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Multi-antigen-targeted T-cell therapy to treat patients with relapsed/refractory breast cancer

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Abstract

Purpose: Adoptively transferred, *ex vivo* expanded multi-antigen-targeted T cells (multiTAA-T) represent a new, potentially effective, and nontoxic therapeutic approach for patients with breast cancer (BC). In this first-in-human trial, we investigated the safety and clinical effects of administering multiTAA T cells targeting the tumor-expressed antigens, Survivin, NY-ESO-1, MAGE-A4, SSX2, and PRAME, to patients with relapsed/refractory/metastatic BC.

Materials and methods: MultiTAA T-cell products were generated from the peripheral blood of heavily pre-treated patients with metastatic or locally recurrent unresectable BC of all subtypes and infused at a fixed dose level of $2 \times 10^7/m^2$. Patients received two infusions of cells 4 weeks apart and safety and clinical activity were determined. Cells were administered in an outpatient setting and without prior lymphodepleting chemotherapy.

Results: All patients had estrogen receptor/progesterone receptor positive BC, with one patient also having human epidermal growth factor receptor 2-positive. There were no treatment-related toxicities and the infusions were well tolerated. Of the 10 heavily pre-treated patients enrolled and infused with multiTAA T cells, nine had disease progression while one patient with 10 lines of prior therapies experienced prolonged (5 months) disease stabilization that was associated with the *in vivo* expansion and persistence of T cells directed against the targeted antigens. Furthermore, antigen spreading and the endogenous activation of T cells directed against a spectrum of non-targeted tumor antigens were observed in 7/10 patients post-multiTAA infusion.

Conclusion: MultiTAA T cells were well tolerated and induced disease stabilization in a patient with refractory BC. This was associated with *in vivo* T-cell expansion, persistence, and antigen spreading. Future directions of this approach may include additional strategies to enhance the therapeutic benefit of multiTAA T cells in patients with BC.

Keywords adoptive T cell therapy, antigen specific T cells, immunotherapy, metastatic breast cancer

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Introduction

Breast cancer (BC) is the most common malignancy worldwide and a leading cause of cancer-related death.[1] Although early detection and advances in conventional chemo-, radio-, and antibody-based therapies[2–5] have substantially increased the cure rates (90% overall 5-year survival in patients with BC), the 5-year survival of those with distant metastases is only 27%,[6] highlighting the need for novel therapies.

A number of refractory diseases have proven responsive to adoptively transferred T cells. For example, in those with CD19+ lymphomas,

durable complete remission rates of 30–50% have been achieved following the adoptive transfer of T cells engineered with a CD19-targeted chimeric antigen receptor (CAR) after lymphodepleting chemotherapy.[7,8] However, disease relapse associated with the emergence of antigen-negative tumors is a frequent occurrence resulting in treatment failure.[9–11] To address the issue of immune escape, our group prepared autologous, polyclonal T-cell lines targeting a spectrum of lymphoma-expressed antigens and when administered to lymphoma patients at high risk of relapse or to treat chemorefractory disease the cell infusions proved safe and induced durable complete remissions (>3 years), without prior lymphodepletion.[12]

Having demonstrated the tolerability and clinical benefit of multi-antigen-targeted T cells (multiTAA-T) in the setting of hematological diseases (lymphoma,[12] multiple myeloma,[13] and leukemia[14,15]), we sought to examine whether a similar strategy might be effective in patients with refractory/metastatic BC when other therapeutic options had been exhausted. Thus, we developed a multispecific T-cell product reactive against the tumor-associated antigens (TAAs), PRAME, SSX2, MAGEA4, NY-ESO1, and Survivin, which in BC are collectively expressed in more than 90% of tumors.[16–30] We hypothesized that the infusion of *ex vivo* expanded autologous T-cell lines (multiTAA T cells) would be safe and promote anti-BC immune activity, and that tumor lysis mediated by the transferred cells would facilitate the recruitment and activation of endogenous immune cells against additional TAAs (i.e. antigen spreading), further extending the breadth and durability of benefit. Here we describe the safety and clinical outcomes achieved in 10 patients who were infused with multiTAA T cells without prior lymphodepletion and in the absence of other therapies.

Methods

Patients

Patients with relapsed/refractory/metastatic BC of all subtypes were included in our Baylor College of Medicine Institutional Review Board-approved protocol (H-39209) conducted under the Food and Drug Administration (FDA)-cleared IND #17586 (ClinicalTrials.gov; NCT03093350, date of registration: March 28, 2017). Inclusion and exclusion criteria are detailed in the protocol (Supplemental Materials). Briefly, any BC patient (of all BC subtypes) between the ages of 18 and 80 years with metastatic or locally recurrent unresectable BC with measurable or evaluable disease as per Response Evaluation Criteria in Solid Tumors (RECIST 1.1) criteria progressing after at least two prior lines of therapy in the advanced setting [those with human epidermal growth factor receptor 2-positive (HER2+) disease had to have failed at least two anti-HER2 agents] without significant organ dysfunction were eligible for participation. All study participants underwent disease assessment at baseline and at least once (~6 weeks) after the second multiTAA T-cell infusion. In all, 12 patients were enrolled in the study. The first patient was enrolled on 1 November 2017 and the last on 30 July 2018. Once enrolled, patients received two infusions at a fixed dose (2×10^7/m^2) 4 weeks apart and were eligible to receive up to six additional infusions if they had stable disease (SD) or a partial response (PR) at disease evaluation. None of the patients received lymphodepleting chemotherapy prior to multiTAA T cells. Complete details on the protocol are available in Supplemental Materials. The data cutoff date for analysis was 5 July 2021.

Generation of multiTAA T cells

MultiTAA T cells were generated as previously described.[31] Briefly, peripheral blood (125–530 ml) was collected from patients following informed consent. Monocyte-derived dendritic cells (DCs) were generated and loaded with peptide mixtures (pepmixes, i.e. 15-mer peptides overlapping by 11 amino acids) spanning Survivin, PRAME, NY-ESO-1, MAGE-A4, and SSX2 (JPT Peptide Technologies, Berlin, Germany). Antigen-loaded DCs were cocultured with autologous peripheral blood mononuclear cells (PBMCs) in T-cell medium supplemented with interleukin 7 (IL-7) (10 ng/ml), IL-12 (10 ng/mL), IL-15 (5 ng/mL), and IL-6 (10 ng/mL). From day 10 and weekly thereafter, responder T cells were restimulated with pepmix-pulsed DCs in the presence of IL-15 (5 ng/mL) or IL-2 (50–100 U/mL) until sufficient numbers were achieved for patient infusion and release testing. The resulting multiTAA T-cell lines were harvested at a median of 26[18–34] days post-T-cell initiation.

MultiTAA T cell characterization studies

Enzyme-linked immunospot assay (ELIspot) analysis was used to determine the frequency of T cells

secreting interferon gamma (IFNγ) in response to TAA pepmixes, as described previously. For phenotypic analysis, we surface-stained multiTAA T cells with phycoerythrin, fluorescein isothiocyanate-, peridinin chlorophyll protein-, allophycocyanin-, Alexa Fluor 700-, phycoerythrin cyanin 7-, Pacific Blue- or Krome Orange-conjugated CD3 (clone SK7), CD4 (SK3), CD8 (SK1), CD56 (B159), CD16 (SJ25C1), CD62L (DREG-56), CD45RA (H100 or 2H4), CD 45RO (UCHL1), CCR7 (3D12), CD69 (L78), CD83 (HB15e), HLA-DR (L243), programmed cell death 1 (PD1; PD1.3.5), and lymphocyte activation gene-3 (LAG3; 874501) (Becton Dickinson [BD], Franklin Lakes, NJ, USA; Beckman Coulter, Pasadena, CA, USA). Control samples labeled with appropriate isotype antibodies were included and a 'fluorescence minus one' strategy was used for multicolor staining. Cells were analyzed using FACScan equipped with a filter set for four fluorescence signals, using CellQUEST software, or FACS-Canto II, using DIVA software (BD). T-cell receptor (TCR vβ) flow cytometric analysis was performed using the IOTest® Beta Mark kit (Beckman Coulter, Brea, CA, USA), as per the manufacturer's instructions. Briefly, 0.5×10^6 cells per tube were surface stained with CD3 and the TCR-vβ-specific monoclonal antibodies provided with the kit and incubated for 20 min at room temperature in the dark. Subsequently, cells were washed, resuspended in 300 µl of phosphate-buffered saline and at least 10,000 live T cells acquired on a Gallios™ Flow Cytometer and analyzed with Kaluza® Flow Analysis Software (Beckman Coulter). The cytotoxic activity of each multiTAA T-cell line toward non-malignant patient-derived PHA blasts (autoreactivity) was measured in a standard ^{51}Chromium release assay at varying effector-to-target (E:T) ratios (40:1, 20:1, 10:1, and 5:1) and the percentage of specific lysis was calculated as [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100.

MultiTAA-specific T-cell in vivo *persistence studies*

To track the infused multiTAA T cells, high-throughput deep sequencing of TCR-vβ CDR3 regions was applied (Adaptive Biotechnologies, Seattle, WA, USA). Deep sequencing was performed on the line infused to pt 9 and on peripheral blood and tumor samples. Those T-cell clones identified within the product but not pre-infusion were coded as line-derived unique clones.

Tumor antigen profiling

As per protocol, tumor biopsies were not mandatory. However, patient 9 underwent a tumor tissue biopsy as standard of care while on-study, which was therefore available for tumor antigen profiling. Tumor tissue slides from this biopsy sample were analyzed by immunohistochemistry (IHC), using a one-step staining technique. Briefly, formalin-fixed paraffin-embedded, unstained slides (4–5 µm thickness) were stained with the following anti-human primary antibodies: MAGE-A4 mouse mAb (clone 6C1, 1:200), NY-ESO1 mouse mAb (clone E978, 1:50) (both Santa Cruz Biotechnology, Dallas, TX, USA), PRAME rabbit pAb (1:200, Bioss Antibodies, Woburn, MA, USA), SSX2 mouse mAb (clone CL3202, 1:500; Atlas Antibodies, Bromma, Sweden), and Survivin rabbit mAb (clone 71G4B7, 1:500; Cell Signaling Technology, Danvers, MA, USA). Standard deparaffinization, rehydration, heat antigen retrieval with citrate buffer (pH 6), elimination of endogenous peroxidase activity by 3% hydrogen peroxide (H323-500, Fisher Scientific, Waltham, MA, USA) and non-specific normal horse serum (2.5%) block were performed with 1X TBST washes. The TAA-specific antibodies were applied overnight at 4°C. The next day an anti-mouse secondary antibody (MP-7402; Ready Vector Labs, Burlingame, CA, USA) or anti-rabbit secondary antibody (MP-7402; Ready Vector Labs) were applied and then 3,3'-Diaminobenzidine (DAB) brown substrate was added to the slides. After 2–5 min of incubation, the slides were washed with water and counterstained with hematoxylin, rinsed in deionized water, dehydrated in serial concentration of alcohol, and cover slipped with Cytoseal 60 mounting medium. Testis with intact spermatogenesis served as a positive and epididymis as a negative control tissue, respectively, for TAAs. Intensity of IHC staining was graded as 1+ (weak), 2+ (moderate), or 3+ (strong) using the combinative semi-quantitative scoring method.[32] Any level of TAA detection by IHC was considered positive in that given tumor sample.

Statistical analysis/sample size considerations

Descriptive statistics were calculated to summarize the clinical and correlative characteristics using mean, standard deviation, standard error of the mean (SEM), median, and range. The primary outcome was defined as clinical benefit (complete response/PR or SD) measured at

6 weeks after the second infusion. Clinical benefit rate was summarized using frequencies/proportions and 95% confidence intervals (CIs). Overall survival and progression-free survival were calculated using the Kaplan–Meier method and summarized with median survival times and 95% CIs.

Results

Patients

In all, 12 patients with BC provided PBMCs from which to generate multiTAA T cells. We successfully generated multiTAA T cells for 11 of these 12 heavily pre-treated patients and 10 were ultimately infused with multiTAA T cells (fixed cell dose: $2 \times 10^7/m^2$). The 11th patient for whom we made cells remained in remission and hence was not infused. One of these patients (patient number 7) received just a single dose before rapidly progressing. The remaining nine patients received two multiTAA T-cell infusions, 4 weeks apart as per protocol. Subsequently, patient number 9, who experienced disease stabilization for a total of 5 months, received three additional doses. BC patients with all subtypes were eligible, nine of the patients treated had HR+ HER2−disease and one had HR+ HER2+ BC. This study included a heavily pre-treated patient population. Patients enrolled in the trial had failed a median of six prior lines of therapy (range 3–10) in both early stage and the advanced metastatic disease setting. Patients had high disease burden with progressing distant metastatic disease. 9/10 patients had visceral involvement at the time of multiTAA treatment, including seven patients with liver metastasis and two patients with treated brain metastasis (Table 1).

MultiTAA-specific T-cell lines

T cells underwent 2–4 rounds of *in vitro* stimulation with pepmix-loaded DCs for an average of 26 (±1) days in culture, resulting in a mean 22 (±2.6) fold expansion (Supplemental Table 1). The 11 multiTAA T-cell lines generated for clinical use comprised CD3+ T cells (mean 98.0 ± 0.4%) with a mixture of CD4+ (mean 56.7 ± 8.0%) and CD8+ (mean 34.1 ± 7.4%) cells that were activated (based on CD69 upregulation – mean 46.8 ± 6.1%) and expressed central (CD45RO+/CD62L+: 11.2 ± 3.4%) and effector memory markers (CD45RO+/CD62L-: 54.4 ± 10.0%). Notably, LAG3 and PD1 were not co-expressed by our T cells, indicating that our expanded populations were not exhausted [Figure 1(a)].[33–36] We further confirmed polyclonality by examining the TCR diversity present in our multiTAA T cells by assessing the TCR-vβ repertoire using a flow cytometric panel that detects more than 70% of all available vβ chains. As shown in Figure 1(b), all measurable vβ families are present in these *ex vivo* expanded cells. The lines recognized the targeted antigens PRAME (range 0–353 SFC/2×10^5), Survivin (range 1–176 SFC/2×10^5), MAGE-A4 (range 0–191 SFC/2×10^5), NY-ESO-1 (range 2–173 SFC/2×10^5), and SSX2 (range 3–191 SFC/2×10^5) by IFNγ ELIspot. None of the lines reacted against non-malignant patient-derived cells (1.1 ± 1.1% specific lysis; E:T 20:1) – a product release criterion to rule out auto-reactivity. The aforementioned multiTAA T-cell line characteristics are summarized in Figure 1.

Clinical outcomes

One patient received one infusion of multiTAA T cells, eight patients received two infusions of multiTAA T cells, while one patient received a total of five infusions. All infusions were well tolerated. There were no instances of cytokine release syndrome, neurotoxicity, or myelotoxicity.

Table 2 summarizes all adverse events observed. Three patients developed grade 2–3 elevations in liver function tests post-infusion but all three had progressing liver metastasis at the time of enrollment and treatment. Patient number 4 experienced a grade 3 elevation of her alanine aminotransferase (ALT), aspartate aminotransferase (AST), and bilirubin levels shortly after her second T-cell infusion and was admitted for work-up. A liver ultrasound showed a diffusely heterogeneous liver, with numerous mass-like lesions suspicious for infiltrative metastasis. A subsequent liver biopsy confirmed the cause of liver failure as BC infiltration and therefore unrelated to T cells. Patient number 7 experienced rapid progression of her disease after the first dose of T cells accompanied by grade 3 elevation of her ALT and AST precluding administration of the second protocol-mandated cell dose. Patient number 9 developed grade 2 elevation of her AST and ALT levels during the course of her treatment without clear association with T-cell infusions. Of note, this patient experienced fluctuation of her AST and ALT levels that ranged between

Table 1. Patient characteristics and clinical outcomes.

Patient ID	Age (years)	BC biomarkers	Organs involved	Prior lines of therapy	No infusions	Response (months post-treatment)	Long-term outcome (months post-treatment)
1	73	IDC and ILC ER+ PR+ HER2–	LNs, bones, brain	- Neoadjuvant anastrazole - Adjuvant Docetaxel + cyclophosphamide - Adjuvant exemestane - Fulvestrant + palbociclib - Exemestane + palbociclib + everolimus - Capecitabine	2	PD (3 months)	Deceased (3.5 months)
2	66	IDC, ER+ PR+ HER2–	Skin, chest wall, lungs, liver, bones	- Anastrazole - Exemestane - Fulvestrant - Letrozole + palbocicilib - Capecitabine - Nab-paclitaxel - Liposomal doxorubicin - High-dose estradiol - Carboplatin	2	PD (2 months)	Deceased (4 months)
3	27	IDC, ER+ PR+ HER2+	LNs, lungs and brain	- Adjuvant FEC × 4 - Paclitaxel + trastuzumab - Tamoxifen - Capecitabine - HP + anastrazole + OS - TCHP × 6 - TDM1 - Liposomal doxorubicin - Navelbine + trastuzumab + pertuzumab - Lapatinib + trastuzumab + exemestane	2	PD (2 months)	Referred to hospice care
4	59	IDC, ER+ PR+ HER2–	LNs, liver, lungs, malignant ascites	- Neoadjuvant paclitaxel - Neoadjuvant doxorubicin + cyclophosphamide - Letrozole + palbociclib - Capecitabine - Eribulin - Exemestane + everolimus	2	PD (2 months)	Deceased (3 months)
5	52	IDC, ER+, PR+, HER2–	Lungs, liver	- Everolimus + tamoxifen - Letrozole - Doxorubicin + cyclophosphamide - Fulvestrant + palbociclib - Capecitabine - High-dose estradiol - Paclitaxel - Eribulin	2	PD (2 months)	Deceased (5.5 months)
6	68	IDC, ER+ PR+ HER2–	Bones, recurrent pleural effusion, liver	- Neoadjuvant docetaxel - Neoadjuvant doxorubicin + cyclophosphamide - Adjuvant anastrazole - Fulvestrant + palbociclib - Nab-paclitaxel	2	PD (2 months)	Deceased (28 months)

(Continued)

Table 1. (Continued)

Patient ID	Age (years)	BC biomarkers	Organs involved	Prior lines of therapy	No infusions	Response (months post-treatment)	Long-term outcome (months post-treatment)
7	63	ILC, ER+ PR+ HER2–	LNs and extensive liver metastasis	- Paclitaxel + cyclophosphamide - Letrozole + palbociclib - Fulvestrant + palbociclib - Liposomal doxorubicin	1	PD (1 months)	Deceased (1 months)
8	61	IDC, ER+ PR+ HER2–	Breast, LNs, bones, liver	- Letrozole - Fulvestrant + palbociclib - Capecitabine	2	PD (2 months)	Deceased (20 months)
9	62	IDC, ER+, PR+ HER2–	Lungs, liver, pleural effusion and bones	- 5FU + doxorubicin + cyclophosphamide - Tamoxifen - Anastrazole + OS - Fulvestrant - Letrozole - Everolimus + exemestane - Letrozole + palbociclib - Capecitabine - High-dose estradiol - Paclitaxel	5	SD (5 months)	Deceased (27 months)
10	63	ILC, ER+ PR+ HER2–	Bones	- Neoadjuvant tamoxifen - Adjuvant anastrazole - Everolimus + Exemestane - Fulvestrant - Fulvestrant + palbocicilib	2	PD (2 months)	Alive (26 months)

ER, estrogen receptor; FEC, 5-flourouracil, epirubicin, cyclophosphamide; HER2+, human epidermal growth factor receptor 2-positive; HP, herceptin, pertuzumab; IDC, infiltrating ductal carcinoma; ILC, infiltrating lobular carcinoma; LN, lymph nodes; OS, ovarian suppression; PD, progression of disease; PR, progesterone receptor; SD, stable disease; TCHP, taxotere, carboplatin, herceptin, pertuzumab; TDM1, trastuzumab–emtansine; 5FU, 5-flourouracil.

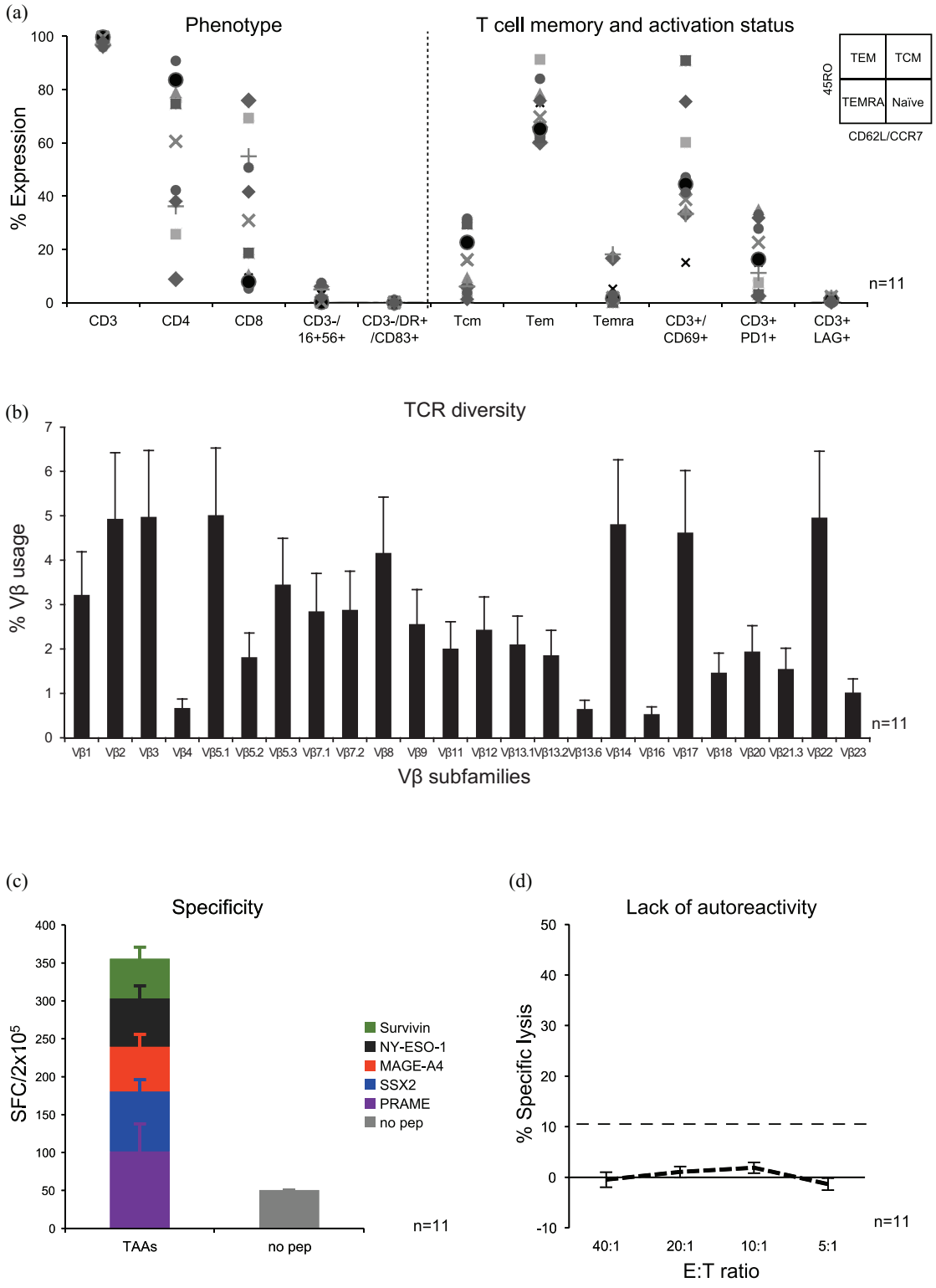


Figure 1. Characterization of autologous multiTAA T cells. (a) Phenotype and memory/activation profile of multiTAA T cells. Each symbol represents an individual patient's T-cell product. (b) TCR-vβ repertoire of multiTAA T cells. Summary data are shown ($n = 11$). (c) MultiTAA T-cell specificity as measured by IFNγ ELIspot for 11 products generated using all five antigens as a stimulus. Data are reported as SFCs ± SEM/2 × 10^5 and each color represents an individual antigenic specificity. (d) Lack of autoreactivity as assessed by co-culturing multiTAA T cells with autologous (non-malignant) targets at effector to target ratios ranging from 40:1 to 5:1. ELIspot, enzyme-linked immunospot assay; IFNγ, interferon gamma; multiTAA T cells, multi-antigen-targeted T cells; SEM, standard error of the mean; SFC, spot forming cells; TCR vβ, T-cell receptor vβ.

Table 2. All adverse events.

Patients, *n*	Event	Max grade	Relationship to Inv. agent	Comments
3	ALT and AST increased	3	Possible	Patient number 4: biopsy of the liver confirming the cause of liver failure as infiltration by BC. Patient number 7: rapid progression of liver metastasis seen on computerized tomography imaging Patient number 9: levels fluctuated between grade 0 and 2 for 2 years prior to enrollment and the 2 years following
1	Cellulitis	3	Unlikely	
1	Blood bilirubin increased	3		Biopsy of the liver confirming the cause of liver failure as infiltration by BC
1	Dehydration	3	Unrelated	
1	Fever	3	Unlikely	Patient diagnosed with cellulitis. Fever resolved with antibiotics
1	Anemia	2	Unlikely	
1	Nausea	2	Unrelated	
1	Pain: sacrum	2	Unrelated	
1	Pain: right arm	2	Unrelated	
1	Back pain	2	Unlikely	
1	Headache	2	Unrelated	
1	Hypoalbuminemia	2	Unlikely	
1	Vertigo	1	Unrelated	
1	Fatigue	1	Unrelated	

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BC, breast cancer.

grade 0 and grade 2 for 2 years prior to enrollment and continued for 2 years after her last T-cell infusion reaching grade 3 levels prior to her death.

No other infusion-related adverse events of any grade were observed. Thus, we demonstrated the safety of targeting five TAAs in patients with relapsed or refractory and metastatic BC at a maximum tested dose of $2 \times 10^7/m^2$ given twice, 4 weeks apart.

The patient who received a single infusion of cells (patient number 7) rapidly progressed, precluding administration of the second protocol-mandated cell dose. The remaining nine patients completed the planned study treatment that included two infusions of multiTAA T cells and thus were evaluable for long-term safety and efficacy. These patients were followed for up to 866 days from treatment initiation. The median progression-free survival time was 106 days (95% CI: 69, Inf). The median overall survival time was 393 days (95% CI: 116, Inf). Eight of the nine patients experienced disease progression within 1–3 months of multiTAA T-cell treatment, while one patient maintained SD at 6 weeks post-infusion. The patient (number 9) who experienced disease stabilization received three additional multiTAA T-cell

infusions at monthly intervals in the absence of all other therapies for a total period of 5 months. During her treatment period, the patient reported improved bone pain control precipitating a decrease in her pain medication and an improvement in her energy levels. She ultimately progressed after her 5th infusion. She went on to live for 22 months after her last T-cell infusion, receiving four more lines of therapy.

Correlative effects

To investigate whether the infused multiTAA T cells initiated any endogenous immune effects, we evaluated the frequency of tumor-reactive T cells in patient peripheral blood pre- and post-infusion. We analyzed T-cell responses to the antigens targeted by the infused line (NY-ESO-1, SSX2, MAGE-A4, Survivin, and PRAME) as well as against a range of other non-targeted BC-expressed antigens including MAGE-A1, -A2B, -A3, -C1, and WT1.[24,27,30,37] We reasoned that the detection of such cells might be indicative of an active antitumor effect mediated by the cells infused, producing *in vivo* antigen spreading, thereby enhancing the antitumor benefits of our therapy. As shown in Figure 2, most patients who experienced disease progression exhibited either a decrease or a transient increase in tumor-specific T cells, which peaked within 8 weeks of infusion and subsequently declined. Interestingly though, in the patient who had prolonged disease stabilization (patient 9), we detected elevated numbers of T cells directed against both targeted [from 2 spot forming cells (SFC) per 2×10^5 input cells pre-infusion to 201 SFC at month 6 post-infusion] and non-targeted antigens (from 4 to 469 SFC), which peaked by month 6 post-infusion and decreased thereafter [Figure 3(a)]. At the same time, we were able to detect multiTAA line-derived T cells in the circulation and infiltrating the patient's tumor, which was shown by IHC to express the TAAs PRAME and Survivin [Figure 3(b)].

Discussion

In this study, we evaluated the feasibility, safety, and clinical effects of administering two infusions of autologous T-cell lines targeting Survivin, PRAME, NY-ESO-1, MAGE-A4, and SSX2 at a fixed cell dose of $2 \times 10^7/m^2$ to 10 patients with advanced BC of various subtypes. The infusions were well tolerated, even with repeat dosing as seen in patient 9 who received a total of five infusions. Furthermore, in patient 9 who also experienced disease stabilization for 5 months, we demonstrate a coincident amplification of T cells directed against both targeted and non-targeted tumor-expressed antigens. Overall, multiTAA T cells can be generated and safely administered to this heavily pre-treated population.

T-cell immunotherapy is rapidly gathering momentum as a cancer treatment. Indeed, the FDA has recently approved five different CAR T-cell therapies for various hematological malignancies: Kymriah™ (tisagenlecleucel) for the treatment of pediatric CD19+ ALL[38]; Kymriah™, Yescarta™ (axicabtagene ciloleucel), and Breyanzi™ (lisocabtagene maraleucel) for adult relapsed/refractory large B-cell lymphoma[7,39]; Yescarta™ for adults with relapsed follicular lymphoma[7,39]; Tecartus™ (brexucabtagene autoleucel) for adults with treatment-resistant or relapsed mantle cell lymphoma[8]; and Abecma™ (idecabtagene vicleucel) for adults with relapsed/refractory multiple myeloma.[40] However, the use of these monospecific T cells has been shown to drive tumor relapse characterized by the outgrowth of malignant cells that lack or have down-regulated the targeted antigen.[9–11] To address this issue and with the goal of developing an effective immunotherapy for BC, we developed a non-engineered T-cell product containing both CD4+ (helper) and CD8+ (cytotoxic) T cells with native TCR specificity for multiple TAAs. Our target antigens were chosen based on the frequency of expression in BC of all subtypes (PRAME: 27–97%; SSX family: 4–65%; MAGE-A4: 4–86%; NY-ESO-1: 8–64%; and Survivin: 26–96%)[16–28,30,37] and immunogenicity to T cells.[24,28,30,31] We hypothesized that the infusion of such multiTAA T cells would be safe and promote anti-BC activity, minimizing the risk for antigen-negative relapses. Finally, we postulated that tumor lysis mediated by the transferred cells would facilitate the recruitment and activation of endogenous immune cells against additional tumor-expressed antigens (i.e. antigen spreading), further extending the breadth and durability of antitumor responses.

There are numerous conventional and recently approved therapies available for the treatment of metastatic BC including chemotherapy, endocrine therapy, and a growing array of targeted agents.[2–5] Novel and investigational therapies such as our multiTAA T cells have a broadly non-overlapping mechanism of action and hence can be

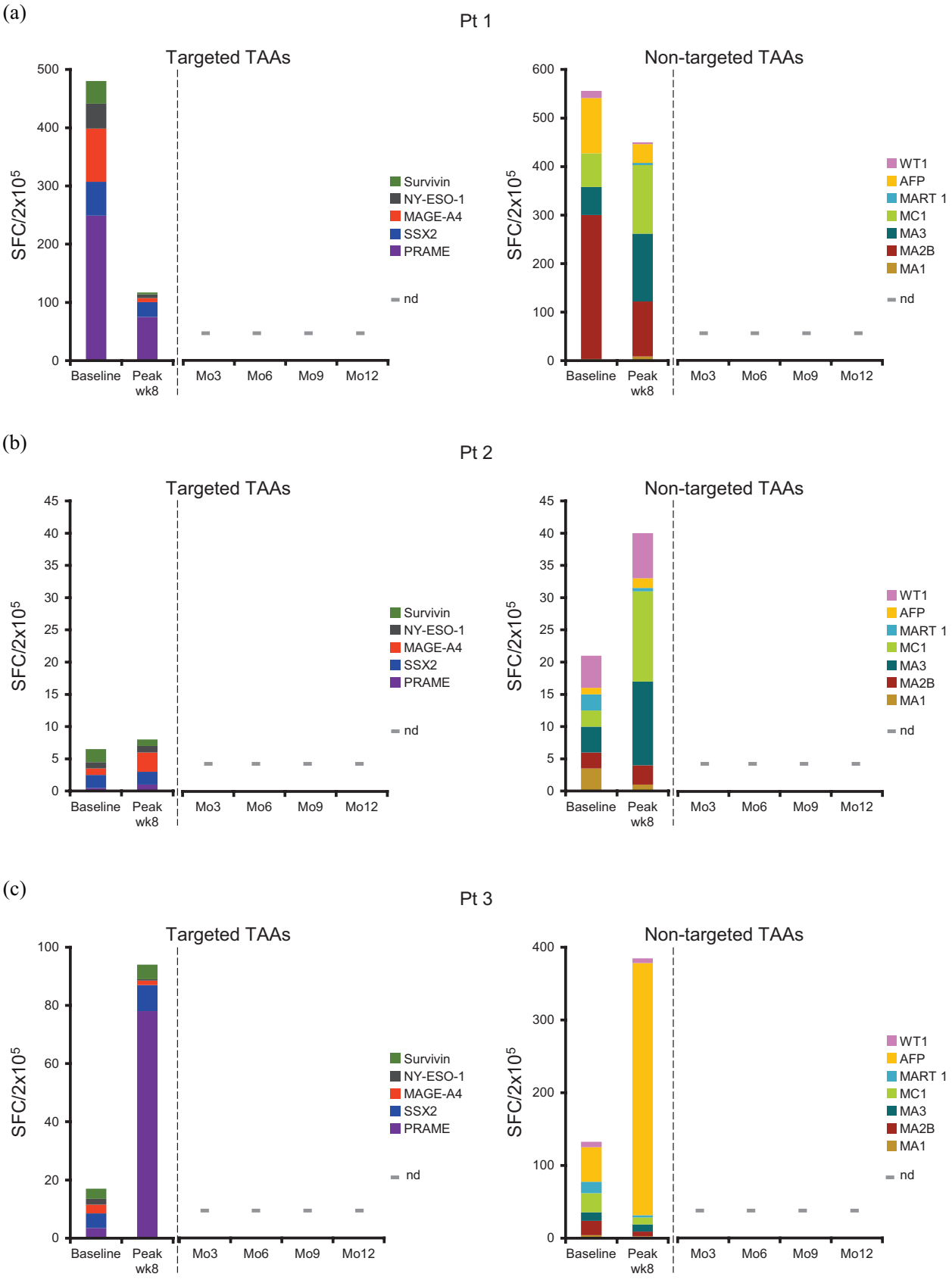


Figure 2. *(Continued)*

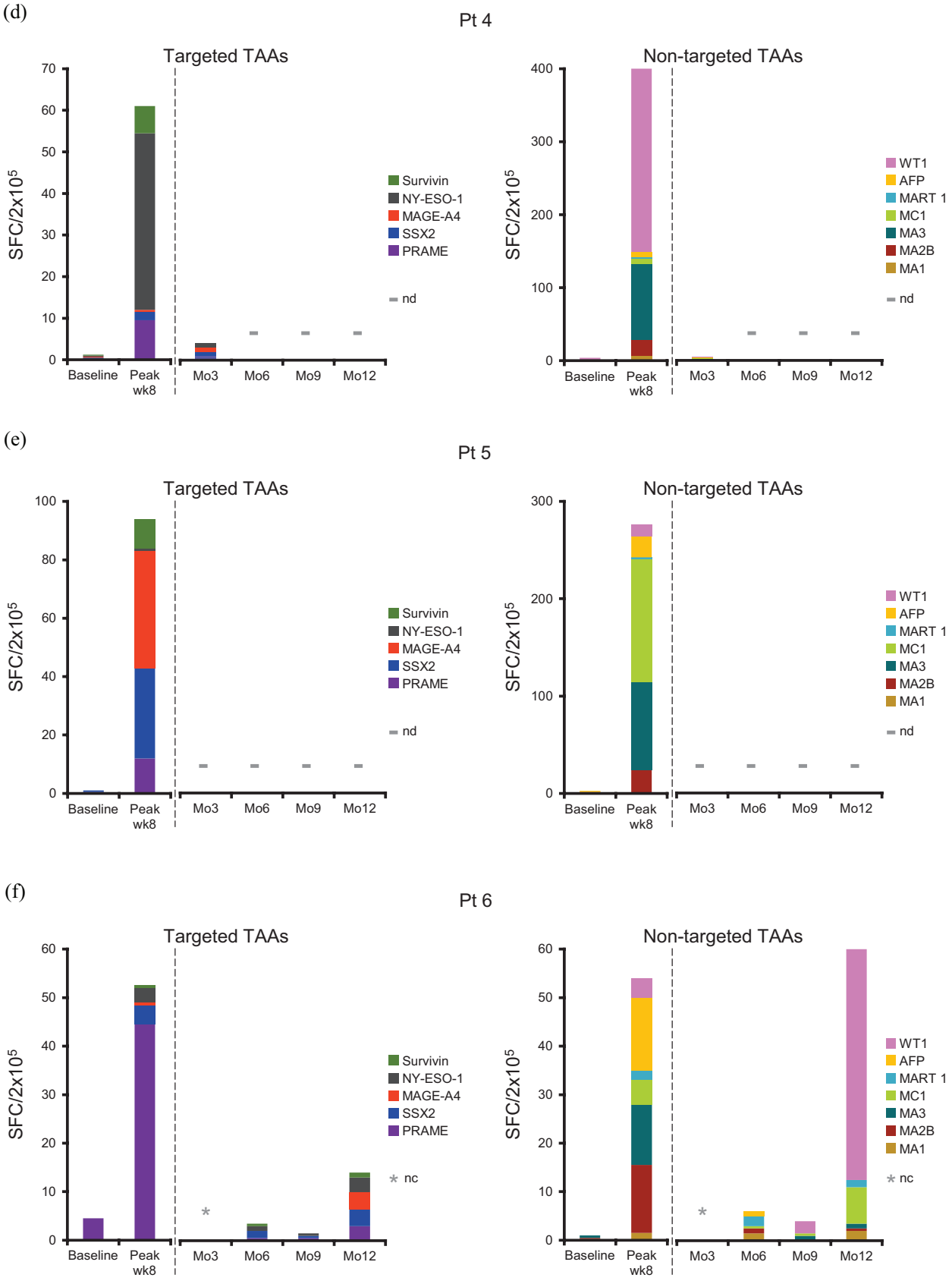


Figure 2. *(Continued)*

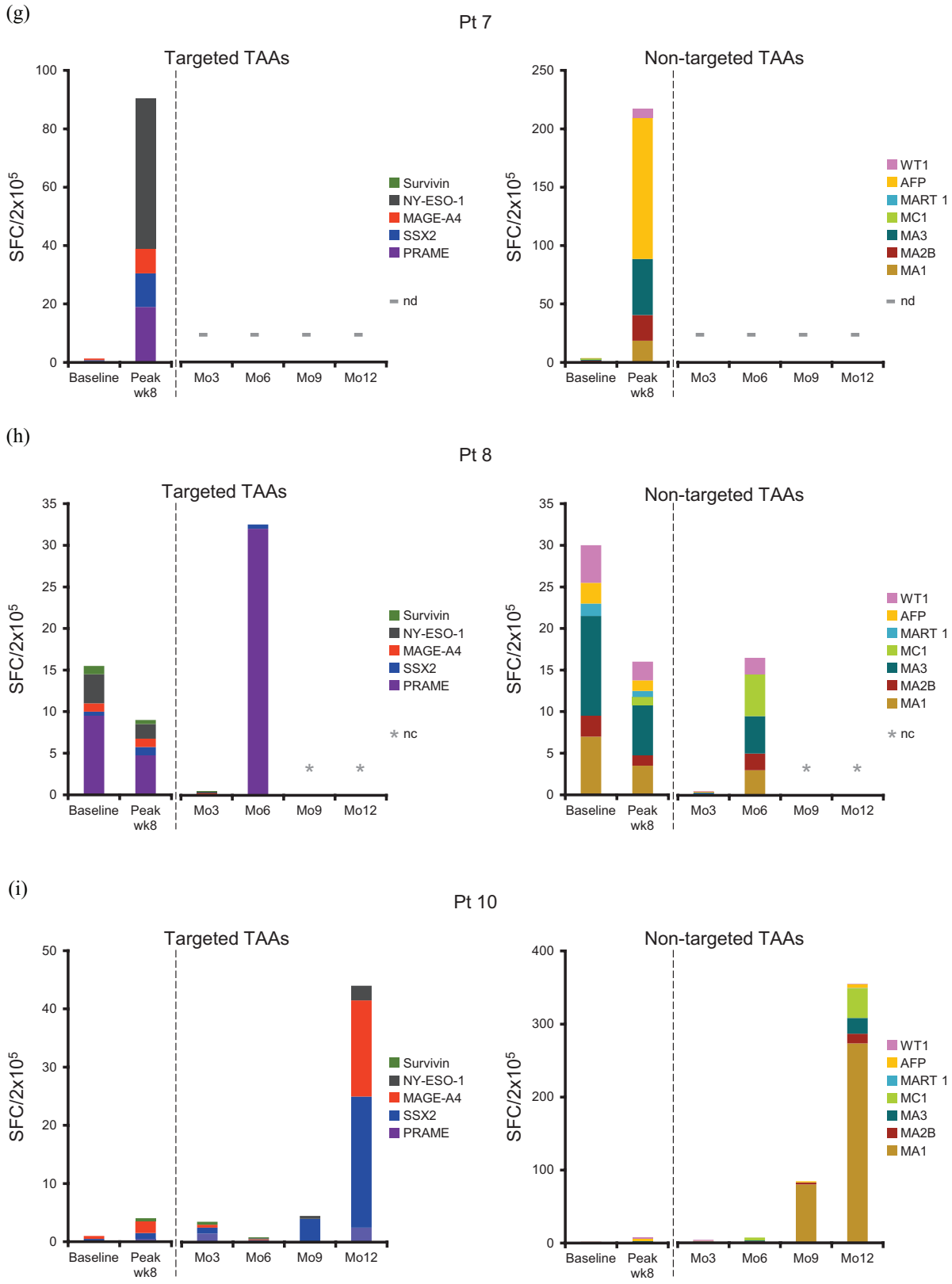
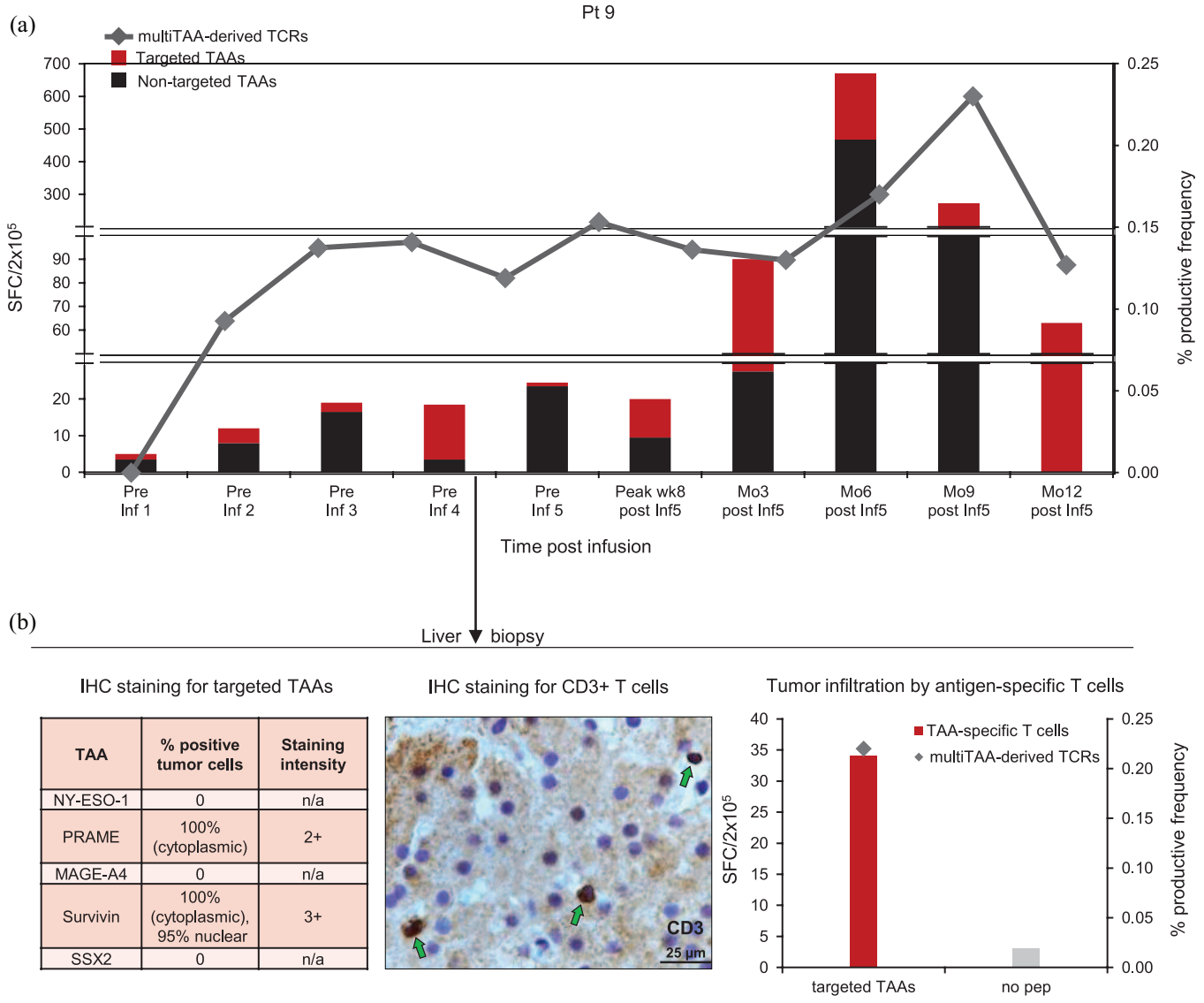


Figure 2. *In vivo* fate of multiTAA T cells in patients who experienced disease progression ($n = 9$). Frequency of T cells specific for targeted TAAs (left) and other non-targeted TAAs (right) in patients who progressed within 1–3 months of initiating multiTAA T-cell treatment. Each panel represents an individual patient. Results are reported as SFC $\pm$ SEM/2 $\times 10^5$ at each specified time point (nd: not done due to disease progression/death, nc: sample not collected).
multiTAA T cells, multi-antigen-targeted T cells; SEM, standard error of the mean; SFC, spot forming cells.



TAA	% positive tumor cells	Staining intensity
NY-ESO-1	0	n/a
PRAME	100% (cytoplasmic)	2+
MAGE-A4	0	n/a
Survivin	100% (cytoplasmic), 95% nuclear	3+
SSX2	0	n/a

Figure 3. Immune correlative studies in patient 9 who experienced disease stabilization following multiTAA T-cell treatment. (a) Immune activity of multiTAA T cells directed against targeted and non-targeted TAAs at the specified time points pre- and post-infusion. Results are reported as SFC ± SEM/2 × 10^5. TCR-vβ deep sequencing of TCRs that were 'unique' to the product infused was used to track the *in vivo* persistence of the introduced multiTAA-specific T cells. Results are reported as the percentage of the circulating productive frequency of unique TCR sequences in peripheral blood, and overlayed on the ELISpot data at each specified time point. (b) MultiTAA T-cell infiltration at the tumor. Left panel: TAA expression (as estimated by IHC) in a liver biopsy sample collected at the indicated time point. Middle panel: detection of CD3+ T cells within the tumor (IHC; original magnification ×200). Right panel: detection of multiTAA line-derived T cells within the tumor as assessed by IFNγ ELIspot with the five targeted antigens used as a stimulus. Results are reported as SFC ± SEM/2 × 10^5.

ELIspot, enzyme-linked immunospot assay; IFNγ, interferon gamma; IHC, immunohistochemistry; multiTAA T cells, multi-antigen-targeted T cells; SEM, standard error of the mean; SFC, spot forming cells; TCR vβ, T-cell receptor vβ.

combined or used sequentially with other therapies. Indeed, in the current study, our patients had a history of multiple relapses, had received a median of six prior lines of therapy, and all had distant metastatic disease with visceral involvement at the time they were enrolled and donated PBMCs for product generation. Despite this, we successfully generated multiTAA T cells for 11 of the 12 consenting patients. The expanded cells demonstrated a polyclonal, Th1-polarized effector profile with a mix of CD4+ (helper) and CD8+ (cytotoxic) cells that expressed a mix of central

and effector memory markers. When administered to patients, we saw an immediate increase in the frequency of circulating T cells directed against the targeted antigens in most patients infused, as well as antigen spreading in 7 of 10. However, this immune effect was transient (observed primarily within the first 8 weeks following infusion) in all but one (patient number 9), who experienced disease stabilization for a total of 5 months (in the absence of other therapies) during which time we could detect tumor-reactive T cells infiltrating her tumor and at steadily elevated levels in her peripheral blood. In the other nine patients, the immune reactivity was short-lived and ultimately all experienced disease progression. Potential reasons for this may include a suboptimal dosing schedule (two infusions of $2 \times 10^7/m^2$, 4 weeks apart) – indeed, in a dose escalation study where tumor-reactive T cells targeting a different spectrum of antigens were administered to patients with a range of solid tumors including Wilm's tumor, neuroblastoma, and a variety of sarcomas, Hont and colleagues reported a dose–response relationship with superior outcomes seen at the highest dose level ($4 \times 10^7/m^2$) with repeat infusions of cells.[41] But at this advanced disease stage, we also cannot rule out other tumor immune evasion tactics. Indeed, in BC, the tumor microenvironment is replete with suppressive cells (e.g. myeloid-derived suppressor cells, M2 macrophages, and T regulatory cells) and cytokines [e.g. granulocyte colony-stimulating factor (CSF), granulocyte-macrophage CSF, IL-10, transforming growth factor-beta, IL-6, and IL-4] as well as expression of checkpoint molecules by tumor and infiltrating immune cells that result in the promotion of tumor growth/survival and suppression of effector function, proliferative capacity, and *in vivo* persistence of infused T cells. Other limitations of this study include the lack of biopsy samples, which would have enabled assessment of both TAA profile and T-cell infiltration post-infusion. Hence, future studies may explore specifically targeting antigens present in patients' tumor profile,[42,43] equipping multiTAA T cells with genetic molecules to enhance their persistence or retain their functionality *in vivo*[44–47] and combining cells with immune-modulating drugs that enhance tumor immunogenicity or overcome evasion strategies.[48,49]

In conclusion, our findings from this first-in-human trial establish the feasibility of making and safely infusing autologous multiTAA T cells for the treatment of BC. In the current study, we have focused on targeting five tumor-expressed antigens (Survivin, PRAME, NY-ESO-1, MAGE-A4, and SSX2), though our platform can accommodate additional specificities to further broaden the spectrum of target tumor antigens. However, this product and manufacturing platform can also be directly applied to the treatment of other solid tumors or the target antigen pool can be customized to specific diseases.

Declarations

Ethics approval and consent to participate

Study was conducted under Baylor College of Medicine Institutional Review Board-approved protocol (H-39209) and Food and Drug Administration (FDA)-cleared IND #17586 (ClinicalTrials.gov; NCT03093350, date of registration: March 28, 2017) with corresponding informed consent from patients.

Consent for publication

Patients included in this study consented to have their study-related data published.

Author contribution(s)

Valentina Hoyos: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Supervision; Writing – original draft.

Spyridoula Vasileiou: Data curation; Formal analysis; Investigation; Methodology; Visualization; Writing – original draft.

Manik Kuvalekar: Data curation; Methodology; Writing – review & editing.

Ayumi Watanabe: Data curation; Methodology; Writing – review & editing.

Ifigeneia Tzannou: Data curation; Methodology; Project administration; Writing – review & editing.

Yovana Velazquez: Data curation; Methodology; Writing – review & editing.

Matthew French-Kim: Methodology; Writing – review & editing.

Wingchi Leung: Visualization; Writing – review & editing.

Suhasini Lulla: Methodology; Writing – review & editing.

Catherine Robertson: Formal analysis; Methodology; Project administration; Resources; Writing – review & editing.

Claudette Foreman: Formal analysis; Methodology; Project administration; Resources; Writing – review & editing.

Tao Wang: Data curation; Formal analysis; Methodology; Writing – review & editing.

Shaun Bulsara: Data curation; Formal analysis; Methodology; Writing – review & editing.

Natalia Lapteva: Resources; Writing – review & editing.

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Charles Kent Osborne: Investigation; Resources; Writing – review & editing.

Angela Coscio: Resources; Writing – review & editing.

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Cliona M. Rooney: Conceptualization; Supervision; Writing – review & editing.

Juan F. Vera: Conceptualization; Writing – review & editing.

Premal Lulla: Funding acquisition; Investigation; Supervision; Writing – review & editing.

Mothaffar Rimawi: Conceptualization; Investigation; Methodology; Supervision; Writing – review & editing.

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Competing Interests

S.V., M.K., and Y.V. are consultants to AlloVir. V.H. holds Marker Therapeutics and AlloVir stock. N.L. is a consultant to Tessa Therapeutics. J.F.V. is a cofounder and equity holder in AlloVir and Marker Therapeutics and an employee of Marker Therapeutics, which aspires to commercialize the described approach. B.J.G. owns QBRegulatory Consulting which has consulting agreements with Tessa Therapeutics, Marker Therapeutics, LOKON, and AlloVir. H.E.H. is a co-founder with equity in Allovir and Marker Therapeutics, has served on advisory boards for Tessa Therapeutics, Kiadis, Novartis, Gilead Biosciences, Fresh Wind Biotechnologies and GSK, and received research support from Kuur Therapeutics and Tessa Therapeutics. C.M.R. has Stock and Other Ownership Interests with Coya, Bluebird Bio, Tessa Therapeutics, Marker Therapeutics, AlloVir, Walking Fish, Allogene Therapeutics, Memgen, Kuur Therapeutics, Bellicum Pharmaceuticals, TScan Therapeutics, Abintus Bio; Consulting or Advisory Role with Abintus Bio, Adaptimmune, Brooklyn Immunotherapeutic, Onk Therapeutics, Tessa Therapeutics, Memgen, Torque, Walking Fish Therapeutics, TScan Therapeutics, Marker Therapeutics, Turnstone Bio; and receives research funding from Kuur Therapeutics. A.M.L is a co-founder and equity holder for AlloVir and Marker Therapeutics and a consultant to AlloVir. P.L. is a member of the advisory board for Karyopharm. J.N. receives research support from Paxman Coolers Ltd. M.R. is a consultant to AstraZeneca, Macrogenics, Seagen and Novartis and receives research support from Pfizer. The remaining authors have no competing financial interests to disclose.

Availability of data and materials

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials. For special requests contact corresponding author.

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Supplemental material
Supplemental material for this article is available online.

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