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Authors

Jang, Hyun J

Caron, Christine

Lee, Calvin K

et al.

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Dual receptor T cells mediate effective antitumor immune responses via increased recognition of tumor antigens

Hyun J Jang, Christine Caron, Calvin K Lee, Lu Wang, Burhan Jama, Jack D Bui , Gerald P Morris

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Department of Pathology, University of California San Diego, La Jolla, California, USA

Correspondence to
Dr Gerald P Morris;
gpmorris@ucsd.edu

ABSTRACT

Background Discovery that ~16% of T cells naturally co-express two T-cell receptor (TCR) clonotypes prompts examining the role of dual TCR cells in immune functions.

Methods Using TCRα-reporter transgenic mice, enabling unambiguous identification of single-TCR and dual-TCR cells, we tested the role of dual TCR cells in antitumor immune responses against immune-responsive syngeneic 6727 sarcoma and immune-resistant B16F10 melanoma.

Results Dual TCR cells were specifically increased among tumor-infiltrating lymphocytes (TILs) in both models, indicating selective advantage in antitumor responses. Phenotype and single-cell gene expression analyses identified dual TCR are predominant during the effective antitumor response, demonstrating selectively increased activation in the TIL compartment and skewing toward an effector memory phenotype. Absence of dual TCR cells impaired immune response to B16F10 but not 6727, suggesting that dual TCR cells may be more influential in responses against poorly immunogenic tumors. Dual TCR cells demonstrated an advantage in recognition of B16F10-derived neoantigens in vitro, providing a mechanistic basis for their antitumor reactivity.

Conclusions These results discover an unrecognized role for dual TCR cells in protective immune function and identify these cells and their TCRs as a potential resource for antitumor immunotherapy.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Antitumor immunotherapy harnessing T-cell reactivity against tumor neoantigens has revolutionized cancer treatment. However, T cell-based cancer immunotherapies are limited by potential immunologic tolerance against neoantigens that are similar to self-antigens and difficulties in identifying T-cell subpopulations capable of recognizing and responding to neoantigen targets.

WHAT THIS STUDY ADDS

⇒ The subset of T cells naturally co-expressing two clonotypic T-cell antigen receptors (TCRs) demonstrate increased participation in in vivo antitumor immune responses in two mouse models. This reactivity is underpinned by dual TCR cells' increased capability to recognize tumor neoantigens.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Discovery of the increased neoantigen recognition capability of dual TCR cells identifies a novel subset of T cells that can be targeted for development of new cancer immunotherapies.

BACKGROUND

The existence of T cells co-expressing two T-cell receptor (TCR) clonotypes as a result of allelic inclusion of TCRα (and to a much lesser extent TCRβ) genes has been recognized for nearly three decades,[1 2] though the physiologic effects of these cells have remained enigmatic. We recently developed the B6.TCRα(TCRA)-green fluorescent protein (GFP)/red fluorescent protein (RFP) transgenic mouse system which enables unambiguous identification of single-TCRα and dual-TCRα cells by flow cytometry[3] to better study dual TCR cells. This model revealed that dual TCR expression is much more common than previously estimated, with ~16% of $CD4^+$ and $CD8^+$ T cells from immunologically naive adult mice co-expressing two TCRα proteins. Given the large portion of the T-cell repertoire now known to co-express two TCR clonotypes, it is of significant interest to understand the roles of dual TCR cells in immune function. Dual TCR cells have been observed to influence and mediate pathogenic immune responses such as autoimmunity and alloreactivity.[4 5] However, we have also demonstrated that dual TCR co-expression can have beneficial effects on T-cell development and function including facilitating thymocyte positive selection,[3 6] promoting peripheral homeostatic proliferation,[7] and association with formation of $CD4^+$ T-cell memory.[3]

The ability of dual TCR cells to contribute to these functions and immune responses is underpinned by the fact that dual TCR cells contain a unique repertoire of TCRs not present in conventional single-TCR cells.[6]

Several studies have demonstrated that dual TCR co-expression during development reduces the stringency of thymic selection, enabling emergence of T cells bearing cross-reactive or autoreactive TCR clonotypes.[6 8–13] While a decreased degree of self-tolerance imparted by thymic selection could be associated with pathogenic autoreactivity, an increased reactivity against self-antigens can also explain the beneficial effects observed in thymic selection,[14] homeostatic proliferation,[15] and memory formation.[16 17]

We hypothesized that the potentially diminished central self-tolerance of dual TCR cells could also have a beneficial effect on the ability to mediate immune responses against tumors. T cells play an essential role in antitumor immune responses, both in the physiologic process of immune surveillance to eliminate neoplastic cells[18–20] and as mediators of therapeutic-induced antitumor immune responses.[21–23] T-cell recognition of transformed cells as targets for elimination depends on T-cell response to tumor antigens. Tumor-specific antigens can arise from either aberrant expression of lineage-stage-specific or developmental-stage-specific proteins, or through generation of novel antigenic epitopes arising from amino acid substitutions encoded by somatic mutations in the tumor cells.[21 24] In both situations, T-cell responses to tumors can be limited by central tolerance-induced ignorance against the root self-antigens. We hypothesized that dual TCR cells, which are known to have reduced negative selection against autoreactive and cross-reactive TCRs and contain unique TCR clonotypes, may have increased ability to recognize tumor antigens and mediate antitumor immune responses.

METHODS

Mice

B6.TCRA-GFP and B6.TCRA-RFP TCRα reporter mice generated by CRISPR/Cas9-mediated knock-in and B6.TCRA-GFP/RFP mice generated by interbreeding were previously described.[3] C57BL/6 (B6), B6.Ly5.1, and B6.Thy1.1 mice were originally purchased from Charles River Laboratories. B6.Thy1.1.TCRα$^{+/-}$ mice genetically deficient for dual TCRα T cells were generated by breeding B6.129S2-Tcra^{tm1Mom}/J mice deficient for *TRAC* gene expression (Jackson Laboratory)[25] with B6.Thy1.1 mice as previously described.[6] Male and female mice of 8–12 weeks of age were used for all studies. All experimental mice were bred and housed in specific pathogen-free conditions in a 12-hour light/dark cycle with ad libitum food and water. All breeding and experiments were performed according to University of California San Diego (UCSD) Institutional Animal Care and Use Committee (IACUC)-approved protocols and under the supervision of the UCSD Animal Care Program.

In vivo tumor models

The 6727 sarcoma cell line was generated from B57BL/6 mice by injection of methylcholanthrene as previously described.[26] B16F10 melanoma cell line[27] was purchased from American Type Culture Collection. Tumor cell lines were cultured as in Roswell Park Memorial Institute medium (RPMI) 1640 with 10% fetal bovine serum (FBS) (HyClone), and confluent cultures were harvested by trypsinization as previously described.[26] For transplantation experiments, 10^6 tumor cells in single-cell suspension in phosphate buffered saline (PBS) were injected subcutaneously into the right flank of recipient mice. Mice were monitored for tumor growth by measurement of tumor length (long axis) and width (short axis). Mean tumor volume was calculated as (length×width^2/2. Animals were sacrificed at indicated time points and spleen, tumor mass, contralateral and draining lymph nodes were collected. Single-cell suspensions were produced from tumor masses by physical disruption and treatment with 1 mg/mL type IA collagenase (Sigma).

Flow cytometry

Flow cytometry was performed to enumerate single-TCR and dual-TCR cells and evaluate T-cell function. Cells were incubated with Zombie Yellow (BioLegend) viability dye prior to labeling with antibodies for identification of T cells (CD4, GK1.5, APC-Cy7; CD8α, 53–6.7, PerCP-Cy5.5; Thy1.1, OX-7, AF700; Ly5.1, A20, AF700), and markers of activation (CD44. IM7, AF700; OX40, OX-86, BV711; CD103, 2E7, Pacific Blue) and exhaustion (PD-1, 29F.1A12, PE-Cy7; LAG-3, C9B7W, APC). Measurement of regulatory T cells (Tregs) and transcription factors was performed by permeabilization and fixation with True Nuclear Fix/Perm buffer and intracellular labeling for FoxP3 (150D, Pacific Blue), Bcl6 (7D1, APC), Tox (TXRX10, eFluor660, Invitrogen), Eomes (Dan11mag, eFluor660) and Nur77 (12.14, AF647). Samples were run with color and fluorescence-minus-one (FMO) controls and where applicable, cells from single-transgenic B6.TCRA-GFP and B6.TCRA-RFP mice were used as FMO controls for gating single-TCR and dual-TCR cells from B6.TCRA-GFP/RFP mice. Flow cytometry analyses were performed using FACSCanto or LSR II instruments (BD Biosciences) with FACSDiva software. Data were analyzed using FlowJo V.10 software.

Single-cell transcriptomic analysis

Single-cell transcriptional analysis of T cells mediating antitumor immune responses against 6727 tumors was performed using Chromium Next GEM Single Cell 5' V.2 reagents (10x Genomics, Pleasanton, California, USA). Tumors were removed 7 days after implantation into B6-TCRA-GFP/RFP recipients, and T cells were recovered by generation of single-cell suspension as described above followed by positive selection enrichment for CD3$^+$ cells (BioLegend), achieving an average 33.9±6.3% purity. Enriched T cells were isolated into GFP$^+$, RFP$^+$, and GFP$^+$RFP$^+$ populations by flow cytometry using an FACSAria instrument (BD Biosciences). Sorted cell populations were not assessed for post-sort purity due to low numbers of cells isolated. Sorted

cells were washed, counted, and labeled with barcoded cell hashtag antibodies against CD45 and major histocompatibility complex (MHC) class I (TotalSeq-C antibodies, BioLegend) for 30 min on ice. Cells were then washed 3× and GFP$^+$, RFP$^+$, and GFP$^+$RFP$^+$ populations from individual mice were combined for GEM emulsion and barcoding. Barcoded libraries were prepared for gene expression analysis and feature analysis according to Chromium Next GEM Single Cell 5' V.2 protocol. Library preparation was confirmed and library concentration measured by TapeStation High-sensitivity D5000 analysis (Agilent, Santa Clara, California, USA) and pooled libraries were sequenced using a NovaSeq S4 (Illumina, San Diego, California, USA). Sequencing data was analyzed using Loupe Browser V.6.2.0 software (10x Genomics).

In vitro tumor antigen stimulation

Responder T cells were isolated from B6.TCRA-GFP/RFP, B6.TCRA-GFP, and B6.TCRA-RFP splenocytes by paramagnetic bead negative selection using MojoSort Mouse CD3 T Cell Isolation kit and labeled with Tag-it Violet tracking dye (BioLegend). Splenocytes from congenically-marked B6.Ly5.1 and B6Thy1.1 mice were irradiated 20 Gy and used as antigen-presenting cells (APCs). Responder T cells were cultured at 10^5 cells/well with 2×10^6 APCs/well in RPMI 1640 supplemented with 10% FBS, 2 mM GlutaMAX, and 0.5 mg/mL gentamicin (Thermo Fisher Scientific) in 96-well flat-bottomed plates at 37°C, 6% CO_2. B16F10 tumor neoantigen peptides (online supplemental table 1), identified from non-synonymous point mutations in protein-coding genes,[28] were synthesized solid-phase peptide synthesis (Peptide V.2.0, Chantilly, Virginia, USA). Peptides were used as unpurified peptides for screening, and high-performance liquid chromatography (HPLC) purified >90% purity for confirmatory studies. Peptides were pooled 10/group and added to cultures at 10 µM final concentration. Anti-CD3/anti-CD28-coated Dynabeads Mouse Activator microbeads (Thermo Fisher Scientific) were used at 5 µL/well as positive control. After 5 days stimulation, cultures were recovered and analyzed by flow cytometry. Proliferation was measured by dye dilution and the frequency of responding cells was evaluated as the Division Index (DI), which calculates the average number of cell divisions of input cells.[29]

Statistical analysis

Data were analyzed using Prism V.7 software (GraphPad, La Jolla, California, USA). Data comparing T-cell subsets and marker expression from individual mice were analyzed non-parametrically using Mann-Whitney test. Comparisons of data from individual mice to reference values for dual TCR frequencies of immunologically naive adult B6.TCRA-GFP/RFP mice[3] were performed non-parametrically using Wilcoxon rank-sign test. Tumor growth was evaluated by comparison of tumor volumes at the end of the experimental period using two-way analysis of variance and by non-linear regression to compare growth kinetics. Hierarchical clustering was performed using the heatmap function in RStudio. Calculated DI values were compared between groups using the unpaired t-test. Two-tailed p values≤0.05 were considered statistically significant.

RESULTS

Dual receptor T cells disproportionately contribute to effective immune rejection of tumor

To evaluate the role of dual TCR cells in mediating antitumor immune responses, we transplanted 10^6 syngeneic methylcholanthrane (MCA)-induced sarcoma 6727 cells into the right flank of B6.TCRA-GFP/RFP reporter mice. The 6727 sarcoma is an immunogenic tumor that initially grows after transplantation, but is ultimately eliminated within 4 weeks in immunologically competent mice.[26] We measured immune responses against the 6727 sarcoma by recovering tumor-infiltrating lymphocytes (TILs), draining lymph node cells (DLN), contralateral lymph node cells (CLN), and splenocytes (SPL) at 1 week after tumor implantation. Single-TCR (GFP$^+$ or RFP$^+$) and dual-TCR (GFP$^+$RFP$^+$) CD4$^+$ and CD8$^+$ T cells were readily identifiable by flow cytometry (figure 1A). Dual TCR cells represented 19.8±2.6% of CD4$^+$ and 22.7±2.7% of CD8$^+$ TILs (figure 1B). This is significantly increased (CD4$^+$ p=0.001, CD8$^+$ p<0.001) compared with the ~16% of T cells expressing dual TCRs in immunologically naive adult mice B6.TCRA-GFP/RFP mice.[3] Dual TCR cells were increased from baseline in all compartments (online supplemental figure 1A), though this increase appeared specifically directed toward the tumor, with dual TCR cells significantly increased among CD4$^+$ DLN and CD4$^+$ and CD8$^+$ TILs compared with the spleen or CLN (figure 1C).

Dual TCR T cells are increased and highly activated in TIL

The increased frequency of dual TCR cells among CD4$^+$ and CD8$^+$ TIL and CD4$^+$ DLN suggests that they may have an increased ability to respond to tumor cells compared with single-TCR cells. To examine this, we measured expression of markers of antigen recognition and T-cell activation (CD44, OX40), tissue homing associated with effective antitumor response (CD103), exhaustion (programmed cell death protein-1 (PD-1), lymphocyte-activation gene-3 (LAG-3)), and Tregs (FoxP3). Comparison of activation markers between single-TCR and dual-TCR TILs revealed that dual TCR CD4$^+$ and CD8$^+$ cells were significantly enriched for activated CD44$^+$ and OX40$^+$ phenotypes than T cells expressing a single TCR clonotype (figure 2A–D). Similarly, dual TCR cells were more likely to express CD103, indicating selective retention in the tumor (figure 2E,F). This activation was specific for reactivity against the tumor, as T cells from spleen, CLN, or DLN did not demonstrate similar activation of either single-TCR or dual-TCR cells (online supplemental figure 1B–D).

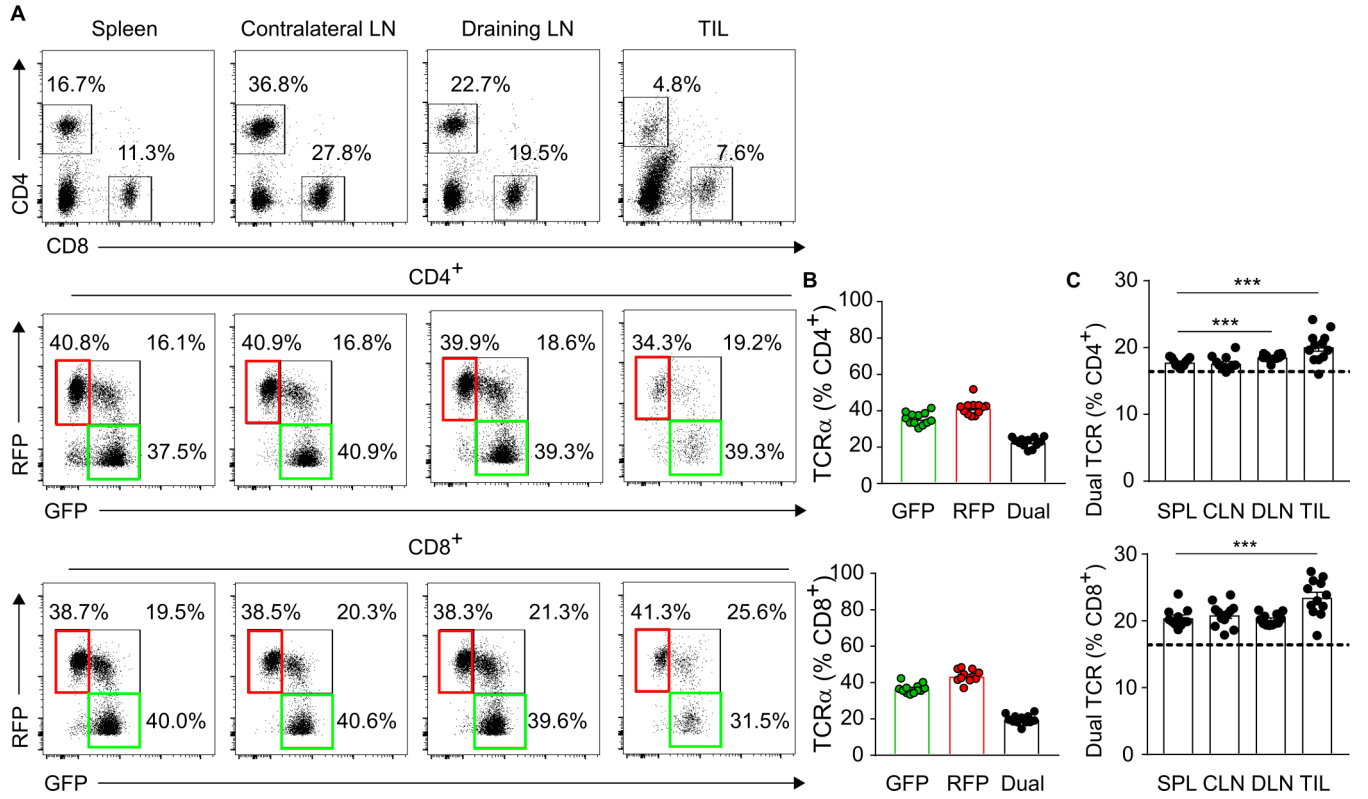

Figure 1 Dual TCR cells are increased in immunogenic tumors and DLN. B6.TCRA-GFP/RFP mice were transplanted with 10^6 MCA-sarcoma 6727 cells in the right flank and immune response was assessed after 1 week by recovery of splenocytes (SPL), contralateral lymph node cells (CLN), draining lymph node cells (DLN), and tumor-infiltrating lymphocytes (TIL) (n=12, 3 independent experiments). (A) Single (GFP$^+$ or RFP$^+$) and dual (GFP$^+$RFP$^+$) T cells were identified by flow cytometry. Data shown is representative example from all compartments of one animal. (B) Frequency of single-TCR and dual-TCR cells among CD4$^+$ and CD8$^+$ TILs. Data shown as dots representing individual mice and box showing group mean values. (C) Frequency of dual TCR cells from all compartments. Data shown as dots representing individual mice and box showing group mean values. Dotted line indicates average frequency of dual TCR T cells from immunologically naive B6.TCRA-GFP/RFP mice. Groups compared with SPL non-parametrically using Mann-Whitney test. ***p<0.005. GFP, green fluorescent protein; LN, lymph node; RFP, red fluorescent protein; TCR, T-cell receptor; TCRA, TCRα; MCA, methylcholanthrine-induced.

Dual TCR cells among 6727 sarcoma TIL also had increased frequencies of PD-1$^+$ and PD-1$^+$LAG-3$^+$ cells among dual TCR TIL (figure 2G–J). Expression of PD-1 by TIL was considered as a marker of antigen-specific reactivity against the tumor.[30 31] However, PD-1 upregulation can indicate both T-cell activation as well as exhaustion. To examine this further, PD-1$^+$LAG-3$^+$ co-expression was measured as an additional indicator of T-cell exhaustion. Similar to T-cell activation, this was specific to the antitumor immune response, as T cells from spleen, CLN, or DLN did not demonstrate increase in expression of these markers (online supplemental figure 1E,F). While this does not unambiguously differentiate exhausted cells from activated cells, both of these populations are considered indicative of T-cell response to tumor.[32] Dual TCR cells in TIL were not more likely to be FoxP3$^+$ Tregs than single-TCR cells (figure 2K,L).

Single-cell RNA sequencing analysis identifies dual TCR cells as predominant effector memory TIL

The activated nature (CD44$^+$, OX40$^+$, PD-1$^+$) of dual TCR TILs compared with single-TCR cells presents non-exclusive possibilities that either dual-TCR cells are better able to recognize tumor antigens and mediate responses similar to conventional single-TCR cells, or that dual-TCR cells may have distinct functional responses. We examined the functional response of dual TCR cells by performing single-cell transcriptional analysis of TILs from B6.TCRA-GFP/RFP mice bearing 6727 tumors. Single TCR GFP$^+$ or RFP$^+$ and dual TCR GFP$^+$RFP$^+$ cells were isolated from five 1-week post-implantation 6727 tumors by paramagnetic bead enrichment for CD3$^+$ T cells followed by flow cytometry cell sorting for GFP$^+$, RFP$^+$, or GFP$^+$RFP$^+$ cells. Isolated populations were labeled with nucleotide-barcoded hashtag antibodies, and barcoded fractions from individual mice were re-pooled for subsequent sample emulsion barcoding and complementary DNA library generation (figure 3A). Libraries were generated for gene expression and barcode feature analysis from each sample, producing a total of 3717 barcoded cells. From these cells, 658 presumptive T cells were identified by filtering for cells expressing *Cd3e* and *Cd3d* and not expressing *Klra1* (Ly49A), *Klra7* (Ly49G) (excluding natural killer (NK) cells), or *H2-Ab1* (excluding B cells and myeloid cells) (online supplemental figure 2A).

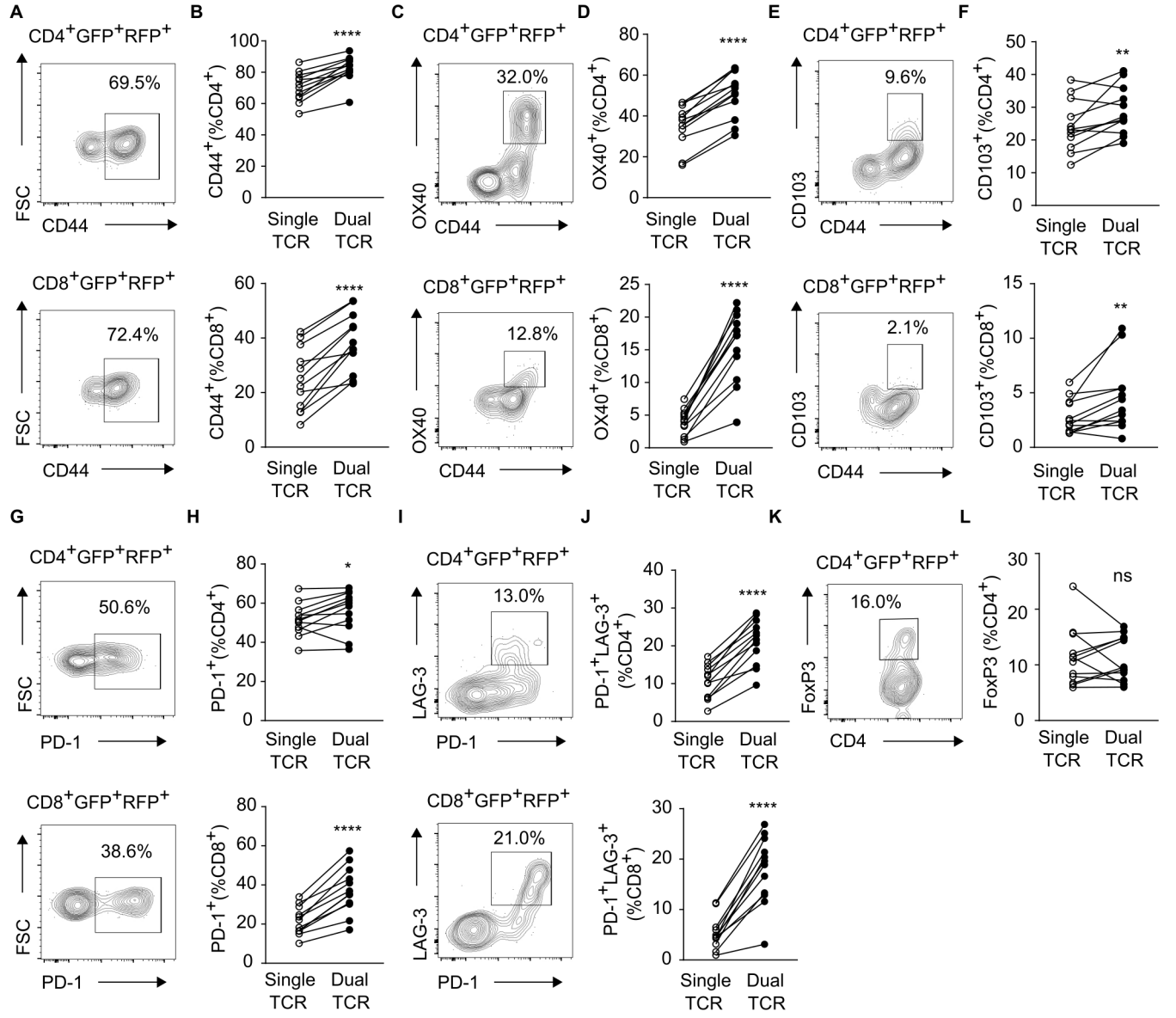

Figure 2 Dual TCR cells are preferentially activated in TILs. Activation and exhaustion of TILs from 6727 sarcoma-bearing B6.TCRA-GFP/RFP mice 1 week after tumor implantation was examined by flow cytometry (n=12, 3 independent experiments). Expression of (A,B) CD44, (C,D) OX40, (E,F) CD103, (G,H) PD-1, (I, J) PD-1 and LAG-3, and (K,L) FoxP3 were compared between single-TCR and dual-TCR $CD4^+$ and $CD8^+$ TILs. (A,C,E,G,I,K) Representative example of all markers expressed by GFP^+RFP^+ TIL from one animal. (B,D,F,H,J,L) Comparison of expression by single-TCR and dual-TCR cells from TIL of individual mice, linked dots representing individual mice. Wilcoxon matched-pairs test. *p<0.05, **p<0.01, ****p<0.001, ns, not statistically significant. GFP, green fluorescent protein; PD-1, programmed cell death protein-1; RFP, red fluorescent protein; TCR, T-cell receptor; TIL, tumor-infiltrating lymphocyte; LAG-3, lymphocyte-activation gene-3; TCRA, TCRα; FSC, forward scatter.

Identified T cells were derived from all five TIL samples (online supplemental figure 2B). K-means analysis of differential gene expression grouped these T cells into four clusters (figure 3B). Clusters 1, 2 and 4 were primarily comprised of *CD8a*$^+$ T cells, while *CD4*$^+$ T cells were contained within cluster 3 and a small subpopulation of cluster 4 (online supplemental figure 2C). Manual review of top-expressed genes identified these clusters as; (1) (32.5%) $CD8^+$ T cells with effector memory, or 'stem-like' capacity (*Tcf7, Il7r, Sell, Bcl2*), (2) (27.8%) terminal effector $CD8^+$ T cells (*Pclaf, Stmn1, Cks1b*), (3) (24.3%) a combination of activated and regulatory $CD4^+$ T cells (*Izumo1r, Tnfrsf4, Tnfsf18, FoxP3*), and (4) (15.3%) exhausted T cells (*Pdcd1, Lag3, Tox, Myb*)[32] (figure 3C).

Cell labeling with barcoded hashtag antibodies was differentiated by feature plot analysis and manual selection for cells with only a single hashtag type present (online supplemental figure 2D). Barcodes segregated in expected groups, with the exception of barcode C0308 (used to label RFP^+ cells sorted from TIL sample 7) which was detected on both GFP^+ and RFP^+ cells from sample 7 (figure 3D). Based on hashtag barcode analysis, 592 (90.0%) of cells were assigned to the sorted input cell subtypes; GFP^+ (n=200, 33.8%), RFP^+ (n=63, 10.6%), and

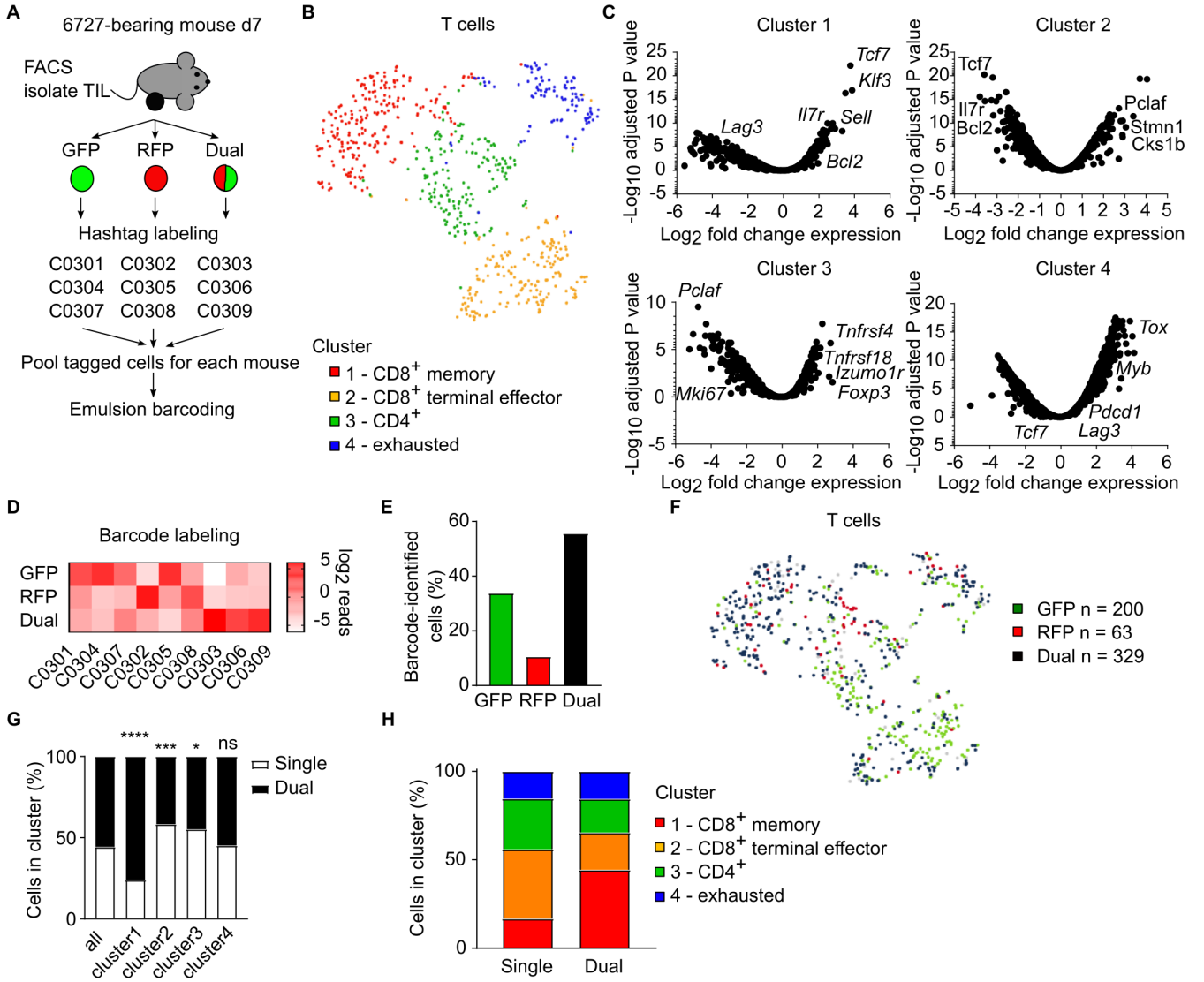

Figure 3 Dual TCR cells predominate effector T-cell response in immunogenic tumors. TILs were recovered from 6727 tumors 7 days after implantation in B6.TCRA-GFP/RFP mice and examined by single-cell transcriptional analysis. (A) Cell hashtag barcoding strategy for GFP$^+$, RFP$^+$, and GFP$^+$RFP$^+$ cells isolated by FACS from individual TIL samples (n=5, 2 independent experiments). (B) T cells (n=658), identified as *CD3e*$^+$*CD3d*$^+$*H2-Ab1*$^-$*Klra1*$^-$*Klra2*$^-$, were clustered by k-means analysis for gene expression. Data represented as t-SNE plot, with cluster identification by manual review of differentially expressed genes. (C) Differential expression of genes plotted against –log10 p value for statistical confidence for each cell cluster. (D) Cluster analysis of expression of cell hashtag barcodes based on manual differentiation (online supplemental figure S2D) of GFP$^+$, RFP$^+$, and GFP$^+$RFP$^+$ input cells. (E) Proportion of cells identified as GFP$^+$, RFP$^+$, or Dual TCR (GFP$^+$RFP$^+$) by barcode analysis. (F) Distribution of cells identified as GFP$^+$, RFP$^+$, or Dual TCR (GFP$^+$RFP$^+$) by barcode analysis among gene-expression based clusters. Data shown as t-SNE plot. (G) Proportion of cells in each cluster identified as single (GFP$^+$ or RFP$^+$) or dual TCR. Proportions of cells in each cluster compared with overall proportion by Fisher's exact test. (H) Percentages of single-TCR and dual-TCR cells in each gene-expression defined cluster. *p<0.05, ***p<0.005, ****p<0.001, ns, not statistically significant. GFP, green fluorescent protein; RFP, red fluorescent protein; TCR, T-cell receptor; TIL, tumor-infiltrating lymphocyte; FACS, fluorescence-activated cell sorting; TCRA, TCRα; t-SNE, t-distributed stochastic neighbor embedding.

GFP$^+$RFP$^+$ dual TCR (n=329, 55.6%) TILs (figure 3E). Given the limited number of cells identified from the RFP$^+$ input cells, as well as the non-exclusive labeling of both GFP$^+$ and RFP$^+$ cells by the C0308 hashtag monoclonal antibody (mAb), GFP$^+$ and RFP$^+$ cells were combined into a single group (single TCR) for downstream analyses. The predominance of dual TCR cells among barcoded cells is consistent with their disproportionate contribution to TIL populations observed by flow cytometry (figure 1).

Single TCR (GFP$^+$ or RFP$^+$) and dual TCR (GFP$^+$RFP$^+$) T cells were present in all four functional clusters (figure 3F). Dual TCR cells were the predominant population among cluster 1 CD8$^+$ effector memory phenotype T cells, enriched beyond their prevalence among all analyzed TIL (76.0%, p<0.001) (figure 3G). Dual TCR cells were also present at increased frequencies among cluster 2 CD8$^+$ terminal effector cells (41.6%, p<0.005) and cluster 3 CD4$^+$ cells (44.7%, p<0.05), though not

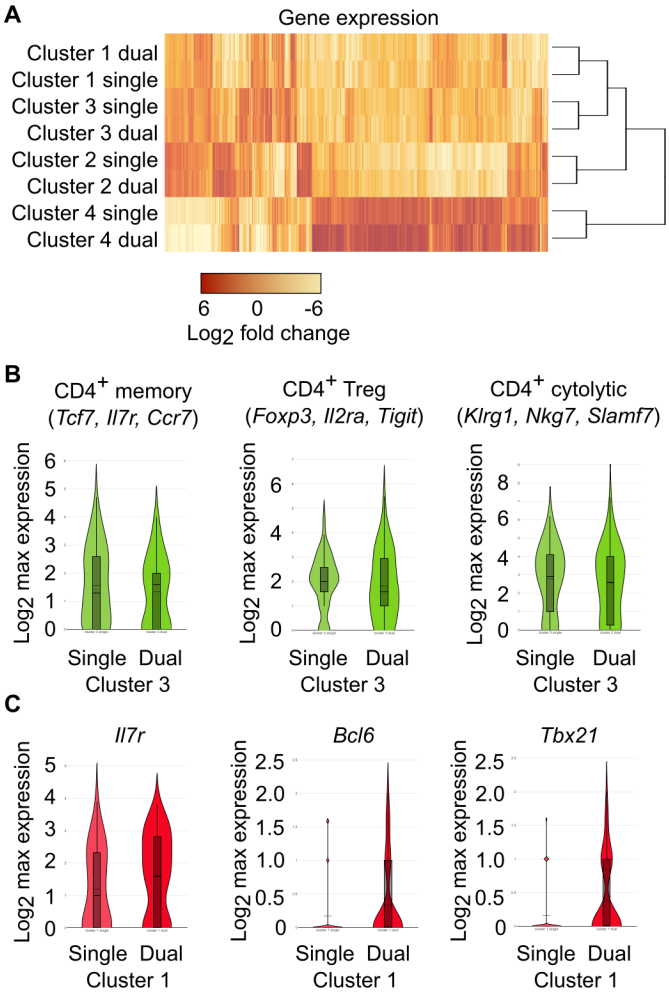

Figure 4 Gene expression of single-TCR and dual-TCR TILs. Gene expression of single-TCR and dual-TCR cells, identified by hashtag barcode (figure 3D) in each functional cluster (figure 3B) was compared. (A) Hierarchical clustering of single-TCR and dual-TCR cells. (B) Differential expression of key genes related to memory (*Tcf7, Il7r, Ccr7*), regulatory (*Foxp3, Il2ra, Tigit*), and cytotoxic (*Klrg1, Nkg7, Slamf7*) $CD4^+$ subsets contained within Cluster 3. (C) Differential expression of key genes related to memory (*Il7r*) and stem-like renewal capacity (*Bcl6, Tbx21*) for $CD8^+$ cells in Cluster 1. Distribution of gene expression shown as violin plot. TCR, T-cell receptor; TIL, tumor-infiltrating lymphocyte.

to the same degree as the effector memory cell population. Notably, dual TCR cells were not disproportionately represented in the exhausted T-cell cluster, suggesting that the significantly increased expression of PD-1 observed by flow cytometry likely reflected T-cell activation, rather than progression to an exhausted cell state. Reciprocal analysis of phenotypes among single-TCR and dual-TCR cells illustrated the propensity of dual TCR cells for the effector memory phenotype during the antitumor response (figure 3H).

Differential gene expression analysis and hierarchical clustering demonstrated that single-TCR and dual-TCR cells within each cluster had comparable gene expression (figure 4A). Across all clusters, single and dual TCR cells demonstrated similar expression of genes associated with T-cell activation (*CD44, CD69*), proliferation (*Mki67*), and TCR signal strength (*Nr4a1*) (online supplemental figure 3A). Single and dual TCR cells in each category had similar expression of genes indicating cytotoxic function (*Gzma, Gzmb, Prf1, Cx3cr1*), terminal effector differentiation (*Tox, Eomes, Prdm1*), or T-cell exhaustion (*Lag3, Havcr2, Tigit*) (online supplemental figure 3B), consistent with the assignment of cluster 2 terminal effector and cluster 4 exhausted phenotypes. Given that clusters 1 and 3 potentially include multiple phenotypes, we attempted to disambiguate the contribution of dual TCR cells by comparison of key genes within those subsets. Cluster 3 contains the majority of $CD4^+$ TILs, which could include memory, Treg, and cytotoxic cells. Comparison of single-TCR and dual-TCR cells did not demonstrate differences in expression of key genes associated with memory (*Tcf7, Il7r, CCR7*), Treg (*Foxp3, Il2ra, Tigit*), or cytolytic (*Klrg1, Nkg7, Slamf7*) TIL phenotypes (figure 4B).[33] Cluster 1 is defined by increased expression of *Tcf7*, *Klf3*, and *Lef1*, genes associated with $CD8^+$ naive, effector memory, and TIL with stem-like renewal capabilities. We did not observe differences in expression of *Il7r*, which has increased expression associated with $CD8^+$ effector memory cells (figure 4C). However, we observed subsets of cells specifically among dual TCR cells in cluster 1 expressing *Bcl6* and *Tbx21* (figure 4C), genes associated with $CD8^+$ TIL with stem-like renewal capability and associated with effective antitumor immune responses.[34 35]

Using these results as a guide, we further investigated expression of key transcription factors by single-TCR and dual-TCR TIL at the protein level by intracellular flow cytometry. We isolated TIL from 6727 tumors 7 days after transplantation and evaluated expression of Bcl6, Tox, Eomes, and Nur77. Consistent with gene expression data (figure 4), a subset of dual TCR $CD8^+$ TIL expressing Bcl6, which is associated with memory and effector memory $CD8^+$ T-cell phenotypes including $CD8^+$ TIL with stem-like renewal capacity [3637], were identifiable (figure 5A). On average, dual TCR $CD8^+$ TIL had increased frequencies of $Bcl6^+$ cells (7.8±3.6%) compared with single-TCR $CD8^+$ TIL (5.5±2.1%), though the difference did not reach statistical significance (figure 5B). We attempted to identify cells co-expressing Bcl6 and Tbet (encoded by *Tbx21*) by flow cytometry, but were unable to identify sufficient numbers of cells for statistical analysis (data not shown).

Examination of Tox expression, associated with an exhausted T-cell state during antitumor responses,[38 39] demonstrated selectively increased expression in dual TCR TIL (figure 5C,D). However, Eomes, another transcription factor associated with T-cell exhaustion [40 41] was not differentially expressed between single-TCR and dual-TCR cells (figure 5E,F). This seeming discrepancy may relate to a recent report indicating that Tox expression can contribute to $CD8^+$ T-cell memory formation,[42] which would be consistent with the increased frequency of $Bcl6^+$ cells and the over-representation among memory

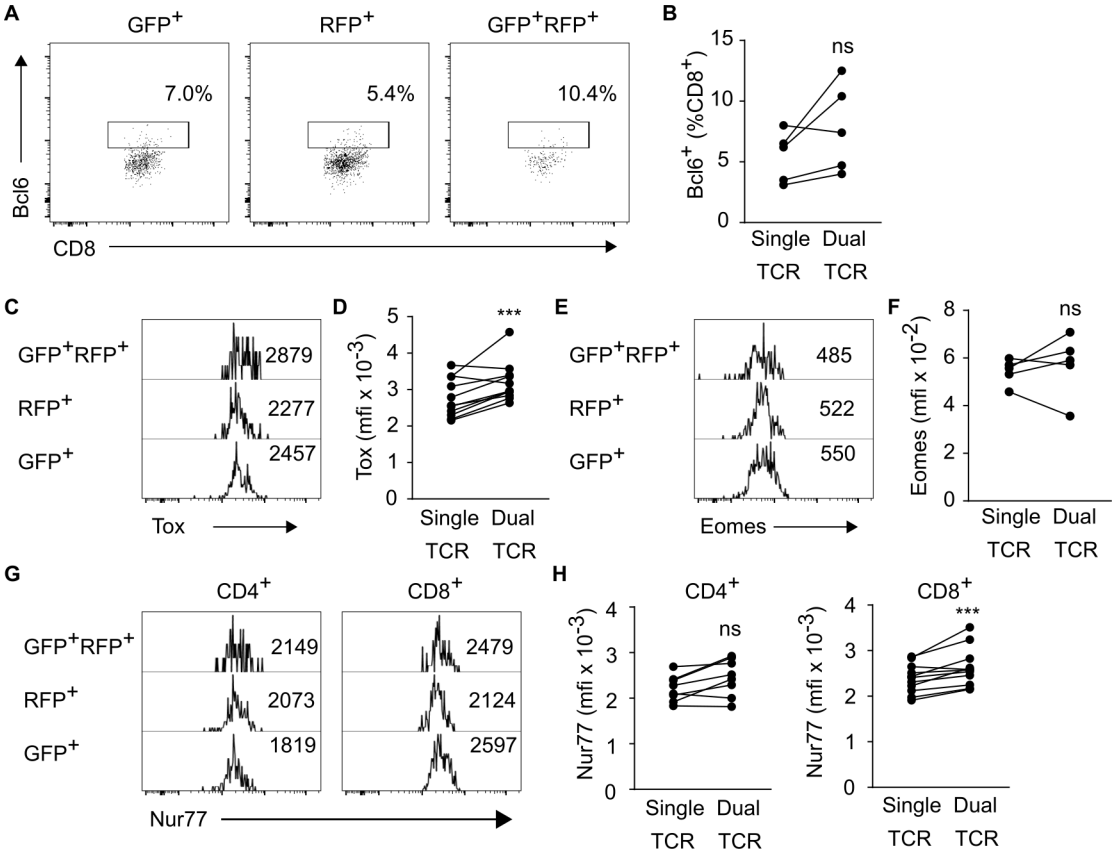

Figure 5 Flow cytometry analysis of transcription factors associated with TIL function. Expression of key transcriptions factors in TILs from 6727 sarcoma-bearing B6.TCRA-GFP/RFP mice 1 week after tumor implantation was examined by intracellular flow cytometry. (A) Identification of Bcl6$^+$ CD8$^+$ GFP$^+$, RFP$^+$, and GFP$^+$RFP$^+$ TIL, representative sample shown. (B) Intrasample comparison of frequency of Bcl6$^+$ single-TCR and dual-TCR TIL for five mice from two independent experiments. (C) Measurement of Tox expression in CD8$^+$ GFP$^+$, RFP$^+$, and GFP$^+$RFP$^+$ TIL, representative sample shown, geometric mean fluorescence indicated. (D) Intrasample comparison of Tox expression by single-TCR and dual-TCR TIL for 11 mice from four independent experiments. (E) Measurement of Eomes expression in CD8$^+$ GFP$^+$, RFP$^+$, and GFP$^+$RFP$^+$ TIL, representative sample shown, geometric mean fluorescence indicated. (F) Intrasample comparison of Eomes expression by single-TCR and dual-TCR TIL for five mice from two independent experiments. (G) Measurement of Nur77 expression in CD4$^+$ and CD8$^+$ GFP$^+$, RFP$^+$, and GFP$^+$RFP$^+$ TIL, representative sample shown, geometric mean fluorescence indicated. (H) Comparison of Nur77 expression by single-TCR and dual-TCR cells from TIL of individual mice, linked dots representing individual mice. All statistical analyses using Wilcoxon matched-pairs test. ***p<0.05, ns, not statistically significant. GFP, green fluorescent protein; RFP, red fluorescent protein; TCR, T-cell receptor; TIL, tumor-infiltrating lymphocyte; TCRA, TCRα.

phenotype cells identified by single-cell gene expression analysis (figure 4). Intracellular flow cytometry measuring Nur77 expression (encoded by *Nr4a1*) demonstrated mixed results, with no measurable difference between single-TCR and dual-TCR CD4$^+$ TIL, but a consistent increase in dual TCR CD8$^+$ TIL (figure 5G,H). Together, these data support the idea that dual TCR expression may influence the quality of antitumor immune responses.

Absence of dual TCR cells impairs immune response against poorly immunogenic tumor

The increased frequencies and activation state of dual TCR cells in TILs from immunogenic 6727 suggests that dual TCR cells are functional contributors to antitumor immune responses. To further test the role of dual TCR cells in antitumor immunity, we transplanted tumor cells into B6.TCRα$^{+/-}$ mice, which lack dual TCR cells due to a heterozygous disruptive mutation in *TRAC*,[25] with transplantation of 6727 tumors. The absence of dual TCR cells did not impair effective rejection of immunogenic 6727 tumors, which elicit robust T-cell responses and are typically rejected within 2–4 weeks after transplantation (figure 6A). However, we hypothesized that the impact of dual TCR cells on antitumor immune responses may be more appreciable in a system with less robust T-cell responses. For this, we used the B16F10 melanoma model, which is a well-characterized poorly immunogenic tumor that grows progressively in immune-competent syngeneic recipients.[27] Indeed, control of B16F10 tumor growth was significantly impaired by the absence of dual TCR T cells (figure 6B). These data indicate that while dual TCR cells are effective contributors to antitumor responses in general, their impact may be most significant in the setting of poorly immunogenic tumors.

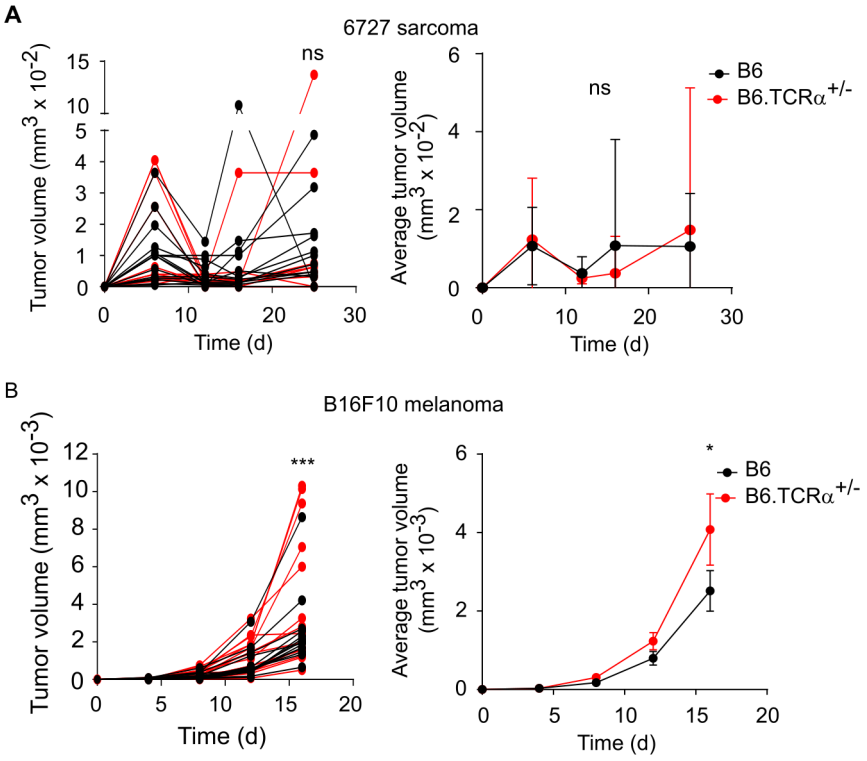

Figure 6 Absence of dual TCR cells impairs control of poorly immunogenic B16F10 tumors. Function of dual TCR cells in mediating antitumor responses was tested by implantation of tumor cells into B6.Thy1.1.TCRα$^{+/-}$ mice, which are genetically deficient in dual TCR cells. Tumor growth was assessed by measurement of tumor length and width and calculation of tumor volume. Tumor volume for mice in each group was compared at each time point using two-way analysis of variance and growth kinetics compared using non-linear regression. (A) B6 (n=16) and B6.Thy1.1.TCRα$^{+/-}$ (n=14) mice were transplanted with 10^6 6727 sarcoma cells in the right flank in three independent experiments. Data shown as individual mice at each time point and mean±SEM for each group with line for non-linear regression analysis. (B) B6 (n=17) and B6.Thy1.1.TCRα$^{+/-}$ (n=17) mice were transplanted with 10^6 B16F10 melanoma cells in the right flank in three independent experiments. Data shown as individual mice at each time point and mean±SEM for each group with line for non-linear regression analysis. *p<0.05, ***p<0.005, ns, not statistically significant. TCR, T-cell receptor.

Dual TCR cells are increased but not effectively activated in a poorly immunogenic tumor

The responses of dual TCR cells against B16F10 tumors was examined in T cells from spleens, CLN, DLN, and TILs, 10–14 days after tumor implantation. Similar to immunogenic 6727 tumors, dual TCR CD4$^+$ (22.6±7.2%, p=0.026) and CD8$^+$ (24.2±6.9%, p<0.001) were significantly increased among TILs isolated from B16F10 tumors (figure 7A). Unlike mice transplanted with 6727 tumors, B16F10-bearing mice did not demonstrate systemic increases in dual TCR cell populations compared with immunologically naive adult B6.TCRA-GFP/RFP mice (online supplemental figure 4A). Within the TIL compartment, both single-TCR and dual-TCR CD4$^+$ and CD8$^+$ TILs exhibited much lower frequencies of CD44$^+$, OX40$^+$, CD103$^+$, PD-1$^+$ and PD-1$^+$LAG-3$^+$ cells (figure 6B–D, online supplemental figure 4B–F) compared with TILs isolated from 6727 tumors (figure 2), indicating a generally ineffective activation in response to B16F10 tumors. The lack of effective activation of both single-TCR and dual-TCR TILs in B16F10 tumors is consistent with the established resistance of the B16F10 melanoma to immune-mediated clearance.

Dual TCR cells have increased ability to recognize tumor neoantigens

Despite the lack of evidence for effective activation of antitumor T cells in B16F10 TILs (figure 7B–D), the increased frequency of dual TCR cells among the B16F10 TILs and the increased growth of B16F10 tumors in the absence of dual TCR cells (figures 6 and 7A) suggests that these cells may have an increased capability of recognizing tumor neoantigens. To test this, we measured in vitro responses against previously identified B16F10 tumor neoantigens (online supplemental table 1).[28] T cells from immunologically naive adult B6.TCRA-GFP/RFP mice were labeled with Tag-it cell proliferation dye and stimulated for 5 days with anti-CD3/anti-CD28 microbeads or B16F10 neoantigen peptides, with responses measured by calculation of the DI based on dye dilution (figure 8A). Given that the response against any individual antigen would be expected to be a relatively low-frequency event in an immunologically naive animal, we pooled neoantigen peptides in groups of 10 to facilitate the detection of a response. In vitro stimulation of T cells with pooled B16F10 neoantigen crude peptides demonstrated that dual TCR GFP$^+$RFP$^+$ cells had selective responses against

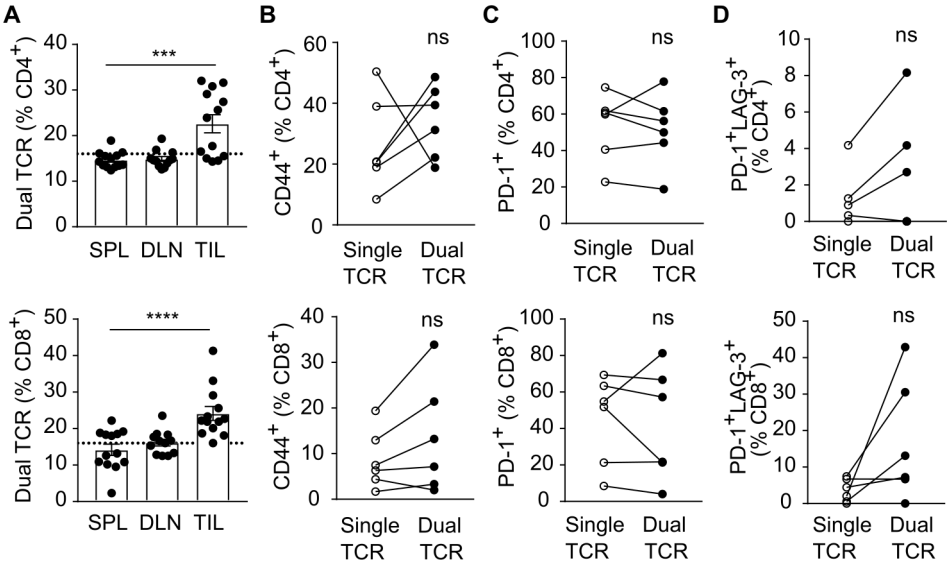

Figure 7 Dual TCR cells are increased but not activated in poorly immunogenic B16F10 tumors. B6.TCRA-GFP/RFP mice were transplanted with 10^6 B16F10 melanoma cells in the right flank and immune response was assessed after 10–14 days by recovery of SPL, CLN, DLN, and TIL. (A) Single (GFP^+ or RFP^+) and dual (GFP^+RFP^+) T cells were identified by flow cytometry as shown in figure 1A. Frequency of single-TCR and dual-TCR cells among $CD4^+$ and $CD8^+$ SPL, DLN, and TILs (n=13, 4 independent experiments). Data shown as dots representing individual mice and box showing group mean values. Dotted line indicates average frequency of dual TCR T cells from immunologically naive B6.TCRA-GFP/RFP mice.[3] Groups compared with SPL non-parametrically using Mann-Whitney test. Frequencies of (B) $CD44^+$, (C) $PD\text{-}1^+$, and (D) $PD\text{-}1^+LAG\text{-}3^+$ single-TCR and dual-TCR TILs was compared between single-TCR and dual-TCR $CD4^+$ and $CD8^+$ TILs (n=6, 3 independent experiments). Data shown as linked dots representing individual mice. Groups compared non-parametrically using Wilcoxon matched-pairs test. ***p<0.005, ****p<0.001, ns, not statistically significant. CLN, contralateral lymph node cells; DLN, draining lymph node cells; GFP, green fluorescent protein; PD-1, programmed cell death protein-1; RFP, red fluorescent protein; SPL, splenocytes; TCR, T-cell receptor; TIL, tumor-infiltrating lymphocyte; LAG-3, lymphocyte-activation gene-3; TCRA, TCRα.

specific neoantigen pools, and that these responses were significantly larger than the responses of single-TCR GFP^+ or RFP^+ cells (figure 8B). Proliferative responses of both single-TCR and dual-TCR cells were strongest against pools 11–20, 21–30, and 41–50 which contain neoantigens associated with the strongest immune responses in a vaccination model against B16F10 melanoma.[28] Testing with HPLC-purified (>90% pure) neoantigen peptides for these pools confirmed the disproportionate capability of dual TCR cells to recognize neoantigen peptides (figure 8C). This data confirms the increased capability of dual TCR cells for responding to tumor neoantigens, and likely represents a mechanism by which they are disproportionately present and activated during in vivo antitumor responses.

DISCUSSION

Here, we present evidence that dual TCR cells have a propensity to act as disproportionate contributors to immune responses against transplanted 6727 MCA sarcoma[26] and the B16F10 melanoma[27] cells. In both models, dual TCR cells were significantly increased (32.8% increase in 6727 TILs, 46.3% increase in B16F10 TILs) among TILs present 7–14 days after tumor implantation (figures 1 and 7), indicating a disproportionate contribution to antitumor immune responses. In the response to immunogenic 6727 tumors, dual TCR cells demonstrated selective and preferential activation against the tumor as evidenced by upregulation of activation markers on the cell surface and expression of effector gene programs (figures 2, 3 and 5). However, dual TCR cells were dispensable for effective immune clearance of 6727 tumors (figure 6A). This data suggests that while dual TCR expression promotes antitumor activity, there is effective antitumor activity among conventional single TCR cells that is sufficient to control highly immunogenic tumors. However, the significant selective expansion of dual TCR cells in these short-term in vivo tumor models as well as the evidence for their enhanced ability to recognize tumor neoantigens (figure 7) suggests that dual TCR cells may be amenable to ex vivo expansion and adoptive transfer or checkpoint blockade approaches to leverage their antitumor reactivity.

Conversely, despite poor activation of both single-TCR and dual-TCR T cells in B16F10 TIL (figure 7), genetic elimination of dual TCR cells significantly impaired immunologic control of B16F10 tumors (figure 6B). The poor activation of dual TCR cells in response to B16F10 likely reflects the intrinsic poorly immunogenic nature of the B16F10 tumor microenvironment, a function of a relatively low mutational burden, low expression of MHC and co-stimulatory molecules, and decreased chemokine production.[43] In addition, the suppressive microenvironment of progressively growing tumors consisting of

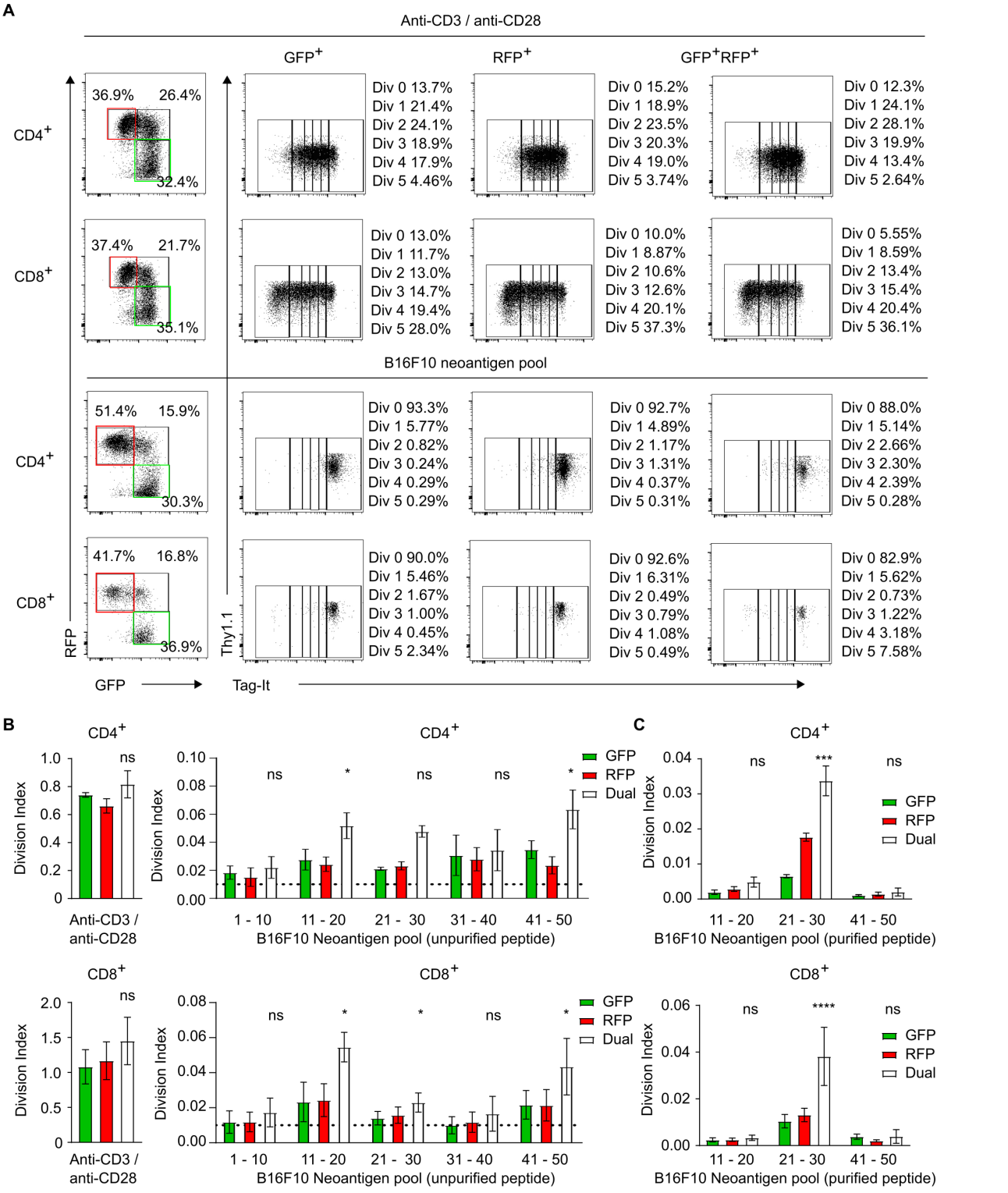

Figure 8 Dual TCR cells have increased ability to respond to tumor neoantigens. T cells isolated from the spleens of immunologically naive B6.TCRA-GFP/RFP mice were labeled with Tag-it cell dye and cultured at 2×10^5 cells/well with 10^6 irradiated congenically-marked syngeneic splenocytes as antigen presenting cells. Cultures were stimulated with anti-CD3/anti-CD28-coated microbeads as positive control or 10 µM pools of 10 B16F10 neoantigens for 5 days and cell proliferation was analyzed by flow cytometry. (A) Representative example of stimulation with peptide pool five. Dye dilution gates defining cell divisions were determined based on positive control proliferative response in the same experiment. Division Index was calculated for GFP^+ or RFP^+ single TCR and GFP^+RFP^+ dual TCR cells for stimulation with (B) anti-CD3/CD28 and 10-peptide pools of unpurified peptides or (C) HPLC-purified neoantigen peptides. Results shown are mean±SEM Division Index from three independent experiments. Single-TCR and dual-TCR results for each condition compared using unpaired t-test. *p<0.05, ***p<0.005, ****p<0.001, ns, not statistically significant. GFP, green fluorescent protein; RFP, red fluorescent protein; TCR, T-cell receptor; TCRA, TCRα; HPLC, high performance liquid chromatography.

increased Tregs, M2-like macrophages, myeloid-derived suppressor cells, inhibitory stromal cells and metabolic competition could thwart the activity of dual TCR cells promptly after their activation.[44–49] It will be important to examine other models, including examination of human TILs across multiple cancer types, in order to evaluate the generalizability of our observations as well as potentially narrow the possibilities of potential tumor-intrinsic factors influencing these responses.

Demonstration that dual TCR cells are more readily capable of responding to B16F10-derived neoantigens (figure 8) provides a mechanistic basis for the observed in vivo reactivity. It should be noted that responses of T cells from immunologically naive animals against tumor neoantigens were low frequency events, and generating measurable responses required both culture of large numbers of T cells against pooled neoantigens. We expect this frequency would be increased in TILs, given the increased number of dual TCR cells and their increased activation/exhausted phenotype in TILs, however, we could not isolate sufficient TILs to perform similar experiments. It is interesting that proliferative responses of bulk cultured cells were strongest against pools 11–20, 21–30, and 41–50 which contain neoantigens associated with effective immune responses in a vaccination model against B16F10 melanoma.[28] These more immunogenic neoantigens were a mixture of conservative (33%) and non-conservative (67%) amino acid substitutions. The similarity in the pattern of reactivity against the neoantigen pools by CD4+ and CD8+ T cells is notable, and presents two distinct possibilities. First and most simply, CD4 and CD8 cells could be coincidentally responding to different peptides within the pools. A second and potentially more interesting possibility is that since the synthesized peptides are 25–28-mers, CD4 and CD8 cells are responding to differentially processed and presented peptides from the same origin peptide. Future studies will need to examine these questions at the clonal level, including generation of neoantigen-specific single-TCR and dual-TCR T-cell clones to better characterize and identify the molecular underpinnings of neoantigen reactivity. It would also be of interest to determine whether these same antigens that elicit the strongest response from immunologically naive T-cell repertories have the same predominance among T cells during an ongoing antitumor immune response, or whether the scope of the antigenic reactivity broadens. It would be important to understand whether dual TCR expression influenced the depth and/or breadth of the antigenic reactivity of T cells during antitumor immune responses.

The potential for differences in the mode of antigen recognition by dual TCR cells stems from important differences in how co-expression of two TCR clonotypes affects development of the TCR repertoire during thymic selection. Dual TCR co-expression facilitates positive selection,[3 6] providing evidence for a beneficial physiologic function of allelic inclusion of TCR genes. However, ample evidence exists that dual TCR co-expression reduces the stringency of negative selection and can promote emergence of T cells with cross-reactive or autoreactive potential.[6 8–13] At the population level, this results in dual TCR cells containing a unique repertoire of TCRs not present in conventional single-TCR cells.[6] This is presumably because these unique dual TCR-associated TCRs are incapable of independently supporting positive selection or would normally be negatively selected. Thus, we propose that an increased ability to recognize tumor neoantigens by dual TCR cells is likely due to a lack of central tolerance-induced ignorance against the root self-antigen, or due to the presence of cross-reactive TCRs, properties that are selected against during negative selection.[14] Again, continued study of dual TCR cell reactivity against these neoantigens at the clonal level may yield significant insight into the mechanisms determining whether the immune system is or is not capable of effective tumor immunosurveillance. Defining the mode of neoantigen recognition by dual TCR cells could have significant impact in designing cancer immunotherapies, particularly against intracellular antigens that are not targetable by chimeric antigen receptor approaches. In these situations, dual TCR cells could serve as a pool for TCR repertoires capable of targeting these neoantigens. Future investigations will be necessary to evaluate the breadth of TCR repertoires contributed to antitumor immune responses by single-TCR and dual-TCR cells.

It will also be of interest to determine whether co-expression of dual TCRs affects the quality of the response against tumors by individual cells. Dissection of 6727 TILs by single-cell transcriptional analysis (figures 3 and 4) not only confirmed the propensity of dual TCR cells to predominate the antitumor immune response, but indicated that dual TCR expression may skew the phenotype of responding T cells. In this case, dual TCR cells demonstrated a significant inclination toward an effector memory phenotype. Further dissection of cells with this phenotype indicated that dual TCR cells contained singular populations of cells expressing *Bcl6* and *Tbx21*, which have been associated with $CD8^+$ TIL with stem-like renewal capability and effective antitumor immune responses.[34 35] This data suggests that dual TCR co-expression may have functional implications beyond expanding the antigenic reactivity of a cell. Recent findings that the affinity of T cells for neoantigen ligands can have deterministic effects on the ability to mediate effective antitumor response[50–52] suggest that dual TCR co-expression could be a contributing factor to this response. Notably, we observed increased expression of Nur77, an indicator of TCR signal strength, by dual TCR cells from TIL (figure 5). Interestingly, the propensity of $CD8^+$ dual TCR cells toward effector memory rather than terminal effector differentiation is similar to our observations of dual TCR cell responses against acute lymphocytic choriomeningitis (LCMV) Armstrong infection, where dual TCR expression promoted $CD4^+$ but not $CD8^+$ terminal effector differentiation, and subsequently promoted effector memory phenotypes in both $CD4^+$ and $CD8^+$ T

cells after the clearance of infection.[3] It will be important to evaluate whether this is a generalizable feature of immune responses by dual TCR cells, and to determine the underlying mechanism. Given that we have recently defined dual TCR co-expression as not the low-frequency anomaly that it was previously presumed to be, but rather a common feature of the immune system representing more than 15% of T cells in mice and humans,[3 53] it is essential to understand how dual TCR co-expression influences effective immune responses.

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Patient consent for publication Not applicable.

Ethics approval Animal experiments were approved by the Institutional Animal Care and Use Committee of the University of California San Diego (protocol #S13152).

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Data availability statement Data are available upon reasonable request. All data will be made available upon reasonable request to the corresponding author.

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ORCID iDs
Jack D Bui http://orcid.org/0000-0002-9324-368X
Gerald P Morris http://orcid.org/0000-0002-1097-4453

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