopsies from different histologies. (A) Core tumor biopsies derived from the specified tumor histologies were cut into six independent 2-3 mm fragments and cultured in high-dose IL-2 from 2 to 5 weeks. TIL expansion from each independent tumor fragment was considered successful when TILs expanded up to 4 confluent wells from a 24-well plate. The percentage of TIL fragments expanded out of the total number of tumor fragments initially cultured in IL-2 was calculated as a percentage and is plotted for each individual biopsy. (B) Percentage of tumor biopsies from which at least one TIL fragment expanded up to 4 wells from a 24-well plate ($n = 154$ tumor biopsies).
IL-2, interleukin 2; TIL, tumor-infiltrating lymphocyte.

that was recognized by TIL culture 8 is yet unknown. The rest of the TIL cultures did not appear to recognize any of the neoantigens tested. Interestingly, TIL cultures 8, 9 and 10 were among the five TIL cultures that displayed the highest frequency of tumor-reactive CD8+ TILs based on the expression of 4-1BB (data not shown).

TIL cultures derived from three additional patients, VHIO-008, VHIO-029 and VHIO-055, were also screened for neoantigen recognition in detail and the results are summarized in Table 2. The number of NSMs identified for these patients' tumors ranged from 183 to 2485. Given the high number of mutations identified in VHIO-008, we selected the top-ranking candidate mutations predicted to bind to the patient's HLA molecules and constructed 12 TMGs encoding for up to 24 mutated minigenes each. VHIO-008 TIL3 and TIL4 contained CD8+ T cells targeting MAGEB2 p.E167Q, encoded by TMG4, and TIL5 contained CD8+ T cells recognizing RPL14 p.H20Y, encoded by TMG3. Hence, three TIL cultures recognized two HLA-I restricted neoantigens detected by WES. VHIO-029 TILs were screened for recognition of all candidate neoantigens identified by WES. In this patient, TIL cultures 3 and 5 were found to recognize

https://doi.org/10.1016/j.iotech.2024.101030

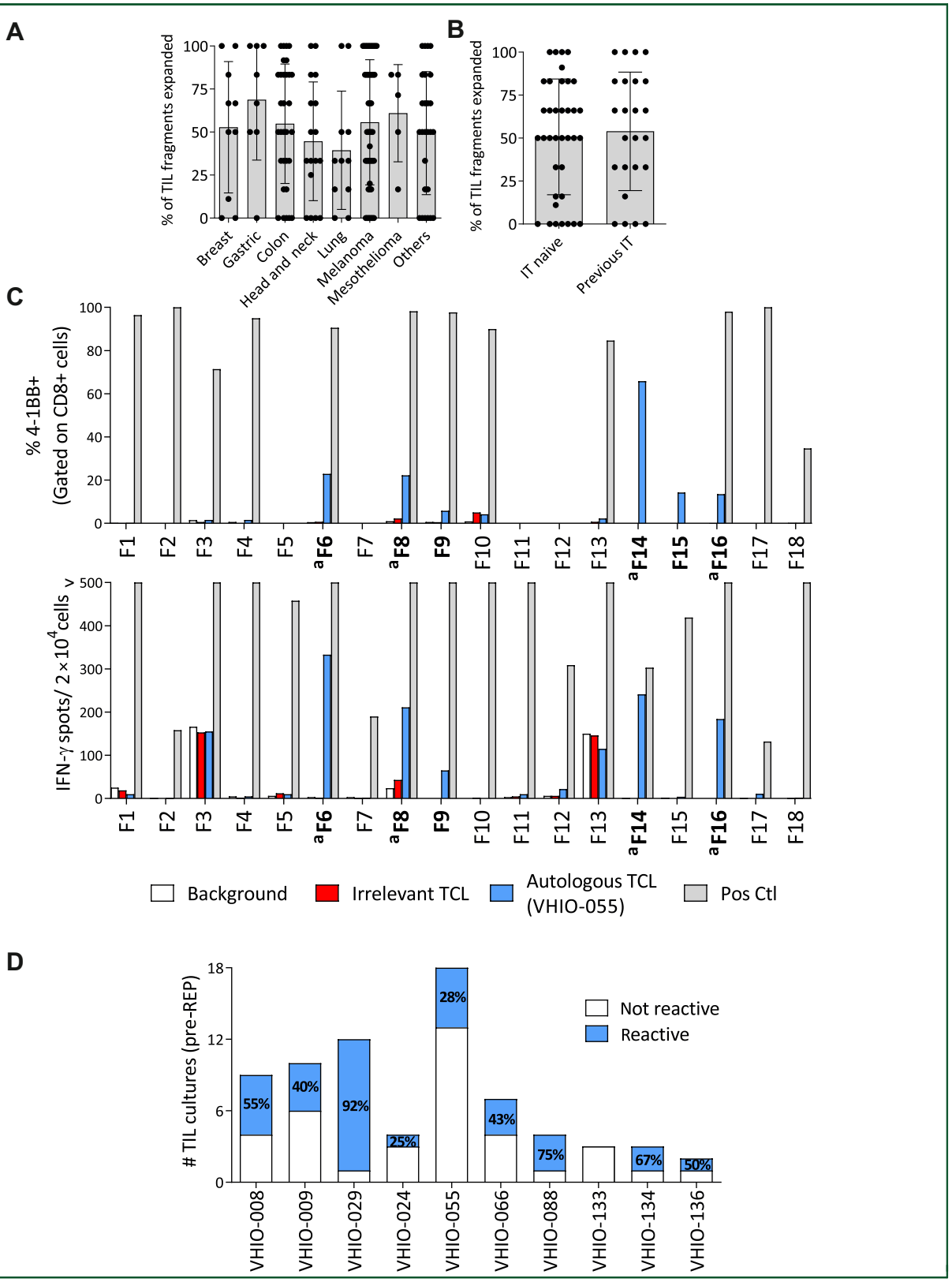

Figure 2. TIL expansion according to tumor histology and prior immunotherapy and reactivity to autologous tumor. (A) Mean percentage of TIL fragments expanded from core biopsies according to tumor histology. Plot displays the mean and the range including the maximum and minimum values. (B) Mean percentage of TIL fragments expanded from core biopsies in IT-naive patients compared with patients who had previously received immune checkpoint blockade combination therapies. In (A) and (B) each dot represents a tumor. Data are expressed as mean ± SD. (C) Representative example of TIL reactivity to autologous TCLs using *ex vivo* expanded pre-REP TILs from patient VHIO-055. TILs were cocultured with the autologous and an irrelevant HLA mismatched TCL. TIL cultures were considered reactive to autologous tumor (highlighted in bold) when the frequency of expression of 4-1BB or OX40 on CD8+ or CD4+ cells, respectively, was ≥0.5% and ≥2× the negative controls, and/or the presence of ≥40 IFN-γ spots and >2× the negative control using IFN-γ ELISPOT. Anti-CD3 stimulated T cells were used as a positive control. Media alone were used as background for the assay. (D) Number and frequency of tumor-reactive TIL cultures expanded from tumor fragments from the evaluated head and neck tumors (VHIO-008, -009), melanoma (VHIO-029, -24, -055, -066, -088, -133 and -136) and renal cell carcinoma (VHIO-134) at the pre-REP phase. TIL

Table 1. Summary of TIL reactivity to autologous tumor cell line

Patient ID	Tumor origin	Number of tumor fragments seeded	Number of TILs expanded from fragments	Number of tumor-reactive TIL cultures	Percentage of tumor-reactive TIL cultures	Type of tumor reactivity (CD4+ and/or CD8+)	Percentage of tumor-reactive TILs in reactive TIL cultures (%4-1BB or OX40)
VHIO-008	Head and neck	12	9	5	55% (5/9)	CD8+	20%-35%
VHIO-009	Head and neck	22	10	4	40% (4/10)	CD8+	3%-22%
VHIO-029	Melanoma	12	12	11	92% (11/12)	CD8+ and CD4+	4%-68%
VHIO-024	Melanoma	6	4	1	25% (1/4)	CD8+	6%-11%
VHIO-055	Melanoma	24	18	5	28% (5/18)	CD8+	4%-67%
VHIO-066	Melanoma	18	7	3	43% (3/7)	CD8+	5%-16%
VHIO-088	Melanoma	6	4	3	75% (3/4)	CD8+	3%-32%
VHIO-133	Melanoma	6	3	0	—	—	—
VHIO-134	Renal cell	6	3	2	67% (2/3)	CD4+	1.5%-3%
VHIO-136	Melanoma	6	2	1	50% (1/2)	CD4+	44%

TIL, tumor-infiltrating lymphocyte.

neoantigen ETV1 p.E455K, while TIL7 recognized GEMIN5 p.S1360L. Finally, TIL cultures derived from VHIO-055 recognized a neoantigen derived from TPD53BPp.629L.

In summary, we were able to detect TIL cultures capable of recognizing neoantigens identified by tumor WES in four out of four patients screened. Consistent with previous data from our laboratory and others, using this approach neoantigen-specific TIL cultures can be detected in ~85% of cancer patients screened.[49,50] Consequently, this technique represents an attractive approach to screen and select TILs for patient treatment given that TCLs or fresh tumor targets are often not available.

Process development and validation of TIL expansion under GMP

For the GMP validations carried out at the classified facilities of the cellular therapy of the BST, patients with different epithelial cancers (colon, lacrimal gland adenocarcinoma, mesothelioma and cervical adenopathy) were enrolled irrespective of their mutational load. Of a total of six additional trucut tumor biopsies processed, a variable number of TIL cultures expanded which constituted the intermediate cell products and were subsequently cryopreserved in independent vials. The median days in culture of the pre-REP phase was 25 and on average, 67% of tumor fragments seeded expanded *ex vivo*, ranging from 11% to 83% depending on the tumor. Moreover, the viability of the expanded TIL cultures during pre-REP before cryopreservation was 87% ± 5% (Figure 4). Three intermediate product batches (TIL19001, TIL19003 and TIL19010) were selected to carry out the GMP validations of the REP based on different frequencies of CD3, CD4 and CD8+ populations and different pre-REP expansion times. Exceptionally, the GMP validations of the REP were carried out without prior assessment of neoantigen recognition in the pre-REP TIL cultures, since the goal of the GMP validations was to ensure that TILs could be expanded to very high numbers and meeting all quality standards established by the regulatory authorities.

In total, six intermediate product batches (TIL19001, TIL19002, TIL19003, TIL19006, TIL19009 and TIL19010) as well as three finished products (REP20002/TIL19001, REP20003/TIL19003 and REP20004/TIL19010) were generated following the validation design defined in the manufacturing process. The generated batches met the established specifications in all cases (Tables 3 and 4). The number of total viable cells obtained in each case was 9.94e9 (REP20002), 1.10e11 (REP20003) and 4.30e9 (REP20004). The finished product consisted of expanded T lymphocytes derived from autologous tumor adjusted to the defined dose range in conditioning solution (Plasmalyte supplemented to 2% w/v with human albumin).

Clinical study design

Based on the aforementioned preclinical data showing detection of neoantigen-reactive TILs in all four patients tested and the literature, as well as the GMP-grade validation of TIL expansion, we decided to design a clinical trial. This single-center, open-label, phase I trial aims to assess the safety and efficacy of neoantigen-reactive *ex vivo* expanded TIL cultures (NEXTGEN-TIL) in patients presenting advanced epithelial tumors and ICB-resistant solid tumors. TIL-ACT with NEXTGEN-TIL product aims at eliminating the tumor cells by using autologous neoantigen-selected TIL cultures expanded *ex vivo,* in combination with HD-IL-2 and preceded by the administration of an NMA-LD regimen.

This study consists of two separate phases depicted in Figure 5, each requiring separate informed consent and

cultures were considered tumor-reactive if they met the previously specified criteria in two consecutive coculture experiments. The percentage of tumor-reactive TIL cultures is specified for each of the patients' samples tested.

HLA, human leukocyte antigen; IFN-γ, interferon-γ; IT, immunotherapy; REP, rapid expansion protocol; SD, standard deviation; TCL, tumor cell line; TIL, tumor-infiltrating lymphocyte.

[a]TIL cultures displaying tumor reactivity in two consecutive coculture experiments.

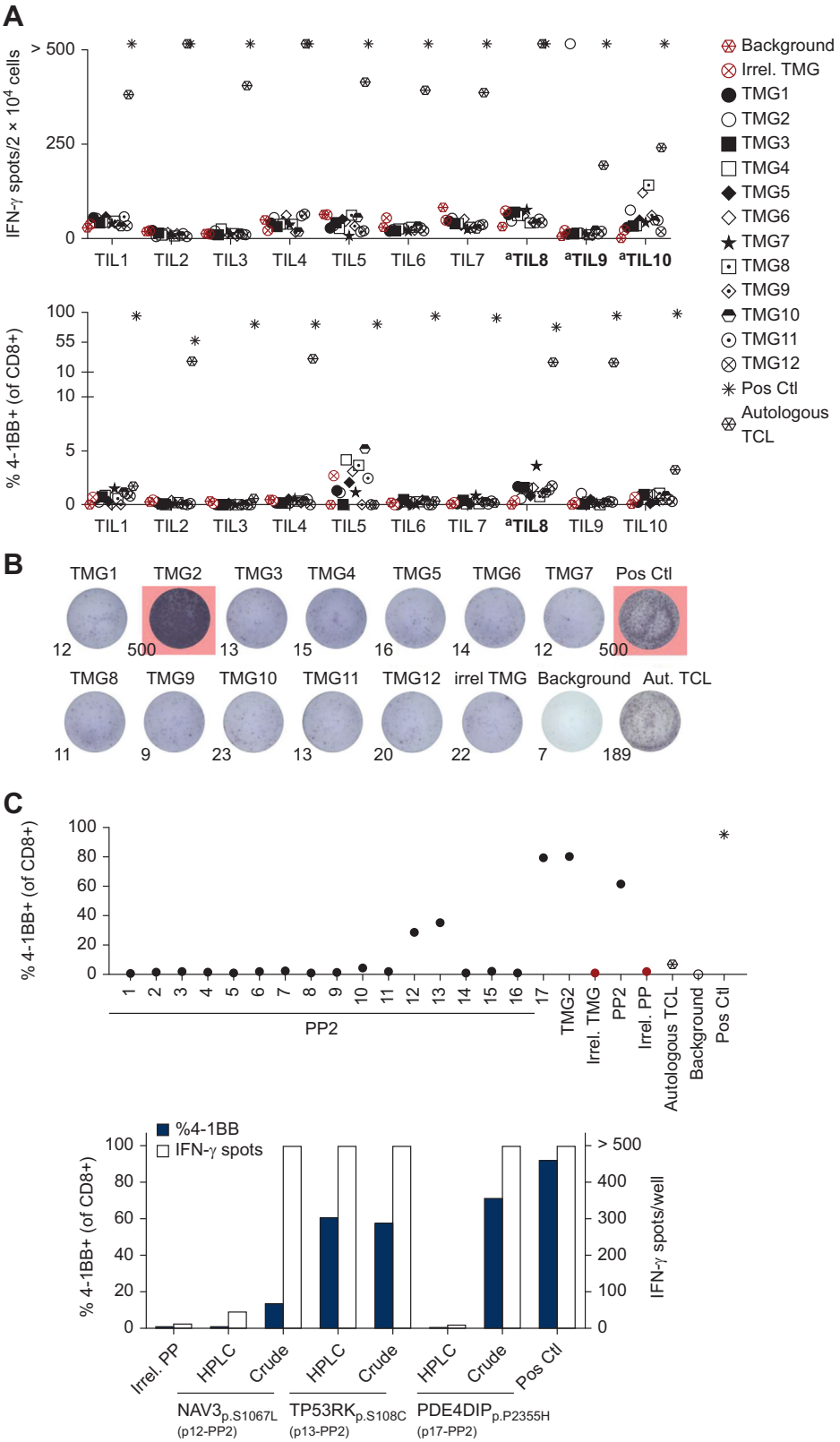

Figure 3. TIL cultures derived from patient VHIO-009 with head and neck cancer recognize at least three neoantigens encoded by TMG2, TMG6, TMG7 and TMG8 identified by tumor whole-exome sequencing. TIL cultures expanded from 10 independent tumor fragments derived from two core biopsies were cocultured with control autologous B cells or B cells electroporated individually with an irrelevant TMG or TMG1-TMG12 IVT RNA encoding all candidate neoantigens identified by tumor WES. (A) Twenty hours after coculture, reactivity to neoantigens was measured using IFN-γ ELISPOT assay (top panel) and detection of 4-1BB activation marker on CD8+ lymphocytes by flow cytometry (bottom panel). Anti-CD3 (OKT3) was used as positive controls. Media alone were used as background for the assay. Recognition of the autologous TCL (TC VHIO-009) by TILs is plotted. TIL cultures were classified as reactive to a specific TMG (highlighted in bold) when the frequency of expression of 4-1BB or OX40 on CD8+ or CD4+ cells, respectively, was ≥0.5% and ≥2× the irrelevant TMG condition (negative control), and/or the presence of ≥40 IFN-γ spots and >2× the negative control using IFN-γ ELISPOT. (B) IFN-γ ELISPOT data after co-culturing expanded TIL9 against the different TMG,

Table 2. Summary of TIL culture reactivity to neoantigens

Patient ID	Tumor origin	Number of NSMs	Reactive TIL cultures[a]	Target TMGs or PPs recognized	# NeoAg reactive TIL cultures/total tested	Neoantigens recognized[b]
VHIO-008	Head and neck	2485	TIL3, TIL4 TIL5	TMG4 TMG3	3/9	$MAGEB2_{p.E167Q}$ $RPL14_{p.H20Y}$
VHIO-009	Head and neck	266	TIL9 TIL10 TIL8	TMG2/PP2 TMG6 TMG8 TMG7	3/10	$TP53RK_{p.S108C}$ $NSMCE1_{p.N156D}$ $GBP_{p.E359K}$ NE
VHIO-029	Melanoma	283	TIL7 TIL3, TIL5	TMG1 and PP1	3/12	$GEMIN5_{p.S1360L}$ $ETV1_{p.E455K}$
VHIO-055	Melanoma	183	TIL8 TIL16	TMG1 TMG1	2/18	$TP53BP_{p.P629L}$

IFN-γ, interferon-γ; NeoAg, neoantigen; NE, not evaluated; NSM, non-synonymous somatic mutation; PP, peptide pool; TIL, tumor-infiltrating lymphocyte; TMG, tandem minigene.
[a]Specific TIL cultures containing TILs reactive to TMG or PP based on 4-1BB/OX40 up-regulation and/or IFN-γ release.
[b]Gene name, position and amino acid change are specified.

meeting specific inclusion criteria. The first phase (Figure 5A) is the pre-treatment/screening phase, where the patient's tumor and blood samples are extracted to select TILs for *ex vivo* expansion (pre-REP), followed by the WES-identified neoantigen recognition. Patients with $\geq$1 neoantigen-reactive TIL cultures and satisfying the eligibility criteria progress to the second phase (Figure 5B), the treatment phase, where selected neoantigen-reactive TIL cultures undergo REP while the patient receives a preparative lymphodepleting chemotherapy. Then, the expanded selected TIL cultures are re-infused back, followed by administration of HD-IL-2. Given that the first phase can take between 1.5 and 3 months and that the patients' clinical condition can deteriorate, patients can receive a bridge treatment meanwhile (between the first and second phase).

The NMA-LD regimen involves cyclophosphamide (60 mg/kg) and fludarabine (25 mg/m^2) on days −5 and −4, and fludarabine alone on days −3 to −1 (Figure 6). Post-regimen, IL-2 is administered intravenously at 720 000 IU/kg every 8 h, up to six doses as tolerated.

This clinical protocol has been approved by both the institutional ethics committee and the national regulatory agency (Spanish Agency of Medicines and Medical Devices). All patients recruited in the trial will be asked to sign an informed consent form, as required. The trial has been published both in the Spanish Trial Registry (EudraCT 2020-005778-90) and in ClinicalTrials.gov (NCT05141474).

Selection of neoantigen-specific TIL cultures for patient treatment

The technical details of the screening phase of this clinical study are depicted in Figure 5. *Ex vivo* expanded TIL cultures, constituting the intermediate pre-REP cell products, will be cryopreserved and, as soon as all the reagents needed are available (B cells, TMGs and PPs), they will be screened against neoantigen-loaded autologous antigen-presenting cells. TIL cultures comprising neoantigen-reactive TILs (specific criteria detailed in the 'Materials and Methods' section) will be selected and pooled for REP up to high numbers and patient treatment. In order to release intermediate pre-REP cell products to undergo REP, the following security specification criteria need to be met: intermediate pre-REP cultures have to be sterile, mycoplasm negative and test for endotoxin $\leq$1 EU/ml. Moreover, at least two pre-REP cultures need to expand from all seeded fragments and have to be neoantigen-reactive.

Selection of patients

To be eligible for the study, patients must have histologically or cytologically proven metastatic or unresectable solid tumors. The disease must have progressed to at least one standard therapy (including at least one prior line with ICB for the group of patients with tumors where ICB is approved), or the patient is unable/unwilling to receive standard therapy, or no standard therapy exists for a particular disease. Additionally, patients must meet all the defined criteria in the clinical study before enrollment.

The population in this study is heterogeneous. Based on literature, we hypothesize that TIL cultures enriched for neoantigen recognition (NEXTGEN-TIL) may be superior to unselected TILs at mediating tumor regression in patients with epithelial tumors and other solid tumors where ICB is approved and used as part of standard therapy.

The primary objective of this study aims to evaluate the safety and tolerability of NEXTGEN-TIL products in patients

anti-CD3 (positive control), media (backgound), irrelevant TMG and autologous TCL. Numbers indicate counted IFN-γ spots. (C) TIL9 reactive against TMG2 recognized a neoantigen derived from TP53RKp.S108C. To determine the mutation specifically recognized, 25-mer peptides encoding the individual NSMs contained in TMG2 were independently pulsed on to autologous B cells overnight. B cells were then washed and cocultured with TIL9. After 20 h, T-cell responses were evaluated by measuring up-regulation of 4-1BB by flow cytometry (top panel). Autologous B cells pulsed with and irrelevant PP and TMG, and anti-CD3 were used as negative and positive controls, respectively. Crude peptide preparations were used for neoantigen screening, and the reactivities were further confirmed with HPLC-grade peptides (bottom panel).
Aut., autologous; HPLC, high-performance liquid chromatography; IFN-γ, interferon-γ; Irrel., irrelevant; Pos Ctl, positive control; PP, peptide pool; TCL, tumor cell line; TIL, tumor-infiltrating lymphocyte; TMG, tandem minigene.
[a]TIL cultures displaying TMG reactivity in two consecutive coculture experiments.

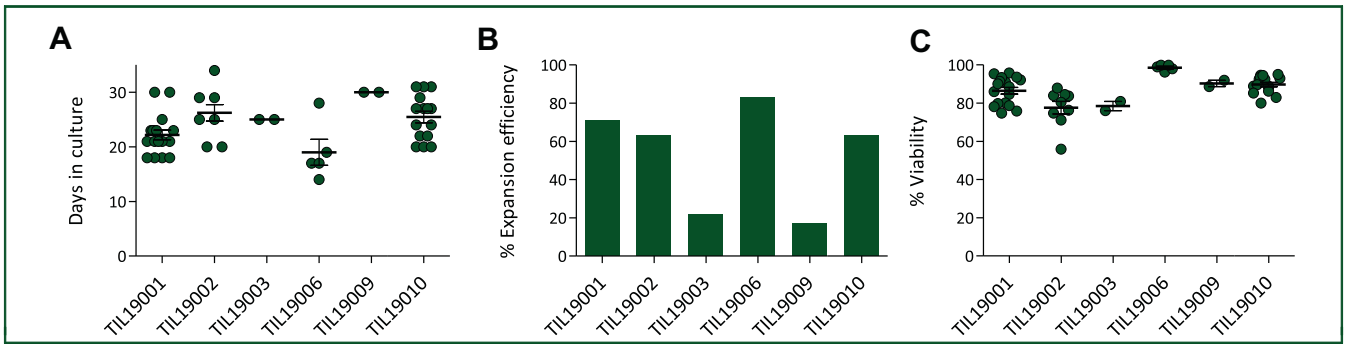

Figure 4. TIL expansion validations of phase I (pre-REP) in the classified facilities of the manufacturer. Core tumor biopsies derived from different tumor histologies [colon adenocarcinoma (TIL19001, 19003, 19010), lacrimal gland adenocarcinoma (TIL19002), cervical adenopathy (TIL19006) and malignant pleural mesothelioma (TIL19009)] were cut into six independent 2-3 mm fragments and cultured in high-dose IL-2 from 2 to 5 weeks. (A) Median days in culture, (B) frequency of TIL fragments expanded of the total number of tumor fragments initially cultured in IL-2 and (C) the viability of the expanded TILs and is plotted for each individual biopsy. IL-2, interleukin 2; REP, rapid expansion protocol; TIL, tumor-infiltrating lymphocyte.

with metastatic or unresectable epithelial tumors and ICB-resistant solid tumors. The secondary objectives are to determine the success of producing neoantigen-reactive TILs and to evaluate the initial clinical activity of the NEXTGEN-TIL products in our target patients.

This study also has several exploratory objectives. Firstly, to study the phenotypic and transcriptomic traits of TIL and their functionality, as well as their persistence in peripheral blood following transfer, and to explore the relationships between these features and clinical outcome. Secondly, to better understand the relationship between the diversity of neoantigens targeted, the clonality of the neoantigens and the diversity and frequency of the T-cell receptors (TCRs) targeting each of these neoantigens. Further, we aim to identify the contribution of heterogeneity of the specific neoantigens targeted or their loss of expression to tumor progression and clinical outcomes. Lastly, we will carefully analyze the economic cost of this therapy at VHIO to determine the feasibility of escalating its application from pilot to a regular health care procedure.

Clinical study data analysis

All safety parameters in the study will be summarized. Safety will be determined by adverse events, laboratory tests, vital signs, electrocardiograms, physical examinations and performance status. Adverse event data will be reported in listings. The first safety evaluation will be carried out with the first six patients. If there is a maximum of one treatment-limiting toxicity (TLT), the study will continue recruiting patients for up to a total of 10 patients. If more than one TLT or any of the other criteria defined in the clinical protocol are observed in the first six patients, an adjustment in TIL product will be needed for further examination.

For categorical endpoints (i.e. ORR), counts and percentages, with 95% confidence intervals (CIs), will be calculated. For the univariate analysis, the logistic regression will be carried out to identify prognostic factors to response. Continuous variables will be summarized with descriptive statistics (mean, standard deviation, range and median). For the ORR analysis, only patients who have measurable disease at baseline and have had their disease re-evaluated will be considered.

Time-to-event variables (i.e. progression-free survival) will be analyzed according to the Kaplan—Meier method. Kaplan—Meier survival curves will be reported, along with associated 95% CIs. Waterfall plots will be used to describe the best variation of the sum of target lesions during the follow-up. Extensive longitudinal data analysis will be used

Table 3. Established specifications of the three intermediate product batches selected to carry out the REP GMP validations

Specifications		Viable cell number	Viability	Identity			Endotoxin	Mycoplasm	Sterility
		9e6	≥70%	≥95% CD45+	% CD4+ (informative)	% CD8+ (informative)	≤1 EU/ml	Negative (neg)	Sterile (stl)
TIL19001	F1	1.64e7	95.8	99	82.4	0.1	≤1 EU/ml	Neg	Stl
	F6	9.84e7	78.1	99.2	30.5	58.3	≤1 EU/ml	Neg	Stl
	F15	2.56e7	90.1	97.4	96	0.5	≤1 EU/ml	Neg	Stl
	F18	2.33e7	93.6	99.1	85.9	12.2	≤1 EU/ml	Neg	Stl
	F24	1.74e7	91.1	97.7	97.2	0.1	≤1 EU/ml	Neg	Stl
TIL19003	F7	1.41e7	76.3	99.4	87.1	8.3	≤1 EU/ml	Neg	Stl
	F10	1.15e7	74.8	99	43.2	50	≤1 EU/ml	Neg	Stl
	F4	2.07e7	83.7	98.1	90.4	4	≤1 EU/ml	Neg	Stl
	F5	2.32e7	84.5	98.9	65.2	32.2	≤1 EU/ml	Neg	Stl
TIL19010	F3	1.73e7	88.9	99.9	51.6	41.7	≤1 EU/ml	Neg	Stl
	F4	1.97e7	94.6	99.9	92.3	2.4	≤1 EU/ml	Neg	Stl
	F6	1.47e7	83	100	68.3	28.3	≤1 EU/ml	Neg	Stl
	F22	1.57e7	92.5	99.5	85.7	10	≤1 EU/ml	Neg	Stl
	F23	2.25e7	88.9	99.9	94.2	3.7	≤1 EU/ml	Neg	Stl

Table 4. Established specifications of the three finished product batches

	Specifications	REP2002	REP2003	REP2004
Viable cell number	5e8-1.1e11	9.94e9	1.1e11	4.3e9
Viability	≥70%	95.6	94.1	88.6
Identity	≥95% CD3+	97.6	96.2	95.1
Potency	≥200 pg/ml IFN-γ and/or TNF-α plus ≥double negative control	575 pg/ml IFN-γ	498 pg/ml IFN-γ	4602 pg/ml IFN-γ
Impurities	≤5% CD3− Unique HLA and matching with patient's HLA	2.4 Ok	3.8 Ok	4.9 Ok
Endotoxin	≤1 EU/ml	≤0.552 EU/ml	≤0.50 EU/ml	≤0.50 EU/ml
Karyotype	No chromosomal alterations	No alterations	No alterations	No alterations
Mycoplasm	Negative (neg)	Neg	Neg	Neg
Sterility	Sterile (stl)	Stl	Stl	Stl

HLA, human leukocyte antigen; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α.

to analyze the percentage of change in tumor size from baseline at 6 and 12 weeks and then every 3 months in the first year and every 6 months in the second year and as per principal investigator's discretion thereafter.

DISCUSSION

Recent publications have determined to what extent patients with common epithelial cancers contain TILs that recognize neoantigens, a key prerequisite for developing

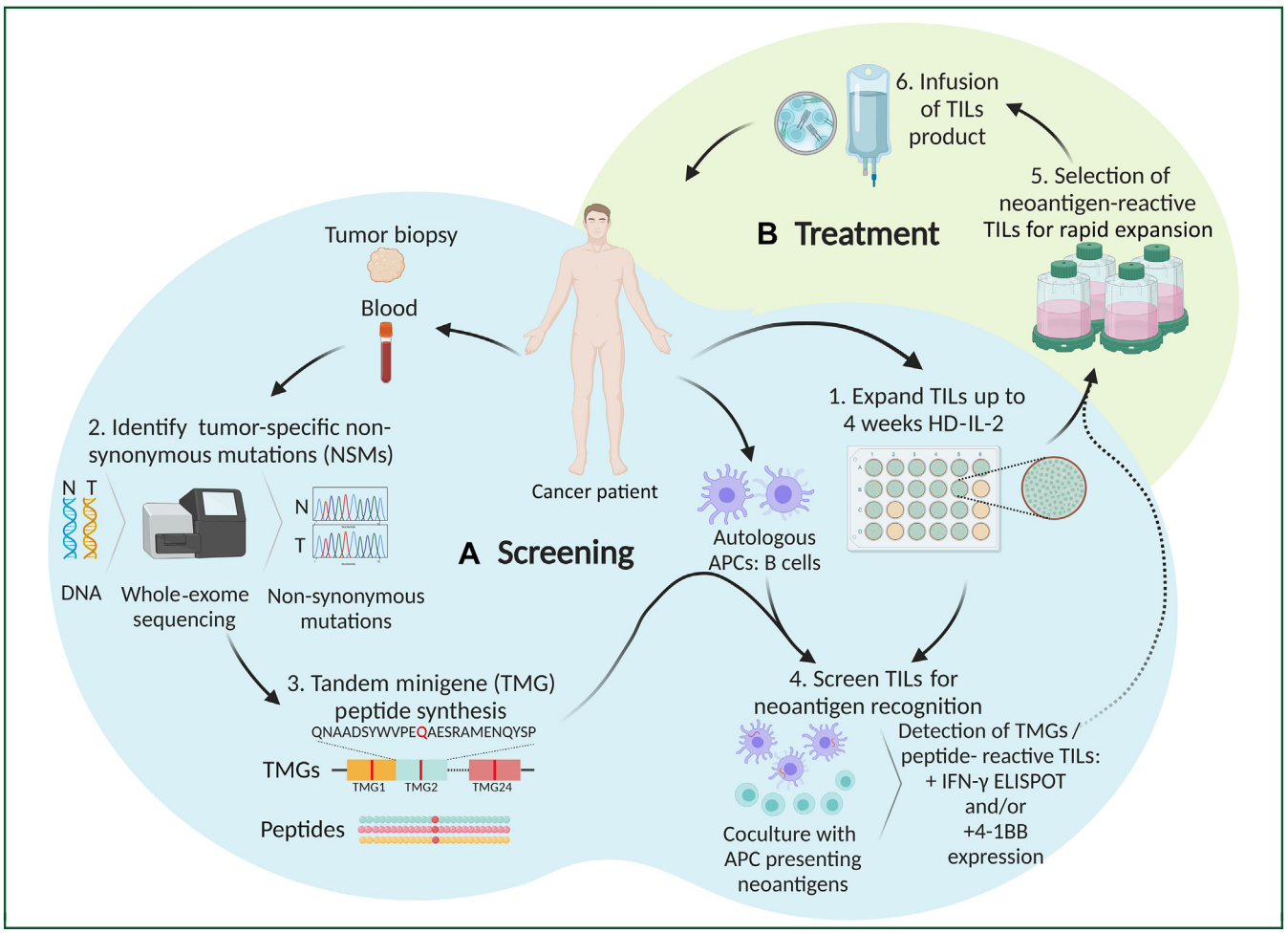

Figure 5. Diagram summarizing the screening and treatment phase of the NEXTGEN-TIL clinical study. (A) Patients sign the informed consent for sample procurement. TILs are expanded from the tumor biopsy by culturing them in HD-IL-2 (pre-REP). DNA extracted from tumor (T) and blood (N) is used to carry out WES and to identify somatic NSMs. This information is used to design TMGs encoding for NSMs and synthetic long peptides which are used to express or pulse the neoantigen candidates on to the autologous B cells expanded from the peripheral blood. B cells are then co-incubated with TILs expanded from the biopsy to identify TIL cultures containing neoantigen reactivities. Patients with one or more TIL cultures containing neoantigen reactivities and meeting the eligibility criteria will sign the inform consent of the treatment phase of the clinical trial. (B) During the treatment phase, neoantigen-reactive TIL cultures are selected for REP. Patients receive a preconditioning lymphodepleting chemotherapy and infusion of TILs as well as up to six doses of HD-IL-2.
APC, antigen-presenting cell; HD-IL-2, high-dose interleukin 2; NSM, non-synonymous mutation; REP, rapid expansion protocol; TIL, tumor-infiltrating lymphocyte; TMG, tandem minigene; WES, whole-exome sequencing.

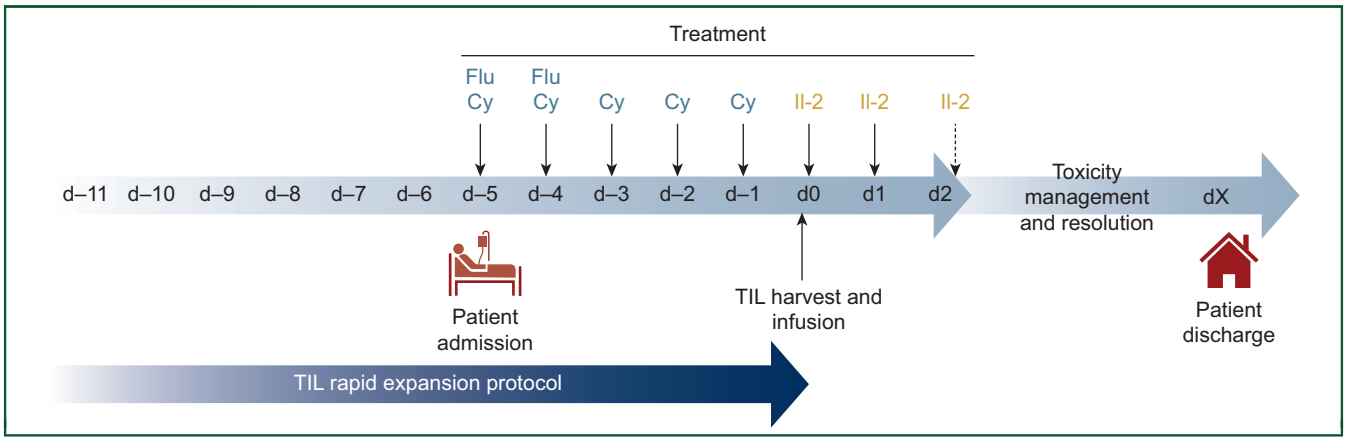

Figure 6. Treatment phase of the clinical study, TIL expansion and patient hospitalization. When neoantigen reactivity is detected and if eligibility criteria for the treatment phase are met, the clinical team together with the manufacturer agree on a date to initiate the rapid expansion of TIL day (d) −11 and to infuse the cell product (d0). Patients are admitted to the hospital at d−5 for initiation of a 5-day lymphodepleting preparative regimen consisting of cyclophosphamide 60 mg/kg and fludarabine 25 mg/m^2, as shown. TILs are harvested and infused at d0, at least 24 h after the last dose of chemotherapy. High-dose IL-2 (720 000 IU/kg) is administered following TIL infusion every 8 h, up to six doses as tolerated. Patients are discharged from the hospital following resolution of acute treatment-related toxicities. Cy, cyclophosphamide; Flu, fludarabine; IL-2, interleukin 2; TIL, tumor-infiltrating lymphocyte.

such personalized T-cell products. In 2019, Parkhurst et al. detected neoantigen-reactive TILs in 62 out of 75 (83%) patients with specific gastrointestinal (GI) cancers using high-throughput immunologic screening of TILs to candidate mutant gene products identified through WES.[50] A total of 124 TIL populations reactive against neoantigens were identified, all of which were private except for one. Furthermore, the results of *in vitro* T-cell recognition studies showed that 1.6% of candidate NSMs are immunogenic. These results indicate that most epithelial cancers induce T-cell responses to neoantigens, making a neoantigen-enriched TIL product a real possibility.

TIL administration was associated with durable tumor regression in one BrCa patient and two patients with HPV+ head and neck or cervical cancer in whom the infusion product showed predominantly neoantigen reactivity.[30,31] Subsequently, in 2022, it was reported that TILs were isolated and grown in culture from the resected lesions of 42 patients with metastatic BrCa, and a median number of 112 (range 6-563) NSMs per patient were identified.[32] Twenty-eight of 42 (67%) patients contained TILs that recognized at least one immunogenic somatic mutation (median 3 neoantigens per patient, range 1-11), and 13 patients demonstrated robust reactivity appropriate for adoptive transfer. In this study, six patients were enrolled on a protocol of ACT of enriched neoantigen-specific TILs, in combination with pembrolizumab ($\leq$4 doses). Objective tumor regression was noted in three patients, including one complete response (now ongoing over 5.5 years) and two partial responses (6 and 10 months). In addition, infusion of TIL products highly enriched for neoantigen recognition induced tumor regression in patients with cholangiocarcinoma and CRC.[33,34]

Based on our preclinical testing of TILs for neoantigen recognition in four patients, only a small fraction of the TIL cultures screened comprised neoantigen reactivity (Figure 3 and Table 2). This, together with previous literature showing tumor regression in selected patients with epithelial cancers following treatment with neoantigen-selected TILs,[30-34] supports the selection of TILs containing reactivity as a means to enrich for neoantigen-reactive TILs to potentially enhance clinical efficacy. Nonetheless, one consideration is that the screening and selection of neoantigen-reactive TIL cultures occurs in the intermediate pre-REP T-cell product, which needs to be further expanded for patient treatment. Unless the TCR clonotype(s) displaying neoantigen recognition is already highly oligoclonal, the rapid expansion of TILs could lead to changes in the TCR repertoire which could either be beneficial or detrimental if they result in an increase or a decrease in the neoantigen-reactive TCR clonotypes. In the case of GI cancers, TIL expansion during the REP was found to decrease the frequency of neoantigen-reactive lymphocytes,[49] but whether this also occurs in TILs derived from other tumors is unknown. Further purifying neoantigen-reactive TCR clonotypes or driving their specific expansion are a few strategies that are being investigated by us as well as other groups, to potentially maintain or enhance neoantigen reactivity during the REP.

Our results, as well as previous data,[29,51] support that TILs can expand from ICB-naive and ICB-refractory tumors and that they can recognize tumor. However, recent data show that anti-programmed cell death protein 1 (PD-1)-experienced patients harbor tumors with lower mutational burden and TILs derived from these patients recognize fewer neoantigens, as compared with anti-PD-1-naive patients.[29] Despite this, TIL products capable of mediating antitumor responses following transfer of TILs in anti-PD-1-experienced patients in this study still recognized more neoantigens, than in non-responders. The decrease in the detection of neoantigen-reactive TILs could represent a challenge for the feasibility of treating anti-PD-1-experienced patients with neoantigen-selected TIL products as described here. On the contrary, it may also help select anti-PD-1-experienced patients who are more likely to respond to TIL therapy.

Concerning safety, in the more recent studies in solid tumors other than melanoma, toxicities were found to be like the ones previously described, and treatment was described as well tolerated with manageable toxicities.[52,53] Following this rationale, we do not anticipate a different safety profile in patients with other solid tumors from patients with metastatic melanoma, because they are treated by the same TIL-ACT standard therapy (i.e. NMA-LD chemotherapy and TIL infusion followed by HD-IL-2). TIL therapy has rarely shown toxicities that could be attributed to the T-cell product. In few instances when these occurred, patients developed autoimmune toxicities associated with infusion of TILs targeting antigens shared by the tumor and normal tissues such as melanoma differentiation antigens.[54] By enriching our TIL product for TIL cultures targeting neoantigens, which are exclusively expressed by the tumor, we intend to make our product safer, but also, potentially, more efficacious. Although the clinical experience with neoantigen-reactive TILs is yet limited, thus far all the neoantigen-selected TIL products have been used in patients with solid cancers (other than melanoma) and have demonstrated to be safe.

The results derived from this project might provide novel therapeutic options for patients with metastatic epithelial tumors and ICB-resistant solid tumors, where there is currently an unmet clinical need, as well as contribute to a better understanding of tumor and T-cell traits influencing the clinical efficacy of ACT in this scenario.

FUNDING

This work was supported by Instituto de Salud Carlos III (ISCIII) [grant number ICI20/00076] and co-funded by the European Union, and by the Comprehensive Program of Cancer Immunotherapy & Immunology II (CAIMI-II) supported by the BBVA Foundation [grant number 53/2021]. AG was the recipient of a Miguel Servet Contract from ISCIII [grant number MS15/00058] and the Investigator Consolidation Award [grant number CNS2023-145343] from the Ministerio de Ciencia, Innovación y Universidades, Spain. AYE was supported by the Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR) [grant number 2021 FI_B 00365]. We are grateful to Ricardo Pujol for revising the manuscript.

DISCLOSURE

AG is a member of the scientific advisory board (SAB) of Achilles Therapeutics plc, SingulaBIO, RootPath, Inc., BioNTech SE and is a consultant advisor for Instil Bio; is a co-inventor of patents licensed (E-059-2013/0 E-085-2013/0, E-149-2015/0) and with royalties from Intima Bioscience Inc., Intellia Therapeutics, Inc., Tailored Therapeutics, LLC, Cellular Biomedicine Group Inc. and Geneius Biotechnology, Inc. EG is a consultant advisor of Roche/Genentech, F. Hoffmann/La Roche, Ellipses Pharma, Neomed Therapeutics Inc, Boehringer Ingelheim, Janssen Global Services, SeaGen, TFS, Alkermes, Thermo Fisher-Bristol-Mayers Squibb, MabDiscovery, Anaveon, F-Star Therapeutics, Hengrui. EMC is a consultant advisor of Bristol Myers Squibb, Merck Sharp & Dohme, Novartis, Pierre Fabre, Roche, Sanofi, Regeneron and received research funding from MSD, Sanofi, BMS. She declares speaking symposiums with Amgen, Bristol Myers Squibb, Merck Sharp & Dohme, Novartis, Pierre Fabre, Regeneron and clinical trial participation (principal investigator) with Amgen, Bristol Myers Squibb, GlaxoSmithKline, Merck Sharp & Dohme, Novartis, Pierre Fabre, Roche, Sanofi, Iovance, Regeneron. PB is a consultant advisor of Allogene, Amgen, Autolus Therapeutics, Bristol Myers Squibb/Celgene, Jazz Pharmaceuticals, Kite/Gilead, Incyte, Miltenyi Biomedicine, Novartis, Nektar, Pfizer and Pierre Fabre. JT reports personal financial interest in the form of scientific consultancy role for Alentis Therapeutics, AstraZeneca, Aveo Oncology, Boehringer Ingelheim, Cardiff Oncology, CARSgen Therapeutics, Chugai, Daiichi Sankyo, F. Hoffmann-La Roche Ltd, Genentech Inc, hC Bioscience, Immodulon Therapeutics, Inspirna Inc, Lilly, Menarini, Merck Serono, Merus, MSD, Mirati, Neophore, Novartis, Ona Therapeutics, Ono Pharma USA, Orion Biotechnology, Peptomyc, Pfizer, Pierre Fabre, Samsung Bioepis, Sanofi, Scandion Oncology, Scorpion Therapeutics, Seattle Genetics, Servier, Sotio Biotech, Taiho, Takeda Oncology and Tolremo Therapeutics; stocks from Oniria Therapeutics, Alentis Therapeutics, Pangaea Oncology and 1TRIALSP; and also educational collaboration with Medscape Education, PeerView Institute for Medical Education and Physicians Education Resource (PER). GV reports speaker's fee from Pfizer, MSD, GSK and Pierre Fabre; advisory role with AstraZeneca; and consultant fees from Reveal Genomics. All other authors have declared no conflicts of interest.

REFERENCES

1. Morad G, Helmink BA, Sharma P, Wargo JA. Hallmarks of response, resistance, and toxicity to immune checkpoint blockade. *Cell*. 2021;184(21):5309-5337.
2. Sharma P, Goswami S, Raychaudhuri D, et al. Immune checkpoint therapy-current perspectives and future directions. *Cell*. 2023;186(8):1652-1669.
3. Granhøj JS, Witness Præst Jensen A, Presti M, Met Ö, Svane IM, Donia M. Tumor-infiltrating lymphocytes for adoptive cell therapy: recent advances, challenges, and future directions. *Expert Opin Biol Ther.* 2022;22(5):627-641.
4. Rosenberg SA, Restifo NP. Adoptive cell transfer as personalized immunotherapy for human cancer. *Science*. 2015;348(6230):62-68.
5. Goff SL, Dudley ME, Citrin DE, et al. Randomized, prospective evaluation comparing intensity of lymphodepletion before adoptive transfer of tumor-infiltrating lymphocytes for patients with metastatic melanoma. *J Clin Oncol*. 2016;34(20):2389-2397.
6. Borch TH, Andersen R, Ellebaek E, Met Ö, Donia M, Svane IM. Future role for adoptive T-cell therapy in checkpoint inhibitor-resistant metastatic melanoma. *J Immunother Cancer.* 2020;8(2):e000668.
7. Dafni U, Michielin O, Lluesma SM, et al. Efficacy of adoptive therapy with tumor-infiltrating lymphocytes and recombinant interleukin-2 in advanced cutaneous melanoma: a systematic review and meta-analysis. *Ann Oncol*. 2019;30(12):1902-1913.
8. Besser MJ, Itzhaki O, Ben-Betzalel G, et al. Comprehensive single institute experience with melanoma TIL: long term clinical results, toxicity profile, and prognostic factors of response. *Mol Carcinog*. 2020;59(7):736-744.

9. Seitter SJ, Sherry RM, Yang JC, et al. Impact of prior treatment on the efficacy of adoptive transfer of tumor-infiltrating lymphocytes in patients with metastatic melanoma. *Clin Cancer Res*. 2021;27:5289-5298.
10. Sarnaik AA, Hamid O, Khushalani NI, et al. Lifileucel, a tumor-infiltrating lymphocyte therapy, in metastatic melanoma. *J Clin Oncol*. 2021;39(24):2656-2666.
11. Orcurto A, Chiffelle J, Ghisoni E, et al. In-depth immune and molecular profiling of melanoma patients receiving adoptive T-cell therapy reveals biomarkers of efficacy in ATATIL study. *J Clin Oncol*. 2021;39(suppl 15):2533.
12. Rohaan MW, Borch TH, van den Berg JH, et al. Tumor-infiltrating lymphocyte therapy or ipilimumab in advanced melanoma. *N Engl J Med*. 2022;387(23):2113-2125.
13. Chesney J, Lewis KD, Kluger H, et al. Efficacy and safety of lifileucel, a one-time autologous tumor-infiltrating lymphocyte (TIL) cell therapy, in patients with advanced melanoma after progression on immune checkpoint inhibitors and targeted therapies: pooled analysis of consecutive cohorts of the C-144-01 study. *J Immunother Cancer*. 2022;10(12):e005755.
14. Mullard A. FDA approves first tumour-infiltrating lymphocyte (TIL) therapy, bolstering hopes for cell therapies in solid cancers. *Nat Rev Drug Discov*. 2024;23(4):238.
15. Martín-Lluesma S, Svane IM, Dafni U, et al. Efficacy of TIL therapy in advanced cutaneous melanoma in the current immuno-oncology era: updated systematic review and meta-analysis. *Ann Oncol*. 2024;35(10):860-872.
16. Creelan BC, Wang C, Teer JK, et al. Tumor-infiltrating lymphocyte treatment for anti-PD-1-resistant metastatic lung cancer: a phase 1 trial. *Nat Med*. 2021;27(8):1410-1418.
17. Stevanović S, Helman SR, Wunderlich JR, et al. A phase II study of tumor-infiltrating lymphocyte therapy for human papillomavirus-associated epithelial cancers. *Clin Cancer Res*. 2019;25(5):1486-1493.
18. Amaria R, Knisely A, Vining D, et al. Efficacy and safety of autologous tumor-infiltrating lymphocytes in recurrent or refractory ovarian cancer, colorectal cancer, and pancreatic ductal adenocarcinoma. *J Immunother Cancer*. 2024;12(2):e006822.
19. Pedersen M, Westergaard MCW, Milne K, et al. Adoptive cell therapy with tumor-infiltrating lymphocytes in patients with metastatic ovarian cancer: a pilot study. *Oncoimmunology*. 2018;7(12):e1502905.
20. Snyder A, Makarov V, Merghoub T, et al. Genetic basis for clinical response to CTLA-4 blockade in melanoma. *N Engl J Med*. 2014;371(23):2189-2199.
21. Rizvi NA, Hellmann MD, Snyder A, et al. Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. *Science*. 2015;348(6230):124-128.
22. Van Allen EM, Miao D, Schilling B, et al. Genomic correlates of response to CTLA-4 blockade in metastatic melanoma. *Science*. 2015;350(6257):207-211.
23. van Rooij N, van Buuren MM, Philips D, et al. Tumor exome analysis reveals neoantigen-specific T-cell reactivity in an ipilimumab-responsive melanoma. *J Clin Oncol*. 2013;31(32):e439-e442.
24. Lauss M, Donia M, Harbst K, et al. Mutational and putative neoantigen load predict clinical benefit of adoptive T cell therapy in melanoma. *Nat Commun*. 2017;8(1):1738.
25. Robbins PF, Lu YC, El-Gamil M, et al. Mining exomic sequencing data to identify mutated antigens recognized by adoptively transferred tumor-reactive T cells. *Nat Med*. 2013;19(6):747-752.
26. Kristensen NP, Heeke C, Tvingsholm SA, et al. Neoantigen-reactive CD8+ T cells affect clinical outcome of adoptive cell therapy with tumor-infiltrating lymphocytes in melanoma. *J Clin Invest*. 2022;132(2):e150535.
27. Lu YC, Yao X, Crystal JS, et al. Efficient identification of mutated cancer antigens recognized by T cells associated with durable tumor regressions. *Clin Cancer Res*. 2014;20(13):3401-3410.
28. Andersen R, Donia M, Ellebaek E, et al. Long-lasting complete responses in patients with metastatic melanoma after adoptive cell therapy with tumor-infiltrating lymphocytes and an attenuated IL2 regimen. *Clin Cancer Res*. 2016;22(15):3734-3745.
29. Levi ST, Copeland AR, Nah S, et al. Neoantigen identification and response to adoptive cell transfer in anti-PD-1 naïve and experienced patients with metastatic melanoma. *Clin Cancer Res*. 2022;28(14):3042-3052.
30. Zacharakis N, Chinnasamy H, Black M, et al. Immune recognition of somatic mutations leading to complete durable regression in metastatic breast cancer. *Nat Med*. 2018;24(6):724-730.
31. Stevanović S, Pasetto A, Helman SR, et al. Landscape of immunogenic tumor antigens in successful immunotherapy of virally induced epithelial cancer. *Science*. 2017;356(6334):200-205.
32. Zacharakis N, Huq LM, Seitter SJ, et al. Breast cancers are immunogenic: immunologic analyses and a phase II pilot clinical trial using mutation-reactive autologous lymphocytes. *J Clin Oncol*. 2022;40(16):1741-1754.
33. Tran E, Turcotte S, Gros A, et al. Cancer immunotherapy based on mutation-specific CD4+ T cells in a patient with epithelial cancer. *Science*. 2014;344(6184):641-645.
34. Tran E, Robbins PF, Lu YC, et al. T-cell transfer therapy targeting mutant KRAS in cancer. *N Engl J Med*. 2016;375(23):2255-2262.
35. Jung Y, Han D. BWA-MEME: BWA-MEM emulated with a machine learning approach. *Bioinformatics*. 2022;38(9):2404-2413.
36. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J*. 2011;17:10-12.
37. Van der Auwera GA, Carneiro MO, Hartl C, et al. From FastQ data to high confidence variant calls: the Genome Analysis Toolkit best practices pipeline. *Curr Protoc Bioinformatics*. 2013;43(1110):11.10.1-11.10.33.
38. Koboldt DC, Zhang Q, Larson DE, et al. VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing. *Genome Res*. 2012;22(3):568-576.
39. Kim S, Scheffler K, Halpern AL, et al. Strelka2: fast and accurate calling of germline and somatic variants. *Nat Methods*. 2018;15(8):591-594.
40. Larson DE, Harris CC, Chen K, et al. SomaticSniper: identification of somatic point mutations in whole genome sequencing data. *Bioinformatics*. 2012;28(3):311-317.
41. Benjamin D, Sato T, Cibulskis K, Getz G, Stewart C, Lichtenstein L. Calling somatic SNVs and indels with Mutect2. bioRxiv. 2019. https://dx.doi.org/10.1101/861054.
42. McLaren W, Gil L, Hunt SE, et al. The Ensembl Variant Effect Predictor. *Genome Biol*. 2016;17(1):122.
43. Kodysh J, Rubinsteyn A. OpenVax: an open-source computational pipeline for cancer neoantigen prediction. *Methods Mol Biol*. 2020;2120:147-160.
44. O'Donnell TJ, Rubinsteyn A, Bonsack M, Riemer AB, Laserson U, Hammerbacher J. MHCflurry: open-source class I MHC binding affinity prediction. *Cell Syst*. 2018;7(1):129-132.e4.
45. Szolek A, Schubert B, Mohr C, Sturm M, Feldhahn M, Kohlbacher O. OptiType: precision HLA typing from next-generation sequencing data. *Bioinformatics*. 2014;30(23):3310-3316.
46. Dobin A, Davis CA, Schlesinger F, et al. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*. 2013;29(1):15-21.
47. Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics*. 2014;30(7):923-930.
48. Gros A, Parkhurst MR, Tran E, et al. Prospective identification of neoantigen-specific lymphocytes in the peripheral blood of melanoma patients. *Nat Med*. 2016;22(4):433-438.
49. Gros A, Tran E, Parkhurst MR, et al. Recognition of human gastrointestinal cancer neoantigens by circulating PD-1+ lymphocytes. *J Clin Invest*. 2019;129(11):4992-5004.
50. Parkhurst MR, Robbins PF, Tran E, et al. Unique neoantigens arise from somatic mutations in patients with gastrointestinal cancers. *Cancer Discov*. 2019;9(8):1022-1035.
51. Andersen R, Borch TH, Draghi A, et al. T cells isolated from patients with checkpoint inhibitor-resistant melanoma are functional and can mediate tumor regression. *Ann Oncol*. 2018;29(7):1575-1581.

52. Pedersen M, Westergaard MCW, Milne K, et al. Adoptive cell therapy with tumor-infiltrating lymphocytes in patients with metastatic ovarian cancer: a pilot study. *Oncoimmunology*. 2018;7:e1502905.
53. Schoenfeld AJ, Lee SM, Doger de Spéville B, et al. Lifileucel, an autologous tumor-infiltrating lymphocyte monotherapy, in patients with advanced non-small cell lung cancer resistant to immune checkpoint inhibitors. *Cancer Discov*. 2024;14:1389-1402.
54. Yeh S, Karne NK, Kerkar SP, et al. Ocular and systemic autoimmunity after successful tumor-infiltrating lymphocyte immunotherapy for recurrent, metastatic melanoma. *Ophthalmology*. 2009;116(5):981-989.e1.