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Selective expansion of T-cell receptor engineered T cells with increased stem-like phenotypes using neoantigen stimulation

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ABSTRACT

Background Adoptive transfer of T-cell receptor-engineered T cells (TCR-T cells) has shown promising efficacy in solid tumor treatment, but achieving clinical benefit typically requires infusion of tens of billions of cells. The commonly used rapid expansion protocol (REP), based on the CD3-agonistic OKT3 antibody and irradiated allogeneic feeder cells, exponentially expands tumor-infiltrating lymphocytes (TILs). However, the effect of REP on TCR-T cell frequency and phenotype remains unclear. This study aimed to evaluate the impact of REP on TCR-T cells and to assess the potential of a neoantigen-specific stimulation platform, NeoExpand, as a strategy to selectively expand TCR-T cells with favorable phenotypes.

Methods We compared the effects of REP and NeoExpand on the frequency, yield, and phenotype of TCR-T cells engineered against shared neoantigens. Various autologous antigen-presenting cell (APC) types, including dendritic cells and bulk peripheral blood mononuclear cells (PBMCs), were tested as stimulators in NeoExpand. Additionally, we combined NeoExpand with CD3 activation, with or without allogeneic feeders, to improve scalability. The role of exogenous interleukin (IL)-21 in shaping TCR-T cell phenotypes was also investigated.

Results REP impaired the frequency and phenotype of TCR-T cells, particularly $CD4^+$ and $CD8^+$ subsets matched to their engineered TCRs. In contrast, NeoExpand selectively increased TCR-T cell frequency and improved their phenotypes but did not consistently achieve higher total yields compared with REP. Among the tested autologous APCs, PBMCs effectively supported selective expansion. Further optimization using PBMCs as APCs revealed that combining NeoExpand with CD3 activation enabled exponential expansion without compromising selectivity. Inclusion of IL-21 during NeoExpand promoted a naïve/stem-like phenotype in $CD8^+$ TCR-T cells, with minimal effect on $CD4^+$ TCR-T cells.

Conclusions These findings demonstrate that NeoExpand enables selective expansion of TCR-T cells while preserving favorable phenotypes and scalability. The approach supports further clinical evaluation of NeoExpand as a strategy to generate high-quality TCR-T cell products for adoptive cell therapy.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ T-cell receptor-engineered T cell (TCR-T) therapies have demonstrated clinical potential in treating solid tumors, but their efficacy depends on infusing large numbers of high-quality, tumor-reactive cells. The rapid expansion protocol (REP), which relies on OKT3 and feeder cells, is widely used for manufacturing these products but can drive T-cell differentiation and expand unengineered cells.

WHAT THIS STUDY ADDS

⇒ This study reveals that REP impairs the frequency and phenotype of TCR-T cells, whereas a neoantigen-specific stimulation platform, NeoExpand, enables selective expansion of TCR-T cells while maintaining favorable naïve/stem-like phenotypes. Autologous peripheral blood mononuclear cells served as effective antigen-presenting cells, and combining NeoExpand with CD3 activation via OKT3 achieved exponential yet selective T-cell expansion.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ NeoExpand provides a scalable, clinically adaptable alternative to conventional REP for manufacturing TCR-T cells. By enriching functional, less differentiated T cells, this approach may improve persistence and antitumor activity in adoptive cell therapy. These findings support the integration of NeoExpand into future clinical manufacturing protocols and could inform regulatory strategies for optimizing next-generation TCR-T cell therapies.

INTRODUCTION

Adoptive cell therapies (ACT) have shown clinical efficacy against advanced solid tumors.[1] Tumor-infiltrating lymphocytes (TILs) have recently been approved for the treatment of checkpoint inhibitor-refractory melanoma.[2] However, the proportion of

tumor-reactive T cells within bulk TILs from solid tumors is often low due to the presence of a large number of non-reactive bystander cells.[3–8] Furthermore, TILs frequently display exhausted phenotypes, a consequence of the immunosuppressive tumor microenvironment and chronic antigen exposure.[3–8] Solid epithelial tumors also tend to have low tumor mutation burdens, resulting in fewer neoantigens and limiting the tumor reactivity of TIL products.[9 10] In addition, not all tumors are resectable to generate TILs, making TIL therapy infeasible in certain patients.

T-cell receptor-engineered T cell (TCR-T) therapies, in which genes encoding tumor-reactive TCRs are inserted into normal circulating lymphocytes, have emerged as a promising alternative to TIL therapy thanks to several potential advantages.[11] TCR-T cells can exhibit less exhausted phenotypes than TILs and can be expanded to contain a large number of tumor-reactive cells. In addition, patients do not need to have a population of T cells inherently reactive to tumor. For example, in a recent clinical trial, a patient with metastatic breast cancer achieved a partial regression following infusion of TCR-T cells targeting the $p53^{R175H}$ neoantigen, even though her TILs exhibited no reactivity to the same neoantigen.[12] Beyond such individual cases, clinical validation has also been achieved: a TCR-T therapy targeting melanoma-associated antigen family A4 was recently approved for the treatment of synovial sarcomas.[13]

Conventional manufacturing of TCR-T cell products involves viral transduction of peripheral blood lymphocytes (PBL) followed by non-specific stimulation of CD3 via the rapid expansion protocol (REP).[14–16] However, REP is known to induce T-cell differentiation and may expand non-specific bystander cells due to the non-specific nature of CD3 activation by the CD3-agonistic OKT3 antibody.[4 17 18] Despite its widespread use, the impact of REP on the number and quality of TCR-T cells is not clearly understood.

To address this, we analyzed clinical infusion products generated for an ongoing clinical trial conducted by the Surgery Branch at the National Cancer Institute.[15] Our analysis revealed that REP decreased the frequency of TCR-T cells co-expressing appropriate CD4 or CD8 co-receptors, potentially diluting the therapeutic products with non-functional cells. Given the dose-responsiveness observed in some ACT trials,[19] and the potential for bystander cells to impair efficacy, we tested whether our previously reported neoantigen-specific stimulation protocol, NeoExpand, could selectively enrich tumor-reactive TCR-T cells.[4 20]

Using a library of TCRs targeting various p53[12] or RAS[21] "hotspot" neoantigens (including all KRAS, HRAS and NRAS mutations sharing the same epitope sequences), we evaluated the ability of NeoExpand to selectively stimulate and expand TCR-T cells. We also compared various antigen-presenting cells (APC) and found that peripheral blood mononuclear cells (PBMCs) were an effective and practical APC for NeoExpand. Although NeoExpand enriched murine TCR $(mTCR)^+$ co-receptor $(CoR)^+$ cells relative to REP, it did not consistently increase their absolute cell numbers. Combination of TCR stimulation with CD3 activation using OKT3 included in REP achieved robust and selective expansion of $mTCR^+CoR^+$ cells. Finally, we compared the function and phenotype of TCR-T cells expanded using NeoExpand versus REP both in vitro and in vivo.

RESULTS

Characterization of the infused TCR-T cells targeting private neoantigens

Parkhurst *et al.* recently described an ongoing phase II clinical trial where patients with advanced solid tumors were treated with autologous PBLs engineered with TCRs specific for neoantigens uniquely present within each patient's tumors.[15] To generate TCR-T cell products, neoantigen-reactive TCRs were identified by screening TILs for neoantigen reactivity (figure 1A). TCR sequences were isolated from the reactive T cells and were re-constructed into a retroviral vector to transduce autologous PBLs. To generate infusion products, transduced T cells were subsequently expanded through REP, which included OKT3 and allogeneic feeder cells.

To examine the effect of the REP on the TCR-T cells, we compared the number and phenotype of transduced cells before and after the REP. Infusion products from 14 patients included 21 unique TCR-T cell products targeting distinct neoantigens.[15] Four of these products, which underwent CD4 or CD8 bead selection due to low $CD4^+$ or $CD8^+$ fractions in the initial PBLs, were excluded from analysis. Each engineered TCR contained the murine constant region sequences (mTCR) to improve α/β pairing, allowing for detection of transduced cells by flow cytometry.[22]

Across the remaining 17 TCR-T products, the average frequency of $mTCR^+$ cells in the infusion products was 59.4% (range: 21.7%–80%) (figure 1B). However, when CD4 or CD8 positivity was taken into account, the mean frequency of the $mTCR^+$ co-receptor$^+$ ($mTCR^+CoR^+$) cells in the infusion products was 22.7% (range: 5.6%–72.8%), whereas the means of TCR-T with the opposite co-receptor ($mTCR^+CoR^-$) and the untransduced T cells ($mTCR^-$) were 33.29% and 40.62%, respectively (figure 1B). Because CD4 and CD8 co-receptors are generally required to augment the activation of TCRs[23 24] and because $CD4^+$ and $CD8^+$ T cells fulfill distinct immunologic roles, these data indicated that fewer than one-quarter of infused cells were appropriately redirected to target neoantigens with full functional potential.

Analysis of TCR-T cells before and after REP allowed us to further evaluate the effect of REP on TCR-T cells. The frequency of the $mTCR^+CoR^+$ cells significantly declined following REP (fold reduction: 0.76, p=0.043) (figure 1C), suggesting that REP might have resulted in a relative loss of $mTCR^+CoR^+$ cells, potentially due to outgrowth of non-transduced or $mTCR^+CoR^-$ cells. In

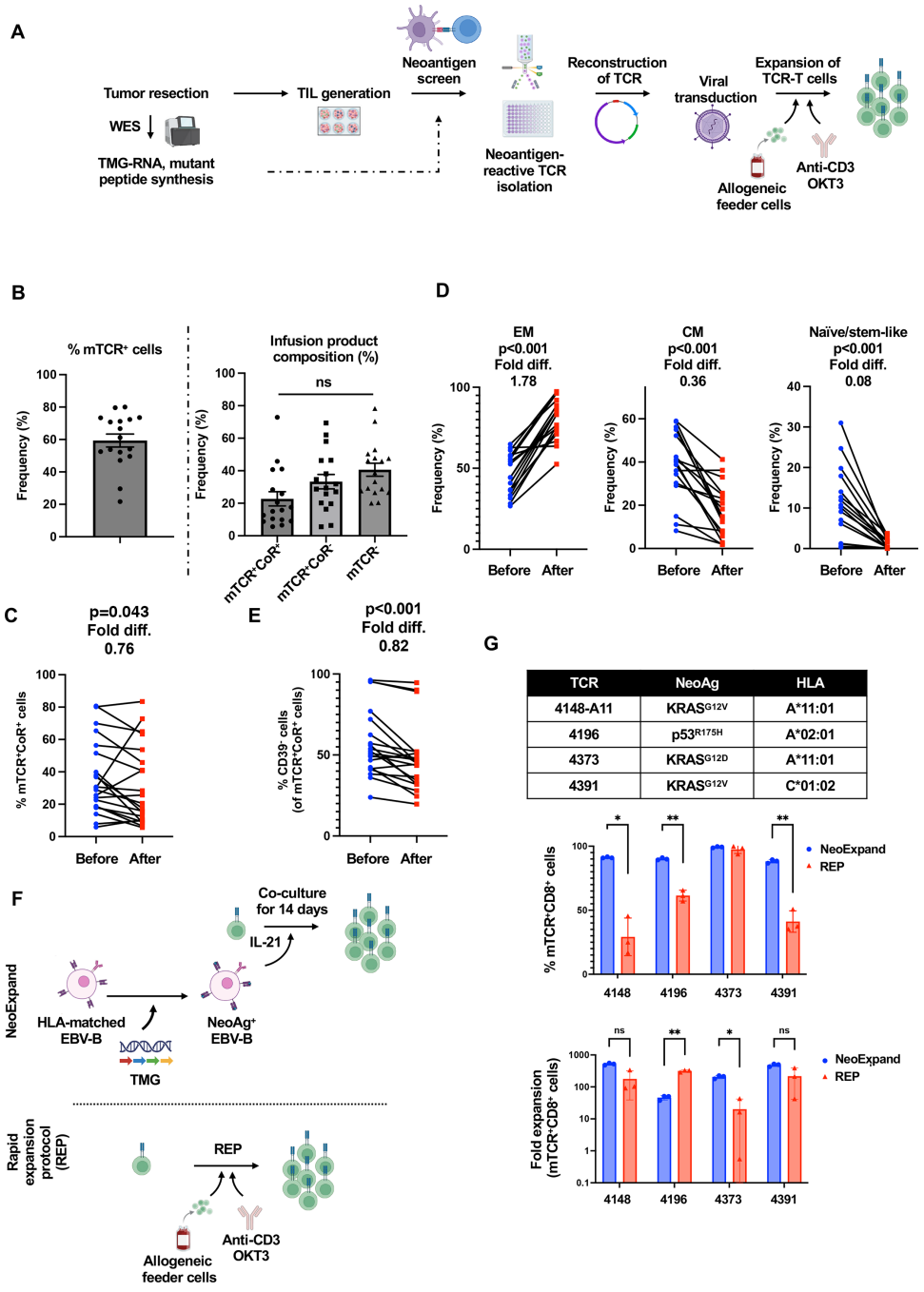

Figure 1 TCR-T cells expanded via REP show deterioration of the frequency and phenotype of mTCR$^+$CoR$^+$ cells. (A) Schematic of the production of TCR-T cells for infusion back into patients as part of clinical trial NCT03412877.[15] The process includes conventional REP. (B) Frequency of T cells expressing neoantigen-reactive TCRs among the 17 TCR-T products infused to patients.[15] Four TCR-T products that had been generated by bead-based CD4 or CD8 selection were excluded from this analysis. The frequency of exogenously expressed TCR (mTCR) was measured by flow cytometry on the day of cell infusion following REP (left panel). Frequency of mTCR$^+$CoR$^+$, mTCR$^+$CoR$^-$, and mTCR$^-$ cells (right panel). The frequencies were determined based on the expression of the CD4 or CD8 co-receptor matching those of the original T-cell clones from which the TCRs were isolated. (C) Frequency of mTCR$^+$CoR$^+$ cells before and after REP. (D) Comparison of the phenotype of the TCR-T cells before and after REP. (E) Frequencies of CD39$^-$ cells out of mTCR$^+$CoR$^+$ cells in the 17 TCR-T products. (F) Schematic of the NeoExpand culture using EBV-transformed B cells engineered with TMG expressing various p53 or RAS "hotspot" mutations. For the design of the TMG, see online supplemental figure S1A. (G) The frequency (middle panel) and the fold expansion (bottom panel) of mTCR$^+$CD8$^+$ cells generated via NeoExpand or REP. Four TCRs specific for KRAS or p53 neoantigens are described (top). The mean±SEM of triplicates is reported. (B, D–E, G) Statistical analyses by two-tailed, paired Student's t-test. *p<0.05, **p<0.01, ns not significant. CM, central memory; CoR, co-receptor; EBV, Epstein-Barr virus; EM, effector memory; HLA, human leukocyte antigen; IL, interleukin; mTCR, murine TCR; NeoAg, neoantigen; REP, rapid expansion protocol; TCR, T-cell receptor; TIL, tumor-infiltrating lymphocyte; TMG, tandem minigenes; WES, whole exome sequencing.

addition to this numerical reduction, phenotypic impairment of TCR-T cells was noted in post-REP samples. The proportion of effector memory (T_{EM}: $CD45RO^+CD62L^-$) cells significantly increased, whereas populations with central memory ($T_{CM:}$ $CD45RO^+CD62L^+$) or naïve/stem-like phenotypes ($T_{N/stem}$: $CD45RO^-CD62L^+$) significantly decreased (figure 1D). While T_{CM} and $T_{N/stem}$ subsets are associated with high proliferative potential, stemness and persistence, the T_{EM} phenotype is associated with enhanced cytotoxicity but reduced persistence.[25 26] In addition, the surface expression of CD39, a marker of T-cell exhaustion,[27] increased during REP, decreasing $CD39^-$ cells (figure 1E). These data point to the possibility that REP, despite its capacity to robustly expand T cells, may induce phenotypic and potentially functional impairment of TCR-T cells, consistent with prior reports.[4 17 18]

Previously, we reported that neoantigen-specific stimulation (termed NeoExpand) selectively enriched neoantigen-reactive TILs.[4] Given the functional similarity between neoantigen-reactive TILs and TCR-T cells transduced with neoantigen-reactive TCRs, we hypothesized that NeoExpand could similarly promote selective expansion of TCR-T cells. To test this, we used human leukocyte antigen (HLA)-matched, Epstein-Barr virus-transformed B (EBV-B) cells engineered with tandem minigene (TMG) RNAs encoding various "hotspot" p53 or KRAS neoantigens (figure 1F, online supplemental figure S1).[12 21] Patient-derived PBLs were transduced with four different TCRs targeting KRAS or p53 neoantigens (figure 1G). All four TCRs were restricted by class I HLAs.[12 21] Following transduction, T cells were expanded either via conventional REP or via NeoExpand, which involved co-culture with TMG^+ EBV-B cells in the presence of interleukin 21 (IL-21), a cytokine known to prevent T-cell differentiation and preserve the proliferative potential of antigen-experienced T cells.[20 28]

In three out of the four TCR-T cell products, the frequency of $mTCR^+CD8^+$ T cells was significantly higher with NeoExpand compared with REP (figure 1F). However, when the fold expansion of $mTCR^+CD8^+$ T cells was assessed, results were mixed: REP exhibited higher fold expansion for one TCR (4373) but lower or comparable expansion for the others (4196, 4148, and 4391). These data suggest that compared with conventional REP, although NeoExpand selectively stimulates TCR-T cells, further optimization is needed to improve absolute cell yields.

Determining the amount of antigens needed for optimal T-cell growth during NeoExpand

Next, we sought to determine the optimal level of antigen stimulation for TCR-T cell expansion. To minimize variability that could be seen with EBV-B cells expressing various irrelevant HLAs, we engineered monkey-derived COS7 cells to express individual HLA alleles, generating monoallelic APC lines (figure 2A). Using these HLA-engineered COS7 cells, we titrated peptide concentrations of minimal epitopes previously identified for TCR reactivity[12 21] (figure 2A, online supplemental figure S2).

Except for the 4391 TCR, which yielded high frequencies of $mTCR^+CD8^+$ T cells across all conditions, NeoExpand consistently resulted in higher frequencies of $mTCR^+CD8^+$ T cells compared with REP or IL-2 alone (figure 2B). No significant differences were observed between 10 and 1,000 ng/mL peptide concentrations, suggesting a wide effective range. Consistent with figure 1G, the fold expansion of $mTCR^+CD8^+$ T cells did not differ significantly between NeoExpand and REP (figure 2C). These results indicate that NeoExpand is compatible with a broad range of peptide concentrations, demonstrating its high antigen sensitivity.

Determining optimal autologous APCs for NeoExpand

Given the regulatory and safety challenges of using cell lines like EBV-B and COS7 in clinical settings, we evaluated alternative autologous APCs for their ability to support NeoExpand (figure 3A). We tested CD40L-stimulated B cells, immature monocyte-derived dendritic cells (DCs), and unmodified bulk PBMCs obtained by leukapheresis. To prevent feeder cell overgrowth, PBMCs were irradiated.

To study clinical scalability of NeoExpand using autologous APCs, an initial experiment testing two class I HLA-restricted TCRs was conducted in a G-Rex 100 flask, which could support expansion of up to 4×10^9 cells per flask (figure 3A, online supplemental figure S3A). APCs were pulsed with peptides for 2–4 hours, washed and co-cultured with TCR-T cells. IL-2 alone and REP conditions were included as controls. At days 7, 10, and 14, an aliquot of cells was collected to assess the absolute cell counts, frequency and phenotype of $mTCR^+CD8^+$ T cells. After initial testing of two class I HLA-restricted TCRs (online supplemental figure S3A) in G-Rex 100 flasks, one class I HLA-restricted and five additional class II HLA-restricted TCRs were tested (online supplemental figure S3B) in smaller G-Rex 24 well plates.

Expansion of 4373 TCR-T cells using PBMCs as APCs was as robust as that with DCs in terms of both the frequency and fold expansion of $mTCR^+CD8^+$ T cells (figure 3C). Notably, the PBMC condition yielded the highest number of $T_{N/stem}$ cells and the lowest number of T_{EM} cells (figure 3D). In an aggregate experiment testing a total of eight class I and II HLA-restricted TCRs (online supplemental figure S3B), which included the 4373 TCR data above, a similar trend was noted: on average, the frequency of $mTCR^+CoR^+$ cells was higher in NeoExpand cultures with autologous APCs compared with REP (figure 3E), although overall fold expansion was comparable across conditions (figure 3F).

Among the eight TCRs, we investigated phenotypic differences in six TCR-T cell products expanded via NeoExpand or REP. NeoExpand with PBMCs consistently yielded higher frequencies of $CD39^-$ and $T_{N/stem}$ cells and lower frequencies of T_{EM} cells than REP ($p<0.05$, figure 3G and online supplemental figure S3C). In T cells

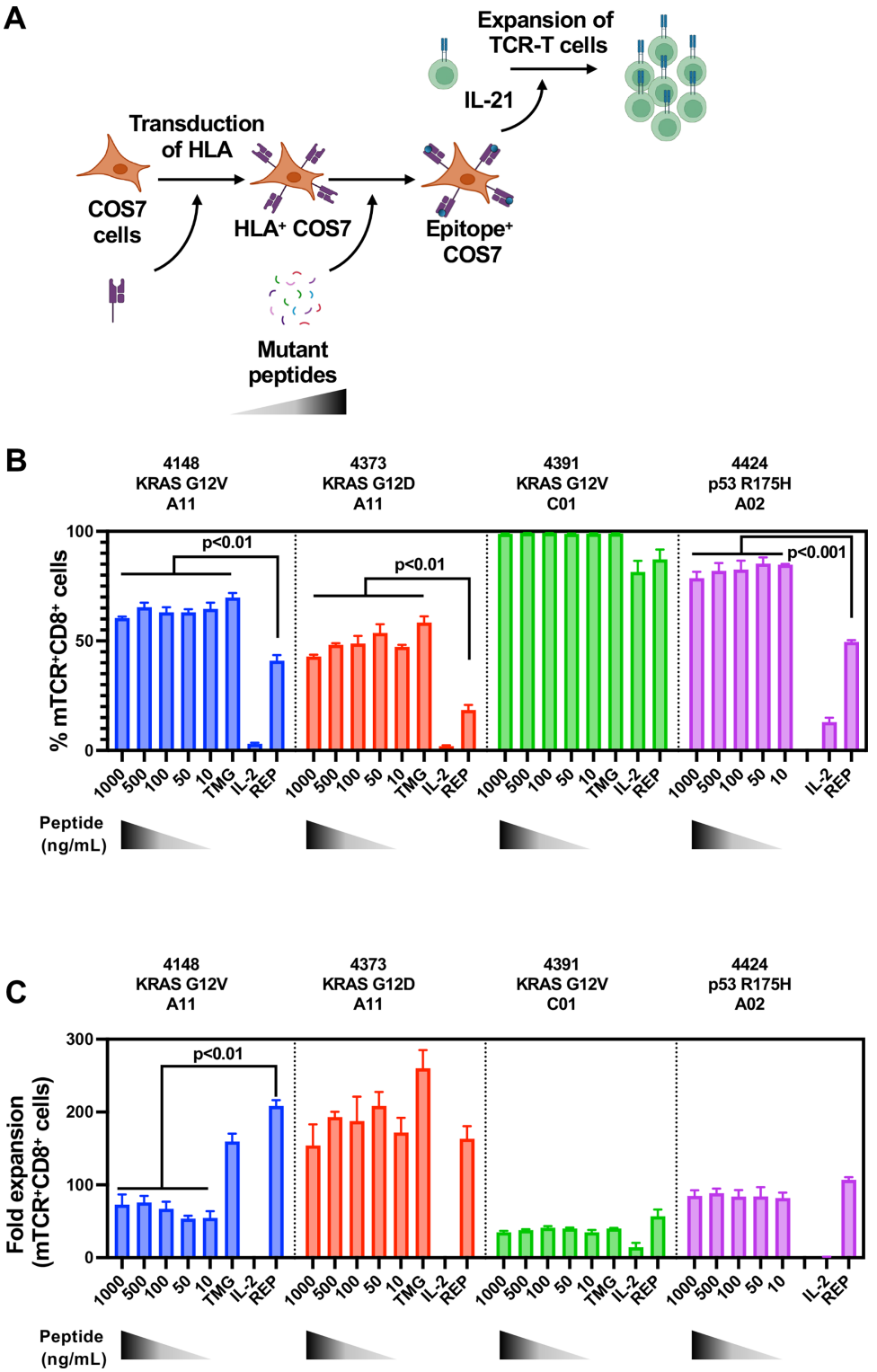

Figure 2 NeoExpand is highly sensitive and allows a wide range of neoantigen concentrations to be used for effective TCR-T cell enrichment. (A) Schematic of NeoExpand using HLA-engineered COS7 cells. Monkey kidney-derived COS7 cells lacking HLA expression were retrovirally engineered to express HLAs of interest, followed by pulsing of cognate peptides. (B) Frequency of mTCR$^+$CD8$^+$ T cells. At day 14 following NeoExpand or REP, the frequency of mTCR$^+$CD8$^+$ T cells was determined by flow cytometry. The IL-2 alone condition where T cells were expanded without any stimulation was included as a control. For the "TMG" condition, which was also included as a control, COS7 cells were constitutively transduced with the mutant TMG construct (online supplemental figure S1A). (C) Fold expansion of mTCR$^+$CD8$^+$ T cells. Fold expansion was calculated based on the frequency reported in (B) and the fold expansion of total T cells at the bulk level. (B–C) The mean±SEM of three replicate cultures is depicted. Statistical analyses by one-way ANOVA with the Geisser-Greenhouse's correction. ANOVA, analysis of variance; HLA, human leukocyte antigen; IL, interleukin; mTCR, murine TCR; REP, rapid expansion protocol; TCR, T-cell receptor; TMG, tandem minigenes.

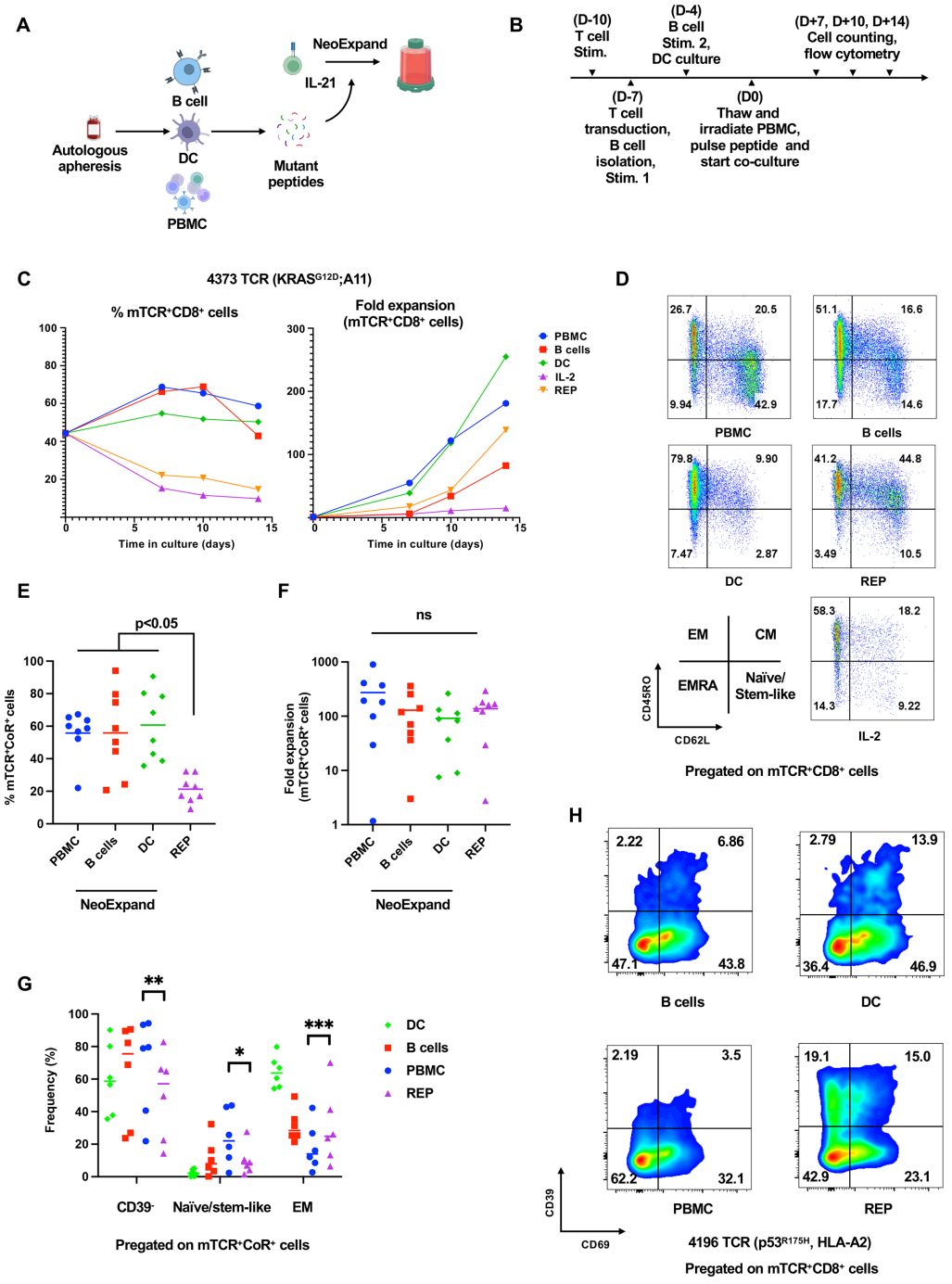

Figure 3 NeoExpand with peptide-pulsed autologous PBMCs as APCs effectively expands mTCR$^+$CoR$^+$ cells. (A) Schematic of NeoExpand testing autologous B cells, DCs and PBMCs. (B) Timeline for APC generation and the NeoExpand co-culture. Stim., T or B-cell stimulation. (C) Representative plots showing the frequency of and fold expansion of mTCR$^+$CD8$^+$ T cells transduced with 4373 TCR. At days 7, 10, and 14 following the initiation of the NeoExpand or the REP, an aliquot of T cells were harvested and analyzed for the frequency (left) and the fold expansion (right) of mTCR$^+$CD8$^+$ T cells. (D) Representative phenotype plots pregated on mTCR$^+$CD8$^+$ T cells. At day 14, cells expressing 4373 TCR were collected for the flow cytometric analysis of T-cell differentiation. (E,F) Comparison of the frequency of mTCR$^+$CD8$^+$ T cells (E) and the fold expansion (F) of mTCR$^+$CoR$^+$ cells. A total of eight TCRs (both HLA class I and II restricted) were used. See online supplemental figure S3A and B for the list of TCRs used. (G) Frequency of CD39$^-$, naïve/stem-like and T_{EM} cells (pregated on mTCR$^+$CoR$^+$ cells). The phenotype of TCR-transduced T cells was analyzed by flow cytometry. For the individual plots, see online supplemental figure S3C. (E,F,G) Bars represent mean values, and each dot represents a TCR-T sample. Statistical analyses by two-tailed, paired Student's t-test. *p<0.05, **p<0.01, ***p<0.001. (H) CD39 and CD69 expression on T cells expressing 4196 TCR expanded by NeoExpand using various autologous APCs or REP. APC, antigen-presenting cell; CM, central memory; CoR, co-receptor; DC, dendritic cell; EM, effector memory; EMRA, terminally differentiaed effector-memory cells; HLA, human leukocyte antigen; IL, interleukin; mTCR, murine TCR; PBMC, peripheral blood mononuclear cell; REP, rapid expansion protocol; TCR, T-cell receptor; T_{EM}, effector memory T cell.

expressing CD8-restricted TCRs (4196 and 4373), we further evaluated CD39 and CD69 expression. A previous study identified $CD39^{-}CD69^{-}$ cells as a stem-like T-cell subpopulation associated with favorable clinical outcomes in melanoma TIL ACT.[29] Among all tested expansion conditions, NeoExpand performed with peptide-pulsed PBMCs yielded the highest number of $CD39^{-}CD69^{-}$ cells (figure 3H and online supplemental figure S3D). These data indicate that NeoExpand using PBMCs preserves stem-like phenotypes and reduces differentiation, in contrast to the phenotypic skewing observed with REP.

Collectively, these findings indicate that leukapheresis-derived PBMCs represent a convenient, clinically applicable APC source for NeoExpand. Based on these results, we further optimized NeoExpand primarily using PBMCs as APCs.

Combination of neoantigen-specific stimulation with CD3 activation by OKT3

Thus far, our data indicated that while NeoExpand effectively enriched TCR-T cells, it did not surpass REP in expanding the absolute numbers of $mTCR^{+}CoR^{+}$ cells. Previous studies have suggested that TCR and CD3 activation by OKT3 may act non-redundantly.[30] For instance, CD3 activation by OKT3 requires co-stimulation by other cells, such as monocytes,[31] or by CD28 co-stimulation[32] to fully activate T cells. Based on this, we hypothesized that combining neoantigen-specific stimulation (NeoExpand) with CD3 activation by OKT3 could maximize the selective expansion of TCR-T cells. We tested this hypothesis by first co-culturing TCR-T cells with autologous PBMCs pulsed with varying concentrations (50–5,000 ng/mL) of mutant peptides. 24 hours later, irradiated allogeneic feeder PBMCs and OKT3 were added to the culture (figure 4A). As in previous experiments, the frequency of $mTCR^{+}CD8^{+}$ T cells (4391 TCR, online supplemental figure S4A) in all of the NeoExpand conditions increased relative to that of REP or the initial TCR-T culture (pre-expansion or PRE) irrespective of the peptide concentration or the additional REP (figure 4B). Surprisingly, NeoExpand combined with allogeneic feeders and OKT3 (referred to as "PBMC+REP") led to T-cell growth at the bulk level far greater than NeoExpand alone ("PBMC") (figure 4C). This bulk-level expansion translated into a greater fold expansion of $mTCR^{+}CD8^{+}$ T cells than either NeoExpand alone without REP (*i.e.*, PBMC) or REP alone (figure 4D).

This pattern was confirmed in an aggregate experiment involving four TCRs restricted by both class I and II HLAs: both the NeoExpand conditions with and without REP increased the frequency of $mTCR^{+}CoR^{+}$ cells more significantly than REP alone (figure 4E). Importantly, with respect to the fold expansion of $mTCR^{+}CoR^{+}$ cells, PBMC+REP led to significantly greater expansion than NeoExpand alone (PBMC) or REP alone (figure 4F, online supplemental figure S4B).

We also assessed the sensitivity of NeoExpand under this setting by testing the 4400 TCR targeting $KRAS^{G13D}$. As little as 50 ng/mL of peptide, shown in earlier assays to activate only~20% of TCR-T cells via 4-1BB upregulation (online supplemental figure S4C), still resulted in significant expansion when combined with NeoExpand (online supplemental figure S4D). This result, consistent with findings in figure 2, further supports the high antigen sensitivity of the NeoExpand approach.

Collecting a large number of PBMCs from healthy donors can be expensive and often logistically difficult. To address this challenge, we tested whether autologous PBMCs used as an APC could simultaneously function as feeder cells. To test this, OKT3 was added to NeoExpand cultures at 24 hours, either with or without irradiated allogeneic feeder cells (online supplemental figure S4E). In an aggregate experiment that included 35 TCR-donor pairs consisting of both class I and II HLA restricted TCRs (online supplemental table 1), all NeoExpand-based conditions, irrespective of OKT3 or allogeneic feeders, showed increased frequencies of $mTCR^{+}CoR^{+}$ cells compared with REP or PRE conditions (figure 4G). With respect to fold expansion, both PBMC+OKT3 (NeoExpand with OKT3 but without allogeneic feeders) and PBMC+REP outperformed NeoExpand alone (PBMC) or REP alone (figure 4H). Although $mTCR^{+}CoR^{+}$ frequencies were similar between PBMC+OKT3 and PBMC+REP (figure 4G), PBMC+REP achieved significantly greater fold expansion than PBMC+OKT3 (figure 4H), suggesting an additive benefit from allogenic feeder cells when used in combination with NeoExpand and OKT3.

The role of IL-21 in preventing T-cell differentiation during NeoExpand

IL-21 was included in the expansion of TILs[4] based on its ability to preserve memory T-cell phenotypes.[33] However, its role in NeoExpand-mediated TCR-T cell expansion remained unclear. To investigate this, we tested the impact of IL-21 alone or in combination with OKT3 on the expansion and phenotype of TCR-T cells during NeoExpand. We included the testing of OKT3 in conjunction with IL-21 to better understand if there were any synergistic effects between the two factors.

In two experiments, including 20 CD8 and 13 CD4 unique TCR-donor pairs (online supplemental table), all four NeoExpand conditions (with or without IL-21 and/or OKT3) yielded higher frequencies of $mTCR^{+}CoR^{+}$ cells (including both CD4 and CD8 TCRs) compared with REP alone (figure 5A, online supplemental figure S5A). Next, we examined fold expansion of $mTCR^{+}CoR^{+}$ cells. For CD8 TCRs, consistent with the previous experiments, OKT3 significantly enhanced fold expansion, regardless of IL-21: both $IL\text{-}21^{+}OKT3^{+}$ and $IL\text{-}21^{-}OKT3^{+}$ conditions showed higher fold expansion than $IL\text{-}21^{+}OKT3^{-}$ or $IL\text{-}21^{-}OKT3^{-}$ conditions, respectively (figure 5B). In contrast, IL-21 played a more pronounced role in modulating phenotype. Specifically, IL-21 significantly

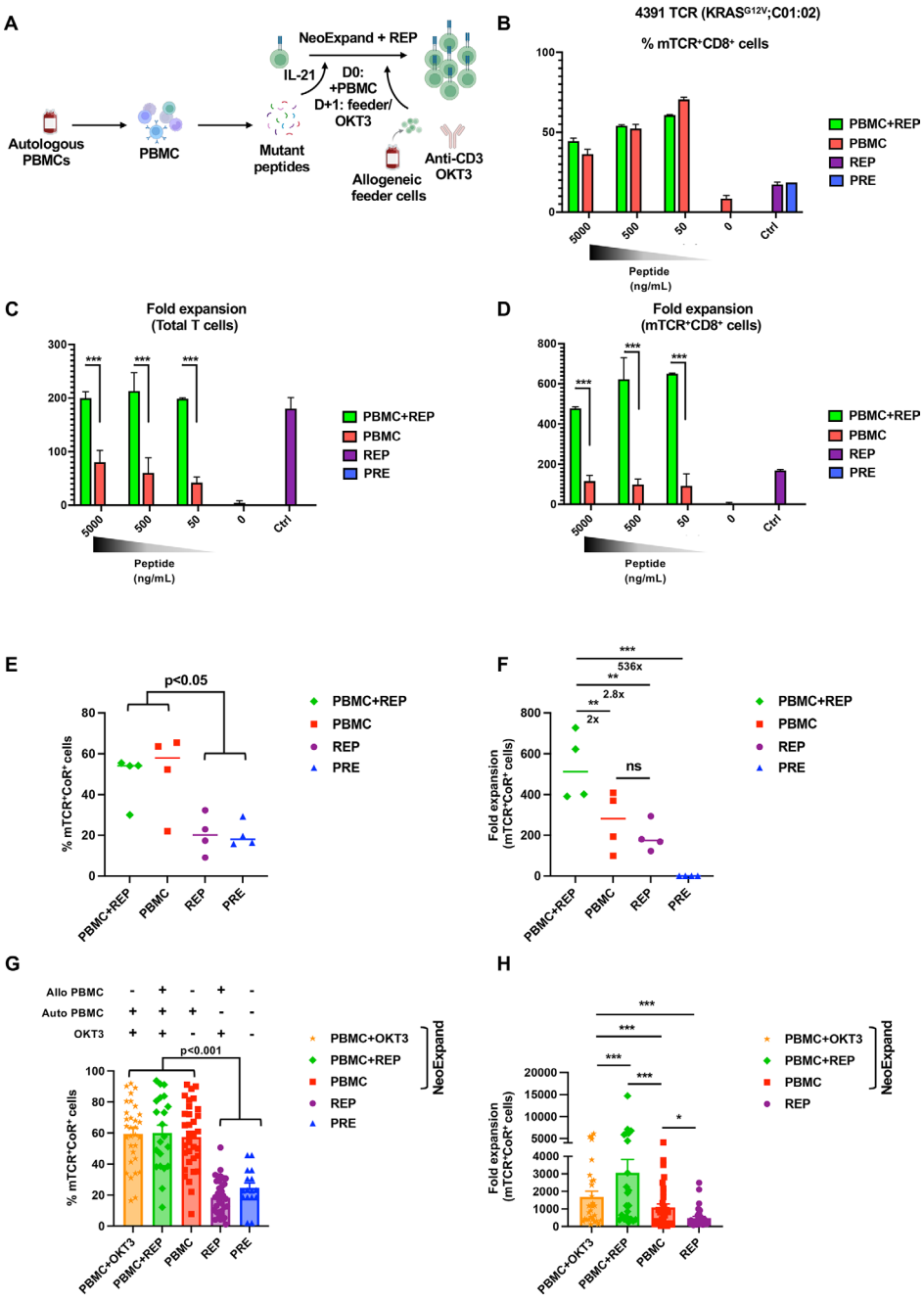

Figure 4 Combination of NeoExpand and CD3 activation by OKT3 with or without allogeneic feeders increases the absolute number of mTCR$^+$CoR$^+$ cells. (A) Schematic of NeoExpand using peptide-pulsed PBMCs in combination with CD3 activation. For the combination, the NeoExpand co-culture was started with autologous, peptide-pulsed and irradiated PBMCs, followed by the addition of an equal number of irradiated allogeneic feeder PBMCs and OKT3 at 24 hours after the initiation of NeoExpand co-culture (PBMC+REP). (B–D) Representative plots for the frequency (B), fold expansion at the bulk T-cell level (C) and the fold expansion of mTCR$^+$CD8$^+$ T cells (D) . T cells transduced with 4391 TCR were expanded via NeoExpand alone (PBMC) or NeoExpand in combination with CD3 activation (PBMC+REP). Autologous PBMCs were pulsed with varying amounts of a minimal epitope peptide containing KRAS^{G12V} (see online supplemental figure S4A). Bar represents the mean±SEM of the three replicate cultures of PBMC or PBMC+REP. Statistical analyses by two-tailed Student's t-test. (E–F) Combination of NeoExpand and REP. The frequency (E) and fold expansion (F) of mTCR$^+$CoR$^+$ cells for four different TCRs are shown. Bars represent mean values, and each dot represents a unique TCR-T sample. See online supplemental figure S4A for the list of the four TCRs and online supplemental figure S4B for the individual plots for the TCRs. (G–H) Comparison between PBMC+REP versus PBMC+OKT3 (without allogeneic feeders) (see online supplemental figure S4E). The frequency (G) and the fold expansion (H) of mTCR$^+$CoR$^+$ cells for 35 unique TCR-donor pairs are shown. Bars represent mean values, and each dot represents a unique TCR-T sample. See the online supplemental table for the list of TCRs. (E,F) Statistical analyses by one-way ANOVA with the Geisser-Greenhouse's correction. (G,H) Statistical analyses by two-tailed, paired Student's t-test. *p<0.05, **p<0.01, ***p<0.001, ns not significant. ANOVA, analysis of variance; CoR, co-receptor; IL, interleukin; mTCR, murine TCR; PBMC, peripheral blood mononuclear cell; PRE, pre-expansion; REP, rapid expansion protocol; TCR, T-cell receptor.

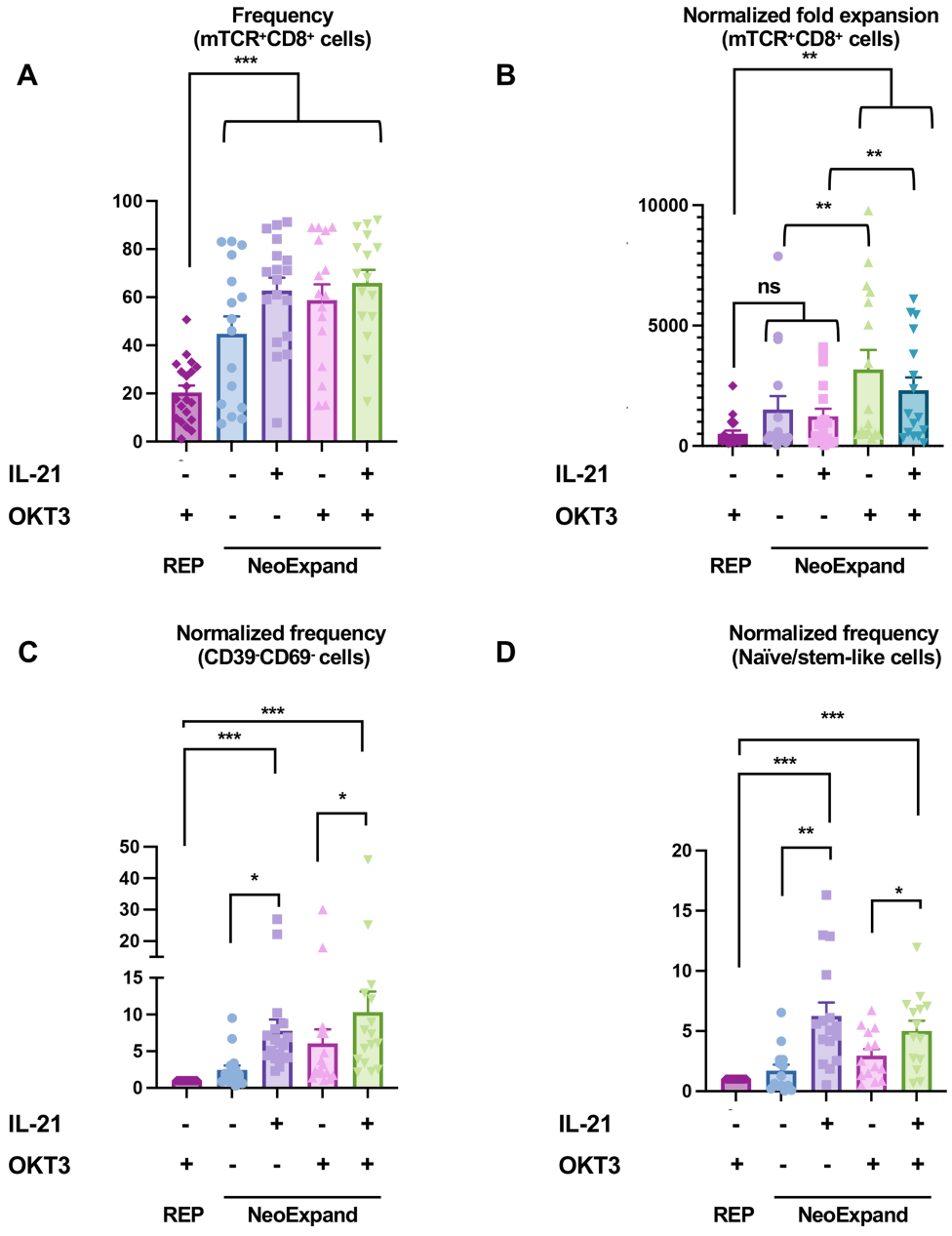

Figure 5 IL-21 maintains the stem-like phenotype of mTCR$^+$CD8$^+$ T cells expanded with NeoExpand. (A–D) 18 unique TCR-donor pairs were expanded using REP or NeoExpand under four conditions with or without IL-21 or OKT3. (A) Frequency of mTCR$^+$CD8$^+$ cells. (B) Fold expansion of mTCR$^+$CD8$^+$ cells. (C,D) Frequencies normalized to REP to account for sample-to-sample variability. (C) Normalized CD39$^-$CD69$^-$ cell frequency (ie, mTCR$^+$CD8$^+$CD39$^-$ cells). (D) Normalized frequency of the naïve/stem-like cell fraction (CD45RO$^-$CD62L$^+$) of mTCR$^+$CoR$^+$ cells. See the online supplemental table for the full list of TCRs. Bars represent mean values±SEM, and each dot represents a unique TCR-donor pair. Statistical analyses by two-tailed, paired Student's t-test. *p<0.05, **p<0.01, ***p<0.001, ns not significant. CoR, co-receptor; IL, interleukin; mTCR, murine TCR; REP, rapid expansion protocol; TCR, T-cell receptor.

increased the proportion of CD39$^-$CD69$^-$ and naïve/stem-like CD8$^+$ T cells, while OKT3 had no significant effect on these subsets (figure 5C and D).

Interestingly, the effects of IL-21 on CD4 TCR-T cells were markedly different. IL-21 appeared detrimental to fold expansion: the IL-21$^+$OKT3$^-$ condition showed significantly lower fold expansion of mTCR$^+$CD4$^+$ cells than the IL-21$^-$OKT3$^-$ condition, and the IL-21$^+$OKT3$^+$ condition showed significantly lower fold expansion of mTCR$^+$CD4$^+$ cells than the IL-21$^-$OKT3$^+$ condition (online supplemental figure S5B). When compared with REP, only the NeoExpand conditions lacking IL-21 (IL-21$^-$OKT3$^-$ and IL-21$^-$OKT3$^+$) showed the greater fold expansion of mTCR$^+$CD4$^+$ cells (online supplemental figure S5B). Phenotypically, unlike the CD8 TCRs, IL-21 and OKT3 had little effect on the phenotype of CD4 TCR-T cells, although all the NeoExpand conditions harbored significantly greater mTCR$^+$CD4$^+$CD39$^-$ or mTCR$^+$CD4$^+$T$_{N/stem}$ cells than REP (online supplemental figure S5C and D).

TCR-T cells expanded via NeoExpand demonstrate enhanced tumor control in vitro and in vivo

To determine whether the improved frequency and phenotype of mTCR$^+$CoR$^+$ cells observed with NeoExpand translated into functional benefits, we evaluated tumor control in both in vitro and in vivo models.

We employed two human neoantigen tumor models: TYK-nu ovarian cancer cells naturally expressing p53^{R175H} and HLA-A*02:01,[12] and the 4391 patient-derived xenograft (PDX) colorectal cancer line expressing KRAS^{G12V} and HLA-C*01:02 (online supplemental figure S6A).[4] TCR-T cells were generated either by conventional REP or NeoExpand using PBMCs (PBMC+OKT3). Consistent with the previous experiments, NeoExpand led to significantly higher frequencies and fold expansion of mTCR$^+$CoR$^+$ cells compared with REP (online supplemental figure S6B and C).

In cytotoxicity assays, TCR-T cells generated via NeoExpand were significantly more effective at killing TYK-nu or 4391 PDX cells than those generated via REP (figure 6A,B). We then tested these TCR-T cells in xenograft ACT models. NSG mice were injected with either TYK-nu or 4391 PDX cells and subsequently treated intravenously with 5 million (4196 TCR) or 10 million (4391 TCR) engineered T cells. In the TYK-nu model, mice treated with REP-expanded 4196 TCR-T cells showed initial tumor regression but quickly relapsed. In contrast, NeoExpand-treated mice experienced sustained tumor control (figure 6D). Similarly, NeoExpand-derived 4391 TCR-T cells led to complete tumor regression, while REP-expanded cells showed minimal effect (figure 6E).

Taken together, these data indicated that the NeoExpand, in particular NeoExpand using PBMC in conjunction with OKT3, enhanced antitumor efficacy of TCR-T cells relative to conventional REP.

DISCUSSIONS

In this study, we employed NeoExpand,[4] a neoantigen-specific stimulation method, to enable the selective and exponential growth of TCR-T cells. Analysis of infusion products generated via the conventional REP revealed a decline in both the frequency and phenotypic quality of mTCR$^+$CoR$^+$ cells. In contrast, NeoExpand conducted using peptide-pulsed autologous PBMCs was effective in enriching mTCR$^+$CoR$^+$ cells, while limiting the upregulation of markers of T-cell differentiation or exhaustion. Despite these improvements, NeoExpand alone did not consistently increase the absolute number of mTCR$^+$CoR$^+$ cells relative to the conventional REP. Surprisingly, combining NeoExpand with CD3 activation via OKT3, with or without allogeneic feeder cells, resulted in both robust and selective expansion of mTCR$^+$CoR$^+$ cells without compromising frequency. These findings suggest that TCR activation and CD3 activation via OKT3 can act synergistically to augment T-cell expansion. We also identified IL-21 as a key factor in maintaining a naïve/stem-like phenotype in CD8$^+$ TCR-T cells during NeoExpand, though its effect on CD4$^+$ TCR-T cells was minimal. Importantly, T cells generated via NeoExpand demonstrated greater anti-tumor activity in vitro and in vivo compared with those expanded via REP.

While 50–100 million CAR-T cells can be sufficient for treating hematologic malignancies,[19] treating solid tumors typically requires ten to hundred times higher numbers of TCR-T cells.[34] Achieving this scale necessitates efficient ex vivo expansion, for which REP is widely used. However, in the TIL context, REP has been reported to reduce the frequency of tumor-reactive T cells,[4 17 18] though its impact on TCR-T cells had remained unclear. By interrogating the pre-REP and post-REP samples from the 17 TCR-T infusion products used in a recent clinical trial,[15] we observed deterioration of the frequency and phenotype of mTCR$^+$CoR$^+$ cells following REP, with fewer than one-quarter of the infused cells retaining mTCR and co-receptor positivity (mTCR$^+$CoR$^+$). The mechanisms driving this loss remain unclear, warranting further investigation into how REP may disproportionately disadvantage functionally relevant TCR-T cells.

We primarily focused on selectively expanding mTCR$^+$CoR$^+$ cells given the importance of CD4 and CD8 co-receptors to the function of class II and class I major histocompatibility complex-restricted TCRs, respectively.[23 24 35] However, there has been a significant amount of literature that describes co-receptor-independent TCRs. In particular, many efforts have been made to enhance the affinity of TCRs to make them co-receptor independent. Nevertheless, the activity of TCRs that were originally derived from CD8$^+$ cells on CD4$^+$ cells or vice versa needs to be further elucidated. Notably, the "mTCR$^+$CoR$^-$" cells may contribute not only to the effectiveness of ACT but also to its side effects, such as the cytokine release syndrome.[36]

As an alternative to non-specific CD3 activation by REP, neoantigen-specific stimulation offers a more selective approach by directly engaging TCRs, thereby enriching mTCR$^+$CoR$^+$ cells. While increasing frequencies, NeoExpand alone failed to significantly increase the absolute number of mTCR$^+$CoR$^+$ cells. Given that CD3 and TCR signals may act through non-redundant pathways,[30–32] we hypothesized that combining NeoExpand with CD3 activation could synergistically promote TCR-T cell proliferation. Indeed, the combination of NeoExpand using peptide-pulsed PBMCs and the CD3 activation (by REP or OKT3 alone) enabled the selective and robust growth of mTCR$^+$CoR$^+$ cells by both the frequency and the absolute cell number. These data indicate that NeoExpand could significantly increase the number of therapeutically relevant TCR-T cells for infusion, which may enhance clinical efficacy as shown in a preclinical model in figure 6.

There is growing interest in improving the frequency and/or phenotype of tumor-reactive cells for use in ACT. While magnetic bead-based methods (e.g., CliniMACS) allow for selective enrichment,[37] they are limited by the number of target markers and can be expensive and technically complex, particularly when processing large cell numbers.

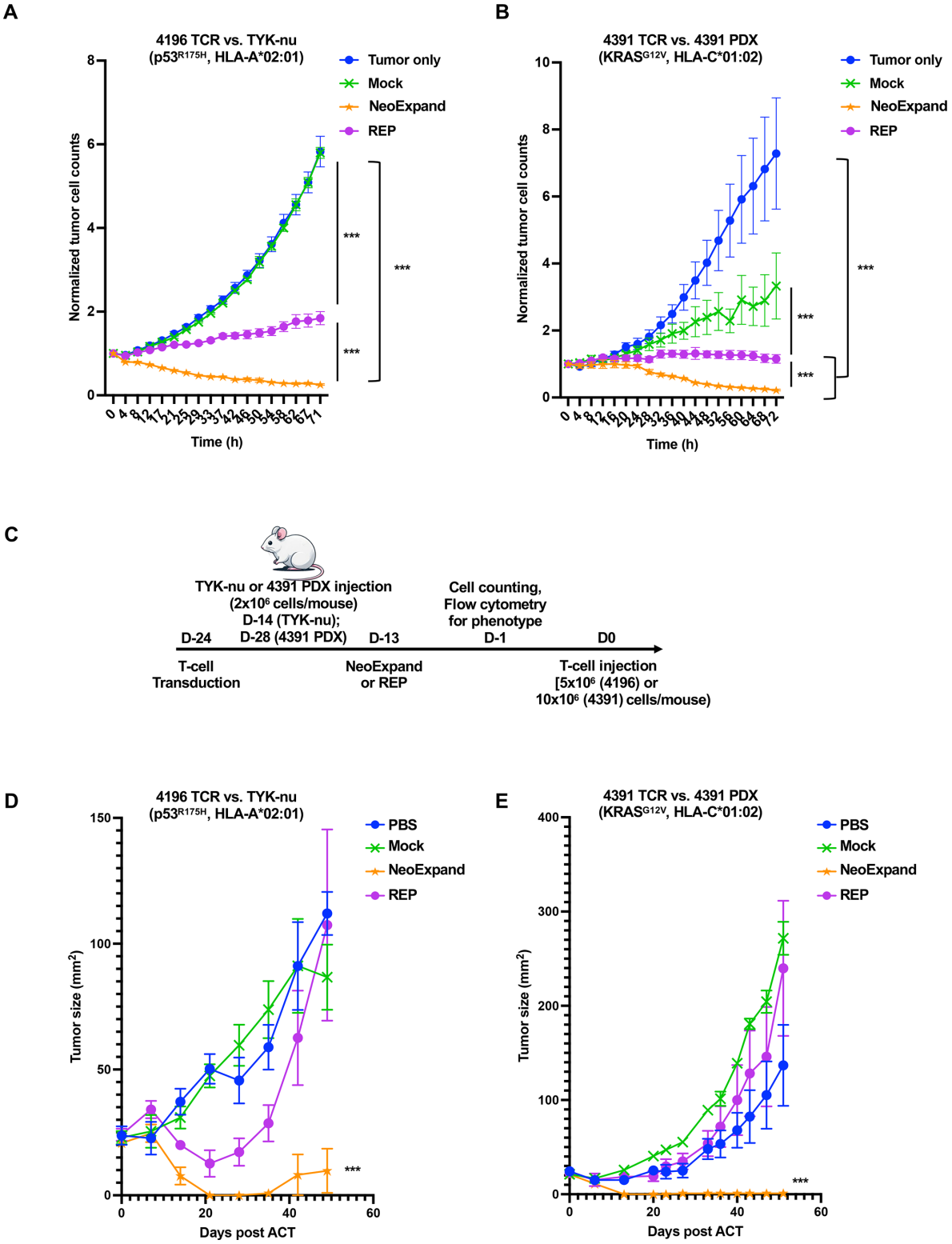

Figure 6 TCR-T cells expanded via NeoExpand exhibit superior tumor control compared with REP in vitro and in vivo. (A–B) In vitro cytotoxicity assays using TCR-T cells targeting either TYK-nu (A; 4196 TCR) or 4391 PDX cells (B; 4391 TCR). Tumor cells were stably labeled with a nuclear-localizing fluorescent protein, and the number of tumor cells was measured over~72 hours and normalized to the 0-hour measurement. Tumor-only and mock-transduced T cells served as negative controls. Data represent mean±SEM (n=4). Effector to target ratio 2:1. (C) Experimental timeline of ACT in mice bearing TYK-nu or 4391 PDX tumors. (D–E) Tumor growth in mice treated with TCR-T cells expanded via REP or NeoExpand. (D) TYK-nu model (4196 TCR). (E) 4391 PDX model (4391 TCR). Each group included five mice (n=5). This experiment was independently reproduced using T cells from a different donor. (A–B, D–E) Statistical analysis by two-way ANOVA. ***p<0.001. ACT, adoptive cell therapies; ANOVA, analysis of variance; HLA, human leukocyte antigen; PBS, phosphate-buffered saline; PDX, patient-derived xenograft; REP, rapid expansion protocol; TCR, T-cell receptor.

Similarly, fluorescence-activated cell sorting (FACS) has limited clinical applicability due to contamination risks, high pressure-induced cell damage, low throughput, and inefficiencies during sorting.[38 39] NeoExpand offers an attractive alternative by naturally enriching for $mTCR^+CoR^+$ cells with favorable phenotypes—such as naïve/stem-like subsets—without requiring extensive manipulation. Among the autologous APCs tested, bulk PBMCs generated via leukapheresis proved to be an effective and convenient APC source, eliminating the need for specialized APC preparations.

Intrigued by the phenotypic differences between REP-expanded and NeoExpand-expanded cells, we further explored the underlying mechanisms. While IL-21 is known to promote memory T-cell phenotypes in vitro,[33 40] its effects on antigen-inexperienced cells, such as TCR-engineered peripheral blood T cells, were unclear. Using both class I HLA-restricted ($CD8^+$) and class II HLA-restricted ($CD4^+$) TCRs, we found that IL-21 clearly increased the frequency of $CD39^-CD69^-$ and $T_{N/stem}$ $CD8^+$ cells, indicating improved phenotypic quality. In contrast, IL-21 significantly reduced the fold expansion of $mTCR^+CD4^+$ T cells and had no measurable effect on their phenotype. Nevertheless, the frequency of $CD39^-$ and $T_{N/stem}$ $CD4^+$ cells in NeoExpand cultures remained significantly higher than that of REP, suggesting other components of the NeoExpand system (e.g., APC-derived co-stimulatory signals) may contribute to this effect. Future studies should explore these possibilities in more detail.

Finally, functional assays demonstrated that NeoExpand-generated T cells were more effective at controlling tumor growth than those expanded via REP. In both in vitro killing assays and in vivo xenograft models, T cells expanded with NeoExpand (PBMC+OKT3) showed superior efficacy. Although the clinical necessity of highly enriched $mTCR^+CoR^+$ T-cell products remains to be determined, our results suggest that their presence correlates with improved functional activity.

A key advantage of NeoExpand is its modularity, allowing integration of different APC formats and neoantigen sources for tailored manufacturing. While the PBMC+REP condition yielded the highest fold expansion (figure 4H), PBMC+OKT3 offers a more practical alternative when allogeneic PBMCs are unavailable. This protocol uses autologous peptide-pulsed PBMCs and OKT3, requiring only minor procedural modifications from REP. In addition, NeoExpand is highly sensitive to low peptide concentrations (online supplemental figure S4C and D), potentially lowering reagent costs. Collectively, these features make NeoExpand a clinically adaptable and scalable alternative to conventional REP for TCR-T cell manufacturing.

MATERIALS AND METHODS

Human subjects and clinical protocols

Written, informed consent was obtained from all study participants, and all studies were conducted in accordance with the Declaration of Helsinki, The Belmont Report, and the US Common Rule. This study was approved by the Investigational Review Board at the National Cancer Institute (NCI) in accordance with an assurance filed with and approved by the US Department of Health and Human Services and was registered at https://clinicaltrials.gov under NCT00068003 and NCT03412877. Leukapheresis was performed to generate PBMCs from patients with chemorefractory metastatic epithelial cancers according to the tissue procurement protocol, NCT00068003. PBMCs were further purified by Ficoll separation, frozen, and kept in liquid nitrogen until use.[15] All the ex vivo cultures were carried out using pheresis products from patients and healthy donor PBMCs were used exclusively as allogeneic feeder cells.

The patient cohort in figure 1 has been previously described.[15] Briefly, adults ages 18–70 with upper or lower gastrointestinal, pancreatic or breast cancer refractory to standard chemotherapy were recruited to NCT03412877. Infusion products from 14 patients who were treated with ACT of autologous TCR-engineered T cells were examined to determine the frequency and phenotype of TCR-T cells in the infusion products.

Generation of autologous antigen-presenting cells

Generation of various autologous APCs has been previously described.[4] To generate immature DCs, peripheral blood monocytes from patient apheresis were isolated using the plastic adherence method. Adherent monocytes were cultured for 4–5 days with DC media consisting of Roswell Park Memorial Institute Medium (RPMI) 1640 (Thermo Fisher, catalog no. 21870092), 5% human serum (Gemini Bio, catalog no. H122013 or Valley Biomedical, catalog no. HP1022HI), 1% penicillin-streptomycin (Thermo Fisher, catalog no. 15070063), 1% GlutaMAX (Thermo Fisher, catalog no. 35050061), 800 IU/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) (Leukine, pharmaceutical grade, Partner Therapeutics) and 200 U/mL IL-4 (PeproTech, catalog no. 200–04). Immature DCs were collected for fresh uses or cryopreserved until use.

Primary autologous B cells were generated from $CD19^+$ cells isolated from autologous apheresis by positive selection using $CD19^+$ microbeads (Miltenyi Biotec, catalog no. 130-050-301) and were co-incubated with irradiated NIH3T3 cells constitutively expressing human CD40 ligand in a B-cell medium (Iscove's Modified Dulbecco's Medium (Thermo Fisher, catalog no. 12440053) supplemented with 10% human serum, 1% penicillin-streptomycin, and 1% GlutaMAX) in the presence of 200 U/mL IL-4. B cells were stimulated every 3–6 days up to three times, cryopreserved or freshly used. When used after cryopreservation, B cells were thawed into B-cell medium 16–24 hours before use.

Transformation of patient-derived B cells (EBV-B) was performed using supernatant from B95-8 cells containing EBV (ATCC, catalog no. VR-1492) according to the manufacturer's instruction without using feeder cells as described before.[4] Either thawed apheresis, blood draw

or CD19+ B cells following bead selection were used for transformation.

A library of COS7 cells expressing various HLAs was previously described.[4] HLA sequences were cloned into an MSGV1 vector for retroviral transduction. COS7 cells were transduced using RetroNectin (Takara Bio, catalog no. T100B), expanded and sorted by FACS (using individual HLA-specific antibodies (Pure Protein) or pan-antibodies against HLA-DP (BD Biosciences, catalog no. 566825), HLA- DQ (BD Biosciences, catalog no. 347453) or HLA-DR (BD Biosciences, catalog no. 347367)) or selected by antibiotics.

For the generation of autologous PBMCs or PBLs, patients' leukapheresis products were once purified with Ficoll and cryopreserved until use.

Flow cytometry and antibodies

T cells following a co-culture with APCs were stained with antibodies specific for the following human markers and mTCR: CD4 FITC (clone RPA-T4; 1:20, catalog no. 555346), OX40 FITC, PE or PE-Cy7 (clone ACT35; 1:20, catalog no. 555837, 555838 or 563663), CD8 FITC, Alexa Fluor 700 or PE-cy7 (clone RPA-T8; 1:25, catalog no. 555366, 561453, or 560917), 4–1BB APC (clone 4B4–1; 1:20, catalog no. 550890), and CD3 Alexa Fluor 700 or APC-Cy7 (clone UCHT1; 1:20, catalog no. 561027 or clone SK7; 1:25, catalog no. 341090).

To study the phenotype of TCR-T cells, the following two panels of antibodies were used:

Panel 1: CD3 APC-Cy7, CD8 PE-Cy7, CD4 FITC (all three same as above), CD39 PE (clone A1; 1:40, catalog no. 328208, BioLegend), CD69 BV650 (clone FN50; 1:25, catalog no. 563835, BD Biosciences), PD-1 BV421 (clone EH12.1, 1:20, BD Biosciences, catalog no. 562516) and mTCR-APC (clone H57-597, 1:20, BD Biosciences, catalog no. 553174).

Panel 2: CD62L BV421 (clone DREG-56; 1:20, catalog no. 304828, BioLegend), CD8 BV650 (clone RPA-T8, 1:20, catalog no. 301042, BioLegend), TIM3 BB515 (clone FN50; 1:20, catalog no. 565568, BD Biosciences), TIGIT PE-Cy7 (clone A15153G, 1:20, catalog no. 372714, BioLegend), CD45RO APC (Clone UCHL1; 1:20, catalog no. 559865, BD Biosciences), CD4 APC-H7 (Clone SK3; 1:20, catalog no. 641398, BD Biosciences) and mTCR-PE (clone H57-597, 1:20, BD Biosciences, catalog no. 561081).

Analytic flow cytometry was performed on LSRFortessa, or FACSymphony (BD Biosciences) with analysis by FlowJo software (V.10.10.0, BD Biosciences). All cells were gated via lymphocytes (forward scatter and side scatter) and live cells by exclusion of cells stained with propidium iodide (catalog no. P1304MP, Thermo Fisher), or 4′,6-diamidino-2-phenylindole (DAPI) (BioLegend, catalog no. 422801).

TCR transduction of PBLs

Transduction of PBLs from donors with various gastrointestinal cancer was performed as previously described.[15] Frozen aphereses were thawed, counted and were stimulated in 50/50 media (RPMI-1640 media containing 10% human serum, 1% GlutaMAX, 12.5 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Thermo Fisher, catalog no. 15630080), 1% penicillin-streptomycin, and 5 µg/mL gentamicin (Quality Biological, catalog no. 120–099–661) mixed with AIM-V (Thermo Fisher, catalog no. 12055083) at 1:1 ratio), supplemented with 50 ng/mL anti-CD3 antibody (Miltenyi Biotec, catalog no. 130-050-301) and 300 IU/mL IL-2 for 48 hours. Retroviral supernatants were freshly collected, loaded into RetroNectin (Takara Bio, catalog no. T100B)-coated 24 or 6-well plates and were spun for 2 hours at 32°C at 2,000 g. Next, stimulated PBLs were added into the virus-loaded plates, spun for 10–20 min at 32°C at 1,500 revolutions per minute (RPM) with minimal acceleration and brake. At days 8–10 post-transduction, T cells were collected and examined for exogenous TCR expression and phenotype analysis by flow cytometry.

NeoExpand

Peptide pulsing of APCs

25 mer peptides containing single amino acid mutations in the middle or minimal epitopes were synthesized and purified to >90% purity by the high-performance liquid chromatography (GenScript, custom synthesis). The concentration of peptides used in NeoExpand ranged from 100 ng/mL to 500 ng/mL except for the titration experiments in figures 2 and 4. APCs were pulsed with peptides for 2–6 hours in APC-specific media and washed two times with phosphate-buffered saline (PBS) before use. Pulsing of PBMCs was performed as follows: on the day of NeoExpand co-culture, autologous PBMCs were thawed into 50/50 media treated with DNase for 30 min, counted, and irradiated (6,000 rad). Finally, PBMCs were pulsed as described above.

NeoExpand co-culture of antigen-loaded APCs and T cells

TCR-engineered T cells were counted and were incubated with antigen-loaded APCs at 1:1 to 1:50 T cell to APC ratio. For the first 3 days of NeoExpand co-culture, cells were cultured in 50/50 media (RPMI-1640 media containing 10% human serum, 1% GlutaMAX, 12.5 mmol/L HEPES (Thermo Fisher, catalog no. 15630080), 1% penicillin-streptomycin, and 5 µg/mL gentamicin (Quality Biological, catalog no. 120–0 99 661) mixed with AIM-V at 1:1 ratio) supplemented with 30 ng/mL IL-21 (PeproTech, catalog no. 200–21) and 0–50 IU/mL IL-2. After initial feeding, the cells were fed every 3 days with 50/50 media containing 30 ng/mL IL-21 and 300 IU/mL IL-2. When NeoExpand was conducted in combination with an REP, 16–36 hours after the initiation of the NeoExpand co-culture with autologous, peptide-pulsed PBMCs, an equal number of allogeneic, irradiated PBMCs were added along with 30 ng/mL OKT3 and 3000 IU/mL IL-2. At the end of 10–28 days of NeoExpand co-culture, T cells were collected to test the frequency of TCR-engineered T cells.

Rapid expansion protocol

Non-specific T-cell stimulation for T-cell expansion through rapid expansion has been described before.[14] Briefly, T cells were incubated with 30 ng/mL OKT3, 3,000 IU/mL IL-2 and irradiated allogeneic feeders (50–100 times the number of T cells) in T-175 flasks, G-Rex 24 well, G-Rex 6 well plates or G-Rex 100 flasks (WILSON WOLF, catalog no. 80192M, 80240M and 80500, respectively). After 5 days, half the media was removed and replaced with fresh 50/50 media containing 300 IU/mL IL-2.

Cell lines

COS7, TYK-nu, and PDX line 4391, have been described before.[4] Tumor cell culture media consisted of RPMI-1640 supplemented with 10% fetal bovine serum (FBS) (Cytiva, catalog no. SH30071.03HI or Gemini Bio, catalog no. 100–106), 1× non-essential amino acid (Thermo Fisher, catalog no. 11140050), 1 mmol/L sodium pyruvate (Thermo Fisher, catalog no. 11360070), 1% penicillin-streptomycin, 1% GlutaMAX, 10 µg/mL gentamicin, and 55 µmol/L 2-mercaptoethanol (Thermo Fisher, catalog no. 31350010). Media were replaced every 2–7 days and cells were passaged when confluence reached 70%.

To introduce the TMG sequences encoding various p53 and RAS neoantigens (online supplemental figure S1)—24–25 mers of 12 RAS mutations and 14 p53 mutations into COS7 cells and EBV B cells, the entire cassette was synthesized and cloned into an MSGV1 vector with a blasticidin resistance gene using NotI and EcoRI (custom cloning by GenScript). For the constitutive expression of the TMG, HLA-engineered COS7 cells or EBV-B cells were retrovirally transduced and selected with 5–10 µg/mL blasticidin.

In vitro tumor killing assays

TYK-nu and 4391 PDX cells were stably labeled with lentivirus (IncuCyte Nuclight Red Lentivirus V.2.0, catalog no. BA-04887, Sartorius, Michigan, USA) to express a red fluorescent protein with a nuclear localization signal, according to the manufacturer's recommendation. For an in vitro killing assay, 10,000 TYK-nu cells or 20,000 4391 PDX cells were incubated with double the number of TCR-T cells (effector:target ratio of 2:1) in a flat bottom 96-well plate. Live cell imaging was performed on CELLCYTE X (Cytena, Germany).

Xenograft tumor treatment by ACT

Animal experiments were approved by the Institutional Animal Care and Use Committees of the NCI and performed in accordance with the National Institutes of Health guidelines. Immunodeficient NSG or NCG (NOD-Prkdce^{m26Cd52}Il2rg^{em26Cd22}/NjuCrl) mice were obtained from NCI or Charles River, respectively. 6–8 weeks old female mice were used for all the xenograft experiments. 2 million TYK-nu or 4391 PDX cells were subcutaneously implanted into the flank of NSG or NCG mice. In 2–4 weeks, when the tumor size reached~30 mm^2, mice were randomized and intravenously injected with TCR-T cells. Following an REP or a NeoExpand, without any further enrichment or sort, T cells were collected for injection. PBS was used as a vehicle for T-cell injection. At the time of T-cell injection and two additional times once daily, mice were intraperitoneally injected with 180,000 IU of recombinant human IL-2 in 500 µL of PBS. Tumor growth was measured once or two times a week, and tumor size was calculated as the product of two perpendicular measurements. All experiments were conducted in a blinded manner.

Statistical analyses

The effect of ACT treatments on the growth of the xenograft tumors in mice was analyzed using two-way ANOVA. All statistical analyses were performed using GraphPad Prism (V.10) and summarized data are presented as mean±SEM. In figure 5 to compare the relative expansion of various conditions while accounting for the differences in the basal levels of various phenotype/differentiation markers, the fold changes were normalized by dividing values to that of REP. P values<0.05 were considered significant. *p<0.05, **p<0.01, and ***p<0.001.

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Contributors SPK and NL designed and executed most of the experiments. YJ, CM, ZY, LL, NRV, MP, SR, MM, SF, MK, HH, SLG, and NDK performed or analyzed experiments. SAR provided oversight and supervised the project. SPK, NL, NDK, and SAR wrote and revised the manuscript. Funding acquisition: SAR. SAR is responsible for the overall content as the guarantor.

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Competing interests SPK, NL, NDK, and SAR have pending patent applications. Surgery Branch, NCI, has collaborative research and development agreements (CRADA) with Iovance Biotherapeutics and Neogene (AstraZeneca).

Patient consent for publication Not applicable.

Ethics approval This study involves human participants and was approved by the Investigational Review Board at the National Cancer Institute (reference numbers: 03-C-0277, 10-C-0166, 18-C-0049). Participants gave informed consent to participate in the study before taking part.

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Data availability statement All data relevant to the study are included in the article or uploaded as supplementary information.

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