

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.

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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 (T_{Mem} 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.

Using human and mouse T cells, we describe here a previously unrecognized T cell–T cell interaction whereby T_{Mem} 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 T_{Mem} 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 T_{Mem} cells enhanced differentiation and impaired antitumor immunity, while isolation of T_{N} cells prior to priming or blockade of Fas signaling prevented T_{Mem} 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 T_{Mem} cells, a finding with direct implications for the design and execution of ACT clinical trials.

Results

T_{Mem} 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_{N} cells ($CD44^{\text{lo}}CD62L^+$), which recognize an epitope derived from the melanoma-associated Ag gp100 (24), alone or in a 1:1 mixture with $Ly5.1^+$ T_{Mem} cells. To generate T_{Mem} 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; $CD44^{\text{hi}}CD62L^+$) and T effector memory cell (T_{EM} cell; $CD44^{\text{hi}}CD62L^-$) subsets from vaccinated mice were isolated by FACS sorting (Figure 1A). Naive pmel-1 cells were subsequently expanded alone or in the presence of T_{CM} or T_{EM} 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 (T_{SCM} cells; $CD44^{\text{lo}}CD62L^+CD122^+Sca-1^+$), T_{CM} cells, and T_{EM} 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 T_{Mem} cells during T_{N} cell priming caused a significant accumulation of T_{EM} cells and an attrition of T_{SCM} cells (Figure 1C). Both T_{CM} and T_{EM} cell subsets were equally capable of augmenting the phenotypic maturation of T_{N} -derived progeny, demonstrating that this prodifferentiation capacity was a property of T_{Mem} 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 T_{Mem} cell population was used, although the magnitude of change was less than that observed using $CD8^+$ T_{Mem} cells (Supplemental Figure 4).

As the frequency of circulating T_{Mem} cells to T_{N} cells can vary widely in patients (18, 29, 30), we evaluated the dose dependency of T_{Mem} cell-induced phenotypic maturation of T_{N} cells. We activated T_{N} cells alone or in titrated ratios with a bulk ($CD44^+$) T_{Mem} cell population. Similar to results using fractionated T_{CM} and T_{EM} cell subsets, we found that priming T_{N} cells in a 1:1 mixture with bulk T_{Mem} cells caused an increase in the T_{EM} cell population and a corresponding loss in T_{SCM} and T_{CM} cells (Figure 1, D and E). As the ratio of T_{Mem} 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 T_{Mem} cells during T_{N} cell priming ex vivo caused dose-dependent phenotypic maturation of T_{N} -derived cells.

T_{Mem} cause precocious differentiation of naive cells. Having established that T_{Mem} cells augment the phenotypic maturation of T_{N} cells during priming, we next evaluated whether T_{Mem} cells influence gene transcription and effector functions of T_{N} -derived progeny. We again primed $Ly5.1^+$ pmel-1 cells alone or in a 1:1 ratio with bulk in vitro-differentiated $Ly5.2^+$ T_{Mem} cells and confirmed that the progeny of T_{N} cells expanded with T_{Mem} cells (T_{N} -derived, mix) were significantly skewed toward T_{EM} cells and away from T_{SCM} cells compared with T_{N} cells primed alone (T_{N} -derived, alone) (Figure 2, A 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_{N} cell-associated lymphoid-homing and costimulatory markers, including *Ccr7* and *Cd27*, was significantly downregulated in T_{N} -derived cells expanded with T_{Mem} cells relative to T_{N} cells expanded alone (Figure 2C). Additionally, we found that T_{N} -derived cells primed with T_{Mem} 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 hpg100_{25–33} peptide (Figure 2, E and F).

To globally assess the influence of T_{Mem} 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 T_{Mem} cells had a gene-expression profile more closely related to T_{Mem} 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 T_{Mem} 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 T_{Mem} 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 T_{Mem} 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 T_{Mem} 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,

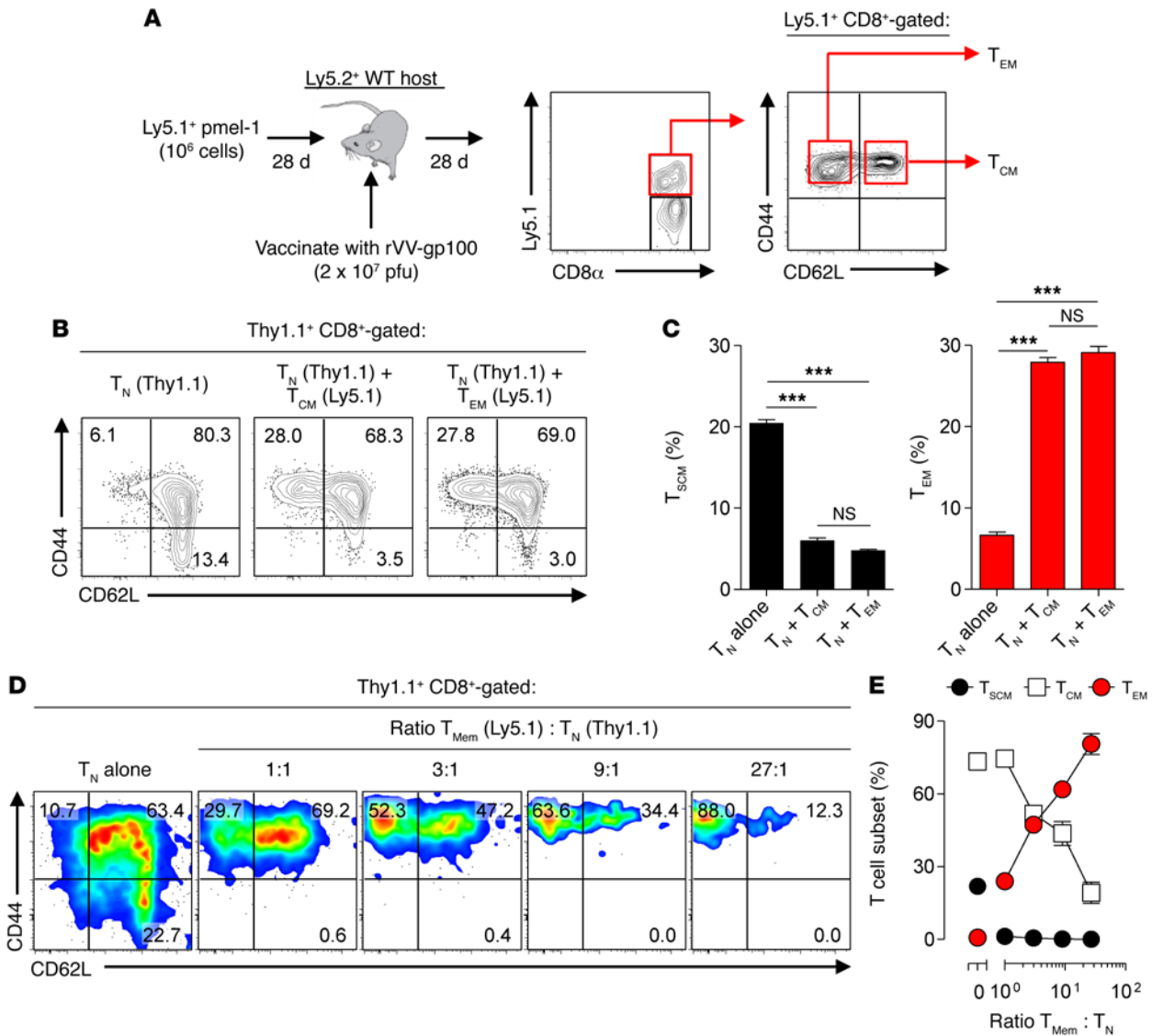


Figure 1. T_{Mem} cells augment naive cell phenotypic maturation during priming. (A) Experimental design and representative FACS plot showing the generation and isolation of vaccine-induced pmel-1 Ly5.1⁺CD8⁺ T_{CM} (CD44⁺CD62L⁺) and T_{EM} (CD44⁻CD62L⁻) cell subsets by adoptive transfer of pmel-1 cells (10⁶) 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⁺ T_N-derived T_{SCM} (CD44^{lo}CD62L⁺Sca1⁺CD122⁺) and T_{EM} cell subsets 6 days following ex vivo expansion alone or in the presence of a 1:1 mixture with Ly5.2⁺ T_{CM} or T_{EM} cells using CD3/CD28-specific antibodies and IL-2. (D) Representative FACS plots and (E) summary graph demonstrating the distribution of Thy1.1⁺ T_N-derived subsets 6 days following ex vivo expansion alone or in the presence of titrated ratios with Ly5.1⁺ bulk (CD44⁺) T_{Mem} cells using CD3/CD28-specific antibodies and IL-2. Results in A–E are shown after gating on live⁺CD8⁺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 T_N cells primed with T_{Mem} cells (Figure 2I). Collectively, these data revealed that T_{Mem} cells globally altered the differentiation program of T_N cells.

Finally, to assess the impact of T_{Mem} cell-induced differentiation of T_N-derived cells on antitumor efficacy, we adoptively transferred T_N cells primed alone or with T_{Mem} cells into mice bearing 10-day established s.c. B16 melanomas. We found that the expansion, persistence, and antitumor efficacy of T_N-derived cells were all significantly impaired in mice receiving T_N-derived cells primed with T_{Mem} cells compared with T_N-derived cells primed alone (P = 0.009) (Figure 2, J and K). We conclude that the presence of T_{Mem} cells dur-

ing T_N cell priming ex vivo caused enhanced phenotypic, functional, and transcriptional differentiation of T_N-derived cells relative to T_N cells primed alone, resulting in impaired antitumor efficacy. Henceforth, we will refer to this process as precocious differentiation.

T_{Mem} cells cause precocious differentiation of T_N cells in vivo. Having established that T_{Mem} augment naive cell differentiation during ex vivo expansion, we next asked whether T_{Mem} cells also influence T_N cell differentiation and antitumor efficacy in vivo after adoptive cotransfer. In prior studies, naive cell expansion and differentiation were impaired when limited numbers (5 × 10²) of T_N cells were transferred into hosts containing a 100- to 1,000-fold greater frequency of T_{Mem} cells (35). Under these conditions, T_N cells are

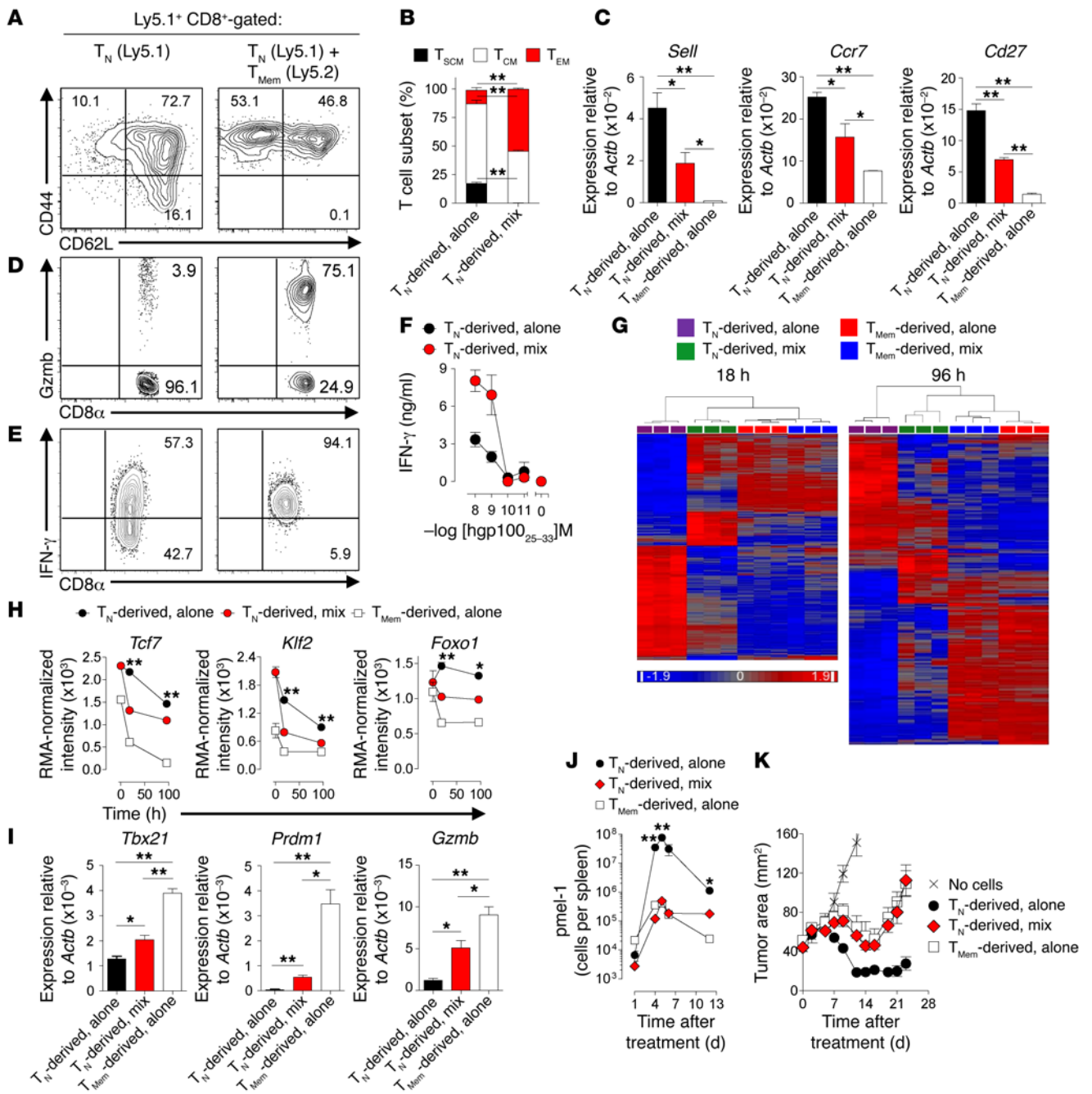


Figure 2. T_{Mem} cause precocious differentiation of naive cells. (A) Representative FACS and (B) bar graph summarizing the distribution of Ly5.1⁺CD8⁺ T_N-derived cell subsets 6 days following priming with CD3/CD28-specific antibodies and IL-2 alone or with Ly5.2⁺CD8⁺ T_{Mem} cells. Data shown after gating on Ly5.1⁺CD8⁺ cells. (C) qPCR analysis of *Sell*, *Ccr7*, and *Cd27* expression in FACS-sorted reisolated T_N cells primed alone, T_{Mem} cells primed with T_{Mem} cells, or T_{Mem} cells primed alone. (D) Granzyme B and (E) IFN-γ intracellular staining in T_N-derived cells stimulated with PMA/ionomycin following expansion alone or with T_{Mem} cells. (F) IFN-γ ELISA of supernatants from reisolated T_N-derived cells expanded alone or with T_{Mem} cells 6 days prior to overnight stimulation with hgp100₂₅₋₃₃ peptide. (G) Heat maps of differentially expressed genes (1-way ANOVA, pFDR < 5%) among T_N-derived cells expanded alone or with T_{Mem} cells at 18 and 96 hours. (H) RMA-normalized intensity of selected T_N-associated genes. (I) Expression of effector cell-associated factors assessed by qPCR at 96 hours from FACS reisolated T_N-derived progeny expanded with or without T_N cells or T_{Mem} cells expanded alone. (J) In vivo expansion and (K) tumor regression following i.v. adoptive transfer of T_N-derived progeny expanded alone or with T_{Mem} cells, or T_{Mem} 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.

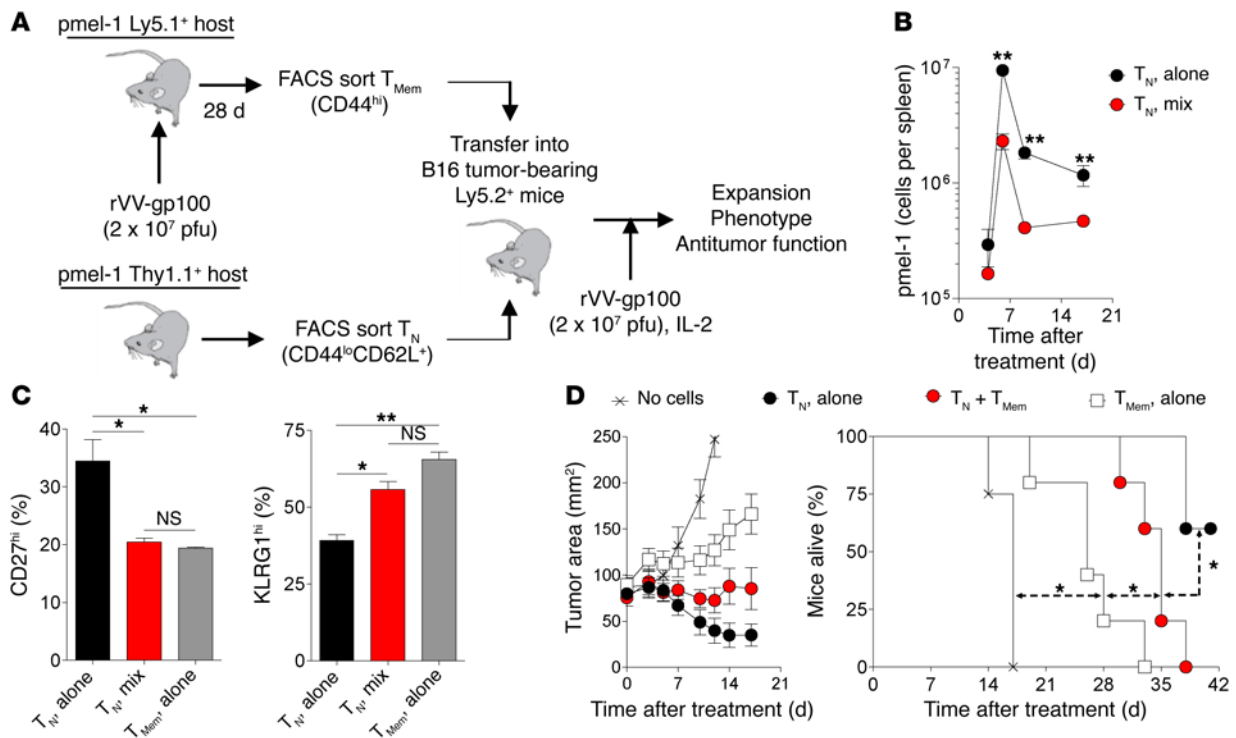


Figure 3. T_{Mem} cells cause precocious differentiation of T_N cells in vivo. (A) Experimental schema showing the generation, isolation, and transfer of Thy1.1⁺ pmel-1 T_N cells (CD44^{lo}CD62L⁺) alone or in combination with FACS-sorted, vaccine-induced Ly5.1⁺ pmel-1 T_{Mem} cells (CD44^{hi}) into Ly5.2⁺ hosts. (B) In vivo expansion and persistence of 1 × 10⁵ adoptively transferred Thy1.1⁺ T_N cells injected alone or in combination with 3 × 10⁵ Ly5.1⁺ T_{Mem} cells into Ly5.2⁺ WT mice bearing 10-day established B16 melanomas. (C) FACS analysis on day 17 of CD27 and KLRG1 expression on CD8⁺Thy1.1⁺ T_N-derived or CD8⁺Ly5.1⁺ T_{Mem}-derived cells. (D) Tumor regression and survival of mice bearing 10-day established B16 melanoma tumors who received 1 × 10⁵ T_N cells alone, in combination with 3 × 10⁵ T_{Mem} cells, or 3 × 10⁵ T_{Mem} cells alone. All treated mice received 6 Gy irradiation, i.v. rVV-gp100, and 3 days of i.p. IL-2. *n* = 3 mice/group/time point (B and C) or *n* = 5 mice per group (D). Results are displayed as mean ± SEM with statistical comparisons performed using an unpaired 2-tailed Student's *t* test corrected for multiple comparisons by a Bonferroni adjustment or log-rank test for animal survival. **P* < 0.05; ***P* < 0.01. Data shown are representative of 2 independently performed experiments.

numerically outcompeted by T_{Mem} cells for limited antigen presented by professional antigen-presenting cells, thereby limiting their priming and differentiation (36). However, whether T_N cell differentiation and antitumor efficacy is influenced when large numbers of cells typically used in adoptive immunotherapies are cotransferred in conjunction with T_{Mem} cells has not been previously evaluated. Therefore, we adoptively transferred (1 × 10⁵) Thy1.1⁺ T_N pmel-1 cells alone or in combination with (3 × 10⁵) FACS-sorted, vaccine-induced, Ly5.1⁺ pmel-1 T_{Mem} cells into sublethally irradiated Ly5.2⁺ hosts bearing 10-day established B16 melanoma tumors (Figure 3A). In addition to cells, recipient mice also received rVV-gp100 vaccination and exogenous IL-2. We found that both the expansion and persistence of T_N cell-derived progeny were significantly impaired when cotransferred with T_{Mem} cells (Figure 3B). In contrast, the recall response of cotransferred T_{Mem} cells was not affected compared with T_{Mem} cells transferred alone (Supplemental Figure 5). These findings were not due to a failure of T_N cells to become primed in vivo, as a significantly larger proportion of the T_N-derived population cotransferred with T_{Mem} cells lost CD27 expression and acquired the senescent marker killer cell lectin-like receptor subfamily G1 (KLRG1) at the conclusion of the immune response compared with T_N cells transferred alone (Figure 3C). Importantly, both tumor regression and animal survival were significantly impaired (*P* < 0.05) in mice receiving T_N cells and T_{Mem} cells together com-

pared with T_N cells alone (Figure 3D). We conclude that T_{Mem} cells can cause precocious differentiation and impaired antitumor efficacy of T_N cell-derived progeny in vivo following adoptive cotransfer.

FasL-Fas interactions mediate precocious differentiation. We next sought to elucidate what T_{Mem} 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 T_{Mem} cells (Supplemental Figure 6). Accordingly, we hypothesized that an activation-induced, cell-surface molecule on T_{Mem} 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 T_{Mem} cells and T_N cells for TNF superfamily members uniquely overexpressed in activated T_{Mem} 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 T_{Mem} cells (Figure 4A and Supplemental Table 4). To determine whether T_{CM} and T_{EM} 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 T_N, T_{CM}, and T_{EM} cell subsets. Consistent with previous reports (38), we found that T_N cells exhibited strong depo-

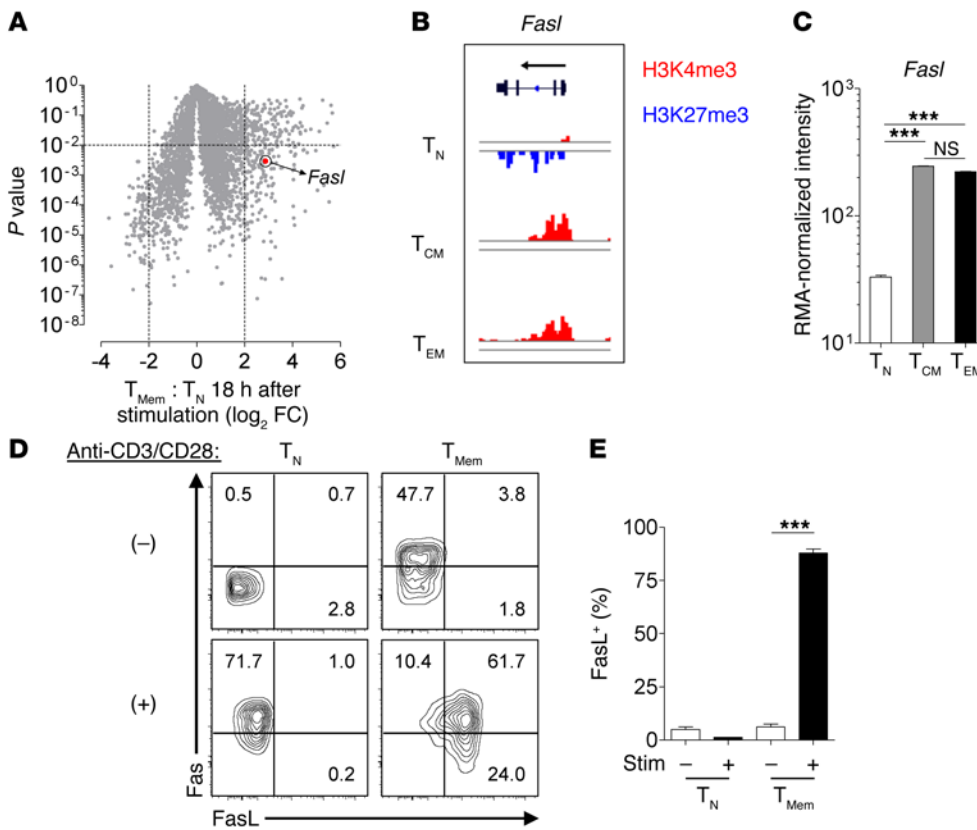


Figure 4. FasL is poised for rapid surface expression in T_{Mem} cell subsets but not T_N cells. (A) Volcano plot indicating differentially expressed genes in T_{Mem} cells versus T_N cells 18 hours after stimulation with CD3/CD28-specific antibodies and IL-2. Dashed lines, $P < 0.01$ and $FC > 2$. (B) 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 T_N , T_{CM} , and T_{EM} cell subsets. (D and E) Fas and FasL surface expression on T_N and T_{Mem} cells at rest or 18 hours after stimulation with CD3/CD28-specific antibodies. All bar graphs shown as mean \pm 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.

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 T_{CM} and T_{EM} 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 T_{CM} and T_{EM} cells compared with T_N cells (Figure 4C). Notably, there were no significant differences in *FasL* gene expression between T_{CM} and T_{EM} cell subsets. We confirmed that T_{Mem} cells but not T_N 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⁺ T_N cells alone or in a 1:1 mixture with Ly5.2⁺ T_{Mem} cells in the presence of a blocking antibody against FasL (α FasL) or isotype control (IgG) and measured changes in T cell differentiation. Provision of α FasL during T_N cell priming with T_{Mem} cells significantly but incompletely limited precocious differentiation (Figure 5, A–C). Consistent with FACS data demonstrating a paucity of FasL expression on recently activated T_N cells, we found no significant differences in the composition of T_N cell-derived progeny when isolated naive cells were primed with α FasL compared with IgG (Supplemental Figure 7).

As antibody blockade may be incomplete, we complemented these findings by genetic means using CD8⁺ T_N cells derived from Fas-deficient *lpr* mice (44). Whereas WT T_N cells underwent augmented differentiation when primed with T_{Mem} cells, T_N cells from *lpr* mice were completely protected from this phenomenon (Figure 5, D and E). We conclude that precocious differentiation results from a contact-dependent interaction in which T_{Mem} cell-provided FasL engages Fas expressed on T_N cells.

Nonapoptotic Fas signaling mediates precocious differentiation. To evaluate whether FasL-delivered signals were sufficient to induce precocious differentiation, we primed T_N cells in the absence of T_{Mem} cells with titrated amounts of recombinant FasL oligomerized through a leucine zipper domain (lz-FasL) (45). We found that lz-FasL caused a dose-dependent increase in the differentiation of T_N cell-derived progeny, as evidenced by an increased frequency of T_{EM} 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 T_{EM} cell phenotype/IFN- γ ⁺ cells following lz-FasL exposure at 33 ng/ml compared with vehicle control (Figure 6D). Importantly, the increase in absolute numbers of T_{EM} cells occurred despite this subset's enhanced susceptibility to lz-FasL-induced apoptosis relative to other subsets (Supplemental Figure 9). The differential

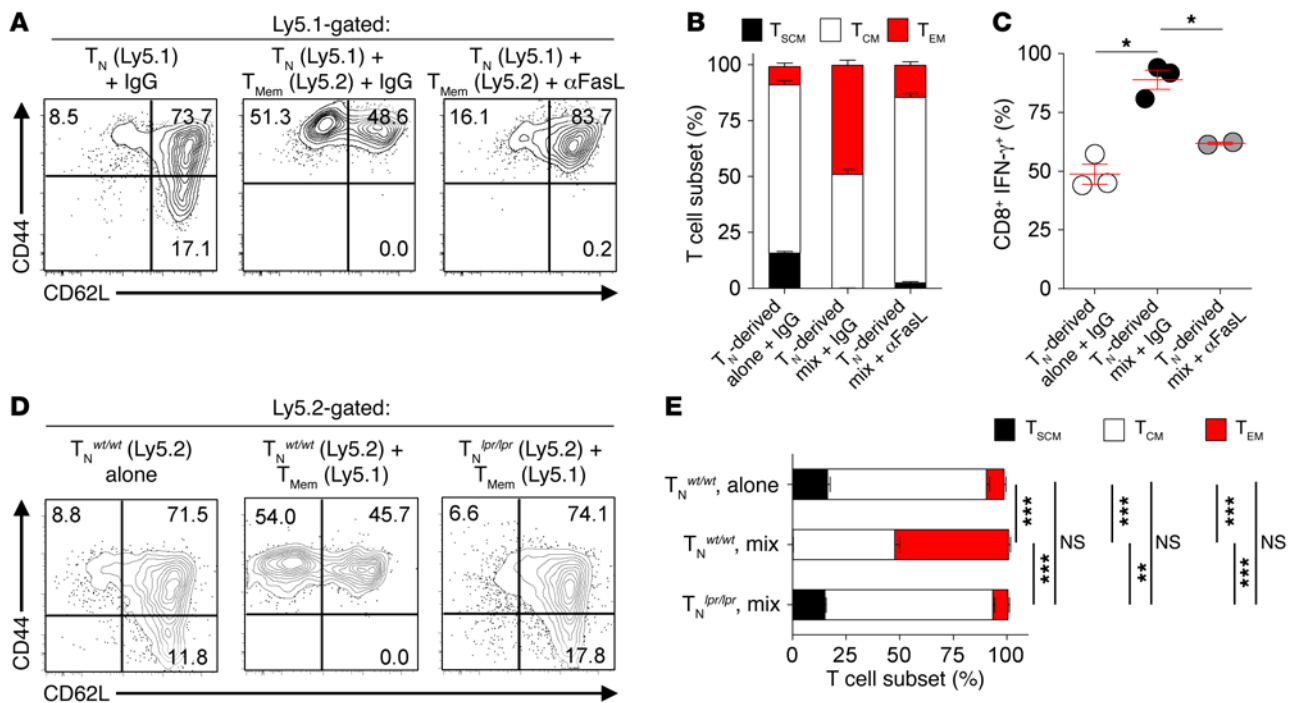


Figure 5. FasL-Fas interactions mediate precocious differentiation. (A) Representative FACS plots, (B) summary bar graph, and (C) scatter plot demonstrating T cell subset frequencies or percentage of IFN- γ ⁺CD8⁺ T cells 6 days following priming of Ly5.1⁺ T_N alone or in a 1:1 mixture with Ly5.2⁺ T_{Mem} with CD3/CD28-specific antibodies, IL-2, and a blocking antibody against FasL (α FasL) or isotype (IgG) control. (D) FACS analysis and (E) bar graph summarizing the distribution of CD8⁺ T cell subsets 6 days following priming of Ly5.2⁺ WT T_N pmel-1 (T_N^{WT/WT}) or *lpr/lpr* T_N pmel-1 (T_N^{lpr/lpr}) cells alone or in the presence of a 1:1 mixture with WT Ly5.1⁺ T_{Mem}. All results shown as mean \pm SEM with $n = 2-3$ per indicated condition or cell type. Statistical comparisons performed using an unpaired 2-tailed Student's *t* test corrected for multiple comparisons by a Bonferroni adjustment. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Data shown are representative of 14 (A and B), 4 (C), and 3 (D and E) independently performed experiments.

death of T_{EM} cells was not attributable to differences in surface Fas expression between memory subsets (Supplemental Figure 10), a finding consistent with published studies of Fas expression on human T_{Mem} cell subsets (45). Collectively, these data suggested that skewing toward the T_{EM} cell phenotype could not be accounted for solely by selective culling of T_{SCM} and T_{CM} 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 apoptosis requires posttranslational palmitoylation of a cysteine residue located within the proximal cytoplasmic region of the receptor (46, 47). In cell lines, transfection of a Fas construct substituting a valine for a cysteine residue at the 194 position (C194V) prevented both Fas incorporation into membrane rafts and Fas-dependent apoptosis (46). Based on these data, a transgenic mouse expressing the FasC194V variant was generated, backcrossed to the *lpr* background (henceforth, FasC194V^{lpr/lpr}), and used to derive CD8⁺ T cells for further analyses. Whereas lz-FasL caused dose-dependent death of WT CD8⁺ T cells, FasC194V^{lpr/lpr} CD8⁺ T cells were significantly protected from apoptosis induction similarly to *lpr* CD8⁺ T cells (Figure 6E). However, unlike cells from the *lpr* mouse, which were also protected from lz-FasL-mediated precocious differentiation, FasC194V^{lpr/lpr} CD8⁺ T cells underwent enhanced conversion to a T_{EM} cell phenotype similar to that of WT cells following priming 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_N cells.

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 phosphatidylinositol 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_N 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_N cells were primed in the presence of congenically distinguishable Ly5.1⁺ T_{Mem} cells, we observed a significant increase in the staining intensity of pAkt T308, pAkt S473, and pS6 in T_N-gated cells. To establish whether increased pAkt content in T_N cells primed with T_{Mem} cells leads to commensurate changes in gene expression, we returned to our microarray analyses. Within 18 hours of T_N cell activation in the presence of T_{Mem} 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 activated T_N cells was sufficient to augment pAkt in the absence of T_{Mem}

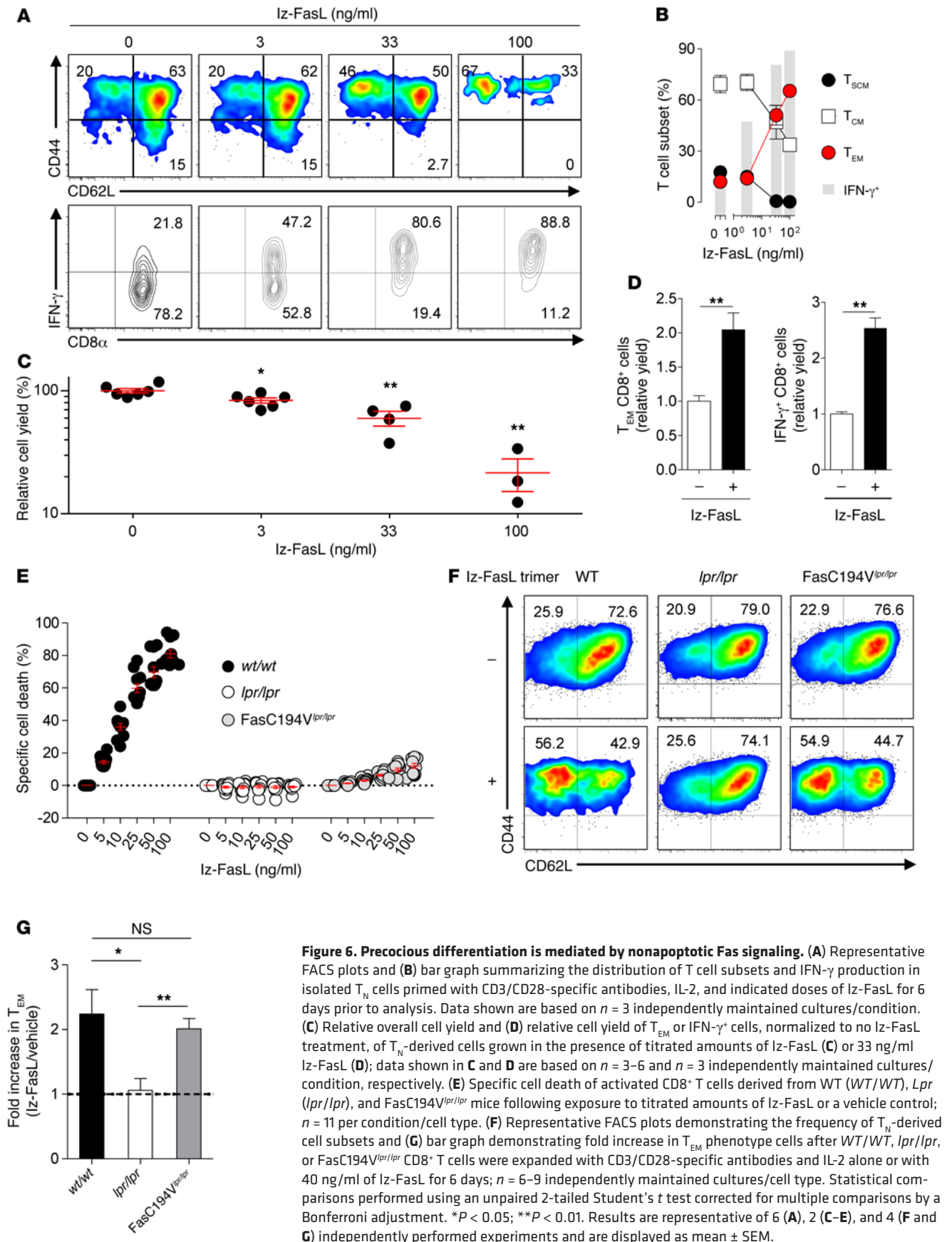


Figure 6. Precocious differentiation is mediated by nonapoptotic Fas signaling. (A) Representative FACS plots and (B) bar graph summarizing the distribution of T cell subsets and IFN- γ production in isolated T_N cells primed with CD3/CD28-specific antibodies, IL-2, and indicated doses of Iz-FasL for 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 T_{EM} or IFN- γ ⁺ cells, normalized to no Iz-FasL treatment, of T_N-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 T_N-derived cell subsets and (G) bar graph demonstrating fold increase in T_{EM} 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 \pm SEM.

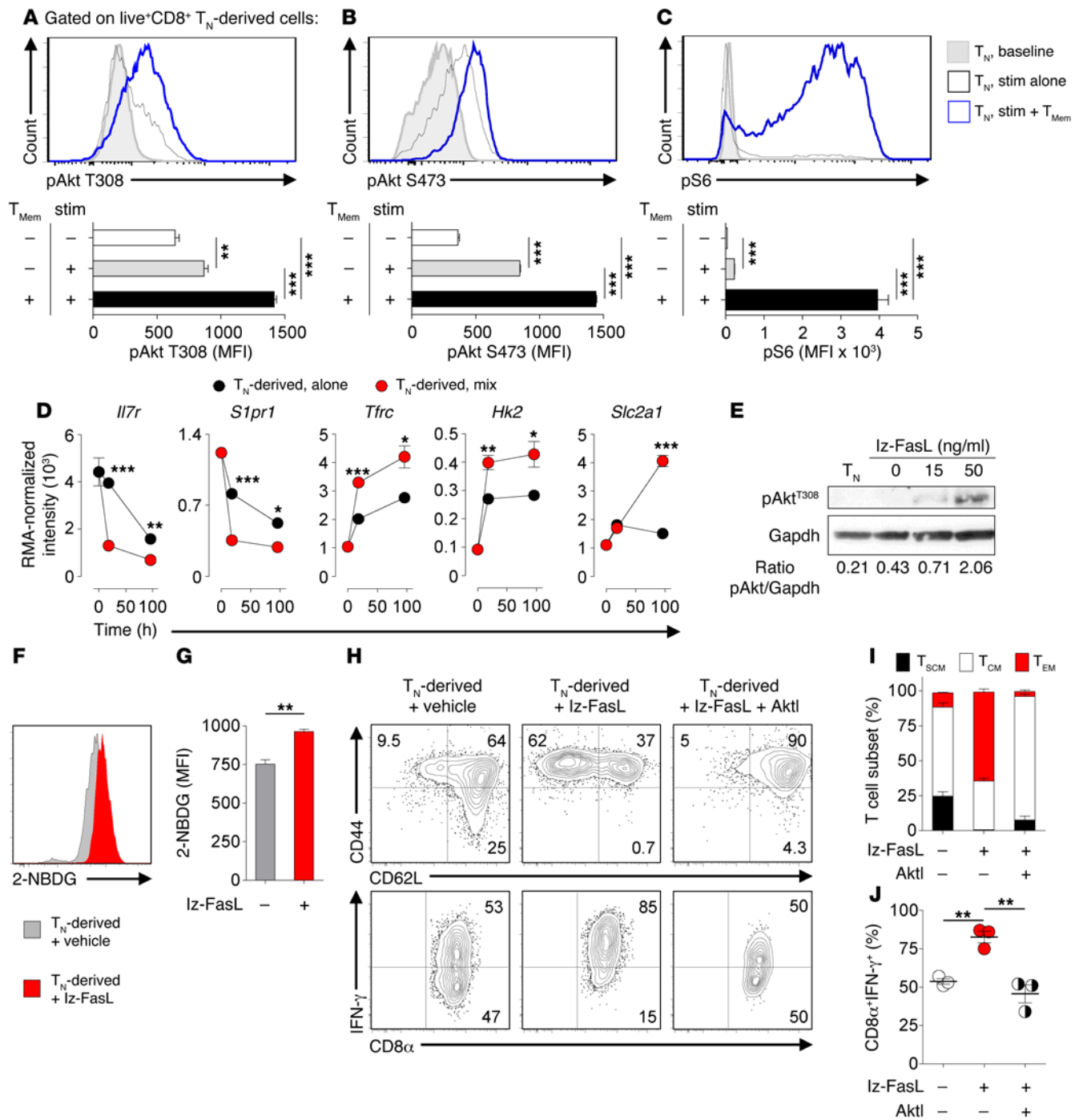


Figure 7. Precocious differentiation is associated with augmented Akt signaling. Representative FACS histograms and summary bar graphs demonstrating the mean fluorescence intensity of (A) pAkt T308, (B) pAkt S473, and (C) pS6 in Ly5.2⁺CD8⁺ T_N cells at rest or 24 hours after stimulation with CD3/CD28-specific antibodies alone or in a 1:1 mixture with Ly5.1⁺ T_{Mem} cells. Data shown after gating on live⁺Ly5.2⁺CD8⁺ cells. (D) RMA-normalized intensity showing expression of *Il7ra*, *S1pr1*, *Tfrc*, *Hk2*, and *Slc2a1* in resting T_N cells, T_N cells primed alone, and T_N cells primed in a 1:1 mixture with T_{Mem} cells. (E) Western blot and densitometry analysis of pAkt T308 and GAPDH in resting T_N or T_N 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 T_N 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 T_N 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.

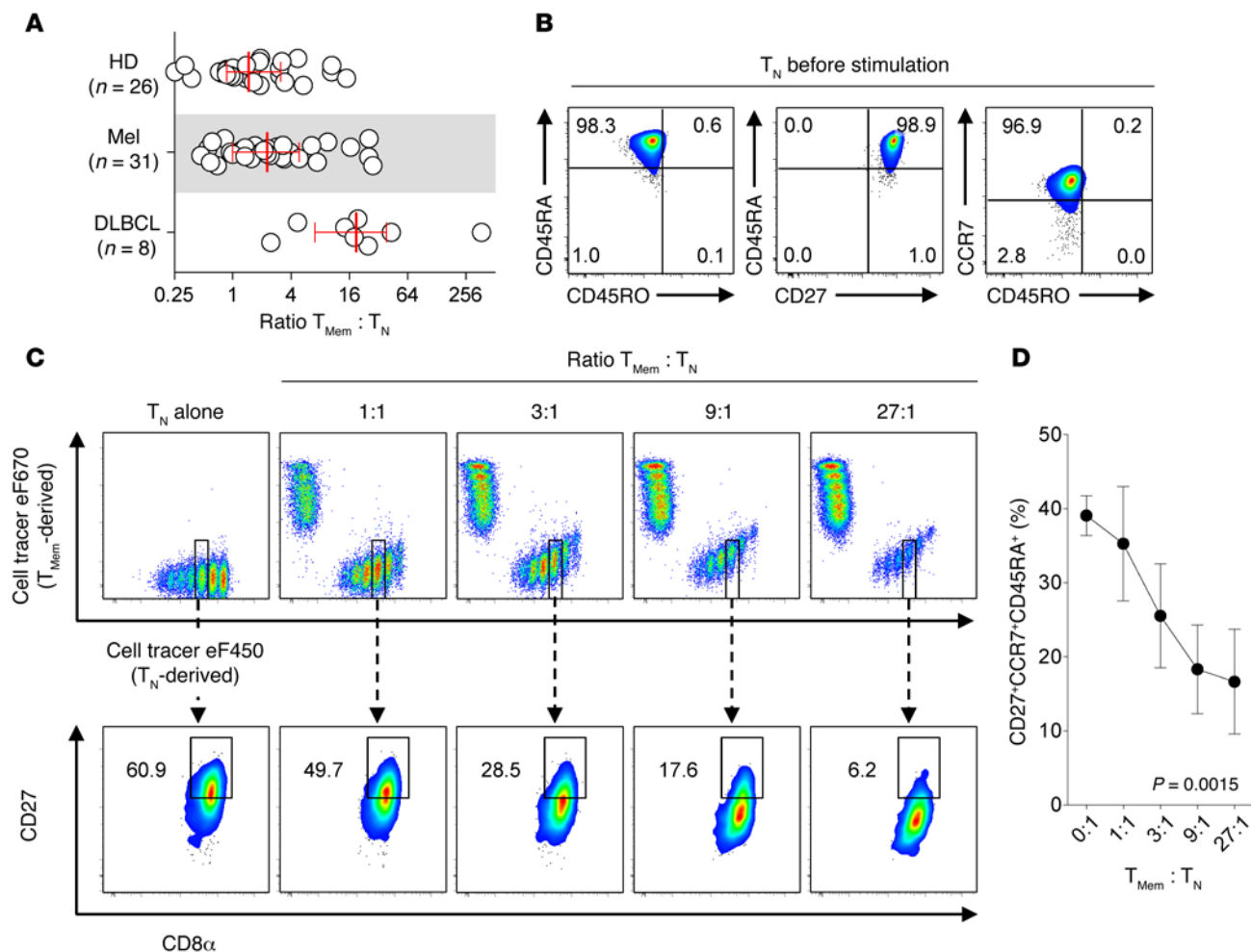


Figure 8. Human T_{Mem} cells induce precocious differentiation of T_N -derived cells. (A) Ratio of memory ($CD3^+CD8^+CD45RO^+$) to naive ($CD3^+CD8^+CD45RO^-CD45RA^+CCR7^+$) T cells in the peripheral circulation of HDs ($n = 26$), Mel patients ($n = 31$), or DLBCL patients ($n = 8$). Results shown as the median \pm interquartile range. (B) Representative FACS plots demonstrating the surface phenotype of isolated T_N cells after membrane labeling with cell tracer eF450 but before stimulation. (C) Representative FACS plots demonstrating the gating strategy used to assess the division-normalized phenotype of human T_N -derived progeny 4 days following activation alone or in the presence of titrated ratios with T_{Mem} cells. Naive and T_{Mem} cells were labeled with cell tracer eF450 or eF670, respectively, and stimulated at indicated ratios using $CD3/CD28$ -specific antibodies and IL-2. Naive-derived cells were subsequently analyzed for the coordinate expression of $CD27$, $CCR7$, and $CD45RA$ using Boolean gating after gating on T_N cells that had diluted an equivalent amount of the cell tracer eF450 dye. (D) Summary graph demonstrating the frequency of T_N cells that coordinately express the markers $CD27^+CCR7^+CD45RA^+$ after undergoing a normalized number of cell divisions plotted as a function of the ratio of T_{Mem} to T_N cells. Results shown as mean \pm SEM for each condition for $n = 3$ independently evaluated donors. $P = 0.0015$ (repeated measures 1-way ANOVA).

cells. Whereas pAkt T308 was undetectable in resting $CD8^+$ T_N cells, we found that lz-FasL caused dose-dependent augmentation of this phosphorylated residue 24 hours following activation with $CD3/CD28$ -specific antibodies (Figure 7E). Consistent with the augmented expression of *Slc2a1* and *Hk2* in T_N cells primed with T_{Mem} cells combined with the capacity of Akt activation to enhance *Glut1* surface expression (53), we measured increased accumulation of the fluorescent D-glucose analog 2-(N-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]amino)-2-deoxyglucose (2-NBDG) in T_N cells activated with lz-FasL (Figure 7, F and G). This indicated a relatively enhanced conversion to a glycolytic metabolism in T_N cells exposed to lz-FasL (54). Finally, to determine whether Akt signaling directly contributed to precocious differentiation, we primed $CD8^+$ T_N cells in the presence of 50 ng/ml of lz-FasL with or without Akt inhibitor VIII (AktI), a well-validated alloste-

ric Akt inhibitor (48, 55). We found that AktI significantly limited lz-FasL-mediated precocious differentiation, as evidenced by a reduced accumulation of T_{EM} cell phenotype and $IFN-\gamma^+$ cells (Figure 7, H-J). We conclude that T_{Mem} cell and lz-FasL-induced precocious differentiation of T_N cells is associated with augmented Akt pathway activation. Pharmacologic inhibition of Akt can partially block the precocious differentiation phenotype.

Human T_{Mem} cells induce precocious differentiation of T_N -derived cells. To evaluate the relevance of precocious differentiation for human ACT trials, we next tested to determine whether human T_{Mem} cells influence the differentiation of T_N cells during ex vivo cell expansion. First, we established the frequency of T_{Mem} cells compared with T_N 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

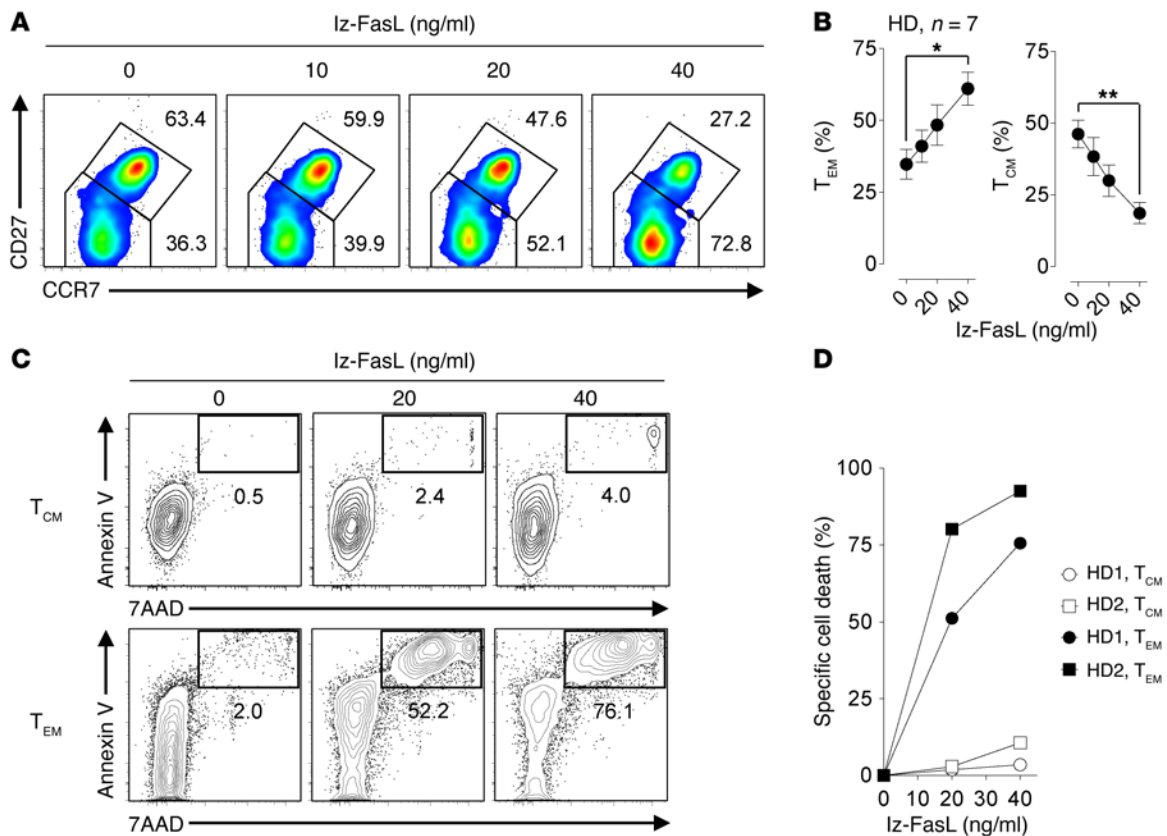


Figure 9. Fas signaling promotes the differentiation of human CD8⁺ T cells. (A) Representative FACS plots and (B) summary graph showing the distribution of CD8⁺ T cell subsets 7 to 10 days after peripheral blood CD8⁺ T cells from HD were stimulated with CD3/CD28-specific antibodies, IL-2, and indicated concentrations of Iz-FasL. Results shown after gating on viable CD8⁺ lymphocytes from $n = 7$ HD. T_{CM} = CD8⁺CD45RO⁺CCR7⁺CD27⁺, T_{EM} = CD8⁺CD45RO⁺CCR7⁻CD27⁻. (C) Representative FACS plots and (D) summary graph displaying the induction of Iz-FasL specific cell death after gating on T_{CM} and T_{EM} cells stimulated with indicated concentrations of Iz-FasL for 12 hours. Cells that coordinately stained 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. * $P < 0.05$; ** $P < 0.01$. Data shown are representative of 7 (A and B) and 2 (C and D) independently performed experiments with results displayed as mean \pm SEM.

adoptively transferred T cells have entered clinical trials (56–58). We found that the T_{Mem} cell to T_N 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 T_N cell-derived progeny in mixed cultures, we isolated T_N (CD8⁺CD45RA⁺CD45RO⁻CCR7⁺) and T_{Mem} (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, T_N cells and T_{Mem} cells lost membrane dye intensity, indicating both subsets had undergone cell division. To determine whether human T_N cells expand at the same rate in the presence or absence of memory cells, we calculated the proliferation index (PI) of T_N-derived cells primed alone or with different ratios of T_{Mem} cells. We discovered that human T_{Mem} cells caused a dose-dependent increase in T_N cell proliferation (Supplemental Figure 11B). To ascertain whether T_{Mem} cells influence T_N cell differentiation independently of cell prolifera-

tion, we gated on T_N-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 T_N cells (CD27⁺CCR7⁺CD45RA⁺) as the ratio of T_{Mem} 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 T_{Mem} cells augmented both the proliferation and differentiation of T_N-derived cells. Further, we conclude that the rate of differentiation is enhanced even when division history is normalized.

In mice, we discovered that T_{Mem} cell-induced precocious differentiation of T_N 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 Iz-FasL. After 9 to 10 days, we found that all expanded cells had assumed a CD45RA^{lo/-}CD45RO⁺ memory phenotype (data not shown). Using coexpression of CD27 and CCR7 (29), we characterized the ratio of T_{CM} (CD8⁺CD45RO⁺CCR7⁺CD27⁺) to T_{EM} cells (CD8⁺CD45RO⁺CCR7⁻

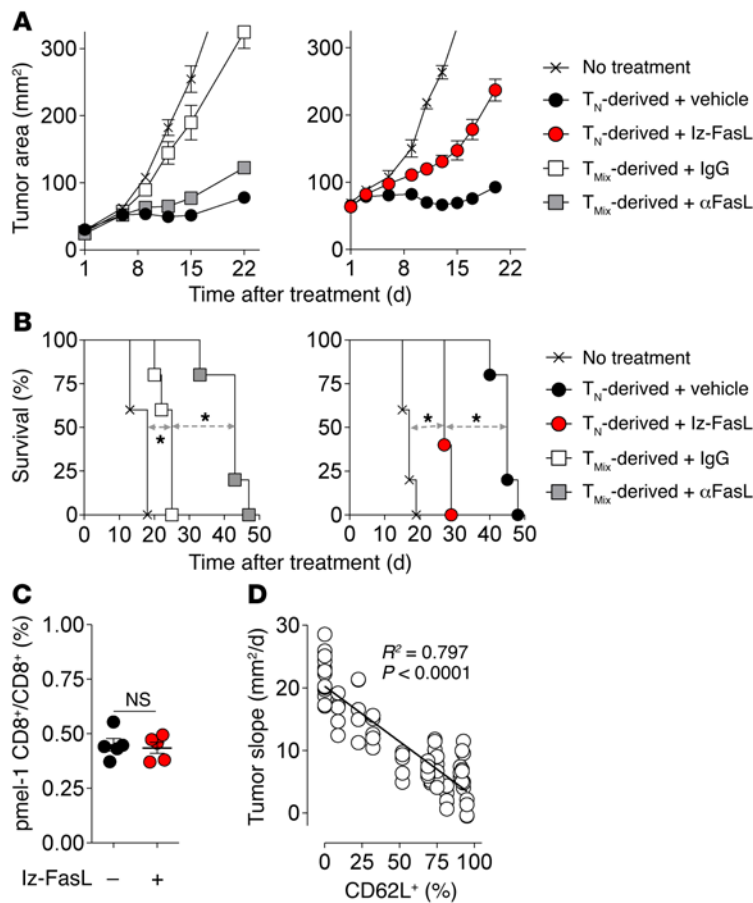


Figure 10. Fas signaling controls T cell differentiation and influences adoptive immunotherapy efficacy. (A) Tumor regression and (B) animal survival of mice bearing 10-day established s.c. B16 melanomas treated with 2.5×10^5 T_N-derived pmel-1 cells primed alone, with lz-FasL (50 ng/ml), or with T_{Mem} in the presence of αFasL or IgG control. Viable cells were isolated using a density separation media before infusion. All treated mice received 6 Gy irradiation prior to cell infusion in addition to i.v. rVV-gp100 and 3 days of i.p. IL-2. Tumor treatment experiments performed with $n = 5$ mice/group. T_{Mix} T_N cells primed with T_{Mem} cells in a 1:1 mixture. (C) Engraftment efficiency of Thy1.1⁺ T_N-derived pmel-1 cells (3×10^5) primed alone or with lz-FasL 18 hours following i.v. adoptive transfer into nonirradiated Ly5.2⁺ hosts; $n = 5$ mice/group. (D) Correlation between the slope of tumor growth and CD62L expression at the time of cell transfer on T_N-derived pmel-1 cells primed alone with either lz-FasL (50 ng/ml), vehicle control, or with T_{Mem} and either αFasL 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 \pm SEM.

CD27). Consistent with experiments using mouse T cells, we found that augmenting Fas signaling using lz-FasL in HD cells caused a significant and dose-dependent accumulation of the more-differentiated T_{EM} cell subset and an attrition of T_{CM} cells (Figure 9, A and B). These changes were not attributable to differences in proliferation, as T cells expanded in lz-FasL and vehicle control diluted a membrane-associated dye similarly (Supplemental Figure 12A). Moreover, analysis of the surface phenotype of T cells that had undergone an identical number of divisions revealed that lz-FasL exposure resulted in a more-differentiated phenotype compared with cells expanded in vehicle control (Supplemental Figure 12B). Consistent with the apoptosis-inducing functions of Fas, lz-FasL resulted in a reduced cell yield at concentrations of 20 ng/ml or more (Supplemental Figure 12C). However, at lower concentrations of lz-FasL, we measured differences in the distribution of T cell subsets while expanding similar numbers of cells. 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 T_{EM} cells were far more sensitive to Fas-induced apoptosis compared with the T_{CM} cell subset. Collectively, we conclude that human T_N cells, like mouse T_N cells, undergo accelerated differentiation when primed in the presence of T_{Mem} cells or augmented Fas signaling.

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 αFasL 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_N-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-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_N-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_N, 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_{Eff}) populations. Despite these data, the vast majority of current ACT trials utilize unfractionated 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_N 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 T_N cells are enriched relative to T_{Mem} cells using magnetic bead isolation of 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-enriched T cells results in superior clinical outcomes compared with transfer of unselected T cell populations.

Mechanistically, we identified the TNF superfamily member FasL, which is expressed on T_{Mem} cells but not T_N cells acutely following activation, as the mediator of precocious differentiation. FasL was both necessary and sufficient to induce the phenomenon. Provision of lz-FasL recapitulated precocious differentiation in the absence of T_{Mem} cells, while antibody blockade of FasL or use of T cells from Fas-deficient *lpr* mice prevented precocious differentiation. Reverse signaling by FasL in T cells has been observed under

some conditions (65). Our findings that T cells derived from *lpr* mice or were protected from precocious differentiation combined with the ability of lz-FasL to mediate the phenomenon excluded reverse signaling as a mechanism in our system.

While Fas is conventionally known as a death receptor, its ability to mediate nonapoptotic functions has been documented in a variety of tissues (39), including T cells (40). We provide several lines of evidence that our findings were not attributable solely to Fas-induced apoptosis. First, we determined that T_{EM} cells, the population that accumulates in response to augmented Fas signaling, was most sensitive to Fas-mediated apoptosis relative to other T cell subsets in both mice and humans. This finding is in agreement with previously reported results (45). Thus, selective culling of T_{SCM} and T_{CM} cells could not account for the skewing toward the T_{EM} cell population. Second, using CD8⁺ T cells derived from a transgenic mouse expressing a FasC194V mutant receptor, we provide genetic evidence that Fas-mediated cell death and cellular differentiation can be uncoupled by impairing the ability of the receptor to undergo posttranslational lipid modification. Although further studies are required to resolve the precise signaling cascade necessary for Fas-induced precocious differentiation, we found that both T_{Mem} cells and lz-FasL augmented Akt signaling in T_N cells. Similarly to previously reported Akt-dependent functions of canonical costimulatory molecules such as CD28 (66), augmenting Fas signaling in T_N cells enhanced cellular differentiation and promoted a glycolytic metabolism. Conversely, inhibition of Akt using a well-validated allosteric inhibitor largely prevented lz-FasL-induced differentiation of T_N cells, further implicating Akt activation in Fas-mediated differentiation. These findings parallel recent reports of Akt-mediated nonapoptotic Fas signaling in glioblastoma (42) and neuronal stem cells (43).

We determined that the capacity to induce precocious differentiation was a generalized property of antigen-experienced 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_N 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_{CM} cells form stable clusters together *in vivo* around antigen-bearing targets (67, 68). Additionally, while lymphoid-trafficking of the CD62L⁺ T_{EM}/T_{Eff} 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_N cells for access to limited antigen and cytokines, thus curtailing the differentiation of T_N cells. However, how T_{Mem} cells influence T_N 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_{Mem} cells caused accelerated T_N cell differentiation into a terminally differentiated $KLRG1^+CD27^{lo}$ phenotype despite an attenuated proliferative burst. The decreased expansion of anti-tumor T_N cells primed with T_{Mem} cells in vivo likely reflected their accelerated conversion to antigen-experienced subsets that are known to have a reduced proliferative capacity relative to T_N cells following poxvirus vaccination (70). Most importantly, cotransfer of T_{Mem} cells impaired the full in vivo antitumor efficacy of T_N cells.

The magnitude of accelerated T_N cell differentiation caused by T_{Mem} cells was remarkably sensitive to the ratio of the 2 subsets such that an exponential relationship existed between the extent to which T_N -derived progeny entered the T_{EM}/T_{EFF} pool and the starting proportion of T_{Mem} to T_N 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 $CD8^+$ T cells drive T_N cell differentiation during priming through a contact-dependent mechanism involving a nonapoptotic Fas-FasL interaction. The net influence of this process synchronizes T_N cell behavior with that of T_{Mem} cells, affecting the functional, transcriptional, and metabolic differentiation of T_N -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_{Mem} cause significantly enhanced differentiation of T_N 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; $Ly5.2^+$), B6.SJL-*Ptprca*^a *Pep3^b*/BoyJ ($Ly5.1^+$), B6.PL-*Thy1^a*/CyJ ($Thy1.1^+$), B6.MRL-*Fas^{lpr}*/J (*lpr*) (44), B6.129S7-*Rag1^{tm1Mom}*/J (Rag), B6.Cg-*Thy1^a*/Cy Tg(*Tcratcrb*)8Rest/J (pmel-1) (24), and B6-Tg(*Tcratcrb*)1100Mjb/J (OT-1) (76) $CD8^+$ 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^{*lpr/lpr*} mice were backcrossed to homozygosity for both the *lpr* Fas allele and the C194V Fas transgene. Where indicated, pmel-1 and OT-1 mice were crossed to *Thy1.1*, *Ly5.1*, *Rag*, or *Rag* × *lpr* 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 4×10^5 B16 (H-2D^b) cells, a spontaneous gp100⁺ 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 $CD8^+$ T cells in combination with 2×10^7 pfu of a previously described recombinant vaccinia virus (rVV-gp100) (24) (rVV-gp100) and 12 μ g 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): $CD8\alpha$ (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- β 1), $CD178$ (clone MFL3), IFN- γ (clone XMG1.2), $KLRG-1$ (clone 2F1), and *Sca-1* (clone D7) (all purchased from BD Biosciences). Fluorochrome conjugates against pS6 S235/236 (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), $CD8$ (SK1) (BD Biosciences) or $CD27$ (M-T271), $CD45RA$ (HI100), 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_N -derived and T_{Mem} -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 $CD8^+$ T cells with 100 μ M 2-NBDG (Invitrogen) for 2 hours before measuring by FACS, as previously described (54). Flow cytometric data were acquired using either BD FACSCanto 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 $CD8^+$ T cell subsets. Mouse T_N cells were isolated either by using a MACS $CD8^+$ negative selection kit (Miltenyi Biotech) in combination with a 1:300 dilution of biotin-conjugated anti- $CD44$ antibody or by FACS sorting $CD44^{lo}CD62L^{hi}CD8^+$ T cells using FACSAria (BD Biosciences). Mouse T_{Mem} cells were generated by in vitro differentiation (as previously described) or by adoptive transfer of congenically distinguishable $Thy1.1^+$ or $Ly5.1^+$ pmel-1 $CD8^+$ T cells into $Ly5.2^+$ WT mice, where recipient mice were vaccinated with rVV-gp100 (2×10^7 PFU) and vaccine-induced T_{CM} ($CD44^{hi}CD62L^+$) or T_{EM} ($CD44^{hi}CD62L^-$) adoptively 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_N cells and T_{Mem} cells were obtained by magnetic bead isolation using the EasySep Human Naive $CD8^+$ T Cell and Memory $CD8^+$ T Cell Enrichment Kits, respectively (STEMCELL Technologies). Fate tracking of T_N and T_{Mem} cells was accomplished by labeling cells with Cell Proliferation Dye eFluor 450 and Cell Proliferation Dye eFluor 670, respectively (eBioscience).

Activation and expansion of $CD8^+$ T cells. Both mouse and human T_N cells with or without T_{Mem} cells were activated and expanded at indicated ratios in 96-well round-bottom plates coated with 2 $\mu\text{g}/\text{ml}$ of $CD3$ -specific and 1 $\mu\text{g}/\text{ml}$ of soluble $CD28$ -specific antibodies (clones 145-2C11 and 37.51; BD Biosciences) in culture media containing 5 ng/ml (mouse) or 20 ng/ml (human) of IL-2 at a final density of 1×10^5 cells/well. Where indicated, T cells were cultured with specified concentrations of lz-FasL, a previously described recombinant oligomerized form of FasL (45), or 10 $\mu\text{g}/\text{ml}$ of either a blocking antibody against FasL (MFL3; BD Biosciences) or IgG (A19-3; BioLegend). In some experiments, cells were also cultured with 1 μM of Akt Inhibitor VIII (Calbiochem) dissolved in DMSO (Sigma-Aldrich). Where indicated, T_N -derived and T_{Mem} -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_N cells alone or in combination with T_{Mem} cells either in the bottom well or in the Transwell insert.

Cytokine release assays. We pulsed B6 splenocytes with indicated concentrations of hgp100₂₅₋₃₃ peptide and incubated cells with T_N -derived pmel-1 $CD8^+$ 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, $CD8^+$ T cell subsets were FACS sorted directly into RNeasy Protect Cell Reagent, and RNA was extracted using the RNeasy Shredders 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*, *Tbx21*, *Tcf7*, *Tfrc*, and *Prdm1* (Applied Biosystems). For Western blot analysis, $CD8^+$ 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 $p\text{FDR} < 0.01$. 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, 2×10^7 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 \pm 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 $CD62L^+$ cells among Fas-modulated $CD8^+$ 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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