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# Neoantigen-specific stimulation of tumor-infiltrating lymphocytes enables effective TCR isolation and expansion while preserving stem-like memory phenotypes

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## ABSTRACT

**Background** Tumor-infiltrating lymphocytes (TILs) targeting neoantigens can effectively treat a selected set of metastatic solid cancers. However, harnessing TILs for cancer treatments remains challenging because neoantigen-reactive T cells are often rare and exhausted, and ex vivo expansion can further reduce their frequencies. This complicates the identification of neoantigen-reactive T-cell receptors (TCRs) and the development of TIL products with high reactivity for patient treatment.

**Methods** We tested whether TILs could be in vitro stimulated against neoantigens to achieve selective expansion of neoantigen-reactive TILs. Given their prevalence, mutant p53 or RAS were studied as models of human neoantigens. An in vitro stimulation method, termed "NeoExpand", was developed to provide neoantigen-specific stimulation to TILs. 25 consecutive patient TILs from tumors harboring p53 or RAS mutations were subjected to NeoExpand.

**Results** We show that neoantigenic stimulation achieved selective expansion of neoantigen-reactive TILs and broadened the neoantigen-reactive $CD4^+$ and $CD8^+$ TIL clonal repertoire. This allowed the effective isolation of novel neoantigen-reactive TCRs. Out of the 25 consecutive TIL samples, neoantigenic stimulation enabled the identification of 16 unique reactivities and 42 TCRs, while conventional TIL expansion identified 9 reactivities and 14 TCRs. Single-cell transcriptome analysis revealed that neoantigenic stimulation increased neoantigen-reactive TILs with stem-like memory phenotypes expressing IL-7R, CD62L, and KLF2. Furthermore, neoantigenic stimulation improved the in vivo antitumor efficacy of TILs relative to the conventional OKT3-induced rapid TIL expansion in p53-mutated or KRAS-mutated xenograft mouse models.

**Conclusions** Taken together, neoantigenic stimulation of TILs selectively expands neoantigen-reactive TILs by frequencies and by their clonal repertoire. NeoExpand led to improved phenotypes and functions of neoantigen-reactive TILs. Our data warrant its clinical evaluation.

**Trial registration number** NCT00068003, NCT01174121, and NCT03412877.

### WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Prior research indicates that tumor infiltrating lymphocytes (TILs) can treat some cancers but challenges arise due to the scarcity and exhaustion of neoantigen-reactive T cells. Current expansion methods, such as the rapid expansion with OKT3, are not selective and can exacerbate the exhaustion of TILs.

### WHAT THIS STUDY ADDS

⇒ This study introduces a neoantigen-specific stimulation method, "NeoExpand," to selectively expand neoantigen-reactive TILs. The method selectively expands neoantigen-reactive TILs, facilitates the identification of neoantigen-reactive T-cell receptors (TCRs) and preserves stem-like memory phenotypes of TILs, leading to improved antitumor efficacy in vivo.

### HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ By selectively expanding neoantigen-reactive TILs and maintaining their less differentiated, stem-like phenotypes, the NeoExpand method could improve the effectiveness of adoptive cell therapies (ACT) and may make TIL ACT available for patients with TILs that contain rare and exhausted neoantigen-reactive TILs.

## BACKGROUND

Neoantigens arising from tumor somatic mutations are highly specific targets for cancer immunotherapies.[1–6] It has been shown that neoantigens can be successfully targeted by adoptive cell therapies (ACT) using tumor-infiltrating lymphocytes (TILs) in patients with advanced solid cancers.[7–12] However, unlike melanoma with a high tumor mutation burden, the number of somatic mutations in common solid

epithelial cancers are low.[13] Except for melanoma,[14] bladder cancer,[15] non-small cell lung cancer,[16] or mismatch repair-deficient tumors,[17] which can respond to immune checkpoint inhibitor treatments, there exist few effective immunotherapies for the majority of solid epithelial cancers, which account for over 90% of cancer-associated deaths in the USA.[18 19] Recent reports show that only 1~2% of somatic mutations in common solid epithelial cancers,[19] including gastrointestinal[5] and breast cancers,[10] are immunogenic. Consistent with that, emerging evidence suggests that among the heterogenous populations of TILs within solid epithelial cancers, neoantigen-reactive T cells are rare and exhibit exhausted phenotypes.[6 20–23] Taken together, the rarity and the exhausted phenotypes of neoantigen-reactive TIL from solid epithelial cancers pose problems in developing effective ACT targeting neoantigens using either T-cell receptor (TCR)-engineered cells or ex vivo-expanded TILs with high neoantigen-reactivity. For example, we recently reported that for various solid epithelial cancers expressing mutated *TP53* (p53), the generation of clinical TIL infusion products with high mutant p53 reactivities was difficult to achieve, ultimately leading to few clinical responses and poor persistence of the infused neoantigen-reactive TIL clones.[24] Alternatively, peripheral blood lymphocytes (PBL) engineered to express TCRs recognizing neoantigens can generate high numbers of neoantigen-reactive cells with less exhausted phenotypes than TILs. However, low frequencies of neoantigen-reactive TILs hinder the identification of neoantigen-reactive TCRs. Furthermore, TCR-engineered T-cell therapies targeting a single neoantigen may lead to tumor escape through loss of human leukocyte antigen (HLA) or antigen(s).[7 10 24–26] In contrast, polyclonal TILs, capable of targeting multiple antigens, have a decreased likelihood of developing escape mechanisms, provided that neoantigen-reactive TILs can be effectively expanded without experiencing excessive exhaustion.[1] In vitro stimulation against tumor antigens has been used to selectively grow T cells of interest, including those targeting tumor-associated antigens and neoantigens.[27–30] However, it remains to be determined whether neoantigen-reactive TIL, given their low frequencies and exhausted phenotypes, can be selectively and effectively stimulated and expanded to a level suitable for patient treatment. The conventional method for T-cell ex vivo expansion using OKT3 (an anti-CD3 antibody) and high dose interleukin 2 (IL-2), also known as the rapid expansion,[31] may cause outgrowth of bystander cells due to its non-specific nature.[32–35] In the current study, we tested whether neoantigen-reactive TILs could be stimulated in a neoantigen-specific fashion to achieve two main goals: first, sensitive identification of neoantigen-reactive TCRs and second, development of TIL ACT products with high neoantigen reactivity. Using p53 and RAS family (ie, KRAS, NRAS and HRAS) mutations as a model, we demonstrate that neoantigenic stimulation could overcome the aforementioned challenges of the conventional TIL expansion by improving reactivities, phenotypes and functions of TILs.

## RESULTS

### Reductions in neoantigen-reactive TIL frequencies during the conventional rapid expansion with OKT3

We have previously reported our methods to identify TIL recognizing neoantigens expressed by solid epithelial cancers and the expansion of those TIL for use in ACT of patients with cancer. Briefly, single or multiple tumor metastases are dissected to establish and expand multiple TIL fragment cultures with high dose IL-2 (6000 IU/mL) for neoantigen screening.[5 10] TIL fragment cultures that recognize neoantigens are then further expanded to $>1\times10^{10}$ cells by the rapid expansion where TILs are stimulated with an anti-CD3 antibody (OKT3), IL-2 and irradiated allogeneic peripheral blood mononuclear cells as feeders (figure 1A), a culture method widely used in the field.[1 7–10 36–39] We tested whether prolonged exposure to high-dose IL-2 and/or non-specific stimulation during the rapid expansion by OKT3 led to the expansion of bystander cells to reduce the frequency of neoantigen-reactive TILs. Ten different neoantigen reactivities identified from three breast cancer TIL infusion products used in clinical trial NCT01174121[10] were analyzed before and after the rapid expansion. All of the 10 reactivities showed a decrease in their frequencies in the infusion products relative to the individual fragment cultures before the rapid expansion (figure 1B). Some reactivities, including those against mutated BTF3, RCL1, TTI2 and p53, showed greater than 10-fold decrease making them nearly undetectable at the end of the expansion.

### Development of NeoExpand for neoantigenic stimulation of TILs

To selectively expand neoantigen-reactive TILs, we developed an in vitro TIL culture method, termed NeoExpand, that involved the specific stimulation of TILs against previously identified or candidate neoantigens. As a starting material, either TIL fragment cultures established individually as described above, a pool of TIL fragment cultures or TILs from fresh tumor digests were used (figure 1C). Fresh tumor digests were either directly used or were briefly cultured for less than a week with IL-2. To provide neoantigen-specific stimulation, a variety of antigen-presenting cells (APC), including autologous dendritic cells (DCs), B cells and HLA-engineered cell lines, such as COS7 cells, were tested (figure 1C). Antigens were introduced into APCs either transiently by transfection of mutated tandem minigene (TMG) RNA or constitutively by virally expressing TMGs. Additionally, APCs were loaded with antigens by pulsing long (24–25 mer) peptides or predicted minimal epitope peptides. From all candidate mutated epitopes identified from whole exome sequencing of tumor versus normal tissues, a small number (<10) of minimal epitopes were

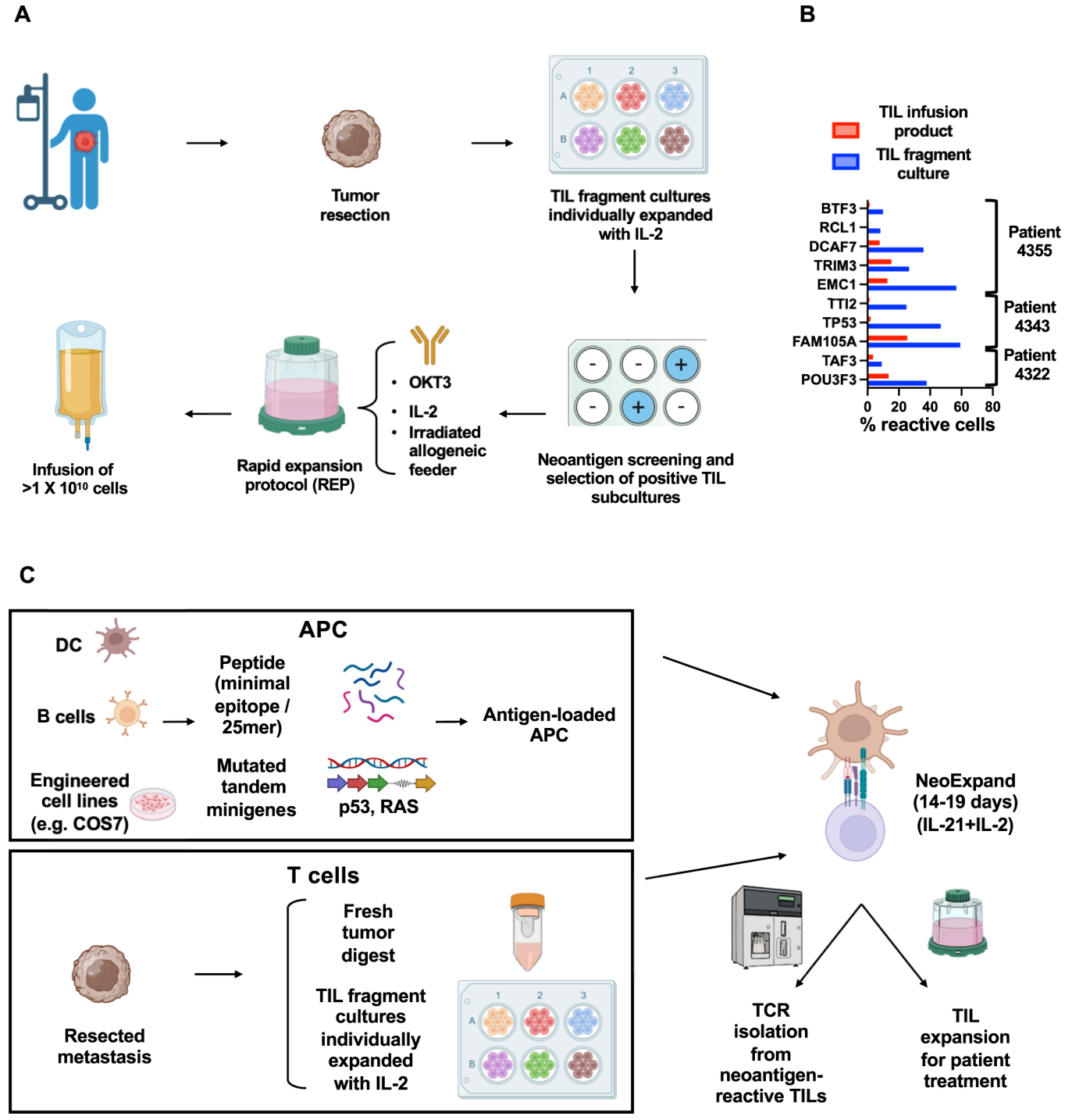


**Figure 1** Conventional TIL expansion and the development of NeoExpand for selective neoantigenic stimulation of TILs. (A) Schematic showing the conventional way of TIL expansion, screening and the rapid expansion for the development of a TIL infusion product. (B) Frequencies of neoantigen-reactive TIL before (ie, TIL fragment culture) and after a rapid expansion with OKT3 (ie, TIL infusion product). TIL fragment cultures with neoantigen reactivities before and after the rapid expansion were co-cultured with autologous dendritic cells pulsed with mutated peptides indicated above. Per cent reactive cells were assessed by flow cytometric measurement of 4-1BB$^+$ or OX-40$^+$ cells. (C) Schematic of NeoExpand using neoantigen-loaded APCs for the expansion of neoantigen-reactive TILs. APC, antigen presenting cell; DC, dendritic cell; IL, interleukin; TCR, T-cell receptor; TIL, tumor infiltrating lymphocytes.

prioritized based on NetMHCpan4.0,[40] MHCflurry1.6,[41] and our own machine learning-based prediction model.[42] Although minimal epitopes were used, the nature of the neoantigenic stimulation was sequence-agnostic as the 24 or 25 amino acids containing mutations in the form of peptides or TMG RNAs were intracellularly processed to be presented by APCs. To determine optimal concentrations of peptides, the efficiency of NeoExpand was

examined on TCR-engineered T cells as a model. Healthy donor PBLs transduced with two different p53 or RAS neoantigen-reactive TCRs identified previously[24 43] were co-cultured with HLA-engineered COS7 cells pulsed with a range of peptide concentrations (online supplemental figure S1). The use of peptide between 10 and 1000 ng/mL appeared to have a negligible effect on the growth of TCR-transduced PBL, indicating flexibility in terms of the amount of antigens required for effective neoantigenic stimulation (online supplemental figure S1). Finally, TILs were co-cultured with antigen-loaded APCs in the presence of IL-2 and IL-21. IL-21 was added during NeoExpand, because it has been shown to preserve the proliferation capacity of antigen-experienced T cells while counteracting differentiation induced by IL-2.[27 28 44 45] For the expansion of TILs targeting shared neoantigens, such as p53 or KRAS, peptides and/or TMGs can be prepared in advance and the entire process of NeoExpand can take approximately 2 weeks. Although not discussed in this study, for the expansion of TILs targeting private neoantigens, newly synthesizing peptides and TMGs can add an additional 4–6 weeks to the timeline.

### Expansion of CD8$^+$ and CD4$^+$ neoantigen-reactive TIL clonal repertoire and sensitive identification of neoantigen-reactive TCRs following neoantigenic stimulation

We tested the effect of neoantigenic stimulation on TILs to facilitate the identification of neoantigen-reactive TCRs and to develop TIL ACT products with improved neoantigen-reactivity, phenotype and functions to potentially replace the conventional rapid expansion with OKT3. As an example, figure 2 shows neoantigenic stimulation of TILs from a patient with colorectal cancer (4,141), whose tumor harbored a p53$^{R175H}$ mutation. A pool of 4,141 TIL fragment cultures were ex vivo expanded with or without neoantigenic stimulation using HLA-engineered COS7 cells as APCs. Following NeoExpand, a dramatic expansion of p53$^{R175H}$-reactive TILs was observed whereas conventional culture with high dose IL-2 did not lead to expansion of p53$^{R175H}$-reactive T cells (figure 2A). From the reactive TILs, one previously identified[46] and one novel TCR were isolated (figure 2B,C). The novel TCR showed specificity for mutant p53 but not wild-type p53 (figure 2D) and was restricted by HLA-A*02:01 (figure 2E). This p53$^{R175H}$-reactive "NeoExpand" clonotype was found at a very low level (<0.01%) in the patient's infusion product (figure 2F), again indicating that the conventional TIL culture, including the rapid expansion with OKT3, failed to expand this neoantigen-reactive TIL clonotype. The therapeutic function of this new TCR was tested using a human ovarian cancer xenograft model.[24] 10 million PBLs from two different healthy donors were transduced with the new 4,141 NeoExpand TCR and injected into immunocompromised NOD-scid IL-2Rg$^{null}$ (NSG) mice bearing human ovarian cancer TYK-nu cells naturally expressing both p53$^{R175H}$ and HLA-A*02 (figure 2G). The two groups of mice that received PBLs engineered with 4,141 NeoExpand TCR showed a significant delay in tumor growth (figure 2H).

Additional examples of neoantigen-reactive TIL identification by NeoExpand are shown in online supplemental figure S2. From a colorectal cancer TIL generated from tumor cells that expressed both HLA-A*02 and A*11 and a KRAS$^{G12D}$ mutation (4,432), NeoExpand was conducted using COS7 cells engineered with HLA-A*02 or A*11 as APCs. KRAS$^{G12D}$-reactive CD8$^+$ T cells were identified only in the TIL stimulated with COS7 cells expressing HLA-A*11 but not with A*02-engineered COS7 cells, indicating that the neoantigenic stimulation led to T-cell expansion in an HLA-specific manner (online supplemental figure S2A). The conventional culture without the neoantigenic stimulation failed to expand this clonotype and no reactivity was identified (data not shown). The TCR isolated from the KRAS$^{G12D}$-reactive clonotype showed specificity for KRAS$^{G12D}$ but not the wild-type peptide (online supplemental figure S2B). To test the in vivo antitumor function of this novel TCR, a new xenograft model was developed using allogeneic pancreatic cancer patient-derived xenograft (PDX) cells (4,069) that naturally expressed KRAS$^{G12D}$ and HLA-A*11:01. NSG mice were injected with 1 million 4069 PDX cells and 2 weeks later received ACT of 6 million healthy donor PBLs expressing 4,432 NeoExpand TCR (online supplemental figure S2C). This RAS$^{G12D}$-reactive TCR showed antitumor efficacy, causing complete tumor regression in this model (online supplemental figure S2D). Collectively, in these two examples, novel neoantigen-reactive TCRs were isolated following neoantigenic stimulation and the TCRs exhibited specificity for neoantigens and in vivo functionality.

Next, we tested whether CD4$^+$ neoantigen-reactive TILs could also be selectively expanded by neoantigen-specific stimulation. Figure 3 shows an example where a CD4$^+$ neoantigen-reactive clonotype that was progressively declining in numbers was expanded by neoantigenic stimulation. Initially by the conventional expansion with IL-2 followed by the neoantigen screening, p53$^{R273C}$-reactive cells were identified in fragment culture 7 of 4,386 breast cancer TIL with robust interferon (IFN)-γ secretion against the mutant p53 TMG or the p53$^{R273C}$ peptide (figure 3A). A TIL infusion product was generated by further expanding reactive cultures, including fragment culture seven, by the rapid expansion with OKT3. When the final infusion product was tested for neoantigen reactivity, however, a loss of the p53 reactivity based on reduced IFN-γ secretion was noted (figure 3B). We examined whether the decreasing p53$^{R273C}$-reactive cells could be expanded by neoantigenic stimulation. From the pool of all the 4,386 TIL cultures, NeoExpand was carried out using autologous DCs as APCs. Following the NeoExpand procedure, expansion of p53$^{R273C}$-reactive cells was noted (figure 3C). From the reactive cells, a single TCR was isolated (figure 3D). When

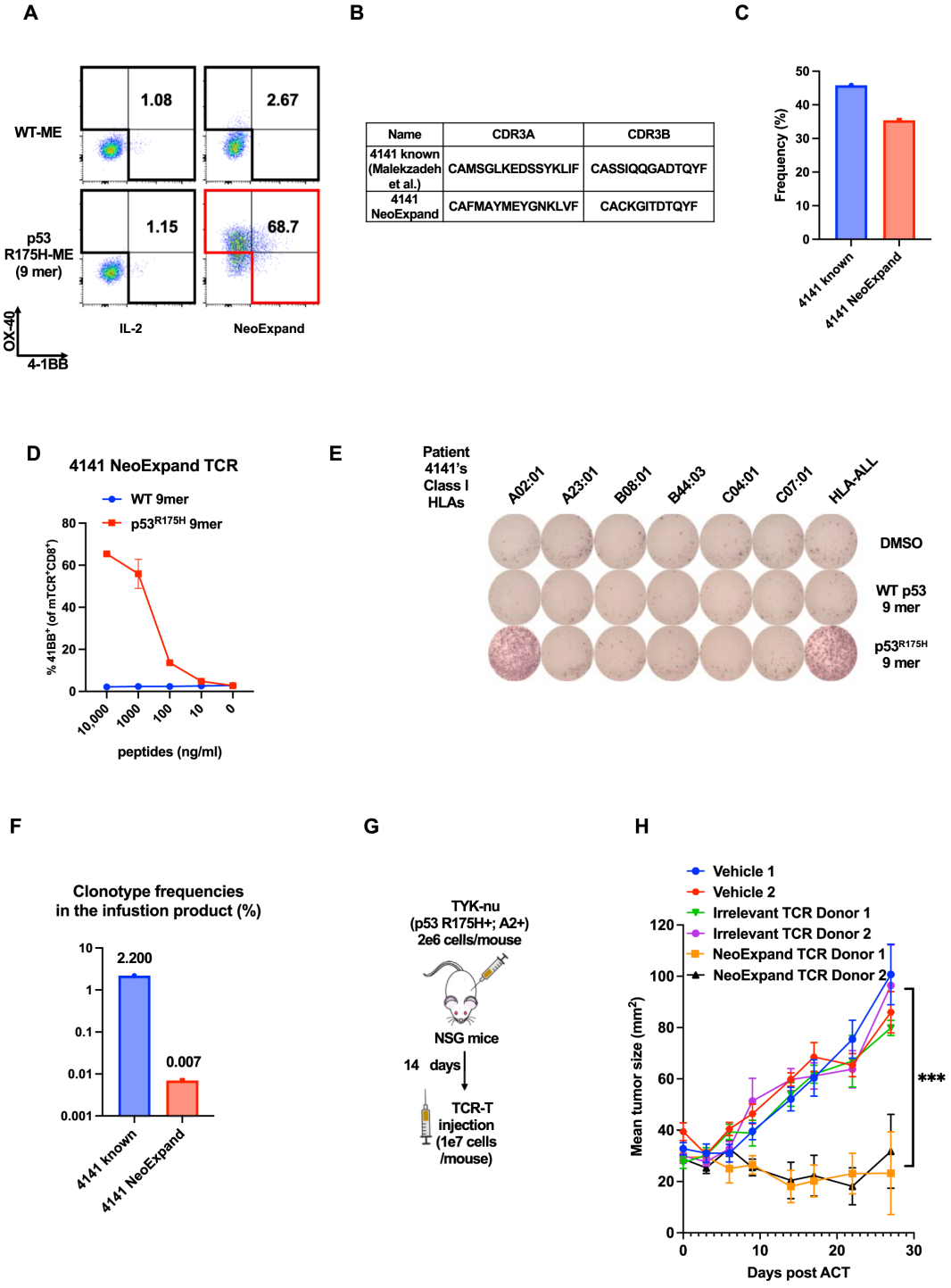


**Figure 2** Expansion of CD8+ neoantigen-reactive TILs targeting a p53 neoantigen following neoantigenic stimulation. (A) Representative example of neoantigen-reactive TIL enrichment by neoantigenic stimulation: enrichment of $p53^{R175H}$-reactive cells from 4,141 TILs via NeoExpand was determined by flow cytometric measurement of 4-1BB and OX-40 following an overnight co-culture. Peptide-pulsed, HLA-engineered COS7 cells were used as antigen-presenting cells. ME minimal epitope. (B) CDR3A and CDR3B TCR sequences of reactive cells in (A). (C) Frequencies of the two $p53^{R175H}$-reactive clonotypes among reactive cells in (A). (D) Peptide titration assay testing specificity of 4,141 NeoExpand TCR isolated in (B). 4-1BB was measured following an overnight co-culture of TCR-engineered healthy donor PBLs with A*02-engineered COS7 cell. (E) HLA testing of 4,141 NeoExpand TCR. Healthy donor PBLs expressing 4,141 NeoExpand TCR were co-cultured with COS7 cells transfected with individual HLAs expressed by patient 4,141. IFN-γ secretion was measured by an ELISpot assay. (F) Frequencies of 4,141 "known" clonotype and 4141 NeoExpand clonotype in the patient infusion product were determined by *CDR3B* sequencing. (G, H) In vivo functional test of 4,141 NeoExpand TCR. Summary diagram (G) and tumor measurement (H) (n=5). Replicates from ACTs of two healthy donor PBLs are shown. Statistical analysis by two-way analysis of variance. ***p<0.001. ACT, adoptive cell therapies; DMSO, dimethyl sulfoxide; HLA, human leukocyte antigen; IFN, interferon; mTCR, murine TCR; PBL, peripheral blood lymphocytes; TCR, T-cell receptor; TIL, tumor infiltrating lymphocytes; WT, wild-type.

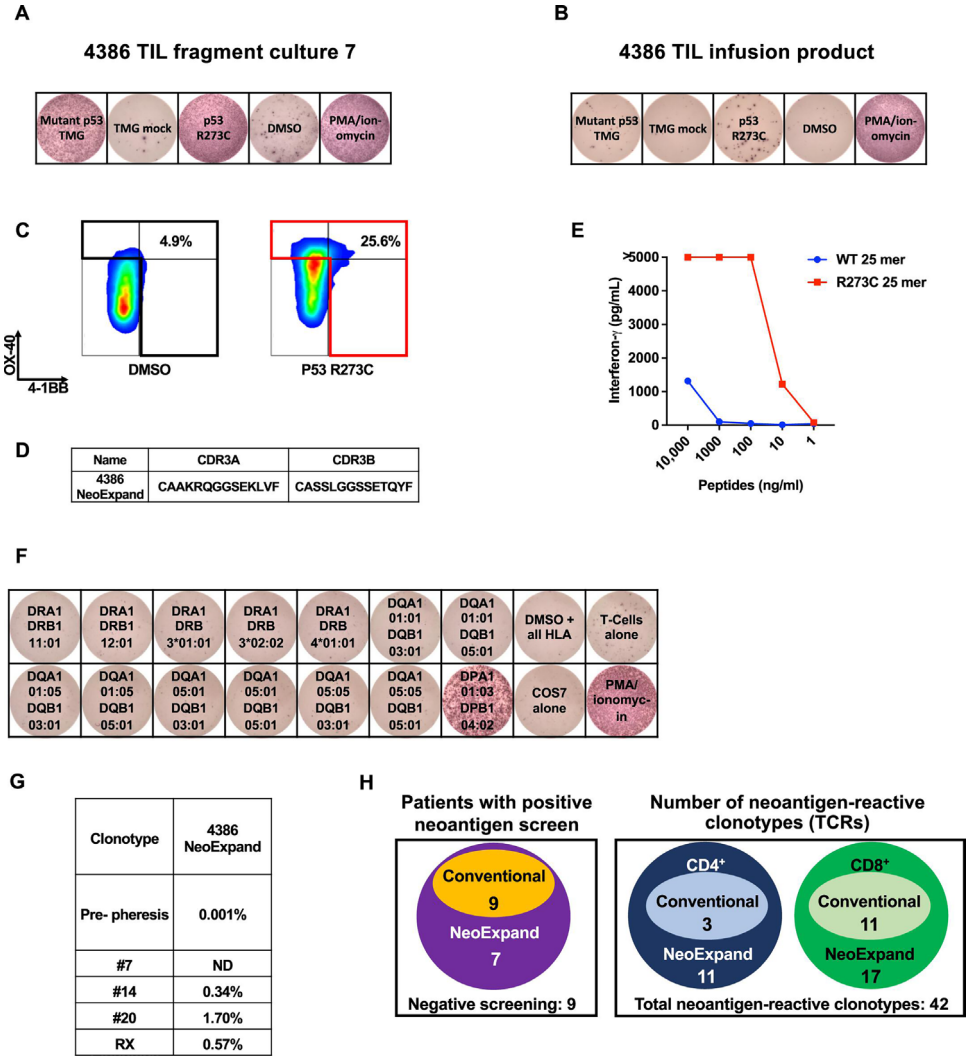


**Figure 3** Neoantigenic stimulation selectively expands neoantigen-reactive TILs, allowing sensitive TCR isolation. (A) IFN-γ secretion of 4,386 TIL fragment culture seven against the $p53^{R273C}$ neoantigen. An ELISpot assay was performed following co-culture of 4,386 TIL fragment culture seven with autologous DCs electroporated with p53 TMG or pulsed with $p53^{R273C}$ 25 mer. The mock transfected (TMG mock) condition and DMSO as the vehicle control for peptide treatment were included as negative controls. (B) IFN-γ secretion of 4,386 TIL infusion product against p53 TMG or the $p53^{R273C}$ peptide was measured as described in (A). (C) Flow cytometric measurement of $p53^{R273C}$-reactive cells following NeoExpand. (D) Isolation of $p53^{R273C}$-reactive TCR. (E) Peptide titration assay testing specificity of 4,386 NeoExpand TCR isolated in (D). IFN-γ secretion was measured following an overnight co-culture of TCR-engineered healthy donor PBLs with 4,386 DCs pulsed with WT or mutant p53 peptides. (F) HLA testing of 4,386 NeoExpand TCR. Healthy donor PBLs expressing 4,386 NeoExpand TCR were co-cultured with COS7 cells transfected with both A and B molecules of class II HLAs expressed by patient 4386. IFN-γ secretion was measured by an ELISpot assay. (G) Frequencies of the 4,386 NeoExpand clonotype before and after the TIL adoptive cell therapy. (H) Venn diagram showing the number of patients with a positive neoantigen screen (left) or the number of neoantigen-reactive $CD4^+$ or $CD8^+$ T-cell clonotypes identified following the conventional TIL expansion or NeoExpand (right). See also online supplemental table S1. DMSO, dimethyl sulfoxide; HLA, human leukocyte antigen; IFN, interferon; PBL, peripheral blood lymphocytes; PMA, phorbol 12-myristate 13-acetate; RX, infusion product; TCR, T-cell receptor; TIL, tumor infiltrating lymphocytes; TMG, tandem minigene; WT, wild-type.

reconstructed and expressed in healthy donor PBLs, the TCR showed specificity for mutant p53 (figure 3E) and HLA restriction of DPA1*01:03-DPB1*04:02 (figure 3F), which is found in over 60% of Hispanic populations in the USA and South American countries.[47] The mutant p53-reactive clonotype was not detected in fragment culture seven following the rapid expansion but was detected in other fragment cultures at a low level, which might have been the source of T cells stimulated by NeoExpand (figure 3G).

## NeoExpand broadens $CD4^+$ and $CD8^+$ neoantigen-reactive TIL clonal repertoire

Iteratively, NeoExpand was performed on 25 TIL samples whose tumor expressed p53 or RAS mutations, and the result was compared with the screening result following

the conventional TIL expansion without neoantigenic stimulation. Out of 25 TIL samples from different patients, the conventional expansion and screening identified 9 reactivities against mutant p53 or RAS, while NeoExpand enabled the identification of 16 reactivities, which included all the 9 reactivities found through the conventional screening (figure 3H and online supplemental table S1). All of the TCR sequences isolated from the neoantigen-reactive TIL clonotypes were reconstructed into retrovirus for functional testing. When all the different, functionally validated neoantigen-reactive TIL clonotypes were enumerated, the conventional screening identified 14 clonotypes (3 CD4; 11 CD8) and NeoExpand identified 42 clonotypes (14 CD4; 28 CD8) (figure 3H). TILs from tumors expressing both p53 and RAS mutations (4,424, 4,426, and 4,430) were stimulated against both neoantigens but only single reactivities against either p53 or RAS neoantigens were identified (online supplemental table S1). This indicated that unlike naïve T cells,[27] neoantigen-reactive TILs could not be induced to generate a novel reactivity. These data in conjunction with the examples in figures 2–3 demonstrate that neoantigenic stimulation can facilitate effective neoantigen-reactive TCR isolation, including both $CD4^+$ and $CD8^+$ TCRs, by expanding the neoantigen-reactive TIL clonal repertoire.

### Effective neoantigen-reactive TIL expansion by neoantigenic stimulation for use in ACT

Next, we investigated the translational potential for NeoExpand as a method to grow TILs for patient treatment by comparing it to the conventional rapid expansion that has been commonly used to generate a large number of T cells for ACT. As exemplified in figure 1B, non-specific stimulation of T cells by OKT3 could reduce the frequencies of neoantigen-reactive TILs. Therefore, we tested whether neoantigenic stimulation could address decreases in frequencies of neoantigen-reactive TIL during ex vivo expansion while achieving exponential growth of TILs. As proof-of-principle, TILs from patients 4,196, 4,385, and 4,391 with metastatic colorectal cancers were used to compare NeoExpand and the conventional rapid expansion. These TIL samples were selected based on their availability as well as compatibility with the existing mouse models for functional testing. As in figure 4A, TILs were grown either by NeoExpand or the rapid expansion with OKT3. The $p53^{R175H}$-reactive cells from 4,196 TILs were counted by staining them with an HLA-A*02 tetramer containing the $p53^{R175H}$ epitope. Due to the unavailability of the HLA-C*01:02 tetramers, to enumerate neoantigen-reactive T cells within 4,385 and 4,391 TILs, the expanded TILs underwent another co-culture with HLA-engineered COS7 cells pulsed with the $RAS^{G12D}$ minimal epitope peptide. The rapid expansion achieved total $CD3^+$ T-cell fold-expansion greater than that of NeoExpand in 4,196 TILs (figure 4B, top right); however, the NeoExpand TILs showed higher frequencies and fold-expansion of neoantigen-reactive T cells than the rapid expansion culture (figure 4B, bottom left and right). Furthermore, following neoantigenic stimulation of 4,196 TILs, four novel $p53^{R175H}$-reactive clonotypes were identified in addition to the three known clonotypes, 6–11, 12–6 and 38–10, that were previously identified following a conventional TIL culture.[48] When reconstructed, these four new TCRs demonstrated in vitro tumor lysis of TYK-nu cells ($p53^{R175H+}$; HLA-A*02$^+$) (online supplemental figure S3). Similar to 4,196 TILs, the rapid expansion led to greater fold-expansion of the bulk $CD3^+$ T cells of 4,385 and 4,391 TILs than NeoExpand (figure 4C,D, leftmost). However, NeoExpand achieved greater fold-expansion of neoantigen-reactive TILs than the rapid expansion (figure 4C,D, middle and rightmost). In the case of 4,391 TIL, following NeoExpand, one previously identified TCR[43] and four novel $RAS^{G12V}$-reactive clonotypes were identified. The four novel TCRs showed mutant RAS specificity with no wild-type reactivity (online supplemental figure S4). In the aggregate of 11 TIL samples tested for NeoExpand and the conventional rapid expansion, including the 3 samples discussed above, the fold-expansion of neoantigen-reactive TILs by NeoExpand was significantly greater than that of the rapid expansion (figure 4E, online supplemental table S3).

### Phenotypic characterization of TILs before and after neoantigenic stimulation or rapid expansion by single-cell transcriptome analysis (single-cell RNA sequencing)

The T-cell cultures from patients 4,196 and 4,391 were further characterized by single-cell RNA sequencing (scRNA-seq) analysis. 4,385 TILs were not analyzed due to their highly monoclonal (80%) composition of neoantigen-reactive TILs following NeoExpand, which lacked the clonal complexity of fresh TILs and would not be representative. The UMAP (Uniform Manifold Approximation and Projection) analysis revealed 14 clusters with distinct transcriptome signatures among the 4,196 TIL (figure 5A, left). The $p53^{R175H}$-reactive neoantigen-reactive TIL clonotypes were mainly found in clusters 3, 4 and 10 (figure 5A, right). Gene Set Enrichment Analysis revealed that the gene expression profile of cluster 4 bore high similarity to the signatures of $CD39^-CD69^-$ stem-like cells described by Krishna *et al*[49] or stem-like memory cells described by Caushi *et al*[50] (online supplemental figure S5 and online supplemental table S4), which also expressed genes associated with stem-like memory T cells, such as *IL7R*, *KLF2*, *SELL* (CD62L), and *TCF7* (TCF1) with little expression of exhaustion markers, such as *ENTPD1* (CD39) or *HAVCR2* (T cell immunoglobulin and mucin domain-containing protein 3 or TIM3) (figure 5B). When $p53^{R175H}$-reactive clonotypes in cluster 4 were enumerated, most of the reactive cells were from either the pre-expansion TIL before NeoExpand/rapid expansion (PRE) or the NeoExpand culture (figure 5C), indicating

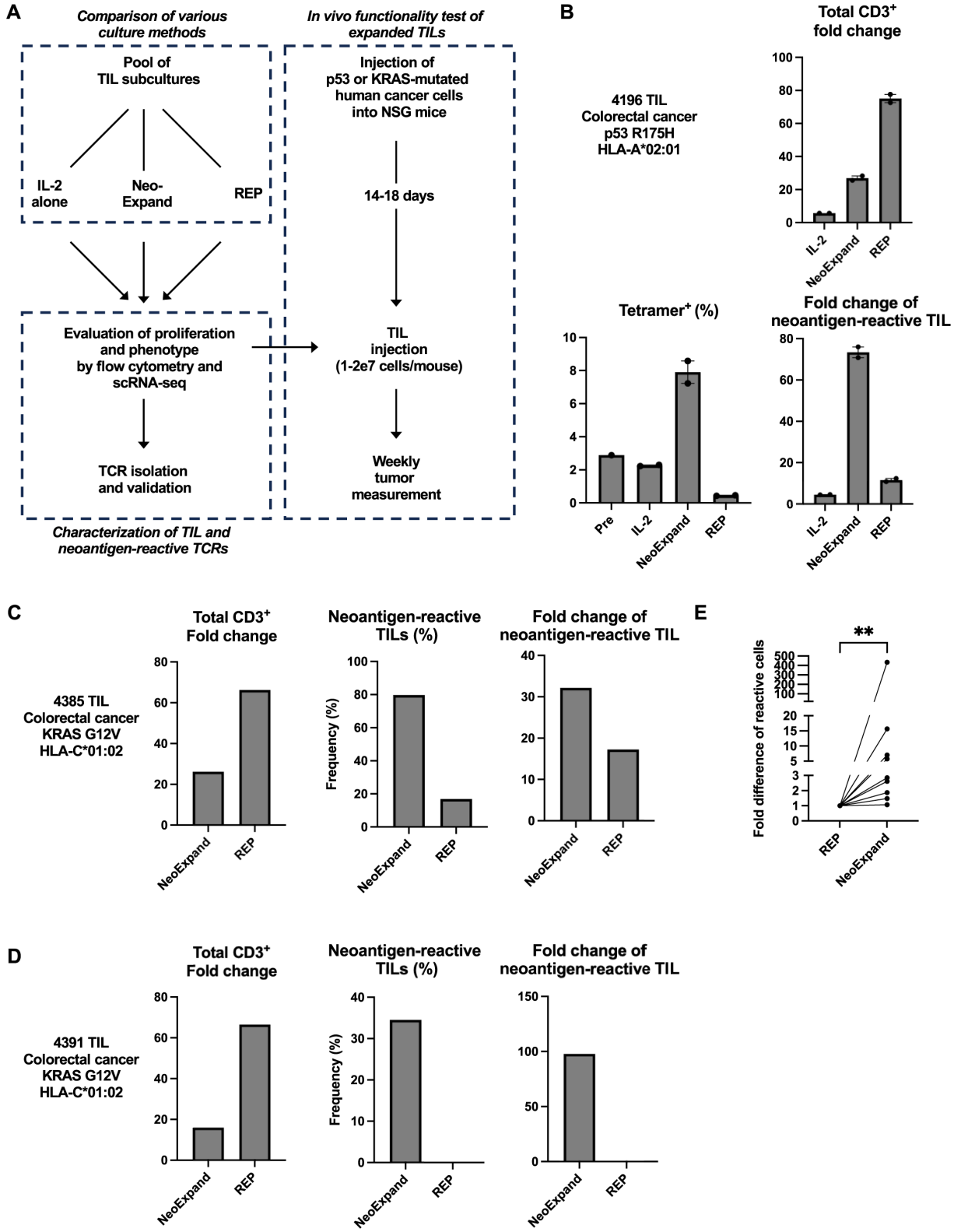


**Figure 4** Superior expansion of neoantigen-reactive TILs by neoantigenic stimulation relative to the conventional rapid expansion. (A) Schematic of 4,196, 4,385, and 4,391 TIL expansion by the rapid expansion with OKT3 or NeoExpand for mouse xenograft adoptive cell therapies studies. (B–D) Comparison of the rapid expansion and NeoExpand. 4,196 TILs (B), 4,385 TILs (C), and 4,391 TILs (D) were expanded by IL-2 alone, rapid expansion or NeoExpand: Fold changes of total CD3$^+$ cells, frequencies of neoantigen-reactive TILs, and fold changes of neoantigen-reactive TILs were evaluated by tetramer staining (B) or by 4-1BB and OX-40 measurement (C,D). Fold changes were calculated based on the number of input cells. (E) 10 TIL samples were tested to compare the efficiency of neoantigen-reactive TIL expansion between the rapid expansion and NeoExpand. Statistical analysis by Wilcoxon matched-pairs signed rank test. **p<0.01. HLA, human leukocyte antigen; IL, interleukin; scRNA-seq, single-cell RNA sequencing; TCR, T-cell receptor; TIL, tumor infiltrating lymphocytes

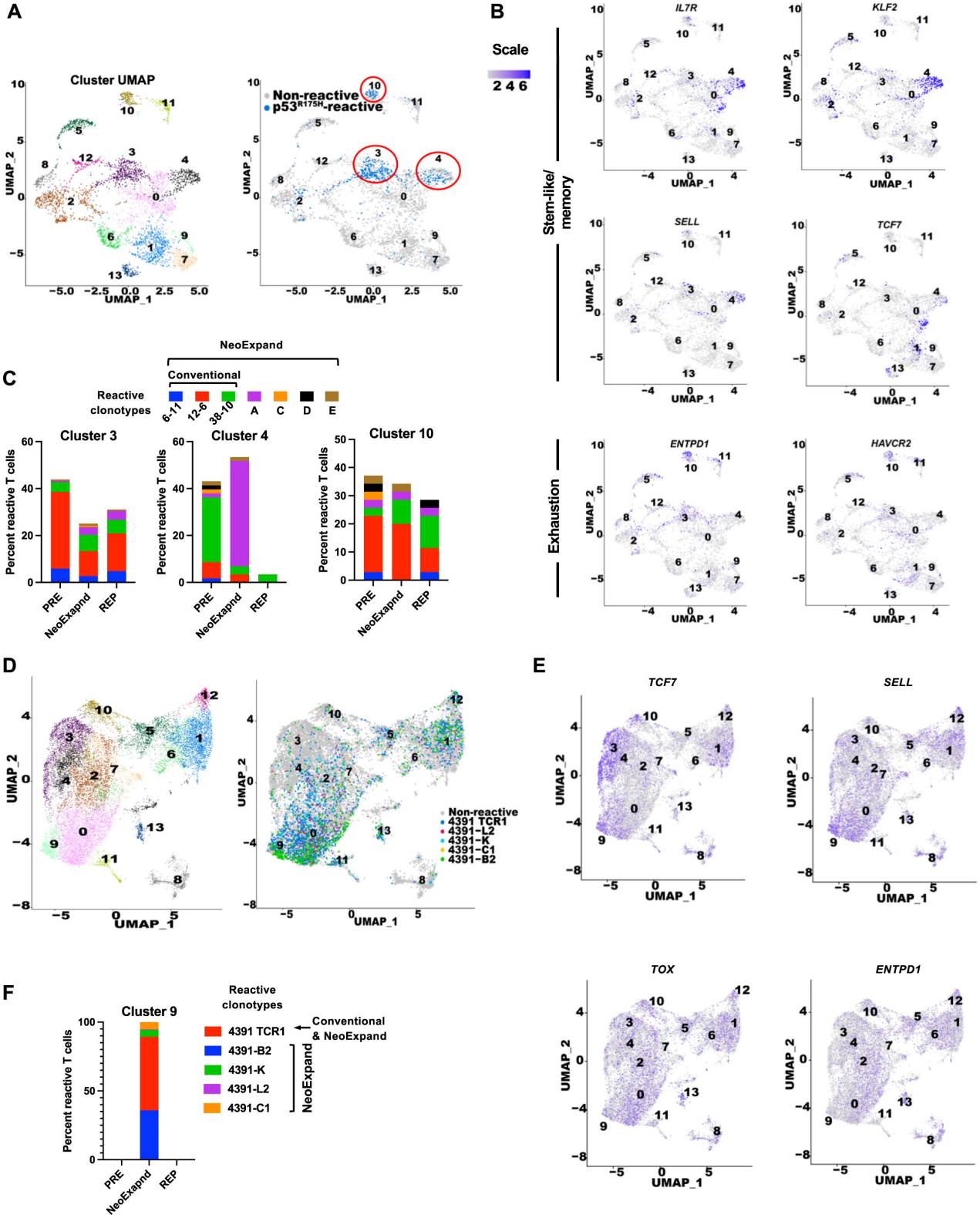


**Figure 5** ScRNA-seq analysis reveals expansion of neoantigen-reactive TILs with stem-like memory phenotypes following neoantigenic stimulation. (A–C) ScRNA-seq analysis of 4,196 TIL following NeoExpand or rapid expansion. (A) UMAP analysis of 4,196 TILs (left) and p53$^{R175H}$-reactive cells (right). Clusters 3, 4, and 10 containing high numbers of p53$^{R175H}$-reactive cells are marked in red. (B) Gene expression of markers of stem-like memory T cells and exhausted T cells. (C) Relative frequencies of p53$^{R175H}$-reactive cells in clusters 3, 4, and 10. (D–F) ScRNA-seq analysis of 4391 TIL following NeoExpand or rapid expansion. (D) UMAP analysis of 4391 TILs (left) and RAS$^{G12V}$-reactive cells (right). (E) Gene expression of markers of stem-like memory T cells and exhausted T cells. (F) Relative frequencies of RAS$^{G12V}$-reactive cells in cluster 9. See also online supplemental table S4 for phenotypic annotation of the clusters. scRNA-seq, single-cell RNA sequencing; UMAP, Uniform Manifold Approximation and Projection; TCR, T-cell receptor; TILs, tumor infiltrating lymphocytes.

depletion of the p53 neoantigen-reactive cells with the stem-like memory phenotype when expanded conventionally by the rapid expansion with OKT3. In contrast, clusters 3 and 10 resembled the gene expression profiles of differentiated effector cells[50 51] (figure 5B and online supplemental figure S5) and contained similar numbers between the different culture conditions (figure 5C). This finding was further substantiated by flow cytometric analysis of tetramer$^+$ 4,196 TILs, which showed expansion of a central memory (CD62L$^+$CD45RO$^+$) population—T cells thought to harbor a long-term repopulating ability with stem-like features[52]—following NeoExpand (online supplemental figure S6). The same population following the rapid expansion was 4.2-fold lower than that of NeoExpand. An scRNA-seq analysis of mutant RAS-targeting 4,391 TILs also identified clusters with high numbers of RAS$^{G12V}$-reactive neoantigen-reactive TILs (figure 5D). Cluster 9 that contained high numbers of the neoantigen-reactive TILs resembled the phenotype of the stem-like memory cells[50] or the CD39$^-$CD69$^-$ stem-like cells[49] (online supplemental figure S7 and online supplemental table S4) and expressed high levels of TCF1 and CD62L and low amounts of CD39 (figure 5E). *TOX*, a transcription factor associated with T-cell exhaustion,[53] was generally high in 4,391 TILs, including cluster 9 and indicated that these cells despite their stem-like gene expression profiles might be different from naïve T cells that do not express high levels of *TOX* (figure 5E). Neoantigen-reactive TILs within cluster 9 were made up almost exclusively of the cells generated through NeoExpand (figure 5F). Expression of *CXCL13*, a recently identified marker for tumor-reactive TILs,[6 20 21] was not detected in 4,196 or 4,391 TILs (online supplemental figure S5D, S7B), indicating that loss of *CXCL13* might occur during an ex vivo culture. Other exhaustion-associated genes that had been considered markers for neoantigen-reactive TILs, such as CD39, TIM3 or programmed cell death protein 1 (PD-1), showed heterogenous patterns of expression and the clusters expanded in response to neoantigenic stimulation tended to express lower levels of these genes, implying their less exhausted phenotypes than the TILs expanded by the rapid expansion (figure 5B and E).

### Functional characterization of TILs expanded by neoantigenic stimulation or the rapid expansion using in vivo xenograft ACT models

The three TILs expanded via NeoExpand or rapid expansion (figure 4A) were functionally compared using in vivo xenograft models. NSG mice were subcutaneously implanted with TYK-nu cells (p53$^{R175H+}$; HLA-A*02:01$^+$) or 4,391 colorectal cancer PDX cells (KRAS$^{G12V+}$; HLA-C*01:02$^+$). These tumor cells naturally expressed the neoantigens and HLA molecules corresponding to 4,196 (p53) or 4,385 and 4,391 TILs (KRAS). When tumors were established, the NSG mice were injected with 4,196, 4,385 or 4,391 TILs expanded through NeoExpand or the rapid expansion with OKT3 (figure 6A). The mice treated with 20 million 4,196 (figure 6B) or 4,385 TILs (figure 6C) expanded via NeoExpand showed significant tumor regression while TILs expanded by the rapid expansion failed to do so when compared with the vehicle controls. The mice treated with 10 million 4,391 TILs expanded via NeoExpand did not show tumor regression but a significant delay in tumor growth relative to that of rapid expansion (figure 6D).

## DISCUSSION

In this study, we explored the selective expansion of neoantigen-reactive TILs through in vitro neoantigen-specific stimulation of TILs. Our data demonstrate that non-specific stimulation, such as the rapid expansion with OKT3, a widely used method for TIL expansion,[1 7–10 36–39] can lead to reduced frequencies of neoantigen-reactive TILs. In contrast, neoantigenic stimulation enabled selective expansion of neoantigen-reactive TILs, not just by their frequencies but also by expanding rare neoantigen-reactive clones and thereby broadening their clonal repertoires. This allowed sensitive detection of previously unidentified neoantigen-reactive TCRs, some of which demonstrated antitumor efficacy in vitro and in vivo. Although the use of the rapid expansion protocol consistently generated more bulk CD3$^+$ T cells than NeoExpand (figure 4B–D), NeoExpand excelled at expanding neoantigen-reactive TILs (figure 4E, online supplemental table S3). ScRNA-seq analysis revealed that neoantigenic stimulation selectively promotes the expansion of neoantigen-reactive TILs with stem-like memory phenotypes, which were largely depleted in the rapid expansion conditions (figure 5C and F). These phenotypic differences could have led to functional consequences. In three different mouse models based on human cancer cells expressing mutant p53 or KRAS, TILs expanded via neoantigenic stimulation effectively controlled the tumor growth, whereas TILs grown by the IL-2 alone or the rapid expansion failed to do so (figure 6).

TILs surrounded by the antagonistic tumor microenvironment can progressively acquire exhausted phenotypes. Recent studies show that neoantigen-reactive TILs express exhaustion-associated molecules, such as CD39, CD103, PD-1 or a combination thereof, which can be used to distinguish neoantigen-reactive TILs from other bystander cells among TILs.[6 20 21 54 55] Further ex vivo expansion of TILs by IL-2 or stimulation like the rapid expansion with OKT3 can exacerbate their already exhausted phenotypes.[32–35] Neoantigenic stimulation appears to address this issue by selectively expanding neoantigen-reactive TILs, including rare neoantigen-reactive TIL clonotypes. When corresponding neoantigen-reactive TCRs were isolated from these rare neoantigen-reactive TIL clonotypes and tested (figure 2H and online supplemental figure S2–4), they demonstrated high avidity and specificity for neoantigens and antitumor efficacy in various in vitro (online supplemental figure S3B) or in vivo (figure 2H and online supplemental figure S2D) models, despite the notion that TCRs isolated following

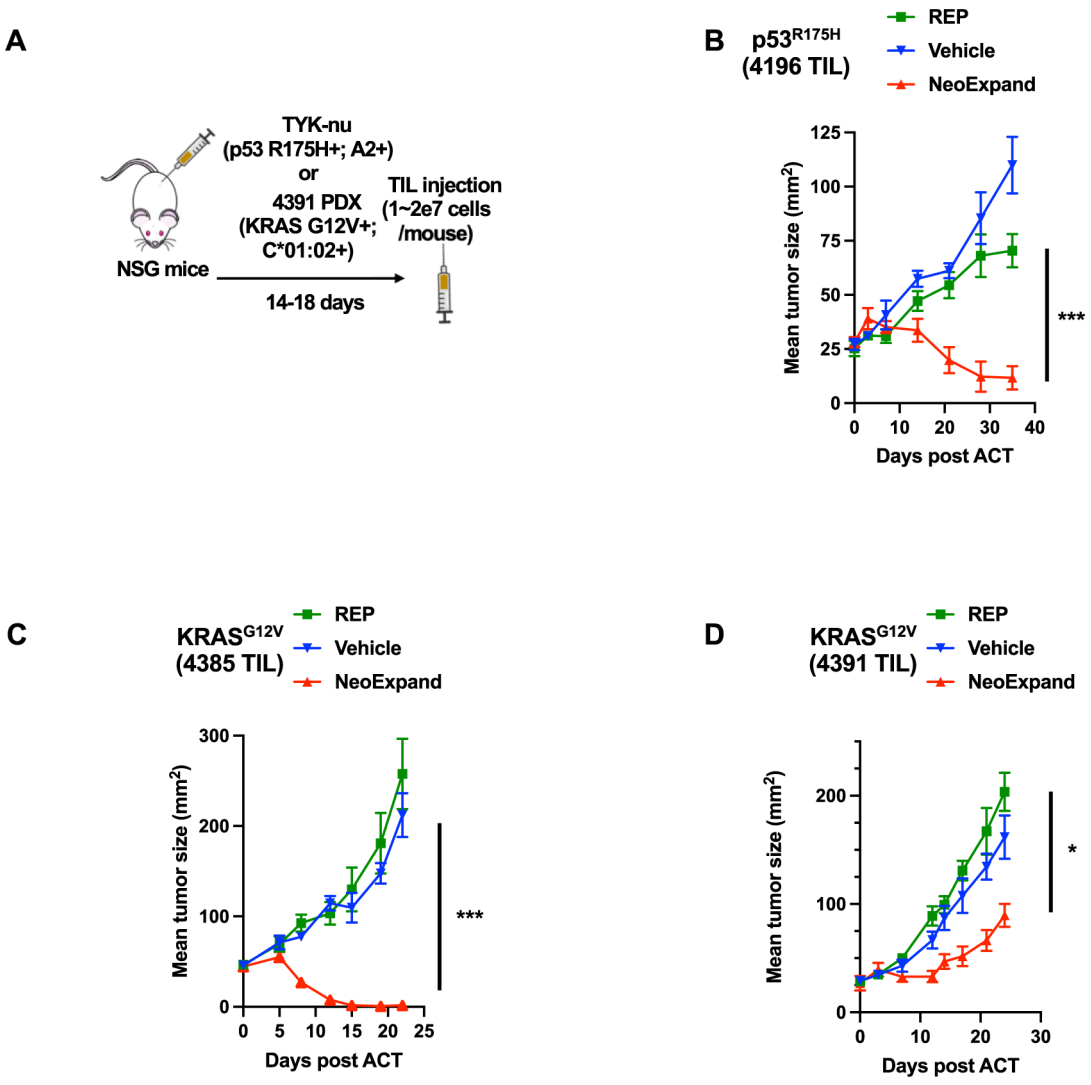


**Figure 6** In vivo functional test comparing the conventional rapid expansion and NeoExpand. (A) Diagram showing in vivo xenograft models testing 4,196 TILs against TYK-nu cancer cells or 4,385 and 4,391 TILs against 4391 PDX cells. (B–D) Tumor growth measurement of mice injected with 4,196 TILs (B) with 4,385 TILs (C) or with 4,391 TILs (C) (n=5). Mice in (B) and (C) were injected with $2\times10^7$ TILs per mouse and mice in (D) were injected with $1\times10^7$ TILs per mouse. The 4196 experiment was independently replicated once. Statistical analysis by two-way analysis of variance *p<0.05, ***p<0.001. ACT, adoptive cell therapies; PDX, patient-derived xenograft; TILs, tumor infiltrating lymphocytes.

in vitro sensitization can have low avidity.[56] The fact that these neoantigen-reactive TCRs are from the tumor, but not the peripheral blood, and that the relatively low concentration was used for the neoantigenic stimulation, may have resulted in no significant expansion of naïve T cells with low avidity for neoantigens. These lines of data point to the possibility that some neoantigen-reactive clones cannot effectively expand under the conventional culture condition. It is possible that the amount of cue for growth is insufficient for some exhausted neoantigen-reactive TILs or it is also possible that tumor cells down-regulate neoantigen presentation on their cell surface, which prevents clonal expansion of some neoantigen-reactive TILs and results in their under-representation in bulk TILs. If neoantigen-reactive TILs are exhausted and no longer functional, autologous PBLs could be engineered with TCRs to develop ACT products with less differentiated phenotypes than TILs. However, given the potential for immune evasion by mechanisms like antigen or HLA loss,[7 24] it may be advantageous to use multiple TCRs targeting different neoantigens/neoepitopes with different HLA restrictions. To that end, NeoExpand can facilitate TCR isolation and the development of therapies containing multiple TCRs. Alternatively, because NeoExpand enables robust growth of neoantigen-reactive TILs and helps them maintain good phenotypes, TIL therapies with heterogenous neoantigen-reactive TIL populations can be an alternative ACT option that can address immune evasion by tumor cells by targeting multiple neoantigens simultaneously.

Tetramers have been widely used to enrich antigen-reactive T cells.[57] However, tetramer-based sorting requires prior knowledge of the epitope sequences, and tetramers based on class II HLAs cannot reliably isolate

antigen-specific CD4+ cells.[58] In addition, fluorescence-activated cell sorting of tetramer+ cells for clinical use is challenging due to the limited availability of current Good Manufacturing Practice-compliant tetramers, time-intensive procedures, and potential sterility issues[59 60] In contrast, NeoExpand enables unbiased expansion of neoantigen-reactive TILs even when the exact sequences of candidate neoantigens are unknown. Even though minimal epitopes were used in this study, 25 mer amino acids or TMGs can be intracellularly processed to generate neoepitopes of any length. NeoExpand requires generation of the APCs, such as DCs and B cells. Although the generation of APCs from patients with cancer for clinical use has been described previously,[61–63] generating ample numbers of APCs for use in neoantigenic stimulation may be challenging and it needs to be addressed in future studies. Three recent reports demonstrated that neoantigen-reactive TILs could effectively be identified based on their transcriptomic signatures using scRNA-seq.[6 20 21] This method is rapid because it does not require upfront neoantigen screening. However, in the follow-up study, Chatani *et al* reported that further expansion of neoantigen-reactive and exhausted TILs was problematic requiring an alternative method for the expansion of the neoantigen-reactive TILs.[64] In contrast, NeoExpand can enable both the effective identification of neoantigen-reactive T cells and their expansion for patient treatment. In practice, the various tumor-reactive TIL isolation methods can be chosen or combined to meet specific needs based on their advantages/disadvantages.

Collectively, our data suggest that neoantigenic stimulation of TILs via NeoExpand enabled sensitive identification of neoantigen-reactive CD4 and CD8 TCRs thanks to the expansion of the neoantigen-reactive TIL clonal repertoire. Notably, NeoExpand led to the expansion of the population of T cells with stem-like memory phenotypes, which in turn led to functional enhancement. Finally, our data warrants the evaluation of NeoExpand for clinical use.

## 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 performed 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. TILs or fresh tumor digests were generated from 25 patients with chemorefractory metastatic epithelial cancers enrolled in tissue procurement protocol NCT00068003. Metastases and leukaphereses were collected from each patient at the time of recruitment. TILs were expanded ex vivo for 2–4 weeks, frozen, and kept in liquid nitrogen until use. Leukaphereses were instantly cryopreserved and kept in liquid nitrogen until use. Healthy donors were recruited under the tissue procurement protocol NCT00068003 and underwent leukaphereses.

Adults ages 18–70 with upper or lower gastrointestinal, pancreatic or breast cancer refractory to standard chemotherapy were recruited to either NCT01174121 or NCT03412877. Infusion products from three patients who were treated with ACT of autologous TILs (NCT01174121) were examined to determine the clonal architecture of neoantigen-reactive TILs, and their neoantigen reactivity has been previously reported.[5 6 10]

### Generation of antigen-presenting cells

#### Primary immature dendritic cells

Generation of autologous immature DCs has been previously described.[7] Briefly, peripheral blood monocytes from patient apheresis were isolated using the plastic adherence method. Frozen apheresis was thawed, washed and resuspended in AIM-V media (Thermo Fisher, Cat. 12055083) with 1 µg/mL DNase (STEMCELL Technology, Cat. 07900) at $10^6$ cells/$cm^2$. After 90 min of incubation at 37°C, 5% $CO_2$, non-adherent cells were removed and adherent cells were vigorously washed three times with phosphate-buffered saline (PBS). After another incubation with AIM-V media for 60 min, adherent cells were washed again and were cultured for 4–5 days with DC media consisting of RPMI 1640 (Thermo Fisher, Cat. 21870092), 5% human serum (GeminiBio, Cat. H122013 or Valley Biomedical, Cat. HP1022HI), 1% Penicillin-Streptomycin (Thermo Fisher, Cat. 15070063), 1% GlutaMAX (Thermo Fisher, Cat. 35050061), 800 IU/mL GM-CSF (LEUKINE; Partner Therapeutics) and 200 U/mL IL-4 (PeproTech, Cat. 200–04). Immature DCs were collected for fresh uses or cryopreserved for further uses.

#### Primary autologous B cells

Primary autologous B cells were generated as previously described.[65] Briefly, B cells were isolated from autologous apheresis by positive selection using CD19+ microbeads (Miltenyi Biotec, Cat. 130-050-301) and were co-incubated with irradiated NIH3T3 cells constitutively expressing human CD40 ligand in the presence of 200 U/mL IL-4. B cells were harvested between days 4 or 6 after the initial stimulation and were restimulated 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. B-cell medium comprised of Iscove's Modified Dulbecco's Medium (Thermo Fisher, Cat. 12440053) supplemented with 10% human serum, 1% Penicillin-Streptomycin, 1% GlutaMAX and 200 U/mL IL-4.

#### Transformation of patient-derived B cells using Epstein-Barr virus

Transformation of patient-derived B cells (Epstein-Barr virus (EBV)-B) was performed using supernatant from B95-8 cells containing EBV (ATCC, Cat. VR-1492) according to the manufacturer's instruction without using feeder cells. Either thawed apheresis or CD19+ B cells following bead selection were used for transformation.

### HLA engineering of COS7 cells

A library of HLAs was individually introduced into an MSGV1 backbone for retrovirus generation. HLA sequences were collected from IPD-IMGT/HLA (versions 3.35–3.51), codon optimized, and cloned into an MSGV1 vector using NheI and EcoRI (custom cloning by GenScript). Class II HLAs were cloned in as a pair and were spaced with a P2A site. Retroviral supernatant was generated in HEK293 cells constitutively expressing Gag and Pol as described previously.[24] COS7 cells were transduced using RetroNectin (Takara Bio, Cat. T100B), expanded and sorted by fluorescence-activated cell sorting (FACS) (using individual HLA-specific antibodies (Pure Protein) or pan-antibodies against HLA-DP (BD Biosciences, Cat. 566825), HLA- DQ (BD Biosciences, Cat. 347453) or HLA-DR (BD Biosciences, Cat. 347367)) or selected by antibiotics.

### TMG engineering of COS7 or EBV-B cells

For constitutive expression of TMG, wild-type (WT) or mutant p53 or RAS TMG sequences from previous studies[24 43] were individually or together (with a P2A site in the middle) cloned into an MSGV1 vector (GenScript) with a blasticidin resistance gene. HLA-engineered COS7 cells or EBV-B cells were retrovirally transduced as described above. Blasticidin-resistant cells were selected under 5–10 µg/mL blasticidin treatment for 1 week and were maintained with 5 µg/mL blasticidin. Selected APCs were functionally validated using T cells expressing known TCRs targeting p53 or RAS neoantigens by co-culturing T cells and TMG-expressing APCs overnight and measuring 4-1BB expression by flow cytometry and/or IFN-γ secretion by an IFN-γ ELISpot assay (Mabtech, Cat. 3420–2H).

### Transient transfection of TMG RNA into APCs

Autologous DCs or B cells, or HLA-engineered COS7 cells were transiently transfected with TMG RNA. The sequence of mutant TP53 and RAS TMGs were previously reported in Malekzadeh *et al*[46] and Levin *et al*,[43] respectively. Synthesis and transfection of TP53 or RAS TMG RNAs were performed as previously described.[24 43] Up to 3.5 million DCs or B cells were centrifuged and resuspended in 100 µL of Opti-MEM (Thermo Fisher, Cat. 11058021) and electroporated with 5–10 µg TMG RNA using 2 mm cuvette and a BTX ECM 830 Square Wave Electroporation System (BTX Cat. 45–2052) at 150 V for 10 ms (DC) or for 20 ms (B cells). Alternatively, COS7 cells or DCs were transfected with TMG RNA using Lipofectamine MessengerMAX (Thermo Fisher, Cat. LMRNA015) according to the manufacturer's instruction. Electroporated or transfected APCs were used the next day for co-culture.

### Peptide pulsing of APCs

24 to 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 10 ng/mL to 500 ng/mL (see also online supplemental figure S1). The sequences of the peptides used throughout the study are available in online supplemental table S2.

### Flow cytometry and antibodies

T cells following a co-culture with APCs were stained with antibodies specific for the following human markers and murine TCR (mTCR): CD4 fluorescein isothiocyanate (FITC) (clone RPA-T4; 1:20, catalog no. 555346), OX40 PE (clone ACT35; 1:20, catalog no. 555838), CD8 PE-cy7 (clone RPA-T8; 1:25, catalog no. 560917,), 4–1BB APC (clone 4B4–1; 1:20, catalog no. 550890), and CD3 APC-Cy7 (SK7; 1:25, catalog no. 341090,) with or without mTCRβ-BV421 staining (catalog no. 562839; all from BD Biosciences). 4,196 TILs were stained with the following two panels of antibodies in conjunction with tetramer staining: panel 1: CD3 APC-Cy7, CD8 PE-Cy7, CD4 FITC (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, BD Biosciences, Cat. 562516) and tetramer-APC (custom generated). Panel 2: CD62L BV421 (clone DREG-56; 1:50, 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 tetramer-PE (custom generated). Analytic flow cytometry was performed on LSRFortessa, or FACSymphony (BD Biosciences) with analysis by FlowJo software (V.10.6.2, TreeStar). All cells were gated via lymphocytes (forward scatter and side scatter) and live cells by the exclusion of cells stained with propidium iodide (catalog no. P1304MP, Thermo Fisher), or DAPI (BioLegend, cat. 422801). For TCR isolation 4–1BB+ and/or OX40+ cells were sorted separately through $CD3^+CD4^+CD8^-$ (for CD4) and $CD3^+CD4^-CD8^+$ (for CD8) gates using SH800S or MA900 (Sony Biotechnology). For single-cell transcriptome analysis of patient 4,196 and 4,391 TILs, $CD8^+$ cells were sorted using MA900 (Sony Biotechnology).

### TCR transduction of healthy donor PBLs

Transduction of healthy donor autologous PBLs was performed as previously described.[24] Healthy donor-aphereses were thawed, counted and were stimulated in 50/50 media (RPMI 1640 media containing 10% human serum, 1% GlutaMAX, 12.5 mmol/L HEPES (Thermo Fisher, Cat. 15630080), 1% Penicillin-Streptomycin, and 5 µg/mL gentamicin (Quality Biological, Cat. 120–099–661) mixed with AIM-V at 1:1 ratio) supplemented with 50 ng/mL anti-CD3 antibody (Miltenyi Biotec, Cat. 130-050-301) and 300 IU IL-2 (Aldesleukin, Clinigen) for 48 hours. Retroviral supernatants were loaded into RetroNectin (Takara Bio, Cat. 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 1500 RPM with minimal acceleration and brake. Transduced T cells were cultured for up to 1 month in 50/50 media supplemented with 300 IU/mL IL-2. At day 4–6 post-transduction, T cells were collected and examined for exogenous TCR expression by flow cytometry.

### Generation of TILs

TILs used in this study were generated using the previously described method.[66] Resected tumors were removed from normal tissues immediately after surgical excision. Areas of firm, solid tumor were selected for processing and sized to about 1–3 mm per section. Individual fragments were placed in a 24-well plate in 2 mL of the T-cell culture media (RPMI 1640 containing 10% human serum, 1% GlutaMAX, 12.5 mmol/L HEPES, 1% Penicillin-Streptomycin, and 5 µg/mL gentamicin without AIM-V) containing high-dose IL-2 (6,000 IU/mL, Chiron). Fragments were cultured at 37°C at 5% $CO_2$ for 5 days. On day 5, culture media were replenished with fresh media and IL-2 (6,000 IU/mL) and reassessed every 2–3 days. When cultures exceeded $10^6$ cells/mL or were nearly confluent, the wells were split 1:1. Each fragment was maintained as a separate culture.

### NeoExpand

#### Antigen loading onto APCs

When peptides were used for antigen loading, 10 ng/mL to 100 ng/mL minimal predicted epitope peptides or 100 ng/mL to 500 ng/mL 24–25 mer-long peptides with a mutation in the middle were pulsed onto APCs for 2–4 hours. Peptide-loaded APCs were then washed with PBS and were used for co-culture. TMGs were either transfected or constitutively expressed in various APCs as described above.

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

TCR-engineered T cells or TILs were counted and were incubated with antigen-loaded APCs at 4:1 to 1:10 effector-to-target ratio. For the first 3 days of NeoExpand co-culture, cells were cultured in 50/50 media supplemented with 30 ng/mL IL-21 (PeproTech, Cat. 200–21) and 0–50 IU/mL IL-2 for TCR-engineered PBLs or 300 IU/mL IL-2 for TILs. 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 for TCR-engineered PBLs or 1,000 IU/mL IL-2 for TILs. At the end of 14–19 days of NeoExpand co-culture, T cells were collected for testing the frequency of TCR-engineered T cells or neoantigen-reactive TILs.

#### Determination of the frequency of TCR-engineered T cells (A) or neoantigen-reactive TILs (B)

##### *TCR-engineered T cells*

During and after NeoExpand, the frequency of TCR-engineered T cells was determined by flow cytometry. Because all the neoantigen-reactive TCRs used in this study contained mTCR constant region sequences, exogenous TCR expression was tracked by staining with an mTCR antibody.

##### *Neoantigen-reactive TILs*

The frequency of neoantigen-reactive TILs was determined by one or more of the following methods:

1. TILs following NeoExpand were subjected to additional co-culture with APCs expressing a candidate antigen(s) for 18 hours. T cells recognizing neoantigens were determined by flow cytometry measuring the upregulation of 4-1BB or OX-40 and by IFN-γ ELISpot assays. 20,000 to 100,000 APCs were co-cultured with 20,000 to 100,000 TILs in IFN-γ ELISpot plates (96-well plates with a polyvinylidene difluoride membrane; EMD Millipore, Cat. MAIPSWU10). Phorbol 12-myristate 13-acetate (81 nmol/L) and ionomycin (1.34 µmol/L) (Thermo Fisher, Cat. 00–4970–93,) were included as a positive control. Co-cultured cells were stained and analyzed by flow cytometry as described below (see antibodies, flow cytometry, and FACS), and IFN-γ ELISpot plates were processed using the Human IFN-γ ELISpot BASIC kit (horseradish peroxidase; Mabtech, Cat. 3420–2H) according to the manufacturer's instructions.
2. Once the sequences of *CDR3B* of neoantigen-reactive TILs clonotypes were identified, their frequencies were determined by *CDR3B* survey sequencing using bulk genomic DNA by Adaptive Biotechnologies.
3. For $CD8^+$ neoantigen-reactive TILs clones, when the HLA restriction element and the minimal epitope sequences were available, tetramers were synthesized. Tetramer generation was previously reported.[24] TILs following NeoExpand were stained with tetramers and were analyzed by flow cytometry.
4. 4,196, and 4,391 TILs described in figure 5 were analyzed using the single-cell transcriptome analysis which included Single Cell Immune Profiling for *TCRA/B* sequence identification.

### Rapid expansion protocol (rapid expansion)

Non-specific T-cell stimulation for T-cell expansion through rapid expansion has been described before.[31] 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 flaks or G-Rex 24-well, G-Rex 6-well plates or G-Rex 100 flasks (Wilson Wolf, Cat. 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 for TCR-engineered T cells or 3,000 IU/mL IL-2 for TILs.

### Cell lines

Commercially available COS7, TYK-nu, and PDX line 4391, have been described before.[24 43] Pancreatic cancer PDX line 4,069 was established as follows. A freshly resected tumor metastasis from patient 4,069

with metastatic pancreatic cancer was dissected into small fragments of 2 mm in diameter. One fragment was implanted subcutaneously into the flank of an NSG mouse using a 20-gage needle. Tumor growth was measured weekly and when the tumor reached 1 cm in diameter, it was harvested and subsequently passaged into another NSG mouse. When the tumor grew in the mouse the second time, it was harvested and mechanically dissociated using gentleMACS Dissociator (Miltenyi Biotec, Cat. 130-093-235) using the "mouse implanted tumor 1.01" program. The resulting cell suspension was filtered through a 100 µm cell strainer and washed once before being placed in a tissue culture flask. Tumor cell culture media consisted of RPMI 1640 supplemented with 10% FBS (Cytiva, Cat. SH30071.03HI or GeminiBio, Cat. 100–106), 1× non-essential amino acid (Thermo Fisher, Cat. 11140050), 1 mmol/L sodium pyruvate (Thermo Fisher, Cat. 11360070), 1% Penicillin-Streptomycin, 1% GlutaMAX, 10 µg/mL gentamicin, and 55 µmol/L 2-mercaptoethanol (Thermo Fisher, Cat. 31350010). Media were replaced every 3–7 days and cells were passaged when confluence reached 70%. The presence of $KRAS^{G12D}$ mutation and HLA-A*11 in 4069 PDX cells was initially determined by whole exome sequencing of the freshly resected tumor and later validated by reverse transcription polymerase chain reaction (RT-PCR) and Sanger sequencing of the RNA from the established PDX line.

### 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-$Prkdc^{em26Cd52}Il2rg^{em26Cd22}$/NjuCrl) mice were obtained from NCI or Charles River, respectively. 6–8 weeks old female mice were used for all the xenograft experiments. 1–3 million tumor cells were subcutaneously implanted into the flank of NSG or NCG mice. In 2–3 weeks, when the tumor size reached ~30 $mm^2$, mice were randomized, intravenously injected with TCR-engineered PBLs or TILs and monitored for tumor growth. PBS was used as a vehicle for T-cell injection. At the time of T-cell injection and two times additionally, the mice were intraperitoneally injected with 180,000 IU of recombinant human IL-2 in 500 µL of PBS. Tumor growth was measured once or twice a week, and tumor size was calculated as the product of two perpendicular measurements. All experiments were conducted in a blinded manner. Retroviral transduction of healthy donor PBLs was performed as described above. The TCR-engineered PBLs in figure 2H were injected at day 14 post-transduction. The TCR-engineered PBLs in online supplemental figure S2D were sorted for $CD8^+mTCR^+$ cells, expanded for 14 days with rapid expansion and injected into mice. Neoantigen-reactive TIL injection in figure 6 was performed on day 15 after rapid expansion or NeoExpand without a sort.

### Sample preparation and sequencing for single-cell transcriptome and TCR sequencing analysis

Single-cell transcriptome analyses were performed as described before.[24] Live $CD8^+$ cells were FACS sorted (see antibodies, flow cytometry, and FACS) from 4,196 and 4,391 TILs following the IL-2 culture, NeoExpand or rapid expansion. The sorted T cells were resuspended in PBS at the concentration of $5\times10^5$ cells/mL, and loaded onto a Chromium Controller (10X Genomics, Cat. 1000204) for single-cell sample preparation. One to two channels per reaction were used to prepare each sample for sequencing following the manufacturer's protocol. 10,000 T cells per channel were loaded onto the Chromium Controller with the target-cell recovery of 6,000 single cells. The single-cell complementary DNA (cDNA) samples were first universally amplified by running 16 cycles of PCR using a thermocycler (Bio-Rad, Cat. T100) and the Chromium Next GEM Single Cell 5′ Reagent Kits V.2 (10X Genomics, Cat. PN-1000265) according to the manufacturer's instructions. cDNAs for TCR (VDJ) sequencing were further amplified by two additional PCR reactions using TCR-specific primers according to the manufacturer's protocols (10X Genomics, Cat. PN-1000252,). The whole transcriptomes from the same cDNA samples were amplified after cDNA fragmentation per the manufacturer's protocol. The processed single-cell cDNA samples were sequenced using an Illumina NextSeq 550 sequencer (High Output Kit V.2.5; Read1: 26 b.p; Read2: 98 b.p.; Illumina, Cat. 20024912). The whole transcriptome libraries were sequenced using the Illumina NextSeq 2000-P3 kit (Read 1: 26 b.p.; Read 2: 90 b.p.; Illumina, Cat. 20040561).

### Statistical analyses

The effect of ACT treatments on the growth of the xenograft tumors in mice in figure 2G, online supplemental figures S2C and S6A were analyzed using two-way analysis of variance. All statistical analyses were performed using GraphPad Prism (V.10) and summarized data are presented as mean±SEM. To compare the relative expansion of neoantigen-reactive TILs in figure 4E, the fold changes were calculated by dividing the absolute numbers of TILs post-expansion with the starting numbers of TILs used for REP or NeoExpand (online supplemental table S4). Next, relative fold differences of neoantigen-reactive TILs (REP vs NeoExpand) were calculated by normalizing each value with the fold change of REP. Statistical significance was assessed by non-parametric Wilcoxon matched-pairs signed rank test. P values<0.05 were considered significant. P<0.05 (*), p<0.01 (**), and p<0.001 (***).

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**Data availability statement** Data are available upon reasonable request. Publicly available data sets were analyzed to characterize the phenotypes of post-expansion TILs. The single-cell RNA sequencing data in Figure 5 are available from the corresponding author (SAR@nih.gov) upon reasonable request.



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