Memory T cell–driven differentiation of naive cells impairs adoptive immunotherapy

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Adoptive cell transfer (ACT) of purified naive, stem cell memory, and central memory T cell subsets results in superior persistence and antitumor immunity compared with ACT of populations containing more-differentiated effector memory and effector T cells. Despite a clear advantage of the less-differentiated populations, the majority of ACT trials utilize unfractionated T cell subsets. Here, we have challenged the notion that the mere presence of less-differentiated T cells in starting populations used to generate therapeutic T cells is sufficient to convey their desirable attributes. Using both mouse and human cells, we identified a T cell–T cell interaction whereby antigen-experienced subsets directly promote the phenotypic, functional, and metabolic differentiation of naive T cells. This process led to the loss of less-differentiated T cell subsets and resulted in impaired cellular persistence and tumor regression in mouse models following ACT. The T memory–induced conversion of naive T cells was mediated by a nonapoptotic Fas signal, resulting in Akt-driven cellular differentiation. Thus, induction of Fas signaling enhanced T cell differentiation and impaired antitumor immunity, while Fas signaling blockade preserved the antitumor efficacy of naive cells within mixed populations. These findings reveal that T cell subsets can synchronize their differentiation state in a process similar to quorum sensing in unicellular organisms and suggest that disruption of this quorum-like behavior among T cells has potential to enhance T cell–based immunotherapies.

Introduction

Adoptive cell transfer (ACT), the ex vivo expansion and reinfusion of antigen-specific (Ag-specific) T cells, represents a potentially curative treatment for patients with advanced cancer (1–4) and viral-reactivation syndromes (1, 5, 6). Recent progress in the ability to genetically redirect patient-derived peripheral blood T cells toward tumor and viral-associated antigens by modification with a T cell receptor (TCR) or chimeric antigen receptor (CAR) has greatly simplified the generation of therapeutic T cells (7–10). Given the clinical efficacy of T cell therapy combined with the ability of T cells to be manufactured according to standardized procedures, ACT is now poised to enter mainstream clinical practice. However, fundamental questions remain regarding the optimal source, expansion, and quality of therapeutic T cells used for transfer.

In mice, ACT of naive CD8+ T cell–derived cells (T N-derived cells) exhibits a superior capacity to expand, persist, and treat cancer compared with normalized numbers of memory T cell–derived cells (TMem cells) (11, 12). Preclinical human studies have confirmed that T N-derived cells maintain higher levels of the costimulatory marker CD27 and the lymphoid homing markers CD62L and CCR7; they also retain longer telomeres (12–15). Each of these parameters has correlated with the likelihood that patients will obtain an objective clinical response following ACT (15–17). Despite these findings, the majority of current T cell therapy clinical trials do not specifically enrich for defined T cell subsets, but rather utilize unfractionated T cell populations (2). As T N cells are in the circulation of most cancer patients (13, 18), the following question arises: is the presence of T N cells in the initial population used to generate therapeutic T cells sufficient to convey their desirable attributes, or is physical separation of T N cells from antigen-experienced subsets required to unleash the full therapeutic potential of T N-derived cells (19, 20)? Prior investigations revealed that T N cells form homotypic clusters during T cell priming that can influence their subsequent maturation (21, 22). However, whether antigen-experienced populations directly interact with and influence naive cell differentiation is unknown.

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Using human and mouse T cells, we describe here a previously unrecognized T cell–T cell interaction whereby TMem cells directly influence T N cell differentiation during priming. This process, which we term precocious differentiation, synchronizes the behavior of T N-derived cells with TMem cells, resulting in accelerated functional, transcriptional, and metabolic differentiation of T N cell progeny. Precocious differentiation was cell-dose, contact, and activation dependent. Mechanistically, the phenomenon was mediated by nonapoptotic Fas signaling, resulting in activation of Akt and ribosomal S6 protein (S6), kinases responsible for cellular differentiation and metabolism (23). Consequently, induction of Fas signaling in the absence of TMem cells enhanced differentiation and impaired antitumor immunity, while isolation of T N cells prior to priming or blockade of Fas signaling prevented TMem cell-induced precocious differentiation and preserved the antitumor efficacy of T N-derived cells. Collectively, our results reveal that unleashing the therapeutic potential of T N-derived cells for adoptive immunotherapy necessitates disruption of intercellular communication with TMem cells, a finding with direct implications for the design and execution of ACT clinical trials.

Results

TMem augment naive cell phenotypic maturation during ex vivo priming. We sought to determine whether antigen-experienced CD8+ T cells influence the differentiation of T N-derived progeny. To indelibly track the fate of T N cells, we primed congenically distinguishable Thy1.1+ pmel-1 CD8+ T cells (CD44loCD62L+), which recognize an epitope derived from the melanoma-associated Ag gp100 (24), alone or in a 1:1 mixture with Ly5.1+ TMem cells. To generate TMem cells, we adoptively transferred Ly5.1+ pmel-1 T cells into WT Ly5.2+ hosts and vaccinated recipient mice with a gp100-encoding recombinant vaccinia virus (rVV-gp100) to generate Ag-experienced CD8+ T cells in vivo. Twenty-eight days later, Ly5.1+CD8+ T central memory cell (T CM cell; CD44hiCD62L+) and T effector memory cell (T EM cell; CD44hiCD62L−) subsets from vaccinated mice were isolated by FACS sorting (Figure 1A). Naive pmel-1 T cells were subsequently expanded alone or in the presence of TCM or TEM cells using anti-CD3/CD28 antibodies and IL-2, which is similar to many current human ACT protocols (15, 25, 26).

Following expansion, the progeny of isolated T N cells had differentiated into all 3 antigen-experienced subsets, including T stem cell memory cells (TSCM cells; CD44loCD62L−CD122+Sca-1+), TCM cells, and TEM cells (refs. 27, 28, Figure 1B, and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI81217DS1). Strikingly, we found that the presence of TMem cells during T N cell priming caused a significant accumulation of TEM cells and an attrition of TSCM cells (Figure 1C). Both TCM and TEM cell subsets were equally capable of augmenting the phenotypic maturation of T N-derived progeny, demonstrating that this prodifferentiation capacity was a property of TMem cell subsets in general. These findings were not attributable to differences in cell expansion, were not TCR specific, and occurred regardless of growth and cytokine conditions tested (Supplemental Figures 2 and 3). Augmented T N cell differentiation was also observed when a CD4+-containing bulk TMem cell population was used, although the magnitude of change was less than that observed using CD8+ TMem cells (Supplemental Figure 4).

As the frequency of circulating TMem cells to T N cells can vary widely in patients (18, 29, 30), we evaluated the dose dependency of TMem cell-induced phenotypic maturation of T N cells. We activated T N cells alone or in titrated ratios with a bulk (CD44+) TMem cell population. Similar to results using fractionated TCM and TEM cell subsets, we found that priming T N cells in a 1:1 mixture with bulk TMem cells caused an increase in the TEM cell population and a corresponding loss in TSCM and TCM cells (Figure 1, D and E). As the ratio of TMem to T N cells was increased, we measured a progressive enhancement in the differentiation of T N-derived progeny. We conclude that the presence of TMem cells during T N cell priming ex vivo caused dose-dependent phenotypic maturation of T N-derived cells.

TMem cause precocious differentiation of naive cells. Having established that TMem cells augment the phenotypic maturation of T N cells during priming, we next evaluated whether TMem cells influence gene transcription and effector functions of T N-derived progeny. We again primed Ly5.1+ T N pmel-1 cells alone or in a 1:1 ratio with bulk in vitro-differentiated Ly5.2+ TMem cells and confirmed that the progeny of T N cells expanded with TMem cells (T N-derived, mix) were significantly skewed toward T N cells and away from TSCM cells compared with T N cells primed alone (T N-derived, alone) (Figure 2A and B). To determine whether CD62L loss on T N-derived cells was attributable to genetic downregulation rather than proteolytic cleavage, we reisolated Ly5.1+ T N-derived cells following expansion by FACS sorting and performed quantitative PCR (qPCR). We found that expression of selectin L (SelL), the gene encoding CD62L as well as other T cell–associated lymphoid-homing and costimulatory markers, including Ccr7 and Cd27, was significantly downregulated in T N-derived cells expanded with TMem cells relative to T N cells expanded alone (Figure 2C). Additionally, we found that T N-derived cells primed with TMem cells were more functionally differentiated, as evidenced by increased granzyme B content (Figure 2D) and enhanced IFN-γ secretion following stimulation with either PMA/ionomycin or hpg10025–33 peptide (Figure 2E and F).

To globally assess the influence of TMem cells on the differentiation of T N-derived progeny, we performed gene-expression analyses of reisolated cells at rest and at serial time points following activation. Hierarchical clustering demonstrated that within 18 hours, T N cells primed with TMem cells had a gene-expression profile more closely related to TMem cells than to T N cells (Figure 2G). We found that 139 and 259 transcripts were differentially expressed (fold change [FC] > 2, positive FDR [pFDR] < 0.05) between T N cells primed alone or with TMem cells at 18 hours and 96 hours, respectively (Supplemental Tables 1 and 2). In contrast, only 0 and 10 transcripts were differentially expressed by these criteria in TMem cells expanded alone or with T N cells at these time points (Supplemental Table 3), indicating that the predominant outcome of this T cell–T cell interaction was TMem cell–induced changes in T N cells. Examination of specific genes revealed that key naive and memory-associated transcription factors (TFs), including transcription factor 7 (Tcf7) (27), Kruppel-like factor 2 (Klf2) (31), and forkhead box O1 (Foxo1) (32), were significantly downregulated in T N cells primed with TMem cells compared with T N cells primed alone (Figure 2H). Conversely, genes encoding effector-associated TFs and molecules, such as T-box 21 (Tbx21) (33), PR domain containing 1,
ing T₈ cells priming ex vivo caused enhanced phenotypic, functional, and transcriptional differentiation of T₈-derived cells relative to T₈ cells primed alone, resulting in impaired antitumor efficacy. Henceforth, we will refer to this process as precocious differentiation.

**Figure 1.** TMem cells augment naive cell phenotypic maturation during priming. (A) Experimental design and representative FACS plot showing the generation and isolation of vaccine-induced pmel-1 Ly5.1+CD8+ TCM (CD44+CD62L+) and TEM (CD44+CD62L−) cell subsets by adoptive transfer of pmel-1 cells (10⁵) into Ly5.2+ WT hosts followed by rVV-gp100 vaccination (2 × 10⁷ pfu). (B) Representative FACS plots and (C) summary bar graphs demonstrating the distribution of Thy1.1+ T₈-derived TSCM (CD44+CD62L+Sca1+CD122+) and TEM cell subsets 6 days following ex vivo expansion alone or in the presence of a 1:1 mixture with Ly5.2+ T₈ or TMem cells using CD3/CD28-specific antibodies and IL-2. (D) Representative FACS plots and (E) summary graph demonstrating the distribution of Thy1+ T₈-derived subsets 6 days following ex vivo expansion alone or in the presence of titrated ratios with Ly5.1+ bulk (CD44+) TMem cells using CD3/CD28-specific antibodies and IL-2. Results in **A-E** are shown after gating on liveCD8+Thy1.1+ lymphocytes and are representative of 2 independently performed experiments. Results in **C** and **E** are presented as mean ± SEM with n = 3 per condition. Statistical comparisons performed using an unpaired 2-tailed Student’s t test corrected for multiple comparisons by a Bonferroni adjustment. ***P < 0.001.

with ZNF domain (**Prdm1**) (34), and granzyme B (**Gzmb**) were differentially overexpressed in T₈ cells primed with TMem cells (Figure 2I). Collectively, these data revealed that TMem cells globally altered the differentiation program of T₈ cells.

Finally, to assess the impact of TMem cell-induced differentiation of T₈-derived cells on antitumor efficacy, we adoptively transferred T₈ cells primed alone or with TMem cells into mice bearing 10-day established s.c. B16 melanomas. We found that the expansion, persistence, and antitumor efficacy of T₈-derived cells were all significantly impaired in mice receiving T₈-derived cells primed with TMem cells compared with T₈-derived cells primed alone (P = 0.0009) (Figure 2J and K). We conclude that the presence of TMem cells during T₂ cell priming ex vivo caused enhanced phenotypic, functional, and transcriptional differentiation of T₈-derived cells relative to T₂ cells primed alone, resulting in impaired antitumor efficacy. Henceforth, we will refer to this process as precocious differentiation.

**TMem cells cause precocious differentiation of T₈ cells in vivo.** Having established that TMem augment naive cell differentiation during ex vivo expansion, we next asked whether TMem cells also influence T₈ cell differentiation and antitumor efficacy in vivo after adoptive cotransfer. In prior studies, naive cell expansion and differentiation were impaired when limited numbers (5 × 10⁶) of T₂ cells were transferred into hosts containing a 100- to 1,000-fold greater frequency of TMem cells (35). Under these conditions, T₂ cells are...
Figure 2. T<sub>Mem</sub> cause precocious differentiation of naive cells. (A) Representative FACS and (B) bar graph summarizing the distribution of Ly5.1<sup>+</sup>CD8<sup>+</sup> T<sub>n</sub>-derived cell subsets 6 days following priming with CD3/CD28-specific antibodies and IL-2 alone or with Ly5.2<sup>+</sup>CD8<sup>+</sup> T<sub>Mem</sub> cells. Data shown after gating on Ly5.1<sup>+</sup>CD8<sup>+</sup> cells. (C) qPCR analysis of Sell, Ccr7, and Cd27 expression in FACS-sorted reisolated T<sub>n</sub> cells primed alone, T<sub>n</sub> cells primed with T<sub>Mem</sub> cells, or T<sub>Mem</sub> cells primed alone. (D) Granzyme B and (E) IFN-γ intracellular staining in T<sub>n</sub>-derived cells stimulated with PMA/ionomycin following expansion alone or with T<sub>Mem</sub> cells. (F) IFN-γ ELISA of supernatants from reisolated T<sub>n</sub>-derived cells expanded alone or with T<sub>Mem</sub> cells 6 days prior to overnight stimulation with hgp100<sub>25-33</sub> peptide. (G) Heat maps of differentially expressed genes (1-way ANOVA, pFDR < 5%) among T<sub>n</sub>-derived cells expanded alone or with T<sub>Mem</sub> cells at 18 and 96 hours. (H) RMA-normalized intensity of selected T<sub>n</sub>-associated genes. (I) Expression of effector cell-associated factors assessed by qPCR at 96 hours from FACS reisolated T<sub>n</sub>-derived progeny expanded with or without T<sub>Mem</sub> cells or T<sub>Mem</sub> cells expanded alone. (J) In vivo expansion and (K) tumor regression following i.v. adoptive transfer of T<sub>n</sub>-derived progeny expanded alone or with T<sub>Mem</sub> cells, or T<sub>Mem</sub> cells grown alone in combination with 6 Gy irradiation, i.v. rVV-hgp100, and 3 days of i.p. IL-2. n = 3 independently maintained cultures/condition or time point for experiments shown in B, C, and F–I. K was performed with n = 5 mice per group. All results shown as mean ± SEM. Statistical comparisons performed using an unpaired 2-tailed Student’s t test corrected for multiple comparisons by Bonferroni adjustment. *P < 0.05; **P < 0.01. Data are representative of 16 (A and B), 3 (D and E), and 2 (C, F, and I–K) independent experiments.
pared with TN cells alone (Figure 3D). We conclude that TMem cells can cause precocious differentiation and impaired antitumor efficacy of TN cell–derived progeny in vivo following adoptive cotransfer. FasL-Fas interactions mediate precocious differentiation. We next sought to elucidate what TMem cell factor or factors caused precocious differentiation. We determined the phenomenon was, in addition to being cell-dose dependent, also cell-contact dependent and activation dependent and could not be reproduced using supernatant transfers from restimulated TMem cells (Supplemental Figure 6). Accordingly, we hypothesized that an activation-induced, cell-surface molecule on TMem cells that can mediate costimulatory-like effects was responsible. Several members of the TNF superfamily satisfy these criteria (37). Therefore, we interrogated our microarray analysis comparing gene expression in TMem cells and TN cells for TNF superfamily members uniquely overexpressed in activated TMem cells to generate a list of candidate ligands. Among the 19 known TNF superfamily ligands (37), only Fasl met criteria (log2 FC > 2, P < 0.01) for being differentially expressed in restimulated TMem cells (Figure 4A and Supplemental Table 4). To determine whether TCM and TEM cells are similarly poised to express FasL following activation, we performed ChIP-seq analysis to assess histone H3 methylation dynamics at the Fasl locus within resting TCM, TEM, and TMem cell subsets. Consistent with previous reports (38), we found that TMem cells exhibited strong depo-
sition of the repressive epigenetic modification trimethylation of histone 3 at lysine 27 (H3K27me3) and minimal deposition of the activating H3K4me3 mark (Figure 4B). In contrast, both TCM and TEm cells acquired permissive H3K4me3 marks and lost all detectable H3K27me3 repressive marks. Correlated with these changes, mRNA expression of Fasl was significantly increased in contemporaneously evaluated TCM and TEM cells compared with TN cells (Figure 4C). Notably, there were no significant differences in Fas gene expression between TCM and TEM cell subsets. We confirmed that TMem cells but not TN cells are poised to express surface FasL at a protein level by performing FACS analysis for this molecule and its corresponding receptor, Fas, on both cell types at rest and 18 hours after activation (Figure 4, D and E).

FasL-Fas interactions can induce apoptosis through Fas-mediated activation of caspase-8 (39). However, Fas signaling can also perform nonapoptotic functions in a variety of tissues, including promotion of T cell costimulation (40), hepatocyte regeneration (41), tumor growth and invasiveness (42), and neuronal differentiation (43). To determine whether FasL can mediate precocious differentiation, we primed Ly5.1+ TN cells alone or in a 1:1 mixture with Ly5.2+ TMem cells in the presence of a blocking anti-FasL antibody (Figure 4C). Notably, there were no significant differences in Fas gene expression between TMem and TEM cell subsets. We confirmed that TMem cells but not TN cells are poised to express surface FasL at a protein level by performing FACS analysis for this molecule and its corresponding receptor, Fas, on both cell types at rest and 18 hours after activation (Figure 4, D and E).

As antibody blockade may be incomplete, we complimented these findings by genetic means using CD8+ TN cells derived from Fas-deficient lpr mice (44). Whereas WT TN cells underwent augmented differentiation when primed with TMem cells, TN cells from lpr mice were completely protected from this phenomenon (Figure 5, A–C). Consistent with the apoptosis-inducing function of FasL, the concentration of lz-FasL was titrated up (Supplemental Figure 7). We found that lz-FasL caused a dose-dependent increase in the differentiation of TMem cell–derived progeny, as evidenced by an increased frequency of TEM cells and enhanced IFN-γ release upon restimulation (Figure 6, A and B). Moreover, we observed commensurate changes in the expression of key differentiation-associated genes, including Sell, Il7ra, Klf2, transferrin receptor (Tfrc), and Gzmb, as the concentration of lz-FasL was titrated up (Supplemental Figure 8). Consistent with the apoptosis-inducing function of FasL, exposure to lz-FasL resulted in reduced cell yields, particularly at concentrations greater than 33 ng/ml (Figure 6C). Despite this effect, we measured a significant increase in the relative numbers of TEM cells versus TN cells 18 hours after stimulation with CD3/CD28-specific antibodies and IL-2. Dashed lines, P < 0.01 and FC > 2. (B) Pattern of activating H3K4me3 and repressive H3K27me3 epigenetic marks within the promoter and gene body of Fasl and (C) RMA-normalized expression intensity of Fasl in resting FACS-sorted TMem, TCM, and TEM cell subsets. (D and E) Fas and FasL surface expression on TN and TMem cells at rest or 18 hours after stimulation with CD3/CD28-specific antibodies. All bar graphs shown as mean ± SEM with n = 3 per indicated cell type or condition. Statistical comparisons performed using an unpaired 2-tailed Student’s t test corrected for multiple comparisons by a Bonferroni adjustment. ***P < 0.001. Data shown in C–E are representative of 2 independently performed experiments.
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significantly protected from apoptosis induction similarly to

that skewing toward the TEM cell phenotype could not be account-

ed for a 1:1 mixture with WT Ly5.1+ TMem. All results shown as mean ± SEM with

human TMem cell subsets (45). Collectively, these data suggested

a finding consistent with published studies of Fas expression on

expression between memory subsets (Supplemental Figure 10),

the distribution of CD8+ T cell subsets 6 days following priming of Ly5.2+ WT TN pmel-1 (TN

CD3/CD28-specific antibodies, IL-2, and a blocking antibody against FasL (\(E\) FACS analysis and (\(\alpha\)D) bar graph summarizing

death of TEM cells was not attributable to differences in surface Fas

augmented differentiation of recently activated TN cells.

We next sought to determine whether Fas-mediated cellular
differentiation and cell death could be uncoupled using a genetic approach. Previous work established that Fas-induced apopto-
sis requires posttranslational palmitoylation of a cysteine residue

located within the proximal cytoplasmic region of the receptor

(46). Based on these data, a transgenic mouse expressing

the FasC194V variant was generated, backcrossed to the

ground (henceforth, FasC194V lpr), and used to derive CD8+ T

cells (Figure 6E). However, unlike cells from the lpr mouse, which

were also protected from lz-FasL-mediated precocious differentia-
tion, FasC194V\(^{\text{lpr/}lpr}\) CD8+ T cells underwent enhanced conversion to a T\(\text{EM}\) cell phenotype similar to that of WT cells following prim-
ing with 40 ng/ml lz-FasL (Figure 6, F and G). We conclude that a

nonapoptotic Fas signal delivered by Fasl is sufficient to induce the augmented differentiation of recently activated T\(\alpha\) cells.

Figure 5. FasL-Fas interactions mediate precocious differentiation. (A) Representative FACS plots, (B) summary bar graph, and (C) scatter plot demonstrating T cell subset frequencies or percentage of IFN-\(\gamma\)/CD8+ T cells 6 days following priming of Ly5.1+ T cells alone or in a 1:1 mixture with Ly5.2+ TMem with CD3/CD28-specific antibodies, IL-2, and a blocking antibody against FasL (\(\alpha\)Fasl) or isotype (IgG) control. (D) FACS analysis and (E) bar graph summarizing

precocious differentiation is associated with induction of Akt signaling. We next sought to determine what signals downstream of Fas contribute to precocious differentiation. Multiple signal transduction pathways have been implicated in nonapoptotic Fas signaling (39), including the phosphatidyl 3-kinase/protein kinase B (also known as Akt) pathway (42, 43). Given the established role of Akt in CD8+ T cell effector differentiation (48), we focused our attention on the induction of phospho-Akt (pAkt) and phospho-ribosomal protein S6 (pS6), a kinase downstream of Akt signaling in T cells (23). Compared with resting T\(\alpha\) cells, naive cells activated for 24 hours with CD3/CD28-specific antibodies exhibited augmented staining with phospho-specific antibod-
ies against both the T308 and S473 activation residues on Akt as well as pS6 (Figure 7, A–C). Importantly, when Ly5.2+ T\(\alpha\) cells were primed in the presence of congenically distinguishable Ly5.1+ TMem cells, we observed a significant increase in the staining intensity of pAkt T308, pAkt S473, and pS6 in T\(\alpha\)-gated cells. To establish whether increased pAkt content in T\(\alpha\) cells primed with TMem cells leads to commensurate changes in gene expression, we returned to our microarray analyses. Within 18 hours of T\(\alpha\) cell activation in the presence of TMem cells, we observed significant alterations in the expression of known targets of Akt signaling, including \(Il7r\) (49), sphingosine-1-phosphate receptor 1 (S1pr1) (50), Tfrc (51), hexokinase-2 (Hk2) (52), and solute carrier family 2 (Slc2a1, also known as Glut1) (ref. 53 and Figure 7D). Based on these data, we next tested to determine whether provision of lz-Fasl to activat-
ed T\(\alpha\) cells was sufficient to augment pAkt in the absence of TMem

precocious differentiation was associated with induction of Akt signaling, we next sought to determine what signals downstream of Fas contribute to precocious differentiation. Multiple signal transduction pathways have been implicated in nonapoptotic Fas signaling (39), including the phosphatidyl 3-kinase/protein kinase B (also known as Akt) pathway (42, 43). Given the established role of Akt in CD8+ T cell effector differentiation (48), we focused our attention on the induction of phospho-Akt (pAkt) and phospho-ribosomal protein S6 (pS6), a kinase downstream of Akt signaling in T cells (23). Compared with resting T\(\alpha\) cells, naive cells activated for 24 hours with CD3/CD28-specific antibodies exhibited augmented staining with phospho-specific antibodies against both the T308 and S473 activation residues on Akt as well as pS6 (Figure 7, A–C). Importantly, when Ly5.2+ T\(\alpha\) cells were primed in the presence of congenically distinguishable Ly5.1+ TMem cells, we observed a significant increase in the staining intensity of pAkt T308, pAkt S473, and pS6 in T\(\alpha\)-gated cells. To establish whether increased pAkt content in T\(\alpha\) cells primed with TMem cells leads to commensurate changes in gene expression, we returned to our microarray analyses. Within 18 hours of T\(\alpha\) cell activation in the presence of TMem cells, we observed significant alterations in the expression of known targets of Akt signaling, including \(Il7r\) (49), sphingosine-1-phosphate receptor 1 (S1pr1) (50), Tfrc (51), hexokinase-2 (Hk2) (52), and solute carrier family 2 (Slc2a1, also known as Glut1) (ref. 53 and Figure 7D). Based on these data, we next tested to determine whether provision of lz-Fasl to activate T\(\alpha\) cells was sufficient to augment pAkt in the absence of TMem
Figure 6. Precocious differentiation is mediated by nonapoptotic Fas signaling. (A) Representative FACS plots and (B) bar graph summarizing the distribution of T cell subsets and IFN-γ production in isolated Tn cells primed with CD3/CD28-specific antibodies, IL-2, and indicated doses of Iz-FasL for 6 days prior to analysis. Data shown are based on n = 3 independently maintained cultures/condition. (C) Relative overall cell yield and (D) relative cell yield of Tn or IFN-γ+ cells, normalized to no Iz-FasL treatment, of Tn-derived cells grown in the presence of titrated amounts of Iz-FasL (C) or 33 ng/ml Iz-FasL (D); data shown in C and D are based on n = 3–6 and n = 3 independently maintained cultures/condition, respectively. (E) Specific cell death of activated CD8+ T cells derived from WT (WT/WT), lpr (lpr/lpr), and FasC194V lpr/lpr mice following exposure to titrated amounts of Iz-FasL or a vehicle control; n = 11 per condition/cell type. (F) Representative FACS plots demonstrating the frequency of Tn-derived cell subsets and (G) bar graph demonstrating fold increase in Tn phenotype cells after WT/WT, lpr/lpr, or FasC194V lpr/lpr CD8+ T cells were expanded with CD3/CD28-specific antibodies and IL-2 alone or with 40 ng/ml of Iz-FasL for 6 days; n = 6–9 independently maintained cultures/cell type. Statistical comparisons performed using an unpaired 2-tailed Student’s t test corrected for multiple comparisons by a Bonferroni adjustment. *P < 0.05; **P < 0.01. Results are representative of 6 (A), 2 (C–E), and 4 (F and G) independently performed experiments and are displayed as mean ± SEM.
Figure 7. Precocious differentiation is associated with augmented Akt signaling. Representative FACS histograms and summary bar graphs demonstrating the mean fluorescence intensity of (A) pAkt T308, (B) pAkt S473, and (C) pS6 in Ly5.2+CD8+ Tn cells at rest or 24 hours after stimulation with CD3/CD28-specific antibodies alone or in a 1:1 mixture with Ly5.1+ Tmem cells. Data shown after gating on live-Ly5.2+CD8+ cells. (D) RMA-normalized intensity showing expression of Il7ra, Stpr1, Tfr, Hk2, and Slc2a1 in resting Tn cells, Tn cells primed alone, and Tn cells primed in a 1:1 mixture with Tmem cells. (E) Western blot and densitometry analysis of pAkt T308 and GAPDH in resting Tn or Tn cells primed for 24 hours with CD3/CD28-specific antibodies and titrated doses of Iz-Fasl. (F) Representative FACS plot and (G) summary scatter plot showing 2-NBDG expression in Tn cells primed alone for 6 days with CD3/CD28-specific antibodies, IL-2, and Iz-Fasl (50 ng/ml) or vehicle control. Data shown after gating on live-CD8+ lymphocytes. (H) Representative FACS plots, (I) summary bar graph demonstrating the frequency of CD8+ T cell subsets, and (J) scatter plots showing IFN-γ production in Tn cells primed with CD3/CD28-specific antibodies, IL-2, and Iz-Fasl (50 ng/ml) in the presence or absence of an inhibitor of Akt1/2 (AktI) for 6 days. Statistical comparisons performed using an unpaired 2-tailed Student’s t test corrected for multiple comparisons by a Bonferroni adjustment. *P < 0.05; **P < 0.01; ***P < 0.001. All data shown are displayed as mean ± SEM. Experiments performed with n = 3 per condition/time point in A–D and F–J. All data shown are representative of 2 independently conducted experiments.
We found that AktI significantly limited IZ-Fasl–mediated precocious differentiation, as evidenced by a reduced accumulation of TEM cell phenotype and IFN-γ+ cells (Figure 7, H–J). We conclude that TEM cell and IZ-Fasl–induced precocious differentiation of TN cells is associated with augmented Akt pathway activation. Pharmacologic inhibition of Akt can partially block the precocious differentiation phenotype.

Human TEM cells induce precocious differentiation of TN-derived cells. To evaluate the relevance of precocious differentiation for human ACT trials, we next tested to determine whether human TEM cells influence the differentiation of TN cells during ex vivo cell expansion. First, we established the frequency of TEM cells compared with TN cells in the circulation of healthy donors (HD) or patients with metastatic melanoma (Mel) and diffuse large B cell lymphoma (DLBCL), 2 malignancies where ex vivo-expanded...
adoptively transferred T cells have entered clinical trials (56–58). We found that the TN cell to TM cell ratio was 1 or greater in the majority of cases, with a median value of 1.5, 2.3, and 18.8 in HD, Mel patients, and DLBCL patients, respectively (Figure 8A). The increased ratio in DLBCL patients was likely due to the influence of prior lymphodepleting chemotherapy (30). To track the fate of human TN cell–derived progeny in mixed cultures, we isolated TN (CD8+CD45RA+CD45RO–CCR7+) and TM (CD8+CD45RA–CD45RO+) cells from the same donor and labeled these subsets with alternative fluorescent membrane dyes (Figure 8B and Supplemental Figure 11A). Labeled T cells were expanded alone or together in titrated ratios using CD3/CD28-specific antibodies and IL-2. Although this approach did not allow for indelible cell tracking, we found we could reliably distinguish each population for up to 6 days. Four days following activation, TN cells and TM cells lost membrane dye intensity, indicating both subsets had undergone cell division. To determine whether human TM cells expanded at the same rate in the presence or absence of memory cells, we calculated the proliferation index (PI) of TM-derived cells primed alone or with different ratios of TM cells. We discovered that human TM cells caused a dose-dependent increase in TN cell proliferation (Supplemental Figure 11B). To ascertain whether TM cells influence TN cell differentiation independently of cell proliferation, we gated on TN-derived cells that had undergone an equivalent number of cell divisions (Figure 8C). We then evaluated the coordinate expression of naive-associated phenotypic markers (CD27, CCR7, CD45RA) by flow cytometry using a Boolean gating strategy. We found across all donors tested that, when normalized for division history, there was a significant dose-dependent loss of TN cells (CD27+CCR7+CD45RA+) as the ratio of TM cells was increased (P = 0.0015) (Figure 8D). Similar conclusions were reached regardless of the generation number evaluated (data not shown). We conclude that human TM cells augmented both the proliferation and differentiation of TN-derived cells. Further, we conclude that the rate of differentiation is enhanced even when division history is normalized.

In mice, we discovered that TM cell–induced precocious differentiation of TN cells was mediated by FasL. To determine whether enhanced Fas signaling augmented the differentiation of human CD8+ T cells, we primed peripheral blood CD8+ T cells from HDs with CD3/CD28-specific antibodies and IL-2 alone or in the presence of titrated concentrations of FasL. After 9 to 10 days, we found that all expanded cells had assumed a CD45RAlo/–CD45RO+ memory phenotype (data not shown). Using coexpression of CD27 and CCR7 (29), we characterized the ratio of TM cells (CD8+CD45RA+CCR7+CD27+) to TEM cells (CD8+CD45RO–CCR7–CD27–) as TN and TM cells stimulated with titrated concentrations of FasL for 12 hours. Cells that coordinate cytokine staining for 7-AAD and annexin V were considered apoptotic. Statistical comparisons performed using an unpaired 2-tailed Student’s t test corrected for multiple comparisons by a Bonferroni adjustment. *P < 0.05; **P < 0.01.
more-differentiated TEM cell subset and an attrition of T CM cells caused a significant and dose-dependent accumulation of the found that augmenting Fas signaling using lz-FasL in HD cells accelerated differentiation when primed in the presence of T Mem
CD27–). Consistent with experiments using mouse T cells, we both our mouse data and previously reported data in human CD4+

To exclude that the observed changes in human CD8+ T cell subset composition were due to selective T CM cell killing, we evaluated the induction of apoptosis (as measured by coordinate 7-AAD and annexin V staining) in T CM and T EM cell subsets following stimulation with titrated amounts of lz-FasL (Figure 9, C and D). Similar to both our mouse data and previously reported data in human CD4+ T cells (45), we found that TEM cells were far more sensitive to Fas–induced apoptosis than T CM cells alone with lz-FasL (50 ng/ml), vehicle control, or with T Mem and either aFasL or IgG. Pooled results from 2 independently performed experiments displayed using n = 4–10 mice per condition. Statistical comparisons performed using an unpaired 2-tailed Student’s t test or log-rank test for animal survival. *P < 0.05. Data shown are representative of 2 independent experiments with results displayed as mean ± SEM.

Fas signaling controls T cell differentiation and antitumor efficacy. T Mem–induced precocious differentiation of T N-derived cells resulted in impaired antitumor efficacy in mice. Given our findings that precocious differentiation is mediated by FasL-Fas interaction, we next tested to determine whether modulation of Fas signaling could influence the therapeutic potential of adoptively transferred T N-derived cells. We therefore primed pmel-1 T N cells in the presence of congenically distinguishable T Mem cells in combination with aFasL or IgG control. In addition, we also primed T N cells alone with lz-FasL or vehicle control. Following expansion, equal numbers of viable CD8+ T cells obtained using a density separation media for each culture condition were adoptively transferred into sublethally irradiated mice bearing 10-day established B16 melanomas in combination with rVV-gp100 and IL-2. We found that FasL blockade during T N cell expansion in the presence of T Mem Cells significantly improved tumor regression (P = 0.009) and animal survival (P < 0.05), nearly rescuing the in vivo antitumor potential of isolated T N cells (Figure 10, A and B). In contrast, FasL blockade had no impact on the antitumor efficacy of isolated T N cells expanded without T Mem cells (Supplemental Figure 13). Conversely, augmenting Fas signaling using lz-FasL during T N cell priming significantly impaired tumor regression (P < 0.01) and animal survival (P < 0.05). The differences in treatment efficacy of lz-FasL primed T N cells were not attributable to impaired cell engraftment, as a similar frequency of cells was recovered 18 hours after transfer (Figure 10C). Overall, we found a significant inverse linear correlation between the state of T cell differentiation, as
measured by CD62L expression on transferred T cells primed with FasL blockade or augmentation, and tumor growth ($R^2 = 0.797$, $P < 0.0001$; Figure 10D). These data suggest that FasL-mediated perturbations in T cell differentiation were highly correlated with the antitumor treatment efficacy of transferred CD8$^+$ T cells. We conclude that the negative influence of T$_{mem}$ cell–induced precocious differentiation on the treatment efficacy of adoptively transferred T$_{naive}$-derived cells in mice is mediated by a FasL-Fas interaction that can be rescued with FasL blockade during cell expansion.

Discussion

Herein, we present evidence using both human and mouse cells that antigen–experience CD8$^+$ T cells directly influence naive cell differentiation both during cell expansion ex vivo and following adoptive cotransfer in vivo. This process, which we have termed precocious differentiation, serves to synchronize the functional, transcriptional, and metabolic state of T$_{naive}$-derived progeny with that of T$_{mem}$ cells. Consequently, precocious differentiation leads to an attrition of the highly potent T$_{SCM}$ and T$_{CM}$ populations and an accumulation of T$_{EM}$ cells, resulting in the impaired proliferation, persistence, and antitumor treatment efficacy of adoptively transferred T cells.

In multiple preclinical models, minimally differentiated T$_{naive}$, T$_{SCM}$, and T$_{CM}$ cell subsets exhibit superior persistence (12, 18, 27, 59–62) and enhanced antitumor (18, 27, 59), antibacterial (62), and antiviral responses following ACT compared with the highly differentiated T$_{EM}$ cell and effector T cell (T$_{naive}$) populations. Despite these data, the vast majority of current ACT trials utilize unfractoned T cell populations (2, 20). Largely, this is based on an assumption that having at least some representation of the less-differentiated subsets in the starting population used to generate therapeutic T cells is sufficient to convey their desirable attributes. Our data directly challenge this notion by demonstrating that T$_{naive}$ cells must either be isolated from T$_{mem}$ cells prior to cell expansion or have their capacity to respond to T$_{mem}$ cell–mediated signaling disrupted to preserve their full therapeutic potential. Accomplishing these goals at a clinical scale might be done using several strategies. These include isolating defined T cell subsets from peripheral blood using serial positive enrichments with reversible Fab streptamers (63), employing a combination of negative and positive magnetic bead isolations (64), and antagonizing Fas or Akt signaling (55). Based on the data in this manuscript, we have initiated a human clinical trial registered at ClinicalTrials.gov (NCT02062359) in which TN cells are enriched relative to T$_{mem}$ cells by a semipermeable membrane pretreatment of naïve CD62L-expressing cells prior to gene engineering with a TCR recognizing the cancer-germline antigen NY-ESO-1. Results from this and other planned clinical trials will determine whether ACT of naive-experienced CD8$^+$ T cells directly influence naive cell differentiation both during cell expansion ex vivo and following adoptive cotransfer in vivo. This process, which we have termed precocious differentiation, serves to synchronize the functional, transcriptional, and metabolic state of T$_{naive}$-derived progeny with that of T$_{mem}$ cells. Consequently, precocious differentiation leads to an attrition of the highly potent T$_{SCM}$ and T$_{CM}$ populations and an accumulation of T$_{EM}$ cells, resulting in the impaired proliferation, persistence, and antitumor treatment efficacy of adoptively transferred T cells.

We determined that the capacity to induce precocious differentiation was a generalized property of antigen–experience T cells, regardless of whether they were T$_{CM}$ or T$_{EM}$ cells. These findings were associated with a permissive epigenetic signature at the Fasl locus in both T$_{CM}$ and T$_{EM}$ cell subsets relative to T$_{naive}$ cells. Further, we resolved that precocious differentiation required both cell activation and direct cell contact between T cell subsets to occur. Separation of T$_{N}$ and T$_{mem}$ cells by a semipermeable membrane prevented the phenomenon and transfer of T$_{mem}$ cell–derived supernatant to T$_{N}$ cells could not reproduce the effect. Recent multiphoton microscopy studies have revealed that T$_{N}$ and T$_{mem}$ cells form stable clusters together in vivo around antigen-bearing targets (67, 68). Additionally, while lymphoid-trafficking of the CD62L$^+$ T$_{EM}$/T$_{naive}$ cell subsets is limited in the steady state, inflamed lymph nodes become permissive to entry of these cells (69). Thus, there is evidence that T$_{N}$ and T$_{mem}$ cells can physically interact with one another in vivo. Whether such T$_{N}$ and T$_{mem}$ cell interactions might also influence the response to intracellular pathogens or vaccines under certain conditions is the subject of future work.

A prior study evaluated the influence of preexisting T cell memory on the recruitment and differentiation of T$_{N}$ cells in vivo (35). Unlike current ACT clinical protocols in which large numbers of T cells are infused in an attempt to gain control over tumor replication, this study evaluated the transfer of a limiting number of T$_{n}$ cells (500 cells) in hosts containing 100- to 1,000-fold greater numbers of T$_{mem}$ cells. Under these conditions, T$_{mem}$ cells numeri-
cally outcompeted T<sub>n</sub> cells for access to limited antigen and cytokines, thus curtailing the differentiation of T<sub>n</sub> cells. However, how T<sub>mem</sub> cells influence T<sub>n</sub> cell differentiation when cells are coin fused in the relatively large numbers and ratios typically used in adoptive immunotherapies has previously not been addressed. Consistent with our ex vivo cell expansion data, we found that cotransfer of T<sub>mem</sub> cells caused accelerated T<sub>n</sub> cell differentiation into a terminally differentiated KLRG1<sup>+</sup>CD27<sup>lo</sup> phenotype despite an attenuated proliferative burst. The decreased expansion of antitumor T<sub>n</sub> cells primed with T<sub>mem</sub> cells in vivo likely reflected their accelerated conversion to antigen-experienced subsets that are known to have a reduced proliferative capacity relative to T<sub>n</sub> cells following poxvirus vaccination (70). Most importantly, cotransfer of T<sub>mem</sub> cells impaired the full in vivo antitumor efficacy of T<sub>n</sub> cells. The magnitude of accelerated T<sub>n</sub> cell differentiation caused by T<sub>mem</sub> cells was remarkably sensitive to the ratio of the 2 subsets such that an exponential relationship existed between the extent to which T<sub>n</sub>-derived progeny entered the T<sub>cm</sub>/T<sub>eff</sub> pool and the starting proportion of T<sub>mem</sub> to T<sub>n</sub> cells. In this manner, more-differentiated T cell subsets have the capacity to synchronize their phenotype, function, and gene expression with less-differentiated subsets. Some unicellular organisms exhibit collective decision making through cell-cell communication once a threshold concentration of members detect an environmental stress (71). This process, termed quorum sensing, allows individual members of a community to exhibit collectivist behaviors in order to coordinate expression of energetically expensive processes involved in virulence and differentiation (71). While quorum sensing has been theorized to be relevant to understanding lymphocyte behavior (72,73), our data add to growing experimental data demonstrating that, under certain circumstances, T cells can also exhibit collectivist behaviors (74,75).

In conclusion, we describe what we believe to be a previously unrecognized T cell-T cell interaction whereby antigen-experienced CD8<sup>+</sup> T cells drive T<sub>n</sub> cell differentiation during priming through a contact-dependent mechanism involving a nonapoptotic Fas-FasL interaction. The net influence of this process synchronizes T<sub>n</sub> cell behavior with that of T<sub>mem</sub> cells, affecting the functional, transcriptional, and metabolic differentiation of T<sub>n</sub>-derived progeny. For adoptive immunotherapies, where there is a strong inverse correlation between T cell differentiation and antitumor efficacy, the implications of our findings are clear: T<sub>n</sub> cells cause significantly enhanced differentiation of T<sub>n</sub> cells, impairing in vivo antitumor efficacy. Therefore, strategies that disrupt quorum-like behavior among T cell subsets might provide a new means of enhancing the effectiveness of T cell-based immunotherapies.

**Methods**

**Mouse strains and animal studies.** Adult (6 to 12 weeks old) female C57BL/6 (B6; Ly5.2<sup>+</sup>), B6.SJL-Ptprc<sup>−/−</sup> Pep3b<sup>−/−</sup>BoyJ (Ly5.1<sup>+</sup>), B6.PL-Thy1<sup>−/−</sup>/C57BL6/J, B6.MRL-Fas<sup>−/−</sup>/J (lpr) (44), B6.129S7-Rag<sup>−/−</sup> (Rag<sup>−/−</sup>) (Rag), B6.Cg-Thy1<sup>−/−</sup>/Cy Tg(TcraTcrb)8Rest/J (pmel-1) (24), and B6-Tg(TcraTcrb)1100Mbj/J (OT-1) (76) CD8<sup>+</sup> TCR transgenic mice were all purchased from the Jackson Laboratory. Transgenic mice harboring the Fas C194V mutant receptor were generated via BAC containing the Fas locus, with the C194V generated by recombineering. These mice were backcrossed to lpr mice on a B6 background. Fas-C194V<sup>lpr/lpr</sup> mice were backcrossed to homozygosity for both the lpr Fas allele and the C194V Fas transgene. Where indicated, pmel-1 and OT-1 T cells were crossed to Thy1.1, Ly5.1, Rag, or Rag<sup>−/−</sup> backgrounds. All mice were maintained under specific pathogen-free conditions.

**Evaluation of vaccine-induced and antitumor immunity.** Adult female B6 mice were implanted by s.c. injection with 4 × 10<sup>5</sup> B16 (H-2D<sup>b</sup>) cells, a spontaneous gp100<sup>+</sup> murine melanoma cell line obtained from the NCI tumor repository. Ten days later, tumor-bearing mice received 6 Gy total body irradiation. Treated mice received i.v. injection of indicated doses and subsets of pmel-1 CD8<sup>+</sup> T cells in combination with 2 × 10<sup>5</sup> pfu of a previously described recombinant vaccinia virus (rVV-gp100) (24) (rVV-gp100) and 12 μg IL-2 (Prometheus) administered twice daily by i.p. injection for a total of 6 doses. All tumor measurements were performed in a blinded fashion.

**Antibodies and flow cytometry.** Mouse cells were stained with fluorochrome-conjugated antibodies against combinations of the following surface and intracellular antigens after Fc receptor blockade (2.4G2): CD8α (clone 53-6.7), CD27 (clone LG.3A10), CD44 (clone IM7), CD45.1 (clone A20), CD45.2 (clone 104), CD62L (clone MEL-14), CD90.1 (clone OX-7), CD90.2 (clone 53-2.1), CD95 (clone Jo2), CD122, (clone TM-β1), CD178 (clone MFL3), IFN-γ (clone XMG1.2), KLRG-1 (clone 2F1), and Sca-1 (clone D7) (all purchased from BD Biosciences). Fluorochrome conjugates against pS235/236 (D57.2.2E), pAkt T308 (C31E5E), and pAkt S473 (D9E) were obtained from Cell Signaling Technologies. Human cells were stained with combinations of the following fluorochrome-conjugated antibodies: CCR7 (150503), CD28 (CD28.2), CD45RO (UCHL1), CD8 (SKI1) (BD Biosciences) or CD27 (M-T271), CD45RA (H100), and CD62L (DREG-56) (BioLegend). Stimulation of T cells for intracellular cytokine staining was accomplished using Leukocyte Activation Cocktail containing phorbol myristate acetate and ionomycin in combination with brefeldin A and monensin solution (BD Biosciences). Apoptosis and specific cell death in defined T cell subsets was assessed using fluorochrome-conjugated annexin V and 7-AAD (both from BD Biosciences), as previously described (45). Where indicated, T<sub>n</sub>-derived and T<sub>mem</sub>-derived subsets were reisolated for additional analyses by FACS sorting to greater than 92% purity using FACS sorting or magnetic bead isolation. Cell viability was determined using PI exclusion in FACS-sorting experiments or fixable live/dead cells (Invitrogen) for diagnostic experiments. For FACS-based glucose uptake assays, we incubated CD8<sup>+</sup> T cells with 100 μM 2-NBDG (Invitrogen) for 2 hours before measuring by FACS, as previously described (54). Flow cytometric data were acquired using either BD FACS Canto II, LSR, or LSRFortessa cytometers (BD Biosciences). FACS data, including calculation of PI, were analyzed with FlowJo Version 9.7 software (TreeStar).

**Isolation and generation of mouse and human CD8<sup>+</sup> T cell subsets.** Mouse T<sub>n</sub> cells were isolated either by using a MACS CD8<sup>+</sup> negative selection kit (Miltenyi Biotech) in combination with a 1:300 dilution of biotin-conjugated anti-CD44 antibody or by FACS sorting CD44<sup>+/-</sup>CD62L<sup>−</sup>CD8<sup>+</sup> T cells using FACS Aria (BD Biosciences). Mouse T<sub>mem</sub> cells were generated by in vitro differentiation (as previously described) or by adoptive transfer of congenic distinguishably Thy1.1<sup>+</sup> or Thy1.2<sup>+</sup> pmel-1 CD8<sup>+</sup> T cells into Ly5.1<sup>−</sup> WT mice, where recipient mice were vaccinated with rVV-gp100 (2 × 10<sup>5</sup> PFU) and vaccine-induced T<sub>mem</sub> (CD44<sup>+</sup>CD62L<sup>−</sup>) or T<sub>mem</sub> (CD44<sup>−</sup>CD62L<sup>+</sup>) adaptively transferred subsets were isolated by FACS sorting more than 28 days later (27,77). Human T cells were obtained either by leukapheresis or
venipuncture and prepared over Ficoll-Hypaque gradient (LSM; ICN Biomedicals Inc.). Human T<sub>n</sub> cells and T<sub>Mem</sub> cells were obtained by magnetic bead isolation using the EasySep Human Naive CD8<sup>+</sup> T Cell and Memory CD8<sup>+</sup> T Cell Enrichment Kits, respectively (STEMCELL Technologies). Fate tracking of T<sub>n</sub> and T<sub>Mem</sub> cells was accomplished by labeling cells with Cell Proliferation Dye eFluor 450 and Cell Proliferation Dye eFluor 670, respectively (eBioscience).

**Activation and expansion of CD8<sup>+</sup> T cells.** Both mouse and human T<sub>n</sub> cells with or without T<sub>Mem</sub> cells were activated and expanded at indicated ratios in 96-well round-bottom plates coated with 2 μg/ml of CD3-specific and 1 μg/ml of soluble CD28-specific antibodies (clones 145-2C11 and 3751; BD Biosciences) in culture media containing 5 ng/ml (mouse) or 20 ng/ml (human) of IL-2 at a final density of 1 × 10<sup>5</sup> cells/well. Where indicated, T<sub>n</sub> cells were cultured with specified concentrations of lz-FasL<sub>s</sub>, a previously described recombinant oligomerized form of Fasl (45), or 10 μg/ml of either a blocking antibody against FasL (MFL3; BD Biosciences) or IgG (A19-3; BioLegend). In some experiments, cells were also cultured with 1 μg/ml of Akt Inhibitor VIII (Calbiochem) dissolved in DMSO (Sigma-Aldrich). Where indicated, T<sub>n</sub>-derived and T<sub>Mem</sub>-derived subsets were reisolated for additional analyses by FACS sorting to more than 95% purity using FACS sorting or magnetic bead isolation. Transwell experiments were conducted in 24-well plates using 0.4 μm inserts (Corning Costar) with T<sub>n</sub> cells alone or in combination with T<sub>Mem</sub> cells either in the bottom well or in the Transwell insert.

**Cytokine release assays.** We pulsed B6 splenocytes with indicated concentrations of hgp100<sub>25-33</sub> peptide and incubated cells with T<sub>n</sub>-derived pmel-1 CD8<sup>+</sup> T cells at a 1:1 ratio overnight at 37°C. Supernatants were analyzed for mouse IFN-γ by ELISA (R&D Systems).

**qPCR and Western blot.** For qPCR analysis, CD8<sup>+</sup> T cell subsets were FACs sorted directly into RNAprotect Cell Reagent, and RNA was extracted using the QiShredders and RNeasy Mini Kits (all from QiAGEN). cDNA was synthesized using the High Capacity RNA-to-cDNA kit (Applied Biosystems), and qPCR was performed on an ABI 7500 Fast Instrument (Applied Biosystems). Gene expression was quantified using probes targeting Ccr7, Cd27, Gzmb, Il7ra, Klf2, Sell, Thbx21, Tf7, Tfrc, and Prdm1 (Applied Biosystems). For Western blot analysis, CD8<sup>+</sup> T cells were sorted into FCS and subsequently lysed in protease inhibitor containing RIPA buffer (Cell Signaling Technology). Protein was quantified using the Bio-Rad protein assay. We separated 30 μg of total protein on a 4% to 12% SDS-PAGE gel followed by standard immunoblotting with antibodies to pAktT308 (C31E5E; Cell Signaling Technology), GAPDH (AB2302; EMD Millipore), and horseradish peroxidase-conjugated goat antibodies to mouse and rabbit IgG (sc-2005 and sc-2004; Santa Cruz Biotechnology Inc.). Blots were developed using chemiluminescence (Thermo Fisher Scientific) and acquired using the ChemiDoc system (Bio-Rad Laboratories).

**Microarray analyses and ChIP-seq.** Gene-expression levels were determined using GeneChip Mouse Gene 1.0 ST arrays (Affymetrix) according to the manufacturer’s protocol. 300 ng of total RNA was used as starting material for cRNA amplification using the WT Expression Kit (Life Technologies) according to the manufacturer’s protocol. cDNA was reverse transcribed, fragmented, labeled using the GeneChip WT Terminal Labeling Kit (Affymetrix), and hybridized on the arrays for 18 hours according to the manufacturer’s directions. Arrays were stained and washed in the Fluidics Station 400 (Affymetrix) and scanned (Affymetrix 7G). Array data were imported into the Partek Genomic Suite using robust multichip analysis (RMA) normalization after background subtraction. One-way ANOVA was used to identify differentially expressed genes among the 4 T cell subtypes with a significant cut-off of pFDR < 0.01. Differentially expressed probe sets were selected using a FDR cut-off of 0.01 without specifying a FC criterion. ChIP-seq assays were performed as previously described (55). Briefly, 2 × 10<sup>7</sup> T cell subsets isolated by FACS sorting were treated with MNase to generate approximately 20% dinucleosomes and 80% mononucleosomes. Antibodies against H3K4me3 (ab8580; Abcam) and H3K27me3 (07-449; Upstate) were used for immunoprecipitation. The ChIP DNA fragments were blunt ended, ligated to Solexa adaptors, and sequenced with the Illumina 1G Genome Analyzer with mapping to the mouse genome (build mm10) using Bowtie software. All original microarray data were deposited in the NCBI’s Gene Expression Omnibus (GEO GSE56344, GSE67825, and GSE67881).

**Statistics.** The products of perpendicular tumor diameters were plotted as the mean ± SEM for each data point, and tumor treatment graphs were compared by using the Wilcoxon rank sum test and analysis of animal survival assessed using a log-rank test. Regression analysis of the slope of tumor regression as a function of CD62L<sup>+</sup> cells among Fas-modulated CD8<sup>+</sup> T cells was performed as described previously (78). For all other experiments, data were compared using either an unpaired 2-tailed Student’s t test corrected for multiple comparisons by a Bonferroni adjustment or repeated measures 1-way ANOVA, as indicated. In all cases, P values of less than 0.05 were considered significant. Statistics were calculated using Prism 5 GraphPad software (GraphPad Software Inc.).

**Study approval.** Animal experiments were conducted with the approval of the NCI and NIAMS Animal Use and Care Committees. All anonymous NIH Blood Bank donors and cancer patients providing human samples were enrolled in clinical trials approved by the NIH Clinical Center and NCI institutional review boards. Each patient signed an informed consent form and received a patient information form prior to participation.

**Author contributions**

CAK, CDS, AJL, TNY, ACC, CO, MR, RR, YJ, RLE, MS, JGC, DCP, ZAB, DC, ZY, PM, AG, SKT, SP, HL, RMS, and NPR designed and/or performed experiments. CAK, CDS, TNY, MR, ACC, FMM, and EW analyzed data. JGC, LG, SAR, and RMS edited the manuscript. CAK and NPR wrote the manuscript.

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