TCR signal strength defines distinct mechanisms of T cell dysfunction and cancer evasion

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T cell receptor (TCR) signal strength is a key determinant of T cell responses. We developed a cancer mouse model in which tumor-specific CD8 T cells (TST cells) encounter tumor antigens with varying TCR signal strength. High-signal-strength interactions caused TST cells to up-regulate inhibitory receptors (IRs), lose effector function, and establish a dysfunction-associated molecular program. TST cells undergoing low-signal-strength interactions also up-regulated IRs, including PD1, but retained a cell-intrinsic functional state. Surprisingly, neither high- nor low-signal-strength interactions led to tumor control in vivo, revealing two distinct mechanisms by which PD1hi TST cells permit tumor escape; high signal strength drives dysfunction, while low signal strength results in functional inertness, where the signal strength is too low to mediate effective cancer cell killing by functional TST cells. CRISPR-Cas9-mediated fine-tuning of signal strength to an intermediate range improved anti-tumor activity in vivo. Our study defines the role of TCR signal strength in TST cell function, with important implications for T cell-based cancer immunotherapies.

Introduction

The affinity of the interaction between TCR and peptide-bound MHC (pMHC) determines the kinetics and magnitude of the T cell response (Skokos et al., 2007; Denton et al., 2011; King et al., 2012; Zikherman and Au-Yeung, 2015; Ozga et al., 2016; Conley et al., 2016). In vitro studies demonstrated that ligand affinity determines the frequency and rate at which naive T cells become activated, but not the course of subsequent differentiation; while high-affinity ligands activate more T cells, all activated T cells commit to the same effector differentiation program and achieve the same cytolytic capacity, regardless of ligand affinity (Altan-Bonnet and Germain, 2005; Balyan et al., 2017; Richard et al., 2018). In support of these findings, during acute infections, high-affinity interactions lead to increased T cell expansion, but both low- and high-affinity interactions result in memory T cell formation (Zehn et al., 2009).

The tumor-reactive CD8 T cell repertoire is highly diverse, with specificity for self-proteins and tumor-specific (mutant and viral) neoantigens. The TCR affinity of T cells specific to tumor self-antigens is generally low (McMahan et al., 2006; Buhrman and Slansky, 2013; Hollingsworth and Jansen, 2019; Hoffmann and Slansky, 2020), and efforts to isolate higher-affinity T cell clones or engineer affinity-enhanced TCR have improved anti-tumor efficacy, albeit with increased risk of off-target and off-target toxicity (Lyman et al., 2005; Schmid et al., 2010; Parkhurst et al., 2011; Bos et al., 2012; Morgan et al., 2013; Zhong et al., 2013; Cameron et al., 2013; Stone et al., 2015; Schmitt et al., 2015; Rapoport et al., 2015; Smit et al., 2017; Chapuis et al., 2019). In contrast, tumor-specific T cells (TST cells) recognize neoantigens generally with high-affinity TCR, yet TST cells become dysfunctional, allowing tumors to develop and progress (Willinsky and Blankenstein, 2005; Zhou et al., 2005; Aleksic et al., 2012; Lu et al., 2013; Tran et al., 2014; Gros et al., 2014; Blankenstein et al., 2015; Leisegang et al., 2016; Gros et al., 2016; Schietinger...
Low- and high-affinity interactions drive robust T cell activation and effector differentiation in tumor-draining LNs (dLNs)

Retroviral vectors were constructed with genes encoding each APL epitope fused to EGFP. MCA205, a C57BL/6-derived fibrosarcoma cell line that expresses high levels of MHC class I (Fig. S1 A), was transduced with retroviral vectors encoding N4-, F6-, or D4-EGFP. MCA-APL cell lines were sorted for EGFP levels to ensure comparable APL expression (Fig. 1 D). APL-expressing MCA cells injected subcutaneously into mice had similar growth rates in vivo (Fig. S1 B). Once tumors were established (~2 wk after tumor inoculation), we assessed the differentiation dynamics of adoptively transferred naive TCR TAG cells (Fig. 2 A). First, we asked whether TCR signal strength impacts T cell priming and activation in dLNs. CellTrace Violet (CTV)-labeled naive, congenically marked (Thy1.1+) TCR TAG cells were adoptively transferred into MCA-APL tumor-bearing hosts. 4 d later, transferred T cells were isolated from the dLN. All MCA-APL tumors elicited robust activation and proliferation of TCR TAG cells, as evidenced by the expression of activation marker CD44 and dilution of CTV (Fig. 2, B and C). Approximately 10% of TCR TAG cells in the dLN of MCA-D4 tumor-bearing mice were still undivided and CD44lo (Fig. 2 C), consistent with previous reports demonstrating that TCR signal strength impacts the frequency and rate of T cell activation and proliferation (Martínez-Usatorre et al., 2018; Zehn et al., 2009; Richard et al., 2018). All TCR TAG cells produced similarly high levels of the effector cytokines IFN-γ and TNF-α (Fig. 2 D) and phosphorylated ERK in response to ex vivo stimulation (Fig. 2 E). Interestingly, despite their similar activation and effector function profiles, TCR TAG cells encountering high-affinity N4 and F6 antigens expressed higher levels of the inhibitory receptors (IRs) PD1 and LAG3, in contrast to TCR TAG cells in the dLN of low-affinity MCA-D4 tumors, which were PD1lo and LAG3lo (Fig. 2 F). Thus, both high- and low-affinity TCR-pMHC interactions trigger the activation, proliferation, and effector differentiation of naive TST cells in dLN, despite differences in IR expression levels.

High-affinity interactions cause TST cell dysfunction, while low-affinity interactions preserve a cell-intrinsic functional state in TST cells

Next, we asked whether TCR signal strength determines TST cell functional states within tumors, the site of chronic tumor antigen encounter and TCR stimulation. Naive, congenically marked (Thy1.1+) TCR TAG cells were adoptively transferred into MCA-APL tumor-bearing hosts and isolated from tumors 7–14 d later. Tumor-infiltrating TCR TAG cells were uniformly CD44loCD62L0 (Fig. S1 C) and expressed similar levels of the activation marker CD69 (Fig. 3 A). In contrast to what we observed in the dLN, tumor-infiltrating lymphocytes (TILs) from all MCA-APL tumors expressed similarly high levels of PD1 and LAG3 (Fig. 3 B), demonstrating that even very-low-affinity TCR-pMHC interactions within the tumor lead to high expression of these canonical IRs. Strikingly, despite their similar activation and immunophenotype, high-affinity N4 and F6 TCR-pMHC interactions led to the rapid loss of IFN-γ and TNF-α production,
To understand if TCR signal sequencing (RNA-seq) on TCRTAG cells isolated from dLN (dLN-N4, dLN-F6, and dLN-D4) and tumors (TIL-N4, -F6, -D4) of MCA-APL tumors were able to be read out for MAPK activity in response to TCR stimulation ex vivo. While TCRTAG cells from all MCA-APL tumors were able to flux calcium (Fig. 3 D), only low-affinity TIL-D4 cells were able to phosphorylate ERK (Fig. 3 E), suggesting potential negative feedback on the MAPK signaling pathway in response to chronic, high-affinity TCR stimulation.

TCR signal strength drives distinct transcriptional and epigenetic programs in TILs

We previously demonstrated that TST cell dysfunctional states are defined by transcriptional and epigenetic programs (Schietinger et al., 2016; Philip et al., 2017; Scott et al., 2019; Philip and Schietinger, 2021). To understand if TCR signal strength regulates transcriptional programs, we performed RNA sequencing (RNA-seq) on TCRTAG cells isolated from dLN (dLN-N4, -F6, and -D4) and tumors (TIL-N4, -F6, -D4) of MCA-APL tumor-bearing hosts; as controls, we included naive TCRTAG cells, as well as effector TCRTAG cells isolated from LN of mice immunized with a recombinant Listeria monocytogenes strain expressing the high-affinity N4 epitope. Principal-component analysis revealed that TCRTAG cells from dLN-N4, -F6, -D4, and effector TCRTAG cells clustered together and were markedly distinct from their TIL counterparts (Fig. 4 A). High-affinity TIL-N4 and -F6 clustered separately from low-affinity TIL-D4, with 2,300 genes differentially expressed between high- and low-affinity TILs (Fig. 4, B and C).

High-affinity TCR–pMHC interactions led to the up-regulation of critical transcription factors and IRs associated with T cell dysfunction and exhaustion, including Tox, Mafdb, Tcf4, Etb1, Cld44 (2B4), and Entpd1 (CD39), and down-regulation of genes associated with stem-like progenitor and memory differentiation states, such as Tcf7 (TCF1), Runx1, Id3, and Il7r (Fig. 4, B–D; and Fig. S2 A). Gene ontology (GO) classification revealed that pathways associated with negative regulation of T cell activation and effector function were enriched in TILs encountering the high-affinity tumor antigens N4 and F6, but not the low-affinity antigen D4 (Fig. 4 E). Moreover, gene programs associated with tumor-specific T cell dysfunction (Philip et al., 2017) or T cell exhaustion during chronic infections (West et al., 2011) were negatively enriched in low-affinity TIL-D4 (Fig. S2 B). Thus, PDL1+ TILs that encounter tumor-specific antigens with lower TCR signal strength retain transcriptional and functional features of effector/memory T cell states. Interestingly, 86% of the genes differentially expressed between high- and low-affinity TILs were not differentially expressed in the dLN (Fig. S2 C), suggesting that the observed transcriptional differences were established de novo at the tumor site and not preestablished during the priming phase in the dLN.

To examine genes and pathways controlling TIL functional states, we categorized the transcriptional program of T cell dysfunction into TCR affinity-dependent and affinity-independent modules. Of the 892 genes that we previously identified as differentially expressed in dysfunctional TILs...
compared with functional effectors (Philip et al., 2017), ∼15% (140 genes) were specifically regulated by TCR affinity and likely dictate the functional state of TST cells (Fig. 4 F). The remaining dysfunction-associated genes, including Pdcd1, Ctl4, Lag3, Havcr2 (TIM3), Cd38, Lef1, and Ikar, were expressed independently of TCR affinity and thus are likely not associated with TIL functionality.

TST cell dysfunction is epigenetically encoded (Philip et al., 2017; Mognol et al., 2017; Sade-Feldman et al., 2018; Bengsch et al., 2018). To understand whether TCR affinity alters TST cell epigenetic programs, we performed an assay for transposase-accessible chromatin with sequencing (ATAC-seq) on TIL from high- (TIL-F6) and low-affinity (TIL-D4) MCA-APL tumor-bearing mice. Percentages of undivided TCRTag cells (CTV?) are shown (right). (D) Production of effector cytokines IFN-γ and TNF-α by TCRTag cells isolated from dLN and stimulated with Nα peptide (0.5 µg/ml). Each symbol represents an individual mouse. For C and D, n = 4–5 per APL; data are representative of three independent experiments. (E) Flow cytometric analysis of phospho-ERK (pERK) by TCRTag cells isolated 4 d after AT from dLN of MCA-F6 (dLN-F6) and MCA-D4 (dLN-D4) tumor-bearing mice. T cells were stimulated ex vivo with MCA-Nα tumor cells (see Materials and methods). Each circle is an individual mouse. n = 4 for dLN-F6, and n = 9 for dLN-D4. Data are representative of two independent experiments. (F) PD1 and LAG3 expression levels on TCRTag cells from dLN (n = 2 per APL). Data are representative of two independent experiments. (B–F) Data are shown as mean ± SEM. *, P < 0.05; **, P < 0.01; ns, P > 0.05; unpaired two-tailed Student’s t test.
loss of MAPK activity (Fig. 3, D and E), supports the enrichment of Nfatc1-binding motifs, as calcium signaling is required for nuclear localization of NFAT, but colocalization of binding partner AP1 is contingent on MAPK activation. While the role of TCR affinity in regulating NUR77 and NFAT activity in thymocytes and mature T cells has been demonstrated (Marangoni et al., 2013; Dolmetsch et al., 1997; Baldwin and Hogquist, 2007; Moran et al., 2011), our data suggest that TCR affinity also regulates the activity of these key transcription factors in the context of tumors. Thus, TCR signal strength drives distinct transcriptional and epigenetic programs that underlie T cell functional heterogeneity in tumors.

Tumor escape results from two distinct mechanisms in the tumor-reactive PD1hi TIL repertoire, depending on TCR signal strength: T cell dysfunction and functional inertness

Given that TST cells encountering neoantigens with low TCR signal strength preserved a cell-intrinsic functional state and the ability to produce effector cytokines ex vivo (Figs. 3 and 4), we hypothesized that these characteristics of T cell function would also correlate with enhanced anti-tumor immunity in vivo. Surprisingly, in vivo anti-tumor effector function of low-affinity TILs was no better than that of dysfunctional, exhausted TILs encountering high-affinity tumor antigens (Fig. 5 A). These results suggest that tumor escape can result from two fundamentally distinct mechanisms operating...
TCR signal strength drives distinct transcriptional and epigenetic programs in TST cells. (A) TCR-TAG cells isolated from dLN (4 d after AT) and tumors (14 d after AT) were subjected to RNA-seq; naive TCR-TAG and effector TCR-TAG cells isolated from LN 4 d after infection with a L. monocytogenes strain.
within the tumor-reactive PDIβ TIL repertoire; high TCR signal strength leads to T cell dysfunction (Figs. 3 and 4; Schietinger et al., 2016; Scott et al., 2019; Philip et al., 2017), while low TCR signal strength results in functional inertness, a state defined by lack of in vivo anti-tumor function, despite retention of an intrinsic functional state of TILs that maintains TST cell function and mediates anti-tumor activity. Given that tumor antigen affinity in patients cannot be modified and that lowering TCR affinity of cancer cells expressing OVA (B16-OVA), a model neoantigen recognized by OVA-specific OT1 CD8+ T cells (TCROT1); TCROT1 cells recognize the H-2Kb-restricted OVA peptide SIINFEKL with high affinity. Similar to the TAG system, CD8α-deficient TCROT1 significantly slowed tumor growth compared with control TCROT1 (Fig. 5 F). Thus, there is a critical range of TCR signal strength for TST cells, which preserves cell-intrinsic functional molecular programs and mediates anti-tumor activity in vivo (Fig. 5 G). Below this Goldilocks affinity range, tumor-reactive PDIβ TILs are functionally inert, while above this range, T cells enter a differentiation state associated with dysfunction and exhaustion.

Therapeutic fine-tuning of TCR signal strength empowers TST cells to eliminate tumors in vivo

Next, we asked whether there is an intermediate “Goldilocks” level of TCR affinity (between dysfunction/exhaustion and functional inertness) that maintains TST cell function and mediates anti-tumor activity. Given that tumor antigen affinity in patients cannot be modified and that lowering TCR affinity of the various clones through manipulation of complementarity-determining regions is unfeasible, we explored alternative and potentially therapeutically applicable strategies to alter TCR signal strength. The CD8αβ coreceptors increase peptide sensitivity by stabilizing the interaction between the TCR and pMHC and by recruiting LCK (Artyomov et al., 2010; Holler and Kranz, 2003); we hypothesized that genetic deletion of CD8α could decrease TCR signal strength without the need to discern or alter the sequence of the TCR itself. We generated CD8α-deficient TCRTAG cells by transducing CD8+ splenocytes of TCRTAG.Cas9 mice with either a single guide RNA (sgRNA) targeting Cd8α (Cd8α sgRNA) or a control (control sgRNA; Fig. 5 C). Indeed, CD8α-deficient TCRTAG cells had significantly lower functional avidity for N4 and F4, as measured by IFN-γ production in vitro, compared with control T cells (Fig. S5 A). CD8α-deficient TCRTAG cells were more functional, producing IFN-γ and TNF-α cells ex vivo and expressing higher levels of CD103 and lower levels of CD39 (Fig. S5 B). RNA-seq revealed that a large number of genes that were differentially expressed between T cells encountering low- versus high-affinity APL tumors were also differentially expressed between Cd8α KO and control TCRTAG cells (Fig. S5 C), and genes expressed in Cd8α KO TILs were enriched for pathways involved in T cell activation, differentiation, and effector function, similar to the TIL-D4 counterparts (Fig. S5 D).

To validate our findings in another tumor/neoantigen model, we used murine B16F10 (B16) melanoma cells expressing OVA (B16-OVA), a model neoantigen recognized by OVA-specific OT1 CD8+ T cells (TCROT1); TCROT1 cells recognize the H-2Kb-restricted OVA peptide SIINFEKL with high affinity. Similar to the TAG system, CD8α-deficient TCROT1 had significantly lower functional avidity for H-2Kb/SIINFEKL compared with control TCROT1 (Fig. 5 D and E). To investigate the anti-tumor efficacy of CD8α-deficient TCROT1 in vivo, B16-OVA cells were injected subcutaneously into wild-type C57BL/6J mice. Once tumors were established, CD8α-deficient TCROT1 or control T cells were adoptively transferred and mice were treated with PD1/PDL1 checkpoint blockade. Strikingly, we observed that CD8α-deficient TCROT1 significantly slowed tumor growth compared with control TCROT1 (Fig. 5 F). Thus, there is a critical range of TCR signal strength for TST cells, which preserves cell-intrinsic functional molecular programs and mediates anti-tumor activity in vivo (Fig. 5 G). Below this Goldilocks affinity range, tumor-reactive PDIβ TILs are functionally inert, while above this range, T cells enter a differentiation state associated with dysfunction and exhaustion.

Discussion

Our study identifies several novel insights into TST cell differentiation and dysfunction. First, we reveal that TST cell dysfunction is the composite of affinity-dependent and affinity-independent molecular programs. Surprisingly, several canonical IRs, including PD1 and LAG3, were similarly expressed on high- and low-affinity TST cells and TILs. While these IRs have been associated with T cell exhaustion, we show that they can also be associated with functional inertness and do not correlate with the intrinsic functional state of TILs. In contrast, the expression of exhaustion markers such as 2B4 and CD39 is dependent on TCR signal strength. This finding is relevant to biomarker-based research using CD39 to demarcate tumor-reactive (CD39+) from nontumor reactive, bystander (CD39−) T cells within tumors (Simoni et al., 2018); our data suggest that CD39+ TILs can be part of the tumor-reactive TIL repertoire that encounters tumor antigens with lower TCR signal strength.

Second, both high- and low-signal-strength interactions lead to failed tumor control in vivo, uncovering two opposing mechanisms within the tumor-reactive PDIβ TIL repertoire that...
Figure 5. Optimal anti-tumor efficacy requires an intermediate range of TCR signal strength. (A) Tumor outgrowth of MCA-APL tumor-bearing mice receiving AT of naive TCR\textsubscript{TAG} cells at day 14. Data show mean ± SEM of \( n = 5\)–7 mice per APL. ns, three-way ANOVA. Data are representative of two independent experiments. (B) Functional inerterness of TIL-D\textsubscript{4}. TIL-D\textsubscript{4} fail to kill D\textsubscript{4} targets but can eliminate N\textsubscript{4} targets. TIL-D\textsubscript{4} were sorted from MCA-D4 tumors 10 d after AT and incubated with MCA-D\textsubscript{4} or MCA-N\textsubscript{4} tumor cells in vitro at a 1:10 effector to target ratio. Killing of tumor cells was assessed 18 h later by flow cytometry (see Materials and methods for technical details). Each circle represents an individual mouse (\( n = 11 \)). Values are mean ± SEM. *, \( P < 0.0001 \), unpaired two-tailed Student’s \( t \) test. Data are representative of two independent experiments. (C) CRISPR-Cas9–mediated deletion of Cd8\textalpha\ in high-affinity Cas9;TCR\textsuperscript{H} T cells to partially lower TCR signal strength. (D) CD8\textalpha expression on TCR\textsubscript{OT1};Cas9 T cells transduced with Cd8\textalpha sgRNA (red) or control sgRNA (black) and reisolated at 30 d after AT into B16-OVA tumor-bearing hosts. Values are mean ± SEM. Each symbol represents an individual mouse. *, \( P < 0.01 \), unpaired two-tailed Student’s \( t \) test. Data are representative of two independent experiments. (E) Functional avidity measured as production of IFN-\( \gamma \) by TCR\textsubscript{OT1};Cas9 T cells transduced with Cd8\textalpha sgRNA (red) or control sgRNA (black) after 4-h stimulation with SIINFEKL peptide at the indicated concentrations. Data represent mean of technical replicates \( n = 2 \) and are representative of two independent experiments. (F) Lowering TCR signal strength through CRISPR-Cas9–mediated deletion of Cd8\textalpha in TCR\textsubscript{OT1} enhances anti-tumor efficacy in vivo. B16-OVA tumor outgrowth in B6 mice that received congenically marked CD8 T cells (Thy1.1/Thy1.2) from TCR\textsubscript{OT1};Cas9 mice transduced with Cd8\textalpha sgRNA (red) or control sgRNA (black) after 4-h stimulation with SIINFEKL peptide at the indicated concentrations. Data represent mean of technical replicates \( n = 2 \) and are representative of two independent experiments. (G) Summary and conclusions of the study. Phenotypic, functional, and transcriptional characteristics of TST cells encountering antigens with distinct TCR signal strength. TF, transcription factor. We propose a Goldilocks signal strength range that allows effective anti-tumor immunity in vivo. TST cells with affinity beyond this range are dysfunctional due to exhaustion (for high-affinity TST cells) or functionally inert (for T cells specific to low-affinity neoantigens and tumor/self-antigens). Affinity tuning for immunotherapeutic interventions through lowering signal strength of high-affinity TST cells or high-affinity chimeric antigen receptor T cells or signal strength enhancement of low-affinity T cells could result in increased anti-tumor effector function.
drive tumor evasion: (1) T cell dysfunction and exhaustion in response to high TCR signal strength, and (2) functional inertness in response to low TCR signal strength, characterized as lack of in vivo effector function despite effector/memory-like transcriptional and epigenetic programs. While low signal strength is sufficient to lead to activation, proliferation, and the up-regulation of certain IRs (PD1 and LAG3), it is too low to mediate in vivo cancer cell killing. Thus, molecular signatures of tumor-reactive PD1hi TILs do not necessarily correlate with or predict T cell anti-tumor efficacy in vivo. While T cell dysfunction and exhaustion has been generally considered to be the major underlying mechanism of PD1hi tumor-specific T cells permitting tumor evasion, we show that functional inertness represents another mechanism of the PD1hi TIL repertoire contributing to tumor escape.

Third, T cells within an intermediate range of TCR signal strength exhibit effective anti-tumor responses, and lowering TCR signal strength in high-affinity TST cells and/or targeting neoantigens within an intermediate range, instead of those with highest affinity, could improve T cell–based immunotherapeutic interventions. In line with this, a recent study assessing the immunogenicity of over 500 predicted neoantigen-derived peptides in lung adenocarcinoma and melanoma patients showed that the epitopes with highest affinity were nonimmunogenic, and the vast majority of the immunogenic peptides were found within an intermediate affinity range (Wells et al., 2020). In addition, another study characterizing successful T cell clones in an adoptive T cell transfer setting targeting KRAS-G12D mutations showed that high-pMHC affinity of over 500 predicted neoantigen-derived peptides in lung adenocarcinoma and melanoma patients showed that the epitopes with highest affinity were nonimmunogenic, and the vast majority of the immunogenic peptides were found within an intermediate affinity range (Wells et al., 2020). In addition, another study characterizing successful T cell clones in an adoptive T cell transfer setting targeting KRAS-G12D mutations showed that TCR–pMHC affinity inversely correlates with in vivo persistence in cancer patients. Specifically, the T cell clones with the highest TCR–pMHC affinity, which made up half of the infusion product, were undetectable after 40 d post-infusion. On the other hand, the clone with the lowest TCR–pMHC affinity that only made up 20% of the infusion product was maintained in circulation at 9 mo after infusion (Sim et al., 2020). Interestingly, in chronic viral infections, another setting of persistent antigen and TCR stimulation, high-affinity virus-specific T cells more readily enter a state of T cell exhaustion, undergo deletion, or become senescent (Probst et al., 2003; Ueno et al., 2004; Lin and Welsh, 1998; Schober et al., 2020). Together, these findings demonstrate that in settings of chronic antigen stimulation, high-affinity neoantigen-specific T cells and high-affinity virus-specific T cells more rapidly differentiate to dysfunction/exhaustion or do not persist, and that paradoxically decreasing signal strength might improve effector function in vivo. Indeed, lowering TCR signal strength through CRISPR/Cas9-mediated deletion of Cd8a improved anti-tumor immunity in vivo. Interestingly, a recent study showed that lowering the signal strength of chimeric antigen receptor T cells, which generally bind to their target tumor antigen with high affinity, led to enhanced anti-tumor effector function in vivo (Feucht et al., 2019). Thus, we propose that there is a critical Goldilocks range of TCR signal strength (Slansky and Jordan, 2010), which allows TST cells to maintain a cell-intrinsic functional molecular program while licensing T cells to execute anti-tumor effector functions in vivo (Fig. 5 G). Outside of this window, T cells are either functionally inert or dysfunctional and exhausted. Future studies into the phenotype and function of human TILs within and outside of this range are needed in order to accurately delineate the range that maximizes anti-tumor function and better guide therapeutic fine-tuning of TCR signal strength.

Materials and methods

Mice

TCR_TAG transgenic mice (B6.Cg-Tg(TcraY1,TcrbY1)416Tev/J), TCR_OTg mice (C57BL/6-Tg(TcraTcrb)1100Mjb/J). Rosa26-Cas9 mice (Gt(Rosa)26Sortm1.1(CAG-cas9*,EGFP)Fezh/J), B6 Thy1.1 mice (B6.PL-Thy1a/Cy/J), and B6 mice (C57BL/6) were purchased from the Jackson Laboratory. TCR_TAG mice and Rosa26-Cas9 mice were each crossed to Thy.1.1 mice to generate TCR_TAG Thy1.1 and Cas9 Thy1.1 mice, respectively. TCR_OTg (Thy1.2) mice were bred to Cas9 Thy1.1 mice to generate TCR_OTg;Cas9 Thy1.1/Thy1.2 progeny. TCR transgenic mice were not on a RAG-deficient background. Both female and male mice were used for experimental studies. Donor and host mice were age and sex matched; mice were 6–12 wk old. All mice were bred and maintained in the animal facility at Memorial Sloan Kettering Cancer Center (MSKCC). Experiments were performed in compliance with the MSKCC Institutional Animal Care and Use Committee regulations.

Antibodies and reagents

The native SV40 large T antigen epitope I (TAG) peptide [SAINNYAQKL (N4)] and TAG APL [SAINNFAQKL (F6) and SAIDNYAQKL (D4)] were purchased from GenScript. Fluorochrome-conjugated antibodies were purchased from BD Biosciences, eBioscience, Cell Signaling Technology, and BioLegend.

Intracellular cytokine staining and CTV labeling

Intracellular cytokine staining was performed using the Cytofix/Cytoperm Plus kit (BD Biosciences) following the manufacturer’s protocol. Briefly, T cells isolated from spleens, LNs, or tumors were mixed with 2 × 10^5 congenerically marked B6 spleenocytes and incubated with 0.5 µg/ml TAG peptide for 4 h at 37°C in the presence of GolgiPlug (BD Biosciences). After staining for cell surface molecules, cells were fixed, permeabilized, and stained with antibodies against IFN-γ (XMG1.2) and TNF-α (MP6-XT22). To assess cell proliferation in vivo, naive TCR_TAG cells were incubated with 5 µM CTV (Thermo Scientific). Excess extracellular dye was quenched with FBS, and cells were washed twice with serum-free RPMI before transfer into host mice.

Flow cytometric analysis

Flow cytometric analyses were performed using Fortessa X20. Cells were sorted using BD FACS Aria (BD Biosciences) at the MSKCC Flow Core Facility. Flow data were analyzed with FlowJo v.10 software (Tree Star).

Generation of plasmids and tumor cell lines

pMFGL-BP-EGFP pMFGL-N4-EGFP, pMFGL-F6-EGFP, and pMFGL-D4-EGFP were constructed by inserting annealed oligonucleotides encoding triple SAINNYAQKL-AAY, SAINNFAQKL-AAY, or SAIDNYAQKL-AAY repeats into the NcoI-linearized pMFG-EGFP
vector. Restriction enzymes were purchased from New England Biolabs. All constructs were verified by sequence analysis. Packaging cells (ATCC) were transfected with APL constructs; supernatants were used to transduce MCA205 cells to generate MCA205-ΔN\(_2\)-EGFP, MCA205-F\(_4\)-EGFP, and MCA205-D\(_4\)-EGFP, respectively, as previously described (Schietinger et al., 2010). Transduced bulk cell lines were sorted for similar EGFP expression levels.

pMIUR
A 1.142-kb fragment encoding turbo RFP in the 5’ to 3’ orientation and an inverted U6-gRNA-scaffold cassette in the 3’ to 5’ orientation was obtained from IDT and cloned into pMIG (Addgene; catalog no. 9044) using standard restriction enzyme–based methods. Briefly, the fragment was amplified using primers that add EcoRI and SalI restriction sites on the 5’ and 3’ regions, respectively, and subsequently verified using Sanger sequencing. The following primer sequences were used: FSR-158, 5’-GGCCAGAATCCGCACCATGTTGTCTAAGGGC-3’; FSR-153: 5’-GGCCAGTGTCCAGGCGCTATTCCCATGATT-3’; vector, EcoRI-trFP-NoBbsI-INV-U6-gRNA-BbsI-SalI cassette.

Listeria infection
The L. monocytogenes ΔactA ΔinIB strain (Brockstedt et al., 2004) expressing the TAG N\(_4\) epitope (SAINNYAQKL; TAG immunization. For AT studies of naive TCRTAG cells into MCA-APL tumor-bearing hosts, 2 × 10\(^6\) CD8\(^+\) splenocytes from cutaneously into TCROT1 mice, whose CD8\(^+\) T cell compartment in PBS, formulated at 1×10\(^8\) CFU/ml into tumor-bearing TCROT1 (Thy1.2) mice. 1 d later, mice were infected with 5×10\(^6\) MCA-N\(_4\), MCA-APL tumor-bearing mice with resultant changes in antigen load and avidity. Once tumors were established, naive TCRTAG cells were isolated from the spleens of B6 host mice at 7 d after TAG immunization; cells were sorted for a CD8\(^+\)CD90.1\(^+\)CD44hi phenotype. (3) Day 4 effector TCRTAG Thy1.1\(^+\) cells from tumor-bearing mice were isolated from the tumor-draining (inguinal) LNs; cells were stained and sorted for a CD8\(^+\)CD90.1\(^+\)CD44hi phenotype. (4) Day 10–14 TCRTAG Thy1.1\(^+\) cells from tumor-bearing mice were isolated from tumors (see above); cells were sorted for a CD8\(^+\)CD90.1\(^+\)CD44hi phenotype. Samples for RNA-seq were directly sorted into Trizol LS reagent (Invitrogen; catalog no. 10296010) and stored at −80°C. Samples for ATAC-seq were resuspended in FBS with 10% DMSO and stored at −80°C.

Calcium flux imaging
8-well chamber slides were coated with 2 μg fibronectin and incubated at 37°C for 1 h. Wells were subsequently washed twice with PBS. MCA-N\(_4\) tumor cells were seeded at 30,000 cells per well in complete DMEM (5% FBS and 100 U/ml penicillin/streptomycin) and incubated at 37°C overnight. The following day, TCRTAG cells were sorted from MCA-APL tumors by FACS and loaded with Fura2-AM (Invitrogen; catalog no. F1221) at 4 μM for 30 min. T cells were then washed and resuspended in phenol red–free complete RPMI (5% FBS and 100 U/ml penicillin/streptomycin) and incubated for an additional 30 min to allow full de-esterification of the intracellular esters. Microplates were washed with phenol red–free complete RPMI and T cells were added to the well immediately before the start of imaging. Imaging was performed with a 20× objective lens (Olympus) using 340-nm and 380-nm excitation every 30 s for 30 min. Emission in the GFP channel was also acquired to localize tumor cells.

Image analysis
Using SlideBook image analysis software (https://www.intelligent-imaging.com/slidebook), the ratio of 340-nm to 380-nm excitation for each cell in the field of view was measured.
calculated. Responses of all cells were aligned based on the initial time of influx and then averaged across the dataset.

**ERK activation assay**

96-well plates were seeded with 100,000 of MCA-N4 tumor cells or parental MCA205 cells as control 4–5 h before the start of the experiment. T cells isolated from tumors (see above) were allowed to rest in prewarmed complete RPMI at 37°C for 1 h. 100,000 T cells were added to each well and incubated at 37°C for 20 min. Ice-cold 4% PFA was then added for a final concentration of 1.6% PFA, and cells were fixed on ice for 10 min. Cells were then spun down and permeabilized with ice-cold 90% MeOH for 20 min on ice. Plates were spun down and washed with FACS buffer (PBS with 3% FBS) and stained for 30 min at room temperature with anti-phospho-p44/42 MAPK (Cell Signaling Technology; clone E10), BV650-conjugated anti-CD8α (BioLegend; clone 53–6.7), and FITC-conjugated anti-Thy1.1 (eBioscience; clone HIS51). Primary antibodies were then washed off before the addition of Alexa Fluor 647-conjugated goat anti-mouse IgG1 (Invitrogen). Cells were incubated for 30 min at room temperature before being washed and analyzed by flow cytometry.

**RMA-S MHC class I binding assay**

TAP-deficient RMA-S cells were incubated with a 1-nM to 1-µM concentration of N4, F6, or D4 peptides for 2 h at 37°C. Cells were then washed and stained for surface expression of H-2Dβ.

**RNA-seq**

RNA from sorted cells was extracted using the RNeasy Mini Kit (Qiagen; catalog no. 74104) according to instructions provided by the manufacturer. After RiboGreen quantification and quality control by an Agilent BioAnalyzer, total RNA underwent amplification using the SMART-Seq v4 Ultra Low Input RNA Kit (Clontech), with 12 cycles of amplification for 2 ng input. Subsequently, 10 ng amplified cDNA was used to prepare libraries with the KAPA Hyper Prep Kit (Kapa Biosystems) using eight cycles of PCR. Samples were barcoded and run on a HiSeq 2500 or HiSeq 4000 in a 50-bp/50-bp paired-end run with the HiSeq SBS Kit v4 (Illumina). An average of 60 million paired reads were generated per sample.

**ATAC-seq**

Profiling of chromatin was performed by ATAC-seq as previously described (Buenrostro et al., 2013). Briefly, 38,000–64,000 viably frozen T cells were incubated in cold PBS and lysed. The transposition reaction was incubated at 42°C for 45 min. The DNA was cleaned with the MinElute PCR Purification Kit (Qiagen; catalog no. 28004), and material was amplified for five cycles. After evaluation by real-time PCR, 7–13 additional PCR cycles were done. The final product was cleaned by AMPure XP beads (Beckman Coulter, catalog no. A63882) at a 1× ratio, and size selection was performed at a 0.5× ratio. Libraries were sequenced on a HiSeq 2500 or HiSeq 4000 in a 50-bp/50-bp paired-end run using the TruSeq SBS Kit v4, HiSeq Rapid SBS Kit v2, or HiSeq 3000/4000 SBS Kit (Illumina). An average of 60 million paired reads were generated per sample.

**Bioinformatics methods**

The quality of the sequenced reads was assessed with FastQC and QoRTs (for RNA-seq samples; Hartley and Mullikin, 2015; Andrews, 2010). Unless stated otherwise, plots involving high-throughput sequencing data were created using R version 3.4.1 (see https://github.com/abcwcm/Shakiba2020 for the code; R Core Team, 2017) and ggplot2 (Wickham, 2016).

**RNA-seq data**

DNA sequencing reads were aligned with default parameters to the mouse reference genome (GRCm38) using STAR (Dobin et al., 2013). Gene expression estimates were obtained with featureCounts using composite gene models (union of the exons of all transcript isoforms per gene) from Gencode (version M16; Liao et al., 2014).

**DEGs**

Based on similarity in phenotype, function (Figs. 2 and 3), and transcriptional profile (Fig. 4 A) of TIL-N4 and TIL-F6, they were treated as replicates for downstream DEG analysis. DEGs were determined with DESeq2 with a q-value cutoff of 0.1.

**Pathway and GO term enrichment analyses**

Gene set enrichment analyses were done using GSEA (Subramanian et al., 2005) on reads per kilobase million values (the seed was set to 149).

GO analysis was performed on up- and down-regulated DEGs using the goseq R package (Young et al., 2010). Only GO categories overenriched using a 0.05 false discovery rate cutoff were considered.

**Heatmaps**

Heatmaps in Fig. 4 C were created using log2-transformed read counts per million of genes identified as differentially expressed by DESeq2. Rows were centered and scaled.

**ATAC-seq data**

**Alignment and identification of open chromatin regions**

The data were processed following the recommendations of the ENCODE consortium (https://www.encodeproject.org/atac-seq/). Reads were aligned to the mouse reference genome (version GRCm38) with BWA-backtrack (Li and Durbin, 2009). Post-alignment filtering was done with samtools and Picard tools (Li et al., 2009) to remove unmapped reads, improperly paired reads, nonunique reads, and duplicates. To identify regions of open chromatin represented by enrichments of reads, peak calling was performed with MACS2 (Liu, 2014). For every replicate, the narrowpeak results of MACS2 were used after filtering for adjusted P values smaller than 0.01. Filtered peaks were annotated using the ChiPseeker package in R (Yu et al., 2015).

**Differentially accessible regions**

Regions where the chromatin accessibility changed between different conditions were identified with DiffBind, with the following options: minOverlap = 5, bUseSummarizeOverlaps = T, minMembers = 3, and bFullLibrarySize = TRUE. 16,264
differentially accessible peaks were identified between high- and low-affinity TST cells. A q-value cutoff of <0.05 was used.

**GO term enrichment analyses**
Enrichment of GO terms was calculated on hyper- or hypo-accessible peaks separately using GREAT v3 (Genomic Regions Enrichment of Annotations Tool) with default parameters (McLean et al., 2010). The consensus peakset identified by DiffBind was used as the background set.

**Coverage files**
Individual coverage files per replicate normalized for differences in sequencing depths between the different samples were generated with bamCoverage of the deepTools suite using the following parameters: -bs 10-normalizeUsing RPGE-effective GenomeSize 2652783500-blackListFile Name mm10.blacklist-ignoreForNormalization chrX chrY-ignoreDuplicates-minFragmentLength 40 -p 1. To create merged coverage files of replicates of the same condition, we used multiBigwigSummary to obtain the sequencing-depth-normalized coverage values for 10-bp bins along the entire genome; i.e., for every condition, we obtained a table with the coverage values in every replicate within the same bin. Subsequently, we chose the mean value for every bin to represent the coverage in the resulting “merged” file (see https://github.com/abcwcm/Shakiba2020 for the actual code that was used). Merged coverage files were used for display in Integrated Genomics Viewer shown in Fig. S3 G.

**Heatmaps**
Heatmaps based on the differentially accessible peaks identified between high- and low-affinity TST cells as shown in Fig. 4 G were created using the dba.plotHeatmap function from the DiffBind package.

**Motif analyses**
To identify motifs enriched in regions with changes of chromatin accessibility, we ran v-4.9.1 (Heinz et al., 2010) with the following flags: -size given -mask. HOMER was run separately on hyper- or hypo-accessible peaks. The consensus peakset identified by DiffBind was used as the background set.

**Combining RNA-seq and ATAC-seq data**
The relationship between RNA-seq and ATAC-seq was explored via “diamond” plots for genes enriched for select transcription factor motifs. Each gene was represented by a stack of diamond-shaped points colored by that gene’s associated chromatin state (blue indicating closing and red indicating opening). The bottom-most point in each stack corresponds to the log2 fold change in expression of that gene.

**Cloning Cd8a-targeting sgRNA into pMIUR**
To target Cd8a locus, three sgRNA were designed using the Broad Institute GFP sgRNA Designer tool (Doench et al., 2016): sgRNA-1 (5’-TGGGTGAGTGTTATCTGTT-3’), sgRNA-2 (5’-ATC CCACAAACAGATAACG-3’), and sgRNA-3 (5’-GACCTGAAGCA GTTGAC-3’). A sgRNA targeting a safe harbor on chromosome 8 was used as control (5’-GACATTCTTTCCACTGG-3’).

sgRNAs were cloned using standard restriction enzyme-based cloning strategies. Briefly, pMIUR was digested with BbsI-HF (New England Biolabs). Annealed and complementary sgRNA pairs were ligated to the linearized backbone. All constructs were sequence verified by Sanger sequencing.

**Anti-tumor efficacy study with Cd8a KO T cells**
For the TAG model, TCROTI mice were injected subcutaneously with 2 × 106 MCA-F6 tumor cells. For the OVA model, C57BL/6J mice were injected subcutaneously with 1 × 106 B16-OVA tumor cells. The sgRNAs targeting the Cd8a locus (Cd8a sgRNA) or a safe harbor on chromosome 8 (control sgRNA) were delivered to TCRTAG;Cas9 Thy1.1/1.2 or TCRQT;Cas9 Thy1.1/1.2 spleenocytes using retroviral transduction. Briefly, Platinum-E cells (ATCC) were transfected with each construct using the Mirus TransIT-LT1 reagent (catalog no. 2305). Viral supernatant was supplemented with polybrene and added to spleenocytes, and the cells were transduced via spinfection on two consecutive days. For spleenocytes transduced with Cd8a sgRNA, live CD4+CD90.1+RFP+CD8+ cells were FACS sorted 48 h after transduction for AT. For those transduced with control sgRNA, live CD4+CD90.1+RFP+CD8+ cells were FACS sorted. C57BL/6J tumor-bearing mice were treated with cyclophosphamide (250 mg/kg) 1 d before AT, and 230,000 T cells were transferred i.v. into each mouse (~14 d after tumor implantation). Mice were treated with anti-PD1 and anti-PDL1 (200 µg each, per mouse) starting day 4 after AT and every other day thereafter. Tumors were measured manually with a caliper. Tumor volume was estimated with the formula (length × width × height)/2.

**Data reporting**
No statistical methods were used to predetermine sample size. The investigators were not blinded to allocation during experiments and outcome assessment. Mice were excluded if donor or tumor-infiltrating T cells could not be found.

**Statistical analyses**
Statistical analyses on flow cytometric data were performed using unpaired two-tailed Student’s t tests (Prism 7.0, GraphPad Software). A P value of < 0.05 was considered statistically significant.

**Online supplemental material**
Fig. S1 shows MHC class I expression levels and growth kinetics of MCA-APL tumors, as well as phenotypic characteristics of tumor-specific T cells in MCA-APL tumors. Fig. S2 shows transcriptional features of tumor-specific T cells. Fig. S3 shows chromatin accessibility changes of MCA-APL tumor-infiltrating T cells. Fig. S4 shows gains and losses of regulatory elements for DEG containing NUR77- or NFAT2-binding motifs. Fig. S5 shows functional, phenotypic, and transcriptional differences between Cd8a-deficient and control T cells.

**Data availability**
The RNA-seq and ATAC-seq data have been deposited in the Gene Expression Omnibus, SuperSeries GSE141818, including GSE141816 (ATAC-seq data) and GSE141817 (RNA-seq data). All
data generated and supporting the findings of the study are available within the paper. Additional information and materials will be made available upon request.

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Figure S1. **MCA205 APL tumor model.** (A) Flow cytometric analysis of MHC class I (H-2Db) expression of MCA-APL cell lines. Histograms are gated on APL-EGFP-expressing MCA205 cancer cells. Data are representative of two independent experiments. Inset numbers show MFI. (B) Tumor outgrowth of MCA-APL cell lines in TCROT1 hosts. Data show mean ± SEM of \( n = 5–8 \) mice per APL; ns, three-way ANOVA. (C–E) Phenotypic characterization of TIL-TCR\textsubscript{TAG} cells isolated from MCA-APL tumors. Flow cytometric analysis of CD44, CD62L (C), CD3\varepsilon (D), and TCR expression levels through tetramer staining (E) of TIL-TCR\textsubscript{TAG} cells isolated 14 d after AT into MCA-APL tumor-bearing hosts. Each symbol represents an individual mouse. NA, naive TCR\textsubscript{TAG} control, tetramer-negative (Tet\textsuperscript{−}) endogenous CD8\textsuperscript{+} T cells; Eff, TCR\textsubscript{TAG} effector CD8 T cells at the peak of response 5 d after *Listeria* (Lm\textsubscript{TAG}) infection. Data are representative of two independent experiments. Data show mean ± SEM; ns, unpaired two-tailed Student’s t test.
Figure S2. **TCR affinity drives distinct molecular programs of tumor-specific T cells.** (A) mRNA expression levels of select genes in TCR\textsubscript{TAG} isolated from the dLN (day 4 after AT) and tumors (TILs; day 10–14 after AT) from high-affinity (blue) or low-affinity (green) MCA-APL tumor-bearing mice. Expression in naive (NA) TCR\textsubscript{TAG} cells is shown as control. (B) Enrichment of gene sets described for tumor-specific T cell dysfunction (from Philip et al., 2017; left), and T cell exhaustion during chronic viral infection (from West et al., 2011 [GSE30962]; right) in TIL-Lo. NES, normalized enrichment score. (C) Venn diagrams showing the degree of overlap between genes up- or down-regulated in TCR\textsubscript{TAG} isolated from dLN or tumors (TILs). Examples of genes in each category are shown.
Figure S3. Chromatin accessibility changes in response to varying TCR signal strength in TST cells. (A and B) Quality control plots for ATAC-seq samples. (A) Fragment length distribution plots (base pairs on x axis and read count on y axis). (B) Number of aligned reads per sample showing the number remaining after mapping and removing duplicated and nonmitochondrial reads. The ENCODE guideline is indicated by the horizontal line. (C) Number of chromatin accessibility changes in TIL encountering high- versus low-affinity tumor antigen. (D) Pie chart showing the proportion of reproducible ATAC-seq peaks in exonic, intronic, intergenic, and promoter regions. (E) Correlation heatmap of peaks that are differentially accessible (false discovery rate <0.05) between TIL-F6 versus TIL-D4. (F) Selected GO terms enriched in peaks opened (red) or closed (blue) in response to high-affinity TCR stimulation in TILs. (G) ATAC-seq signal profiles of loci of affinity-dependent genes, including Tox, Tcf7, Cd244, and Itgae, and affinity-independent genes, including Cd69, Ctls4, and Havcr2. Vertical bars at the bottom of each plot represent regions with statistically significant changes in accessibility in TIL-F6 versus TIL-D4.
Figure S4. Transcription factor binding motif analysis for peaks with differential accessibility based on TCR signal strength. (A and B) Gains and losses of regulatory elements for the most DEGs containing NUR77 (A) or NFAT (B) binding motifs. Plots are divided into top and bottom genes with the highest and lowest respective log₂ fold change (FC) of gene expression (shown on the y axis). Each gene is illustrated by a stack of diamonds representing peaks gained (red) or lost (blue) in high-affinity TILs.
Figure S5. **Lowering TCR signal strength of high-affinity TCRTag enhances anti-tumor effector function.** (A) Functional avidity measured as production of IFN-γ by TCRTagCas9 CD8 T cells deficient of CD8a (transduced with CD8a sgRNA) after 4-h stimulation with N4 (red) or F6 (blue) peptides at the indicated concentrations. EC50 of control CD8α-sufficient TCRTag (transduced with control sgRNA) encountering each APL is shown with asterisks. Data represent mean of technical replicates (n = 2) and two independent experiments. (B) TCRTag cells were transduced with either CD8α-targeting or control sgRNA to generate CD8α-deficient (blue) or CD8α-sufficient control (black) TCRTag cells. CD8α-deficient or control TCRTag cells were injected into MCA-F6 tumor-bearing mice. Ex vivo cytokine production and expression levels of CD103 and CD39 of TCRTag cells isolated from tumors were assessed 10–11 d after transfer. MCA-F6 tumor cells were used in this experiment, because the EC50 of CD8α-deficient T cells to F6 is between that of the CD8α-sufficient control to D4 and F6. Each dot represents an individual mouse. Values are mean ± SEM. Significance is calculated by Student’s t-test. *, P < 0.05; **, P < 0.01; ***, P < 0.001. (C) Hierarchical clustering of genes differentially expressed (log2 fold change >1) both in CD8α-deficient TCRTag TILs versus control cells and in low-affinity TILs (TCRTag cells isolated from MCA205-D4; TIL-Lo) versus high-affinity TILs (TCRTag cells isolated from MCA205-N4 and MCA205-F6 tumors; TIL-Hi). Selected genes within each cluster are shown. (D) Selected GO terms enriched in genes up-regulated in CD8α-deficient TCRTag TILs.