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Clinical effects of administering leukemia-specific donor T cells to patients with AML/MDS post-allogeneic transplant.

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Abstract:
Relapse after allogeneic hematopoietic stem-cell transplantation (HCT) is the leading cause of death in patients with acute myeloid leukemia (AML) or myelodysplastic syndrome (MDS). Infusions of unselected donor lymphocytes (DLIs) are used to enhance the graft-versus-leukemia (GVL) effect, as treatment for relapsed disease. However, as the infused lymphocytes are not selected for leukemia-specificity, the GVL effect is often accompanied by life-threatening graft-versus-host disease (GVHD) due to the concurrent transfer of allo-reactive lymphocytes. Thus, to minimize GVHD and maximize GVL we selectively activated and expanded stem-cell donor-derived T cells that were reactive to multiple antigens expressed by AML/MDS cells (PRAME, WT1, Survivin, NY-ESO-1). Products were successfully generated from 29 HCT donors, and they demonstrated multi-leukemia antigen specificity (mLSTs). In contrast to DLIs, mLSTs selectively recognized and killed leukemia-antigen-pulsed cells with no activity against recipient-derived normal cells in vitro. We have now administered escalating doses of these mLSTs ($0.5\text{-}10\text{x}10^7$ cells/m^2) to 25 trial enrollees with AML/MDS after HCT, 17 of whom were at high risk for relapse and 8 of whom had relapsed disease. Infusions were well tolerated with no grade >2 acute or extensive chronic GVHD up to a dose of $10\text{x}10^7$ cells/m^2. We observed anti-leukemia effects in vivo that translated into not yet reached median LFS and OS at 1.9 years of follow-up among survivors, evidence of sustained immune pressure and objective responses in the active disease cohort (1 CR and 1 PR). In conclusion, mLSTs are safe and promising for the prevention or treatment of AML/MDS following HCT.

Conflict of interest: COI declared - see note

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Title: Clinical effects of administering leukemia-specific donor T cells to patients with AML/MDS post-allogeneic transplant.

Short Title: Leukemia-specific T cell immunotherapy for AML/MDS

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KEY POINTS:

- Donor-derived T cells with native specificity for multiple myeloid leukemia antigens (mLSTs) can be expanded ex vivo
- Infusions of mLSTs to patients with AML/MDS post-HCT is well tolerated and produces anti-leukemia effects

ABSTRACT

Relapse after allogeneic hematopoietic stem-cell transplantation (HCT) is the leading cause of death in patients with acute myeloid leukemia (AML) or myelodysplastic syndrome (MDS). Infusions of unselected donor lymphocytes (DLIs) are used to enhance the graft-versus-leukemia (GVL) effect, as treatment for relapsed disease. However, as the infused lymphocytes are not selected for leukemia-specificity, the GVL effect is often accompanied by life-threatening graft-versus-host disease (GVHD) due to the concurrent transfer of allo-reactive lymphocytes. Thus, to minimize GVHD and maximize GVL we selectively activated and expanded stem-cell donor-derived T cells that were reactive to multiple antigens expressed by AML/MDS cells (PRAME, WT1, Survivin, NY-ESO-1). Products were successfully generated from 29 HCT donors, and they demonstrated multi-leukemia antigen specificity (mLSTs). In contrast to DLIs, mLSTs selectively recognized and killed leukemia-antigen-pulsed cells with no activity against recipient-derived normal cells in vitro. We have now administered escalating doses of these mLSTs (0.5-10x10^7 cells/m^2) to 25 trial enrollees with AML/MDS after HCT, 17 of whom were at high risk for relapse and 8 of whom had relapsed disease. Infusions were well tolerated with no grade >2 acute or extensive chronic GVHD up to a dose of 10x10^7 cells/m^2. We observed anti-leukemia effects in vivo that translated into not yet reached median LFS and OS at 1.9 years of follow-up among survivors, evidence of sustained immune pressure and objective responses in the active disease cohort (1 CR and 1 PR). In conclusion, mLSTs are safe and promising for the prevention or treatment of AML/MDS following HCT.

Keywords: Acute myeloid leukemia, myelodysplastic syndrome, adoptive immunotherapy, allogeneic hematopoietic cell transplantation, graft-versus-leukemia effect

INTRODUCTION

Post-transplant relapse of acute myeloid leukemia (AML) or myelodysplastic syndromes (MDS) are devastating diagnoses with very few effective treatment options. Cellular therapies in the form of donor-lymphocyte infusions (DLIs) and/or a second allogeneic hematopoietic stem cell transplantation (HCT) have proven to be more effective than chemotherapy alone in treating post-HCT relapse[1-4]. However, the toxicities of conditioning chemotherapy, graft-versus-host disease (GVHD), and the need for chronic immunosuppression are significant and life threatening, greatly limiting the number of eligible patients. Furthermore, only a minority of relapsed patients (<30%) will achieve long-term remissions post-DLI or second HCT[3,5]. Thus, there is a clear need both to minimize toxicities and improve the anti-leukemia effects of available cellular immunotherapies post-HCT.

In order to develop a safe and effective cellular therapy for patients with relapsed AML/MDS, we designed a T cell therapy that targets multiple leukemia-associated antigens and thereby attempts to mimic the GVL effect mediated by donor T cells but with a lower risk of inducing GVHD. To this end, we took peripheral blood from hematopoietic stem cell (HSC)-donors and expanded naturally occurring T cells whose native T cell receptors (TCR) were specific for leukemia-expressed antigens (WT1, PRAME, NY-ESO-1 and Survivin). Here we report on safety and the clinical effects mediated by these stem-cell donor-derived multi-leukemia antigen-specific T cells (mLSTs) when infused to 25 HCT recipients with AML/MDS, 17 of whom were at high risk of relapse (in CR at time of infusion, including 5 who had relapsed post-HCT but were back in CR after salvage therapy immediately prior to infusion) and 8 with active AML that had failed HCT as well as subsequent salvage treatments.

METHODS

Patients

Patients with AML or MDS following post-HCT were eligible on a Baylor College of Medicine (BCM) Institutional Review Board (IRB)-approved protocol (H-36346, NCT02494167) to treat refractory/relapsed disease (active arm) or to maintain remission post-HCT (adjuvant arm). Inclusion criteria were (i) life expectancy of ≥6 weeks, (ii) undergone HCT at our center, (iii) Karnofsky (or Lansky) ≥ 50, (iv) informed consent, (v) adequate organ function (bilirubin, serum creatinine ≤2x and AST ≤3x upper limit of normal, Hemoglobin ≥7 g/dl, pulse oximetry >90% on room air), (vi) use of birth control, (vii) available mLSTs and (viii) off investigational antineoplastic therapy for ≥one month. Exclusion criteria were (i) grade ≥2 acute or extensive stage cGVHD, (ii) use of corticosteroids ≥0.5 mg/kg prednisone equivalents, (iii) use of anti-T cell antibodies (eg. alemtuzumab) ≤30 days of infusion, (iv) ongoing infection or (v) pregnancy. Calcineurin inhibitors like tacrolimus were permitted. Patients were required to have engrafted and be at least 30 days post-HCT. None of the enrolled patients received lymphodepleting chemotherapy prior to mLSTs. All patients had to undergo a disease assessment (at a minimum bone marrow exam) within 4 weeks of T cell infusion. Those with active disease had no therapy for their AML/MDS from the time of marrow exam confirming relapse or, if they received intervening therapy, had to demonstrate treatment-failure on infusion day. Bone marrow assessments were repeated at least once post-infusion. Once enrolled, patients received a single infusion of mLSTs at one of the dose levels (DL) (DL1 - 0.5 x 10^7, DL2 - 1 x 10^7, DL3 - 2 x 10^7, DL4 - 5 x 10^7 & DL5 - 10 x $10^7/m^2$) and were eligible to receive up to 6 additional infusions at the same dose if they remained in continuing complete remission (high risk arm) or achieved clinical benefit (active arm) at their subsequent evaluation. The dose escalation schema (based on m^2 rather than kg) closely mirrored doses of Epstein Barr virus-specific T cells (EBVSTs) that were shown to be safe and effective in prior clinical trials at our center[6-9]. The full protocol is provided in **Supplementary Materials**. To compare the frequency of immune evasion tactics at the time of relapse between the mLST cohort and those who were not treated with mLSTs, a

cohort of consecutive adult AML/MDS patients who had successfully engrafted by day+30 postmatched sibling donor-HCT or DLI at our center from 2012-2018 and were not treated with mLSTs were retrospectively analyzed on IRB-approved studies (H-41604 and H-43439).

Generation of mLST products

mLSTs were generated as previously described[10]. Briefly, monocyte-derived DCs were generated from donor peripheral blood, loaded with peptide mixtures (pepmixes - panels of 15-mer peptides overlapping by 11 amino acids) spanning Survivin, WT1, PRAME and NY-ESO-1 (JPT Peptide Technologies, Berlin, Germany) and co-cultured with peripheral blood mononuclear cells (PBMCs) in the presence of a Th1-polarizing cytokine cocktail [IL7 (10ng/ml), IL12 (10ng/ml), IL15 (5ng/ml), IL6 (10ng/ml)]. From day 10, responder T cells were re-stimulated weekly with pepmix-pulsed DCs in the presence of IL15 (5ng/ml) or IL2 (50-100 U/ml) until sufficient numbers were achieved for patient infusion and requisite release testing.

mLST Characterization and Immune Monitoring

Full details are found in **Supplementary methods**. Briefly, Enzyme-linked Immunospot Assay (ELIspot), immunophenotyping, intracellular cytokine staining and ^{51}Chromium release cytotoxicity analyses were performed on aliquots of mLSTs (based on availability of material).

Tumor antigen profiling

Pre-infusion and/or pre-HCT AML/MDS tissue blocks or slides were obtained when available (on protocol H-15280). Immunohistochemistry (IHC) was performed with a one-step staining technique. Briefly, formalin-fixed paraffin-embedded, positively charged, unstained tumor biopsy slides (or, where applicable, clot sections) were obtained and underwent IHC staining (see **supplementary methods**).

Statistical Analysis

Descriptive statistics were calculated to summarize clinical characteristics using mean, standard deviation (SD), standard error of mean (SEM), median and range. Dose escalation was performed independently and concurrently within the two arms (adjuvant and active disease) using the modified continual reassessment method (mCRM) (see protocol attached as **Supplementary**) to determine the maximum tolerated dose (MTD) of mLSTs, with MTD defined as the highest dose level at which the probability of a dose limiting toxicity (DLT) was at most 20%. Patients could be "enrolled" more than once to receive additional T cell infusions if they did not qualify to get additional cells per-protocol and the investigator(s) believed they could benefit from additional infusions, after appropriate regulatory approvals. However, per CRM each patient was counted only once (at the highest DL) for dose escalation calculations. On this study a DLT was defined as grade 3-4 GVHD or NCI CTC grade 3-5 toxicity within 4 weeks of mLSTs. Leukemia-free survival and overall survival (LFS and OS) was calculated from the time of the first mLST infusion to the date of relapse or death or censored at last follow-up. August 5, 2020 was the data cut-off date for analysis. Survival curves and median survival times were estimated by the Kaplan-Meier method. Comparisons in the rates of relapses between groups were made using Fisher's exact tests. Graphic works were performed with GraphPad Prism 6. Reasonable requests for any raw/trial data is available upon request by emailing: lulla@bcm.edu.

RESULTS

Patients

Twenty-nine patients with a diagnosis of AML or MDS who received an HCT and whose donors provided PBMCs were eligible and we were able to generate mLSTs for infusion in all. Two donors had to undergo two separate procurements to achieve pre-determined cell doses for infusion. Thus, a total of 31 products were generated from 29 donors. Nine patients were not

infused while 20 patients [both adults (n=17) and children (n=3)], with 5 patients enrolled and treated twice, representing a total of 25 enrollments were administered mLSTs on study. See CONSORT diagram in **Supplementary Figure 1** for details.

Adjuvant arm: Seventeen enrollees (including 2 patients, Pt#6 and Pt#9, who were enrolled and treated again after additional receiving additional therapy for relapse) received mLSTs while in CR (adjuvant group) (referral patterns listed in **Supplementary results**). The characteristics of treated patients are detailed in **Table 1**. Patients referred to the adjuvant arm of this trial were enriched for those considered to have a high-risk for relapse post-HCT because they had one or more of the following characteristics: prior history of relapse post-HCT (n=5), induction failure or ≥ CR2 prior to HCT (n=7) and/or high risk molecular features (n=12) (**Table 1**, **Supplementary Table 1**). The median time to mLSTs post-HCT was 117 days post-HCT and a timeframe that was driven primarily by the time of donor procurement for mLST manufacture (detailed in **Supplementary results**).

Active disease arm: All 8 enrollees to the active disease cohort (Pt#1 crossed over to the active arm and was re-enrolled as Pt#A1 after FDA approval, while Pt#'s A5 and A6 were enrolled twice, all 3 had active AML that failed another line of salvage therapy or HCT after their first mLST infusion) had relapsed post-HCT with disease that was resistant to subsequent salvage therapy. Patients in this cohort received a median of 5 prior lines of therapy (range: 4 to 10) **(Table 2).** All had measurable active leukemia at the time of mLST infusion with a median of 30% (30 to 70%) marrow blasts pre-infusion.

mLST characterization.

Tumor-specific T cells are present in the circulation of healthy individuals including HSC donors, but at low levels. Indeed, as shown in **Figure 1A** the mean frequency of circulating T cells reactive against WT1, PRAME, NY-ESO-1 and Survivin in our donors was just 2±1 spot forming cells (SFC)/$5x10^5$ PBMCs for each antigen as determined by IFNγ ELIspot assay. Thus,

to maximize therapeutic benefit and minimize the risk of GVHD from alloreactive T cells we selectively activated and expanded the leukemia-reactive population using our previously described methodology[10]. After 34 (±7) days in culture and 2-4 rounds of in vitro stimulation, we achieved a mean 16±2 fold increase in cells, which were enriched for tumor-reactive populations. Of the 31 lines that were generated the mean frequency of mLSTs was 210±58 SFCs/2x10^5, with PRAME inducing the strongest activity (114±35 SFC/2x10^5 cells), followed in descending order by WT1 (53±23 SFC), NY-ESO-1 (34±11 SFC), and Survivin (10±3 SFC) (**Figure 1B**). These expanded cells exhibited no alloreactivity against patient-derived non-malignant PHA blasts (mean 3.0±0.5% specific lysis, E:T 20:1, n=31). Of note, <10% specific lysis of patient-derived PHA blasts at an E:T of 20:1 was set as a study safety release criterion and was met by all products (**Figure 1C**). In addition, these mLSTs were able to kill antigen-loaded targets (mean 24±7% specific lysis, E:T 20:1, n=11 in whom sufficient residual material was available) (**Figure 1D**). The expanded mLSTs were polyclonal as assessed by both TCR vβ deep sequencing (**Figure 1E**) and phenotypic analysis (**Figure 1F**), with a mixture of CD4+ (32.4±4.5%) and CD8+ (44.6±3.9%) T cells that were activated (CD3+/CD69+: 35.1±2.7%) and expressed central (CD45RO+/CD62L+: 16.6±2.5%) and effector memory markers (CD45RO+/CD62L−: 50.8±3.4%). Lines with >50% CD4+ cells (n=7) did not demonstrate any statistically significant differences in magnitude of TAA specificity or killing capacity when compared with those that had <50% CD4+ cells (n=20).

In vivo safety of mLSTs.

Forty-eight infusions were administered to 25 enrollees across both arms and only 3 (12% of all enrollees) developed de novo (grade 1) or worsening (grade 2) acute GVHD post-infusion and 4 (16% of all enrollees) developed de novo mild chronic GVHD (**Table 3**). Only one patient [with grade 2 upper gastrointestinal (GI) GVHD] required systemic steroid treatment. Importantly, there were no cases of cytokine release syndrome (CRS), neurotoxicity or

persistent myelotoxicity (>28 days). Hepatitis (characterized by aspartate and alanine transaminase (ALT/AST) elevations only) was deemed a treatment-related because of its occurrence in 6 patients (who were infused at the 3 lowest DLs) and temporally associated with infusions. Except for one case of grade 3 hepatitis requiring systemic steroids, all others were ≤ grade 2 and self-limited (**Table 4**). No liver biopsies were performed to confirm or refute atypical GVHD as a cause for hepatitis.

Outside of the aforementioned events there were no other grade ≥3 AEs noted on the adjuvant arm of the study. Of the 8 enrollees treated on the active arm of the study all had one or more grade ≥3 cytopenias (anemia, leukopenia or thrombocytopenia) related to their active AML/MDS with no or only transient worsening post mLSTs; all recovered to baseline by day 28 post-infusion.

Adjuvant arm outcomes.

Seventeen enrollees with high-risk AML/MDS were infused with mLSTs; 5 recipients had experienced relapse post-HCT but achieved a CR with subsequent salvage regimens prior to mLST infusion (**Table 1**). Six of 17 enrollees relapsed (**Figure 2**) at a median of 9.5 months (range: 5 to 12.3 months) post-mLSTs. Notably, 1 patient had only molecular relapse without detectable blasts on marrow pathology, while 3 other relapses occurred in immune-privileged extra-medullary sites without bone marrow involvement although all 3 had previously had marrow-only disease. Two patients with CNS-only relapse received intrathecal chemotherapy and cranio-spinal radiation therapy with complete resolution of disease; one of these (pt#6) was subsequently re-enrolled and received mLSTs at a higher dose level (DL4). Both patients are alive and in CR >3 years since their initial mLST infusion. A third patient (pt#1) who experienced multiple foci of relapse within vertebral bodies (4 cortical bony sites) but without involvement of the marrow, failed decitabine salvage therapy, but achieved a CR after transitioning to the mLST active disease arm (as pt#A1) and receiving an additional dose of cells. The patient who

experienced only molecular relapse (pt#9) was treated with 4 cycles of decitabine with dasatinib after which he achieved a molecular CR. He also re-enrolled on the adjuvant arm of this trial and received another infusion of mLSTs at a higher dose level (DL4). Finally, the remaining 2 patients with frank marrow relapse went on to receive salvage systemic chemotherapy, but neither responded. Overall, 11 of 17 enrollees never relapsed post mLSTs (median LFS not reached at a median follow-up of 1.9 years) and 11 of 15 patients remain alive (estimated 2-year overall survival of 77%, **Supplementary Figure 2**) at a median follow-up of 1.9 years post-infusion (range: 6 to 51 months, **Figure 2A** and **2B**), which compares favorably with HCT outcomes for risk-matched AML/MDS patients post-HCT [median LFS of 9 to 15 months and 2-year survival probability of 42% (38 to 46%)][11,12] (**Figure 2B**).

Evidence of immune escape post mLST infusions.

Of six patients who relapsed post mLST infusions, five had available material enabling correlative studies to assess the mechanism of disease relapse (the sixth had evidence of molecular disease only). All five demonstrated one or more known mechanisms of immune escape (**Figure 3A**); upregulation of PD-L1[13] in 3 of 5 (**Figure 3B**) along with tumor relapse in immune-privileged sites (CNS and bone compared with marrow disease at initial treatment) in 3 of 6 (**Figure 3C**)[14-16], decreased MHC class II expression in 4 of 5 (HLA-DR dim/loss at relapse compared with pre-infusion or diagnosis sample, as assessed by flow cytometry, **Figure 3D**) and/or loss of target antigen expression in 3 of 5 (**Figure 3E**)[13,17,18]. When compared with 12 who experienced post-HCT relapses out of 21 consecutive AML/MDS patients who underwent matched sibling donor-HCT or DLI at our center, but who did not receive mLSTs, only 1 experienced extramedullary relapse (p=0.09, ns), and 1 in 9 (of 12 in whom HLA-DR status was known) had HLA-DR dim/loss at relapse (p=0.023) (**Supplementary Table 1**).

In patients treated with mLSTs the frequency of infused cells, as measured by TCR vβ clone tracking (**Supplementary Figure 3**) appeared to decline in those who relapsed compared with those who did not, although small numbers prohibited statistical comparisons. However, at

relapse the frequency of functional TAA-reactive T cells (IFNγ ELIspot) was markedly lower than early post-infusion (**Supplementary Figure 4**). Notably, no statistical differences were seen in proportions of CD4+, CD8+, PD1+, LAG+, central or effector memory subsets in mLST products administered to those who relapsed vs those who remained in CR. Of those who relapsed, 3 were re-enrolled and successfully re-treated with mLSTs (1 on the active disease arm and 2 on the adjuvant arm) after addressing potential mechanisms of immune escape (see "adjuvant arm outcomes" for details). All 3 remained in CR post-retreatment for at least as long or longer (>1 year in 2 cases, ongoing) than post-initial mLST infusion.

Direct anti-AML/MDS effects.

To demonstrate direct anti-tumor effects of mLSTs, 8 enrollees with HCT-resistant AML/MDS were treated with mLSTs. **Table 2** demonstrates that all 8 had relapsed post-HCT with disease that was resistant to salvage measures. Two achieved an objective response post-mLSTs (**Figure 4**). In those who did not respond to therapy we saw a decline in the expression of target TAAs on paired pre- and post-treatment biopsy samples (n=3, **Supplementary Table 2**, **Supplementary Figures 5A** and **6A**) and loss/downregulation of HLA-DR (1 of 4 with known HLA-DR status) (**Supplementary Table 2**). Circulating frequencies of TAA-specific T cells declined coincident with declining TAA expression on progressing tumors (**Supplementary Figures 5B** and **6B**).

Patient #1 was initially infused on the adjuvant arm but relapsed with discrete bone lesions coincident with loss of mLST-derived T cell clones following receipt of systemic steroids for grade 3 hepatitis. Post-relapse the patient received hypomethylating therapy (decitabine), with no response, and was subsequently re-enrolled on the "active" disease arm (re-enrolled as pt#A1, at DL1) after discontinuation of systemic steroids. Within 4 weeks of a single infusion of cells she entered a CR (**Figure 5A** and **B**) and subsequently received 3 additional infusions (all at DL1) of mLSTs 4-6 weeks apart with no recurrence of hepatitis. Prior to mLST infusions,

biopsies of a bone lesion demonstrated a dense CD3+ infiltrate surrounding CD33+ blasts indicative of present but inactive anti-leukemic T cells (**Figure 5C**). Concomitant with achievement of CR, we detected an increase in both CD8+ and CD4+ T WT1-reactive T cells as assessed by both IFN-γ ELISpot (on PBMCs) (**Figure 5D**) and ICS (gated on CD3+ cells) (**Figure 5E**), and derived from infused mLSTs (**Figure 5F**).

Similarly, Pt#A4 who had refractory AML, had a >50% reduction in marrow blast infiltration (from 40% pre-infusion to 15% by week 4 post-infusion) concomitant with rising blood counts (ANC from 250 to >1000 by month 4 without growth factors), which ultimately enabled a second HCT. At the time of infusion this patients' blasts expressed WT1, PRAME and Survivin and the observed clinical benefit occurred coincident with a detectable increase in the frequency of WT1-, PRAME-, and Survivin-reactive T cells. The expanded TAA-reactive T cells were 98% donor post-infusion compared with 5% pre-infusion indicating that they were of mLST origin)(**Supplementary Figure 7**).

DISCUSSION

In this trial we demonstrated the safety and activity of an allogeneic AML/MDS-targeted T cell therapy. When administered to 25 enrollees at high-risk or those that had relapsed post-HCT, mLSTs at doses of 0.5 -10 x 10^7 cells/m^2 were well tolerated, with no cases of grade ≥3 aGVHD or any instance of extensive cGVHD. We observed anti-leukemia effects in both cohorts, evidenced by long post-HCT remissions in the adjuvant group (median LFS not reached at a median follow-up of 1.9 years, estimated 2-year OS of 77%) and objective responses (1 CR and 1 PR) seen in the HCT-refractory, active disease cohort. Thus, outcomes from this trial demonstrate that mLSTs may be a safer yet effective alternative to DLIs.

Prophylactic DLI administrations have been shown to reduce the incidence of relapse among those with high risk AML [30% (DLI) vs >46% (no DLI) in one registry study][19]. . Unlike

DLIs, which frequently induce severe GVHD (≥grade 3 seen in up to 30% of all recipients) (2-5) due to the concomitant presence of alloreactive T cells, we saw no grade ≥3 aGVHD and only 1 episode of grade 2 aGVHD in our 25 recipients, supporting the safety of mLSTs. In addition, we saw none of the toxicities associated with engineered T cells (e.g. CAR T cells), including prolonged myelotoxicity, cytokine release syndrome or neurotoxicity[20-22]. One patient infused with mLSTs at a cell dose of 0.5×10^7 cells/m^2, developed grade 3 hepatitis and 5 others had transient grade ≤ 2 hepatitis following infusions at doses up to 2×10^7 cells/m^2, which was an unexpected toxicity. Only Pt#1 required systemic corticosteroids (0.8 mg/kg prednisone) for the treatment of hepatitis, while another (Pt#8) with grade 2 hepatitis, received 0.5 mg/kg prednisone to treat gastrointestinal GVHD. Both patients responded and corticosteroids were eventually discontinued. Notably, Pt#1 subsequently received 4 additional infusions of the same product at the same dose level (Pt#A1) without recurrent hepatitis on the active disease arm. This suggests that the initial event may have been hepatitic pattern of acute GVHD, which is observed post-transplant[23,24] as doses of calcineurin inhibitors are weaned. Thus, overall, mLSTs, infused at doses comparable with DLIs, were well tolerated and demonstrated a superior safety profile to DLIs and CAR-T cells. Standardization of donor procurements could ensure availability of mLSTs to all patients by day+30 post-HCT for pivotal trials.

Prior attempts to harness the potential of donor-derived leukemia-reactive T cells have mainly focused on targeting a single antigen or a single epitope restricted to one HLA-type[25-29]. While effective at preventing relapse in select reports, these approaches have limited effectiveness in the treatment of frank relapse post-HCT. For example, Chapuis and colleagues administered donor-derived EBVSTs engineered to express a TCR specific for an HLA-A2-restricted WT1 epitope to 11 patients with refractory AML and 12 patients in remission. While restricted to HLA-A2+ individuals, patients in remission at the time of infusion impressively remained in remission longer than a comparator cohort[29]. However, no survival advantage was observed in those who received T cells with active leukemia[28]. In the current trial mLSTs

targeted multiple leukemia antigens/epitopes, thereby permitting inclusion of all individuals irrespective of HLA-type, and produced responses in select patients with active disease. By targeting naturally presented epitopes we were also able to access “leukemia-selective” antigens that have minimal normal HSC expression in contrast with CAR-targets. With safety established, immediately available strategies can be combined (eg: epigenetic modifiers, salvage chemotherapy) to potentially improve the efficacy of mLSTs.

In 5 patients who relapsed following receipt of mLSTs as adjuvant therapy, and in contrast to a matched cohort of consecutive patients post-HCT/DLI, we found evidence of tumor evolution as a countermeasure to T cell attack. Observed signatures included upregulation of PD-L1 and decrease/loss of MHC class II and/or target antigen expression on malignant cells, as well as disease relapse in immune-privileged sites such as the CNS and bone[13-18]. Other mechanisms may also have been responsible for relapses and it should be noted that our findings are constrained by small samples and heterogeneity in patient profiles. These escape phenomena can be addressed with the application of clinically available agents including checkpoint inhibitors[30,31], interferons (upregulate HLA-expression)[32] or epigenetic therapies to alter TAA expression[33-37] combined with higher doses of mLSTs. Indeed, DLIs and epigenetic modifiers have been previously combined. For example, administration of 5-azacytidine[36] or panabinostat[37] with DLIs in two separate trials demonstrated lower than expected relapse rates of 27% and 20%, respectively. In the current study we re-enrolled and successfully re-treated 3 of these patients with mLSTs (1 at the same dose level, while 2 others at higher dose levels). In all 3 cases prior to mLST infusion we addressed potential mechanisms of failure: intrathecal chemotherapy in one with CNS-only disease (Pt#6), withdrawal of corticosteroids in another (Pt#A1) and higher doses of cells in the third (Pt#9).Additionally, our platform can support the generation of mLSTs targeting a broader spectrum of TAAs such as the MAGE family of antigens[35], PR1[38], and/or Cyclin A1[39,40], especially since inter- and intra-patient variability in the expression of individual leukemic blasts is expected[41 39,40].

In conclusion, administration of mLSTs at doses comparable to DLIs proved safe and produced direct anticancer effects in chemo-resistant AML/MDS relapses post-HCT. These results will have to be validated in randomized, multicenter trials, which are currently underway. Nevertheless, this study provides compelling evidence that mLSTs can be safely administered as a single agent or in combination to effectively produce and/or sustain long-term remissions irrespective of patient HLA-type. Given the simplicity and robustness of mLST manufacture (sans gene modification) that can be made available to all AML/MDS patients undergoing HCT as well as the demonstrated anti-leukemia effects, we believe that mLSTs addresses a major unmet need in the management of AML/MDS post-HCT.

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Data sharing: The clinical protocol, individual level patient data and correlative analysis is provided with available raw data for all. Reasonable requests for any additional raw/trial data is available upon request by emailing: lulla@bcm.edu.

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TABLES

Table 1 **Characteristics of patients treated on the adjuvant arm (morphological CR and no detectable disease on flow or genetic analysis pre-infusion)**

Never relapsed post HCT								
ID	**Donor**	**DL**	**Age/G**	**Adverse-risk features**	**Prior Treatments**	**Time to T cells post HCT**	**Donor chimerims (blood or marrow)**	**Ongoing Tacrolimus on day of infusion**
1	MRD	1	57/F	FLT3-ITD	CIA→ Sorafenib→ CIAx2→ RIC-HCT	117	100%	Yes
2	MRD	1	18/F	FLT3-ITD	AAML 1031 (Arm C-sorafenib)→ MAC-HCT	155	90%	Yes
3	MRD	1	55/F	MLL-*r*	7+3→ HiDAC→ MAC-HCT	76	100%	Yes
5	MRD	2	53/F	DNMT3A mut	7+3→ HiDAC→ MAC-HCT	63	100%	Yes
8	MRD	2	65/M	MLL-*r*	7+3x2→ 5-Azax11→ RIC-HCT	156	100%	Yes
9	MRD	3	45/M	*Ph*+AML	7+3+imatinib→ MAC-HCT	106	100%	yes
10	MRD	3	51/F	AML CR2	7+3→ HiDAC→ ***Relapse***→ FLA→ HiDAC→ MAC-HCT	106	100%	Yes
4	MRD	1	54/F	Complex-rIPSS: Int-2	5-azax11→ Transf-dep→ RIC-SCT	66	100%	Yes
11	MRD	3	53/F	CR2 (MRD+ at HCT)	7+3→ HiDAC→ ***Relapse***→ FLA→ ***MRD+***→ MAC-HCT	112	100%	None
14	MRD	3	18/F	FLT3-ITD (MRD+ at HCT)	AAML1031→ ***Relapse***→ CPX-351→ FLA(G)→ Ara-C/Peg/Midostaurin→ ***refractory***→ Venetoclax/Decitabine→ ***MRD+***→ MAC-HCT	132	100%	Unknown
13	MRD	3	26/M	TP53 mut MDS-EB2	5-aza+Venetoclax→ MAC-HCT	132	100%	Yes
15	MUD	5	67/M	CMML→ AMML (PIF)	7+3 → residual disease→ Venetoclax+5-aza→ RIC-HCT	230	91%	None
Relapsed post HCT but in CR after salvage therapy								
ID	**Donor**	**DL**	**Age/G**	**Adverse-risk features**	**Prior Treatments**	**Time to relapse post HCT**		
6	MRD	2	70/F	AML CR3	7+3→ HiDAC→ CIA→ RIC-HCT-***Relapse***→ 7+3	800	100%	None
12	MRD	3	55/M	Ph+, t-AML	7+3→ RIC-HCT→ ***Relapse***→ 7+3	2130	100%	None
7	MRD	2	58/M	RAEB-1 rIPSS: Int-2 → t-AML in CR2	Decitabine→ RIC-HCT→ ***Relapse with RAEB***→ CIA→ ***relapse as MDS***→ DLI(x4)	356	100%	None
9	MRD	4	47/M	*Ph*+AML in CR2	7+3+imatinib→ MAC-HCT→ mLST→ ***Molecular Relapse***→ Decitabine-dasatinib	460	100%	None
6	MRD	4	73/F	AML CR5	7+3→ HiDAC→ CIA→ RIC-HCT-***Relapse***→ 7+3→ mLST→ ***Relapse***→ IT chemo and XRT→ ***Relapse***→ IT chemo and XRT	1330	100%	None

ID: patient ID number, DL: dose level, G: gender, M= Male, F= Female, FLT3-ITD: fms like tyrosine kinase 3 receptor – internal tandem duplication, *MLL-r*: mixed lineage leukemia-1 gene rearrangement, DNMT3A mut: DNA Methyltransferase 3A mutation, Ph+: Philadelphia chromosome positive (BCR-ABL rearranged), CR2-5: complete remission #2 to #5, rIPSS: revised International prognostic system for MDS, Int-2: intermediate-2, MRD+: measurable residual disease present, TP53 mut: mutated TP53 gene, MDS-EB2: Myelodysplastic syndrome, excess blasts 2, CMML→AMML: acute from chronic myelomonocytic leukemia. PIF: primary induction failure, t-AML: therapy related AML, CIA:Clofarabine, idarubicin and cytarabine, RIC: reduced intensity pre-HCT conditioning chemotherapy, MAC: fully myeloablative pre-HCT conditioning chemotherapy, HiDAC: high dose (> 1g/m^2 cytarabine), 7+3: 7 days of cytarabine infusion and 3 days of idarubicin, 5-aza: 5 azacytidine, FLA(G): fludarabine and cytarabine with G-CSF, Ara-C: Cytarabine, Peg: pegasparaginase, AAML 1031: up front pediatric treatment protocol, DLI: standard donor lymphocyte infusions, XRT: radiotherapy, IT chemo: intrathecal chemotherapy only, mLST: donor-derived multi-leukemia antigen-specific T cells.

Table 2 **Characteristics of patients treated on the active disease arm (Any measurable disease pre-infusion)**

ID	Donor	DL	Age/G	Disease	Prior Treatments	Donor chimerims (blood or marrow)	Ongoing Tacrolimus on day of infusion
A2	MRD	1	70/M	IDH1*mut*	7+3→ decitabine→ IDH inhibitor→ cutis relapse→ CIA→ RIC-HCT→ ***Relapse***	100% (skin relapse)	No
A3	Haplo	1	16/M	MDS→ AML	Double cord HCT→ AML ***Relapse***→ C→ haplo-HCTx2→ ***Relapse***	<20%	No
1* & A1	MRD	1	57/F	FLT3-ITD	CIA→ Sorafenib→ CIAx2→ RIC-HCT→ mLST→ steroids→ ***Relapse***	100% (bone relapse)	No
A4	MRD	2	55/M	PIF	7+3→ HiDAC x4→ RIC-HCT→ ***Relapse***→ DLIx4→ MEC→ 5-aza→ ***Relapse***	Not checked	No
A5*	Haplo	2	23/M	Del 17p	CIAx3→ haplo-HCT→ ***Relapse***→ CIA-decitabine→ haplo-HCT→ 5-aza→ Nivolumab→ CD123 BiTE→ MEC-decitabine→ midostaurin→ ***Relapse***	Not checked	No
A5*	Haplo	2	23/M	Del 17p	CIAx3→ haplo-HCT#1→ ***Relapse***→ CIA-decitabine→ haplo-HCT#2 → 5-aza → Nivolumab→ CD123 BiTE→ MEC-decitabine→ midostaurin→ ***Relapse***→ mLST→ haplo-HCT#3→ ***Relapse***	Not checked	No
A6*	MRD	3	20/F	FLT3-ITD	7+3→ HiDAC→ MAC-HCT→ ***Relapse*** → CIA→ ***Relapse***	45%	No
A6*	MRD	3	20/F	FLT3-ITD	7+3→ HiDAC→ MAC-HCT→ ***Relapse*** → CIA→ ***Relapse***→ mLST→ CIA+decitabine→ mLST	Not checked	No

ID: patient ID number, DL: dose level, G: gender, M= Male, F=Female, FLT3-ITD: fms like tyrosine kinase 3 receptor – internal tandem duplication, IDH *mut*: IDH mutation, PIF: primary induction failure, del 17p: deletion of short arm of chromosome 17, CIA:Clofarabine, idarubicin and cytarabine, MEC: mitoxantrone, etoposide and cytarabine, Haplo: haploidentical, RIC: reduced intensity pre-HCT conditioning chemotherapy, MAC: fully myeloablative pre-HCT conditioning chemotherapy, HiDAC: high dose (> 1g/m^2 cytarabine), 7+3: 7 days of cytarabine infusion and 3 days of idarubicin, 5-aza: 5 azacytidine, CD123 BiTE: CD123-CD3 bispecific T cell engager on an investigational protocol, DLI: standard donor lymphocyte infusions, mLST: donor-derived multi-leukemia antigen-specific T cells.*re-enrolled after relapse to the active arm.

Acute GVHD						
Dose level	Organ	# of enrollees	Onset in relation to Infusion	Max overall grade	Systemic steroids	Outcome
DL2 and DL5	Skin	2 of 24 (8%)	7 days post-infusion for both	1	No	Resolved in 1 week for both
DL2	Upper GI	1 of 24 (4%)	14 days post-infusion	2	Yes	Resolved in 2 weeks, steroid treatment discontinued
Chronic GVHD						
Dose Level	Organ	# of enrollees	Onset in relation to infusion	Max overall severity	Systemic treatment	Outcome
DL1	Vaginal	1 of 24 (4%)	1 year post-infusion	Mild	No	Ongoing
DL3	Skin	1 of 24 (4%)	9 months post-infusion	Mild	No	Ongoing

Table 3

Adverse events of special interest

DL3	Joint	1 of 24 (4%)	9 months post-infusion	Mild	No	Ongoing
DL3	Eyes	1 of 24 (4%)	9 months post-infusion	Mild	No	Ongoing

CRS ***None at any dose level***
Neurotoxicity ***None at any dose level***
Myelotoxicity (Cytopenias persisting >28 days) ***None at any dose level***

DL: dose level, GVHD: graft versus host disease, Max: Maximum grade seen in any of the patients at that dose level, CRS: Cytokine release syndrome.

All other treatment related AE's

DL	Incident	# of enrollees	Onset in relation to infusion	Max grade	Treatment, if any	Status
DL1	Hepatitis	1	7 days	3	Prednisone 0.5 mg/kg	Resolved 54 days later
	Nausea/ vomiting	3	5 days, 2 days, 1 month	1	None	Resolved in 7 days
	Skin dryness	1	7 days	1	None	Resolved in 7 days
DL2	Incident	# of patients	Onset in relation to infusion	Max grade	Treatment, if any	Status
	Dry eyes	2	7 days and 9 days	1	Topical treatments	Stable, Schirmer test not done
	Hepatitis	1	26 days	2	Coincident with grade 2 aGVHD above prednisone 0.5 mg/kg	Resolved in 2 weeks
	Anemia	1	32 days	1	Coincident with grade 2 aGVHD above prednisone 0.5 mg/kg	Resolved in 7 days
	Fatigue	1	27 days	2	Coincident with RSV infection	Resolved in 7 days
	Anorexia	1	27 days	2	Coincident with RSV infection	Resolved in 7 days
	Dizziness	1	9 days	1	None	Resolved in 7 days
DL3 **Table 4**	Incident	# of patients	Onset in relation to infusion	Max grade	Treatment, if any	Status
	Diarrhea	1	3 days	1	None	Resolved in 1 day
	Fatigue	2	7 days and 14 days	1	None	Resolved by day 28
	Hepatitis	4	7 days, 7 days, 5 days, 26 days	1	None	Resolved by day 60 in all
DL4	Incident	# of patients	Onset in relation to infusion	Max grade	Treatment, if any	Status
	Diarrhea	1	14 days	1	None	Resolved in 7 days
	Lymphocytosis	1	28 days	2	None	Ongoing without symptoms
DL5	None					

DL: dose level, Max: Maximum grade seen in any of the patients at that dose level, CRS: Cytokine release syndrome, aGVHD: acute graft versus host disease, RSV: respiratory syncytial virus

FIGURE LEGENDS:

Figure 1. In vitro characteristics of mLSTs. Leukemia TAA directed activity of non-manipulated donor lymphocytes (**A**) and ex vivo expanded mLSTs (**B**) (data shown as

mean±SEM). Inability of mLSTs to kill normal recipient cells (**C**) (each individual product is represented as a symbol) but killing of TAA-pulsed normal cells tested at E:T ratios from 80:1 to 5:1 (**D**) (data shown as mean±SEM). Polyclonality of mLSTs as assessed by TCR-vβ deep sequencing (**E**) and by immunophenotyping (**F**). Each symbol represents an individual product.

Figure 2. Clinical Outcomes - Adjuvant Arm. Swimmers plots depicting outcomes after infusion of mLSTs in patients with AML/MDS who were in remission at the time of infusion (**A**) individuals who relapsed post-HCT but were in morphological remission at the time of T cell infusion are denoted by *. **(B)** Kaplan-Meier estimates of leukemia-free survival (LFS) in the adjuvant group.

Figure 3. Mechanisms of immune escape at relapse. Summary of mechanisms of immune escape observed in each relapsing patient (**A**). Expression of PD-L1 as estimated by IHC in relapsing tumors obtained from pt# 3 and #6 (**B**, 400x). MRI imaging in 3 individuals demonstrating extramedullary (immune privileged anatomic site) relapses (**C**). Representative flow cytometry plot from pt #1 depicting loss of HLA-DR expression at relapse (**D**). Downregulation of expression of target TAAs (WT1, PRAME, Survivin - IHC, 400x) on relapsing AML/MDS cells in pt #7 (**E**).

Figure 4. Clinical Outcomes - Active Disease Arm. Swimmers plots depicting outcomes after infusion in patients with AML/MDS who had refractory disease at the time of infusion.

Figure 5. Complete remission from active AML after mLST infusion. MRI images of AML invading bone pre- (**A**) and post- (**B**) mLST infusion. **C** shows Hematoxylin and Eosin stain depicting relapsed AML cells (CD33+ blasts by IHC at 400x) surrounded by a dense infiltrate of CD3+ (by IHC) T cells (10x). Changes in frequency of circulating mLSTs post-infusion as

measured by IFNγ ELISpot on fresh PBMCs (Panel **D**) as well as polyclonality of circulating WT1-specific T cells as estimated by ICS performed on fresh PBMCs at the month 6 timepoint (**E**). Change in the frequency of mLST-derived TCR clones represented as fold change from baseline in the repertoire frequency (**F**).

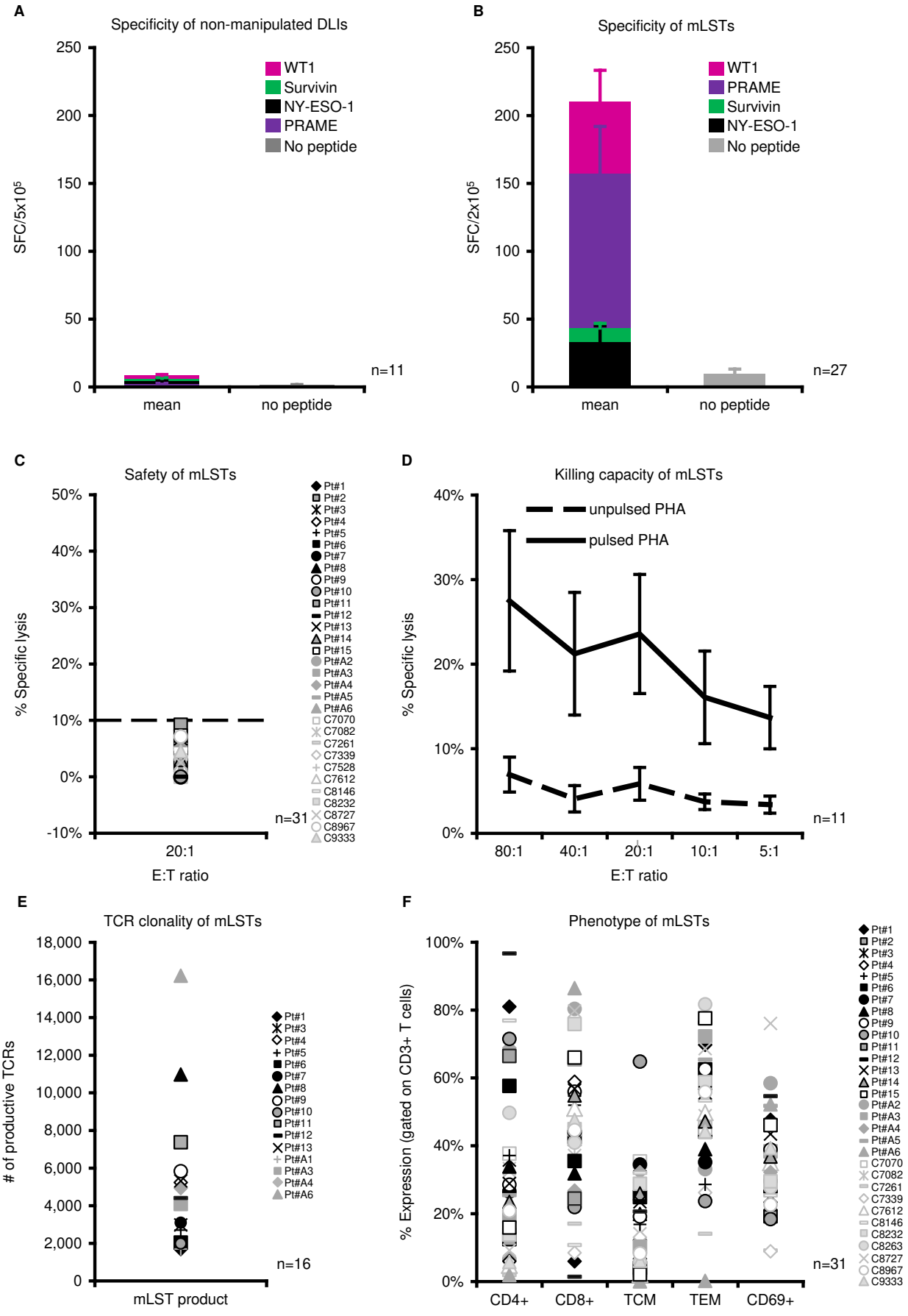


Figure 1. In vitro characteristics of mLSTs. Leukemia TAA directed activity of non-manipulated donor lymphocytes (**A**) and ex vivo expanded mLSTs (**B**) (data shown as mean±SEM). Inability of mLSTs to kill normal recipient cells (**C**) (each individual product is represented as a symbol) but killing of TAA-pulsed normal cells tested at E:T ratios from 80:1 to 5:1 (**D**) (data shown as mean±SEM). Polyclonality of mLSTs as assessed by TCR-vβ deep sequencing (**E**) and by immunophenotyping (**F**). Each symbol represents an individual product.

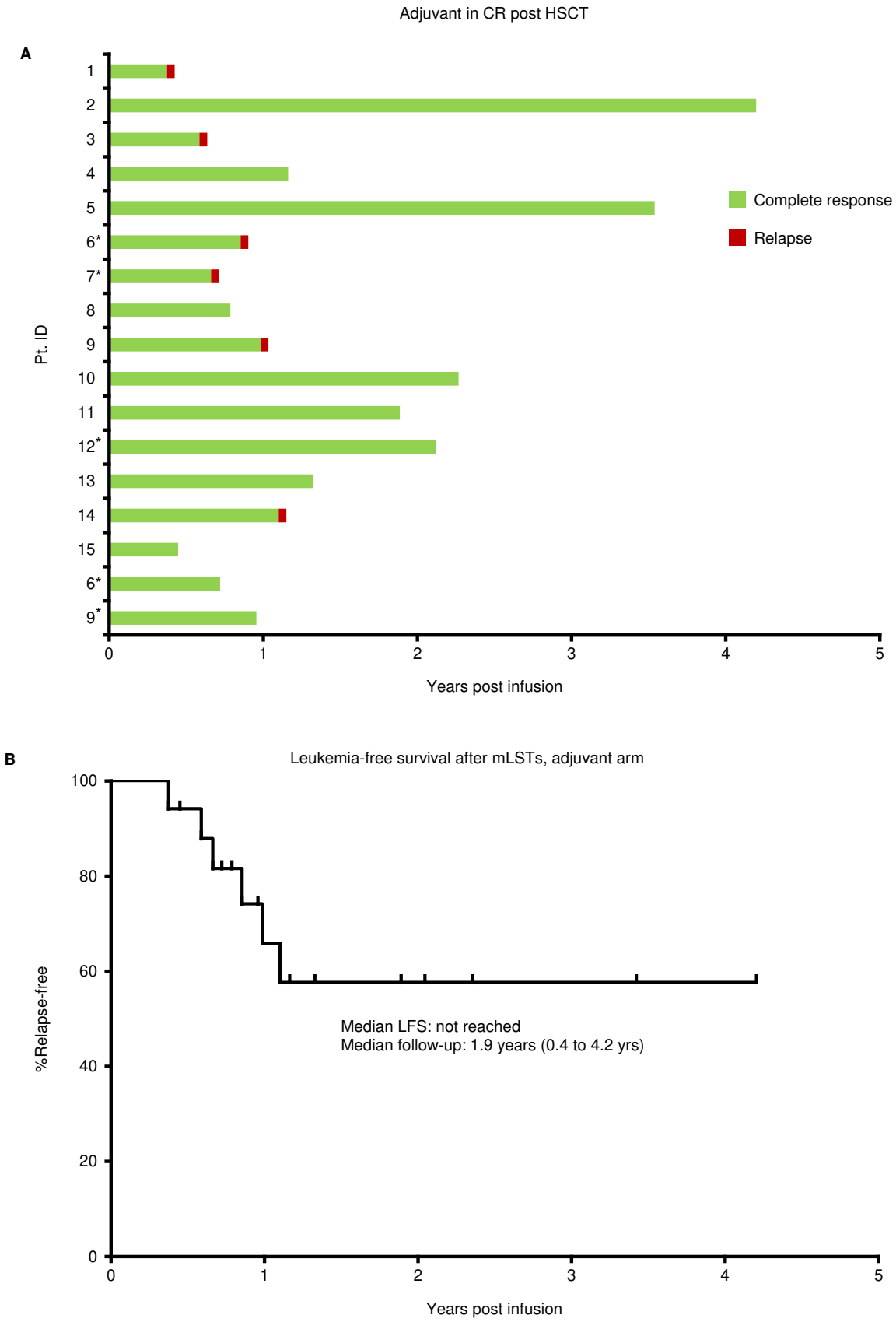


Figure 2. Clinical Outcomes - Adjuvant Arm. Swimmers plots depicting outcomes after infusion of mLSTs in patients with AML/MDS who were in remission at the time of infusion (**A**) individuals who relapsed post-HCT but were in morphological remission at the time of T cell infusion are denoted by *. **(B)** depicts Kaplan-Meier estimates of leukemia-free survival (LFS) in the adjuvant group.

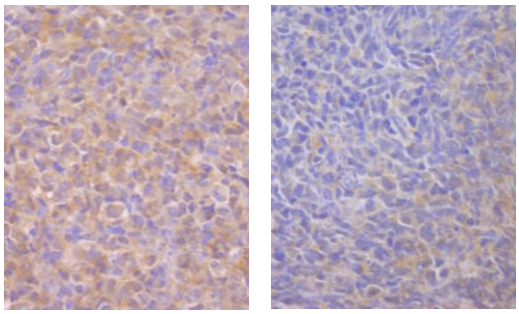
A

Immune escape related to tumor changes

ID	PD-L1 expression on relapse	Anatomic Site	HLA-DR expression on relapse (flow)	Leukemia antigen expression (IHC)
				Pre → Post
1	Negative	Bone, Marrow negative	Dim/Loss	WT1: 5% → 75% PRAME: 100% → 10% Survivin: 75% → >75% NY-ESO-1: 0% → 0%
3	Yes (99%)	CNS, Marrow negative	Dim/Loss	WT1: 10% → 0% PRAME: 75% → 0% Survivin: 90% → 99% NY-ESO-1: 5% → <1%
14	Yes (25%)	Marrow	Dim/Loss	WT1: 5% → 0% PRAME: 60% → 100% Survivin: 40% → 100% NY-ESO-1: 0% → 100%
7	Negative	Marrow	No change	WT1: 10% → 0% PRAME: 99% → 50% Survivin: 60% → 30% NY-ESO-1: 0% → 0%
6	Yes (99%)	CNS, Marrow negative	Dim/Loss	WT1: 25% → 0% PRAME: 75% → 25% Survivin: 75% → 35% NY-ESO-1: 0% → 0%

B

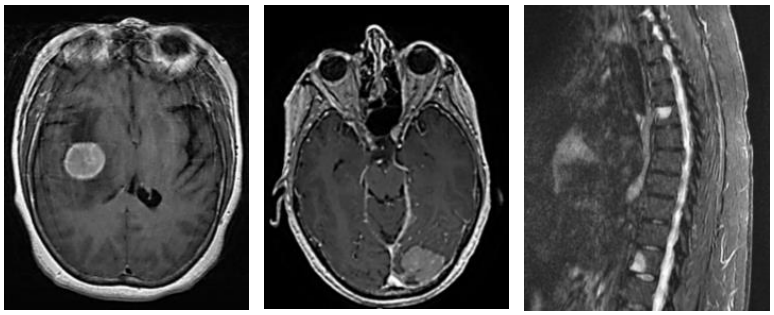
PD-L1 (by IHC)



Pt# 3 Pt# 6

C

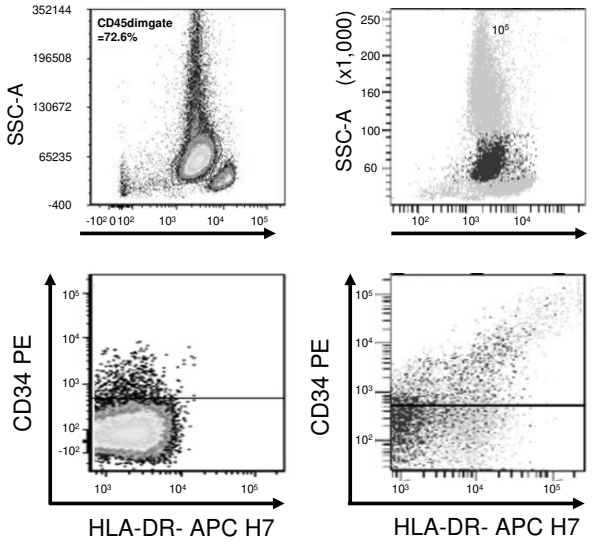
Relapse at immune privileged site



Pt# 3 Pt# 6 Pt# 1

D

HLA-DR change at relapse (Pt#1 as representative)



E

Leukemia antigen expression change at relapse (Pt#7 as representative)

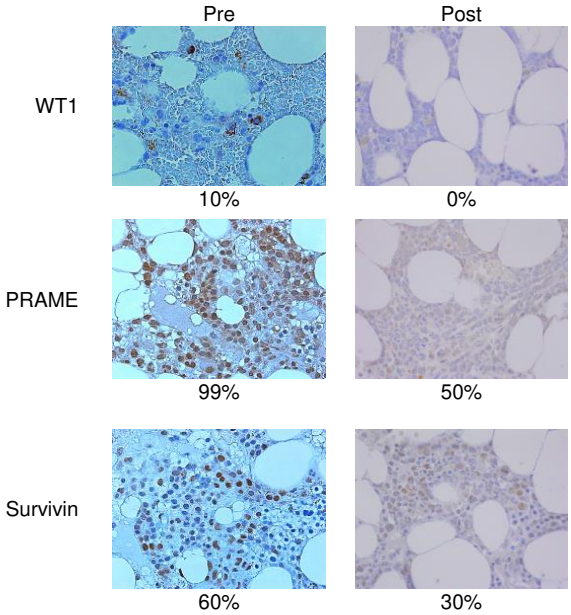


Figure 3. Mechanisms of immune escape at relapse. Summary of mechanisms of immune escape observed in each relapsing patient (**A**). Expression of PD-L1 as estimated by IHC in relapsing tumors obtained from pt# 3 and #6 (**B**, 400x). MRI imaging in 3 individuals demonstrating extramedullary (immune privileged anatomic site) relapses (**C**). Representative flow cytometry plot from pt #1 depicting dim/loss of HLA-DR expression at relapse (**D**). Downregulation of expression of target TAAs (WT1, PRAME, Survivin - IHC, 400x) on relapsing AML/MDS cells in pt #7 (**E**).

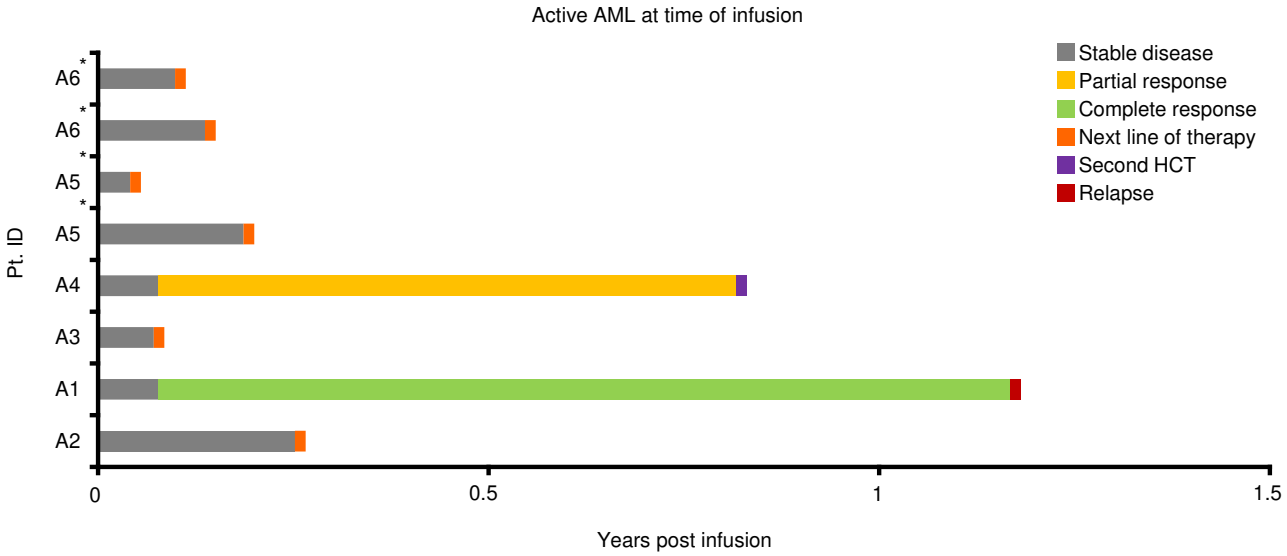


Figure 4. Clinical Outcomes - Active Disease Arm. Swimmers plots depicting outcomes after infusion in patients with AML/MDS who had refractory disease at the time of infusion.

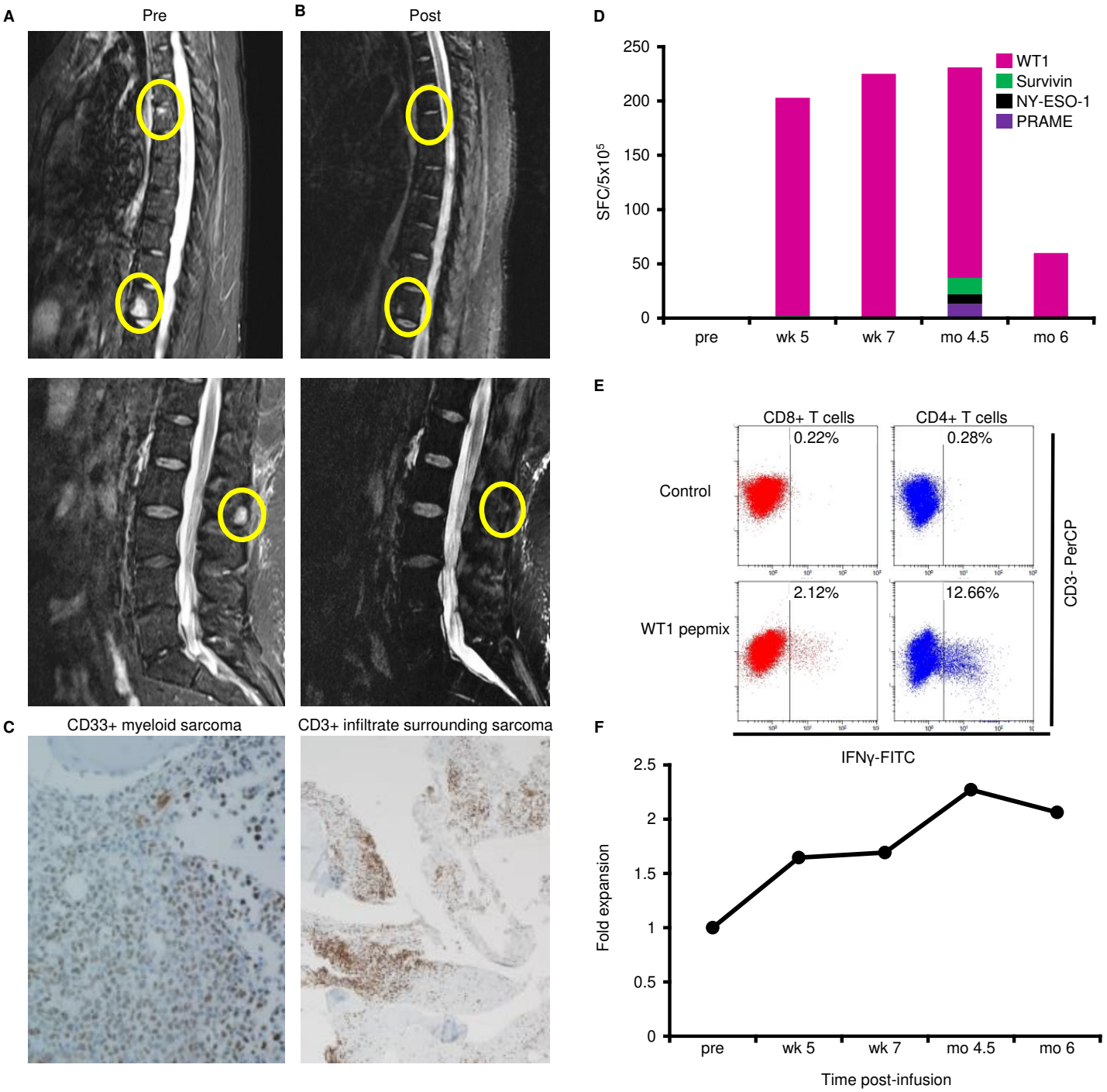


Figure 5. Complete remission from active AML after mLST infusion. MRI images of AML invading bone pre- (**A**) and post- (**B**) mLST infusion. **C** shows AML cells invading bone (CD33+ blasts by IHC at 400x) surrounded by a dense infiltrate of CD3+ (by IHC) T lymphocytes (10x). Changes in frequency of circulating mLSTs post-infusion as measured by IFNγ ELISPOT on fresh PBMCs (Panel **D**) as well as polyclonality of circulating WT1-specific T cells as estimated by ICS performed on fresh PBMCs at the month 6 time point (**E**). Change in the frequency of mLST-derived TCR clones represented as fold change from baseline in the repertoire frequency (**F**).