

T cells genetically engineered to overcome death signaling enhance adoptive cancer immunotherapy

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Title: T Cells Genetically Engineered to Overcome Death Signaling Enhance Adoptive Cancer Immunotherapy

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ABSTRACT:

Across clinical trials, T cell expansion and persistence following adoptive cell transfer (ACT) have correlated with superior patient outcomes. Herein, we undertook a pan-cancer analysis to identify actionable ligand/receptor pairs capable of compromising T cell durability following ACT. We discovered that *FASLG*, the gene encoding the apoptosis-inducing ligand FasL, is overexpressed within the majority of human tumor microenvironments (TMEs). Further, we uncovered that Fas, the receptor for FasL, is highly expressed on patient-derived T cells used for clinical ACT. We hypothesized that a cognate Fas-FasL interaction within the TME might limit both T cell persistence and anti-tumor efficacy. We discovered that genetic engineering of Fas variants impaired in the ability to bind FADD functioned as dominant negative receptors (DNRs), preventing FasL-induced apoptosis in Fas-competent T cells. T cells co-engineered with a Fas DNR and either a T cell receptor or chimeric antigen receptor exhibited enhanced persistence following ACT, resulting in superior anti-tumor efficacy against established solid and hematologic cancers. Despite increased longevity, Fas DNR-engineered T cells did not undergo aberrant expansion or mediate autoimmunity. Thus, T cell-intrinsic disruption of Fas signaling through genetic engineering represents a potentially universal strategy to enhance ACT efficacy across a broad range of human malignancies.

INTRODUCTION:

Adoptive cell transfer (ACT) using genetically engineered T cells has entered the standard of care for patients with refractory B cell malignancies, including pediatric acute lymphoblastic leukemia (ALL)(1) and adult aggressive B cell lymphomas(2, 3). The efficacy of ACT in hematologic lymphoid malignancies has been consistently observed across clinical trials, regardless of institution, gene vector, or cell composition(4-9). However, responses to adoptive immunotherapy in patients with solid malignancies—collectively the leading cause of adult cancer-related deaths(10)—have been comparatively modest(11-15). Additionally, relapse is increasingly being recognized as a major clinical challenge following ACT for hematologic malignancies despite initially high overall response rates(1, 8). New strategies which enhance the potency of transferred T cells without increasing toxicity are therefore urgently needed if cell therapy is to serve a broader role in the treatment of human cancers(16).

Multiple variables may influence the success or failure of transferred T cells to mediate cancer regression in patients whose tumor cells uniformly express a target antigen(17). These can include the state of T cell differentiation(18) and local immune-suppressive factors present within the tumor-bearing host(19). Despite these complexities, one of the most consistent correlates of response observed in both hematologic(2, 4-6, 8) and solid cancers(11, 15, 20-22) has been the expansion and/or persistence of transferred T cells following infusion. Building upon this observation, we hypothesized that disruption of factors which negatively regulate T cell proliferation and survival might represent potentially actionable pathways to enhance adoptive immunotherapies. Several clinical trials have tested whether cell-extrinsic approaches can improve the persistence of adoptively transferred T cells, including co-administration of an immune-checkpoint inhibitor(23, 24). However, these agents may not always efficiently enter the solid tumor microenvironment(25) and can cause non-specific immune activation resulting in

systemic toxicities that do not contribute to efficacy(26). We therefore pursued a cell-intrinsic strategy to enhance the function of tumor-specific T cells, thereby containing the risk of systemic toxicities and taking full advantage of the ability to reliably genetically engineer human T cells for clinical applications.

Using a pan-cancer analysis to identify candidate ligands which might limit the ability of T cells to expand and persist within the tumor-bearing host, we discovered that the canonical apoptosis-inducing ligand *FASLG* is preferentially expressed in the majority of human tumor-microenvironments (TMEs). Further, we found that most therapeutic T cells used for adoptive immunotherapy for both hematologic and solid cancers constitutively express Fas, the cognate receptor for FasL. Based on these findings, we developed a series of Fas dominant negative receptors (DNRs) which function in both primary mouse and human T cells to prevent FasL-induced apoptosis. Adoptively transferred, Fas DNR-engineered T cells showed enhanced T cell persistence and anti-tumor immunity when co-engineered with either a T cell receptor (TCR) or chimeric antigen receptor (CAR) for the treatment of a solid or liquid cancer, respectively. Despite causing enhanced T cell persistence, this approach did not lead to uncontrolled T cell lymphoproliferation or cause off-target autoimmunity. Collectively, these results provide a potentially universal strategy to enhance the durability and survivability of adoptively transferred T cells for the treatment of a wide range of human malignancies following ACT.

RESULTS:

Human TMEs overexpress the death-inducing ligand *FASLG*

Across human ACT clinical trials for both hematologic and solid cancers, in vivo T cell expansion and persistence have positively correlated with clinical responses(4-6, 11, 21). These observations led us to hypothesize that disruption of pathways that impair T cell proliferation and survival might represent exploitable targets for improving outcomes following adoptive transfer. To determine whether ligands that negatively modulate T cell proliferation and survival are enriched within human TMEs, we compared RNA-sequencing data using tumor-containing samples from the TCGA database (<https://cancergenome.nih.gov/>) relative to matched normal tissues of origin. Given recent evidence that tissues adjacent to resected tumors possess an inflamed transcriptomic profile reflective of an intermediate state between transformed and non-transformed tissues(27), we used expression data from the Genotype-Tissue Expression (GTEx) database(28) as a normal control. In total, we analyzed 9,330 samples obtained from 26 different cancer types for which an appropriate matched tissue of origin was available (**Supplemental Table 1**). Raw data from each dataset was extracted and normalized in an identical fashion using the RNA-Seq by Expectation Maximization (RSEM) method(29).

We discovered that expression of *FASLG*, the gene encoding the canonical inducer of cellular apoptosis FasL (CD178), was overexpressed in the majority of evaluated cancer types relative to normal tissues (**Figure 1A**). This included both immunotherapy responsive cancers, such as cutaneous melanoma (SKCM), renal clear cell carcinoma (KIRC), lung adenocarcinoma (LUAD), and gastro-esophageal carcinomas (STAD/ESCA); as well as cancers relatively recalcitrant to current immunotherapies, such as breast carcinoma (BRCA), colorectal adenocarcinoma (READ/COAD), glioblastoma multiforme (GBM), ovarian cancer (OV), pancreatic adenocarcinoma (PAAD), and prostate adenocarcinoma (PRAD). In total, we

discovered that 73% (19/26) of the human tumor types evaluated exhibited significantly higher expression of *FASLG* within the tumor mass relative to normal tissue controls ($P < 0.05$ to $P < 0.001$; Mann-Whitney t test, Bonferroni-corrected). By contrast, only 19% (5/26) of cancer types did not exhibit significant differential expression and only a minority (8%; 2/26) showed evidence of reduced *FASLG* expression in tumor samples versus normal tissue.

To gain greater insight into the nature of *FASLG* expression within human TMEs, we performed gene-set enrichment analysis (GSEA)(30) using genes positively correlated with *FASLG* across all 26 evaluated cancer types (**Figure 1B**). We found that expression profiles for many immune-related pathways, including NK cell cytotoxicity, antigen processing and presentation, TCR signaling, primary immune deficiency, and apoptosis, were each significantly enriched (nominal P -value < 0.001 , FDR q value < 0.001). Consistent with these findings, examination of the top 200 genes positively correlated with *FASLG* revealed a predominance of markers associated both with lymphocyte activation, such as *IFNG*, *PRF1*, *41BB*, and *ICOS*, and immune counter-regulation, including *PDCD1*, *LAG3*, and *IL10RA* (**Figure 1C** and **Supplemental Table 2**). Taken together, these data indicated that a death-inducing ligand which might compromise T cell survival is significantly overexpressed in the majority of human cancer microenvironments and is highly correlated to expression signatures of immune activation and regulation.

We next sought to determine whether Fas (CD95), the cognate receptor for FasL, is expressed on the surface of T cells used for clinical adoptive immunotherapy. Previously, we and others found that Fas is expressed on all non-naïve human T cell subsets from healthy donors (HD), including central memory (T_{CM}), effector memory (T_{EM}), and effector memory T cells co-expressing CD45RA (T_{EMRA})(31, 32). Using apheresis products used to generate therapeutic T cells for ACT from patients with melanoma (MEL) or aggressive B cell lymphomas (DLBCL),

we analyzed both the distribution of CD8 α^+ T cell subsets and the frequency of Fas expression on these subsets. Comparison was made to circulating T cells obtained from a group of age-matched HDs. Consistent with previous reports, we found high expression of Fas on the T_{CM}, T_{EM}, and T_{EMRA} subsets (**Figure 1, D and E**). Additionally, we discovered that the frequency of Fas⁺ naïve CD8 α^+ T cells (T_N) in cancer patients is significantly lower than found in HDs (**Figure 1F**). The depletion of T_N cells likely reflected the influence of prior immune-stimulating and lymphodepleting therapies in the cancer patients analyzed(6, 33, 34). Thus, a significant proportion of human T cells used for ACT express a known death receptor and will likely encounter its cognate ligand abundantly expressed in TMEs.

Fas dominant negative receptors prevent T cells from undergoing FasL-mediated apoptosis

Our findings indicated that patient-derived T cells used for adoptive immunotherapy are skewed towards Fas-expressing subsets, which are subsequently transferred into *FASLG*-enriched TMEs. Based on these data, we next investigated whether disruption of Fas signaling within adoptively transferred T cells might prevent their apoptosis and improve in vivo persistence. In addition to triggering T cell apoptosis, FasL is also an essential effector molecule for T cell-mediated tumor killing(35). Further, systemic administration of either an anti-FasL antibody or Fas-Fc fusion protein might induce toxicities, including development of a lymphoproliferative syndrome and accumulation of an abnormal population of double-negative (DN) CD3⁺B220⁺CD4⁺CD8⁺TCR α/β ⁺ lymphocytes(36, 37). For these reasons, we pursued a cell-intrinsic genetic engineering strategy to disable Fas signaling only within tumor-reactive T cells to maintain anti-tumor potency and minimize the risk of systemic toxicity.

Physiologically, FasL initiates apoptotic signaling by first inducing oligomerization of Fas receptors into trimers or larger oligomers at the cell membrane (**Figure 2A**)(38). Fas

oligomers recruit the intracellular adaptor molecule Fas-associated via death domain (FADD) through homotypic death domains (DD) present in each molecule(39, 40). Aggregation of FADD recruits the cysteine-aspartic acid protease pro-Caspase 8(41) through homologous death effector domains in each molecule, forming the death inducing signaling complex that can initiate the apoptotic signaling cascade(42). Based on this mechanism of action, we hypothesized that overexpression of mutated Fas variants genetically altered to prevent FADD binding would function as dominant negative receptors (DNRs) when expressed in Fas-competent wild type (WT) T cells used for adoptive immunotherapy. Presently, virus-based constructs are the most commonly used methods to stably modify human T cells for clinical application(43). We created a series of retroviral constructs encoding the murine Fas sequence in which either an asparagine residue was substituted for an isoleucine at position 246 of the DD (Fas^{I246N}; a naturally occurring mutant of murine Fas which is unable to bind FADD(44)), or a Fas mutant in which the majority of the intracellular DD was truncated (del aa222-306; Fas^{ADD}) to prevent FADD binding (**Supplemental Figure 1A** and **Figure 2A**). As controls, we generated both an empty vector construct as well as a construct encoding the complete WT sequence of Fas (Fas^{WT}). To identify transduced cells, all vectors contained a Thy1.1 reporter separated from Fas using a T2A “self-cleavage” sequence.

T cells were isolated from Fas-competent WT mice, activated by anti-CD3/CD28 antibodies in the presence of IL-2, and transduced with the empty, Fas^{WT}, Fas^{I246N}, or Fas^{ADD} constructs (**Figure 2B**). Phenotypic analysis 6d following activation and transduction revealed high transduction efficiencies for all constructs as measured by Thy1.1 reporter expression (**Supplemental Figure 1, B and C**). Notably, ectopic Fas expression was measurably higher than endogenous levels of Fas expression for constructs containing either the WT (6.8-fold higher Fas

MFI) or mutant Fas variants (43-fold and 98-fold higher Fas MFI for Fas^{I246N} or Fas^{ADD}, respectively; **Supplemental Figure 1, B and D**).

After 6d in culture, transduced T cells were stimulated with recombinant FasL molecules oligomerized through a leucine zipper domain (lz-FasL) to mimic the function of membrane-bound FasL(45) or left untreated as controls. In the absence of lz-FasL, T cells transduced with each of the constructs remained similarly viable (**Figure 2C**). However, following exposure to lz-FasL, a significant proportion of Thy1.1⁺ T cells transduced either with the empty vector control or Fas^{WT} converted to an apoptotic Annexin V⁺PI⁺ population (**Figure 2, C and D**; $P<0.001$). Interestingly, overexpression of Fas^{WT} consistently resulted in higher levels of apoptosis relative to empty vector-transduced T cells, indicating that expression of Fas above physiologic levels further sensitized T cells to FasL-mediated cell death. By contrast, T cells transduced either with the Fas^{I246N} or Fas^{ADD} vectors were almost completely protected from lz-FasL-induced apoptosis. Among pools of T cells transduced with Fas^{I246N} or Fas^{ADD}, protection from apoptosis was most evident in the transduced Thy1.1⁺ populations. However, we also noted a relative increase in the viability of non-transduced Thy1.1⁻ T cells in cultures containing cells transduced with Fas^{I246N} or Fas^{ADD} (**Supplemental Figure 2**). This suggested that Fas^{I246N} and Fas^{ADD} may also protect neighboring T cells from apoptosis, likely by functioning as a “sink” for local FasL.

In T cells modified with Fas^{I246N}, we found neither functional nor genetic evidence of reversion to the WT sequence. We measured selective enrichment for T cells modified with Fas^{I246N} compared with Fas^{WT} following serial in vitro re-stimulations, indicating that the DNR remained functionally intact over time (**Supplemental Figure 3, A and B**). Further, Sanger sequencing of serially re-stimulated, Fas^{I246N}-transduced T cells showed no evidence of reversion of the I246N point mutation to the WT Fas sequence (**Supplemental Figure 3, C and D**). Thus,

overexpression of Fas variants disabled in their ability to bind FADD function in a dominant negative manner to prevent FasL-mediated apoptosis in WT T cells.

Finally, we sought to ascertain whether the Fas DNRs afforded protection from other apoptosis-inducing stimuli which adoptively transferred T cells might encounter in vivo. These include activation induced cell death (AICD), cytokine withdrawal, and proximity to tumor cells. For these assays, we utilized pmel-1 T cells specific for the cancer antigen gp100 and B16 melanoma engineered to express human gp100 (B16). Although B16 cells did not express FasL at rest, FasL expression was measurably upregulated following incubation with IFN γ (**Supplemental Figure 4**). We found that pmel-1 T cells transduced with Fas^{I246N} or Fas^{ADD} provided equal protection from apoptosis triggered by either lz-FasL or tumor co-culture (**Supplemental Figure 5**). By contrast, T cells transduced with Fas^{ADD} resulted in significantly greater cell viability following AICD induction through anti-CD3/CD28 re-stimulation or acute cytokine withdrawal relative to cells modified with Fas^{I246N}. These findings were potentially attributable to the ability of the Fas^{I246N} variant to bind to FADD with reduced efficiency under certain conditions(46). We therefore subsequently focused exclusively on the Fas^{ADD} DNR for all in vivo experiments given its superior functional attributes. This permitted us to more clearly determine the influence of removing Fas signaling on the in vivo function of adoptively transferred T cells.

Adoptive transfer of T cells engineered with Fas DNR results in superior persistence

Having established that modification with Fas^{ADD} prevented T cell apoptosis in vitro across a diverse range of apoptosis-inducing stimuli, we sought next to determine whether engineering with this Fas DNR resulted in superior in vivo persistence following adoptive transfer. We therefore adoptively transferred congenically marked, gene-modified pmel-1 T cells

into sublethally irradiated Thy1.1⁻ C57BL/6 (B6) mice to induce homeostatic proliferation and measured the expansion and persistence of transferred cells over time. T cells transduced with Fas^{ADD} or empty vector control were identified by expression of the Thy1.1 reporter gene. To measure T cell proliferation, we co-stained for the cellular proliferation marker Ki67.

One day after transfer, we found that Fas^{ADD}- and empty vector-modified pmel-1 T cells engrafted at similar levels and almost uniformly expressed Ki67 (**Figure 3, A-C**). Beginning within three days of transfer, we measured a multi-log expansion of both populations of modified cells. However, at the peak of expansion, we noted a ~50-fold greater increase in the numbers of Fas^{ADD}-modified T cells relative to control-modified cells. This in turn led to a >10-fold higher level of persistence of Fas DNR-modified T cells at d30 (**Figure 3, A and B**). Over time, we measured a comparable reduction in Ki67 expression on both engineered T cell populations (**Figure 3C**) which correlated with reconstitution of the host's endogenous T cell compartment. These data suggested that the in vivo proliferation was comparable between the two engineered T cell populations. However, Fas DNR-modified T cells demonstrated superior overall expansion and intermediate-term persistence, likely through a reduction in apoptosis.

We next sought to ascertain if genetic modification with the Fas DNR resulted in superior T cell persistence within the TME. To ensure that modified T cells were exposed to the same microenvironmental factors within any given tumor, we performed a co-infusion experiment. Congenically distinguishable pmel-1 T cells were obtained from either a Ly5.1⁻/Thy1.1⁻ or Ly5.1⁺/Thy1.1⁻ background. Cells were transduced with the Fas^{ADD} DNR or a Thy1.1-expressing empty vector control, respectively. Transduced T cells were subsequently purified using anti-Thy1.1 microbeads, recombined in a roughly 1:1 ratio, then co-infused into sublethally irradiated Ly5.1⁻/Thy1.1⁻ mice bearing 10d established B16 melanoma tumors (**Figure 3D**). As is currently done in many ACT clinical trials for solid tumors, treated mice received a limited course of IL-2

following transfer(14, 15, 20, 47, 48). Seven days following infusion, both the spleens and tumors of recipient mice were harvested and analyzed for the presence of adoptively transferred, genetically modified, Thy1.1⁺ pmel-1 T cells. We consistently found significant enrichment of Ly5.1⁺ Thy1.1⁺ Fas^{ADD}-modified T cells relative to Ly5.1⁻ Thy1.1⁺ empty vector-modified T cells in both the spleen and tumor of recipient mice (**Figure 3E**; $P<0.001$). Together, these results indicated that genetic engineering with a Fas DNR enhanced T cell persistence in vivo in both the spleen and TME following adoptive cell transfer.

ACT of Fas DNR-modified T cells does not result in an acquired ALPS phenotype

Mice and humans with germline defects in components of apoptotic signaling can develop profound alterations in normal lymphocyte homeostasis and development. These abnormalities, collectively referred to as autoimmune lymphoproliferative syndrome (ALPS), include the accumulation of an aberrant DN lymphocyte population, the development of auto-antibodies, and impaired survival (49, 50). Given the potential safety concerns related to disabling normal Fas signaling in mature T cells, we performed long-term immune-monitoring of animals that received Fas^{ADD} DNR-modified T cells more than 6 months prior (**Figure 4A**). This time point was chosen as mice with germline defects in Fas typically develop overt clinical manifestations within the first 3.5-5 months of life, depending on the background strain(51, 52). Using unmanipulated B6 WT and Fas-deficient lpr (B6-lpr) mice as respective negative and positive controls for the ALPS phenotype, we assessed the frequency of CD3⁺B220⁺ lymphocytes in the spleens of mice who had previously received ACT of Thy1.1⁺Vβ13⁺ pmel-1 T cells modified with the Fas^{ADD} DNR or empty vector control. As expected, the spleens of B6-lpr mice exhibited a significant accumulation of abnormal CD3⁺B220⁺ lymphocytes relative to WT controls (**Figure 4, B and C**; $P<0.05$, $P<0.001$). By contrast, neither of the mice receiving T

cells modified with the empty vector or Fas DNR exhibited a significant increase in this population. To exclude the possibility of transformation of the modified T cell population, we assessed the long-term persistence and phenotype of transferred V β 13⁺Thy1.1⁺ engineered T cells. Consistent with our findings at intermediate time points, we found that T cells engineered with Fas^{ADD} DNR persisted at higher numbers than cells modified with the empty vector after >200d (**Figure 4, D and E**; $P<0.05$). Long-term persisting Fas DNR-modified T cells maintained a conventional CD3⁺B220⁻ phenotype. These data indicated that adoptively transferred pmel-1 T cells expressing the Fas DNR did not undergo abnormal lymphoproliferation in B6 hosts.

It was previously shown that expression of a transgenic TCR crossed to a Fas-deficient *lpr* background can limit the development of ALPS(53). Additionally, the B6 strain manifests lymphoproliferative symptoms at a slower rate compared with other strains(51, 52, 54). We therefore performed additional experiments to assess the safety of the Fas^{ADD} DNR modification by adoptively transferring an open T cell repertoire genetically engineered with either Fas DNR or empty control into the ALPS-susceptible MRL-Mp strain. Fas-deficient mice on an MRL background (MRL-*lpr* mouse) develop autoantibodies, nephritis, and splenomegaly more severely and many months earlier than B6-*lpr* mice (**Supplemental Figure 6A**)(51, 52, 54).

To induce activation and expansion of adoptively transferred T cells in this model, we co-transduced open repertoire T cells from the MRL-Mp mouse with a previously described second generation anti-CD19 28 ζ chimeric antigen receptor (CAR)(55) and the Fas^{ADD} or control vector. Use of the anti-CD19 CAR in these experiments promoted strong *in vivo* proliferation of T cells through recognition of host CD19⁺ B cells. Of note, recently published data indicate that T cells modified with a CAR are still able to undergo stimulation through their TCR(56, 57).

We analyzed the spleens of MRL-Mp mice that received no cells (PBS), or anti-CD19 CAR⁺ T cells transduced with Fas^{ADD} or empty control and compared these to the spleens of age-

matched Fas-deficient MRL-lpr mice (**Supplemental Figure 6C**). We found that spleens from the age-matched MRL-lpr mice were significantly greater in weight when compared to all other treatment groups. Importantly, we measured no difference in spleen sizes between PBS treated mice and mice that received anti-CD19 CAR-transduced cells modified either with the Fas^{ADD} or control. Flow cytometry analysis of splenocytes demonstrated a robust expansion of unusual double-negative, CD3⁺B220⁺ lymphocytes in the spleens of MRL-lpr mice which collectively accounted for >30% of all lymphocytes (**Supplemental Figure 6, D and E**). By contrast, the frequency of CD3⁺B220⁺ lymphocytes in the empty vector and Fas^{ADD} T cell-treated mice were similar to levels observed in the PBS control mice.

To assess for the development of autoimmunity, we performed serum analysis of all treated animals using samples from MRL-lpr mice as a positive control. We found that mice which received anti-CD19 CAR⁺ T cells modified with Fas^{ADD} or empty vector had low anti-nuclear and anti-dsDNA antibody titers comparable to the PBS control (**Supplemental Figure 6F**). In contrast, serum from the MRL-lpr positive control mice demonstrated high titers of both types of auto-antibodies.

In the absence of uncontrolled lymphoproliferation and the formation of auto-antibodies, we found that anti-CD19 CAR⁺ T cells co-transduced with Fas DNR persisted at significantly higher levels in the spleens of recipient MRL-Mp mice compared with control modified anti-CD19 CAR⁺ T cells (**Supplemental Figure 6G**). Further, the persistent Fas DNR-modified CAR⁺ T cells did not acquire a greater proportion of aberrant CD3⁺B220⁺ cells compared with control modified CAR⁺ cells (**Supplemental Figure 6H**). These results directly mirrored our findings using Fas^{ADD}-modified pmel-1 T cells transferred into B6 hosts (**Figure 4, D and E**).

Finally, to assess whether the ALPS-susceptible MRL-Mp recipient mice developed lung pathology following adoptive transfer of Fas DNR-modified T cells, we performed a blinded

pathologic assessment of H&E stained lung specimens. Consistent with previous reports(36), we found that the Fas-deficient MRL-lpr mice developed a dense mononuclear cell inflammatory lung infiltrate in the perivascular and peribronchiolar regions (**Supplemental Figure 7, A and B**). By contrast, mice treated with Fas^{ADD}- or control-modified T cells did not display evidence of an increased inflammatory infiltrate relative to PBS-treated control injection. Further, we observed no evidence of pulmonary fibrosis.

Together, these data in both the B6 and MRL-Mp strains demonstrate that despite the augmented survival of the Fas^{ADD} DNR T cells, we measured no evidence of uncontrolled lymphoaccumulation, formation of a Thy1.1⁺CD3⁺B220⁺ population, or evidence of autoimmunity. Based on these data, we conclude that infusion of mature T cells impaired in Fas signaling does not result in an acquired lymphoproliferative phenotype.

T cell-intrinsic disruption of Fas signaling enhances anti-tumor efficacy following ACT

Having established that adoptively transferred T cells engineered with a Fas DNR results in enhanced persistence without long-term toxicity, we next evaluated the anti-tumor efficacy of these cells. We recently discovered that Fas stimulation can induce non-apoptotic Akt/mTOR-signaling, resulting in augmented T cell differentiation(58-60). Consistent with our previous results, we found that exposure to lz-FasL caused a dose-dependent increase in phosphorylated (p) Akt^{S473} and pS6^{S235,S236} in T cells transduced with an empty vector control (**Supplemental Figure 8, A and B**). Expansion of control-modified cells resulted in an accumulation of T_{EM}-like cells with a reduced capacity to produce IL-2 (**Supplemental Figure 8, C and D**). By contrast, T cells transduced with a Fas DNR did not increase Akt or S6 phosphorylation following lz-FasL stimulation and were protected from augmented Akt-mediated T cell differentiation. Consequently, Fas DNR-modified T cells retained a predominantly T_{CM}-like phenotype and the

capacity to produce IL-2. In several different animal models(32, 61-64) and human clinical trials(11, 65), transfer of T_{CM}-like cells was associated with superior tumor regression compared to transfer of T_{EM}-like cells.

To control for the variable of T cell differentiation status, pmel-1 T cells transduced with Fas^{ADD} or an empty vector were enriched to >96% purity for T_{CM}-like cells by FACS sorting for Thy1.1⁺CD44^{high}CD62L⁺ cells immediately prior to cell infusion (**Figure 5A**). Purified T_{CM}-like cells were subsequently transferred into sublethally irradiated B6 mice bearing established B16 tumors as described in **Figure 3C**. Treated mice also received IL-2 by i.p. injection. We found that adoptive transfer of tumor-specific T_{CM} cells modified with the Fas DNR resulted in superior tumor regression and animal survival compared with control-modified T cells (**Figure 5, B and C**; $P<0.05$).

We next sought to extend our treatment findings using an independent tumor model in which a hematologic malignancy was targeted with a CAR. We utilized a recently developed, syngeneic B-cell ALL (B-ALL) line driven by the physiologically relevant E2a-PBX translocation in a treatment model using a murine second generation 28 ζ anti-CD19 CAR(57, 66). We specifically chose a syngeneic model over the more commonly used xenogeneic anti-CD19 CAR treatment models for two reasons. First, we wished to ensure that the transferred T cells were fully responsive to host-derived FasL in addition to FasL expression by tumor cells and the adoptively transferred T cells. Second, we wished to avoid the potentially confounding influence of xenogeneic reactivity on AICD induction in the transferred T cells.

We first established that dual transduction of B6 CD8 α ⁺ T cells with retroviral constructs containing the Fas^{ADD} or empty vector and anti-CD19 CAR was feasible (**Figure 6, A and B**). Using Protein L to identify CAR-transduced T cells(67), we found that co-transduction efficiencies were similarly efficient using Fas^{ADD} and the empty vector control following Thy1.1-

bead enrichment. Next, we measured how the co-transduced anti-CD19 CAR T cells responded to various apoptosis-inducing stimuli, including exogenous FasL, cytokine withdrawal, AICD, and exposure to antigen-expressing B-ALL tumor cells (**Figure 6C**). Similar to our results using TCR-expressing pmel-1 T cells, we found that the expression of Fas^{ADD} protected CAR-modified T cells from each of these death-inducing stimuli relative to empty vector control-transduced CAR⁺ T cells.

We next tested if co-expression of a Fas DNR in CAR-modified T cells resulted in higher persistence and superior anti-tumor efficacy as we observed in the pmel-1 TCR transgenic/B16 melanoma model. We infused Fas^{ADD} or empty Thy1.1⁺ anti-CD19 CAR⁺ CD8 α ⁺ T cells into sublethally irradiated B6 mice bearing 4d established E2a-PBX B-ALL (**Figure 6D**). Treated mice received daily IL-2 injections for 3d to support expansion of the adoptively transferred T cells. Fourteen days following cell infusion, we analyzed the spleens and bone marrow, two sites of disease for the E2a-PBX B-ALL, for persistence of the adoptively transferred cells. We measured higher levels of Thy1.1⁺ Fas^{ADD} cells in both disease sites in comparison to mice that received empty vector-transduced T cells (**Figure 6E**). The E2a-PBX leukemia expresses classic pre-B-ALL markers, including CD19, B220 and CD93(66). As seen in **Figure 6F**, we found that the bone marrow in untreated (PBS) and empty vector treated mice contained roughly 70% leukemia cells 14d after T cell treatment. However, the mice that received Fas^{ADD}-modified cells contained less than 1% leukemia cells in the bone marrow. This data indicated that CAR⁺ T cells expressing the Fas DNR cells were able to mediate superior leukemia clearance relative to empty vector-transduced T cells.

Finally, we analyzed the survival of leukemia-bearing mice after adoptive transfer of two different doses of second generation 28 ζ anti-CD19 CAR-transduced T cells co-modified with Fas^{ADD} or empty control. In order to provide for a treatment window, we intentionally transferred

doses of CAR-modified T cells previously shown to be subtherapeutic in this model(57). At a relatively higher cell dose (3×10^5 CAR⁺ cells), we found that adoptive transfer of either control- or Fas^{ADD}-modified CAR⁺ T cells resulted in significantly improved animal survival compared with mice which did not receive treatment (**Figure 6G**, left). However, whereas all mice who received the Fas DNR-modified CAR⁺ T cells survived, mice that received control-modified CAR⁺ T cells did not survive longer than 55d. At a further de-escalated dose of CAR⁺ cells (2×10^5), Fas DNR-modified T cells continued to provide long-term survival in 100% of treated mice while control-modified T cells entirely lost efficacy (**Figure 6G**, right). Previous reports have demonstrated that 4-1BB-containing second generation CARs express higher levels of anti-apoptotic proteins compared with CARs containing a CD28 domain(68). We did not test in these experiments whether the Fas DNR could also enhance the function of a 4-1BB-containing CAR. Nevertheless, these data in the solid cancer B16 melanoma and hematologic E2a-PBX leukemia models indicate that Fas DNR expression in adoptively transferred T cells results in superior in vivo cellular persistence and anti-tumor efficacy regardless of whether the antigen targeting structure is a TCR or 28 ζ CAR.

Genetic engineering with Fas DNRs protects human T cells from Fas-mediated apoptosis

To determine the feasibility of engineering primary human T cells with Fas DNRs, we designed retroviral constructs encoding the human Fas sequence mutated to prevent FADD binding. This included a human Fas variant containing a point mutation substituting a valine for an aspartate residue at position 244 (hFas^{D244V})(69, 70), and human Fas with the majority of the intracellular death domain truncated (del aa 230-314; hFas^{ADD}) (**Figure 7A**)(69, 70). CD8⁺ T cells were isolated from HD peripheral blood mononuclear cells (PBMC) and stimulated with anti-CD3/CD28 and IL-2, followed by transduction with hFas^{D244V}, hFas^{ADD}, or an empty vector

control containing a Thy1.1 reporter (**Figure 7B**). In the absence of additional stimulation, both untransduced Thy1.1⁻ and transduced Thy1.1⁺ T cells remained similarly viable as measured by Annexin V and PI staining (**Figure 7C**). However, when these cells were cultured in the presence of increasing doses of lz-FasL, we found that T cells transduced with the empty vector exhibited a significant and dose-dependent increase in the frequency of Annexin V⁺ apoptotic and necrotic cells (**Figure 7, C and D**). By contrast, T cells modified with either hFas^{D244V} or hFas^{ADD} were significantly protected from lz-FasL-mediated apoptosis. This protection was predominantly T cell intrinsic as non-transduced Thy1.1⁻ cells exhibited significantly higher frequency of Annexin V⁺ cells relative to Thy1.1⁺ T cells transduced with hFas^{D244V} or hFas^{ADD}. Thus, we conclude that genetic engineering with a Fas DNR protects primary human T cells from FasL-induced cell death, providing a new method to potentially protect adoptively transferred T cells within the human TME.

DISCUSSION:

Herein, we report the results of a pan-cancer analysis which suggested that a canonical death-inducing ligand, *FASLG*, is overexpressed within the majority of human cancer microenvironments. We further discovered that a significant proportion of human T cells used for adoptive immunotherapy express Fas, the cognate receptor for FasL. Based on these findings, we developed a novel cell-intrinsic strategy to ‘insulate’ Fas-competent mouse and human T cells from FasL-induced apoptosis using genetic engineering with a series of Fas DNRs. Functionally, adoptively transferred Fas DNR-modified T cells exhibited superior persistence in both the periphery and tumors of tumor-bearing animals, resulting in superior tumor regression and overall survival in both solid and liquid syngeneic cancer models. Importantly, while T cells modified with Fas DNR exhibited enhanced survival relative to control-modified T cells as late as 6 months following transfer, we detected no evidence of uncontrolled lymphoproliferation or autoimmunity. These findings therefore provide a novel, potentially universal gene engineering strategy to enhance the function of adoptively transferred T cells against a broad range of human malignancies, including both solid and hematologic cancers.

We previously reported that in addition to its canonical apoptosis-inducing functions, Fas can also promote mouse and human T cell differentiation in an Akt-dependent manner(58, 59). Consistent with these findings, we discovered that T cells transduced with Fas DNRs were protected from lz-FasL-mediated induction of pAkt^{S473} and pS6^{S235,S236}. Consequently, this block in Akt/mTOR signaling minimized T cell differentiation, promoting the accumulation of T_{CM}-like cells which retained expression of the lymphoid homing marker CD62L and the capacity to produce IL-2. In multiple pre-clinical models(32, 61, 62, 64) and in retrospective analyses of human clinical trials(11, 65), infusion of T_{CM}-like cells was associated with superior anti-tumor outcomes compared with T_{EM}-like cells. We therefore compared the anti-tumor efficacy of

phenotypically matched, FACS-sorted, T_{CM}-like cells modified with a Fas DNR or an empty vector control. We found that Fas DNR-modified T_{CM} exhibited superior treatment efficacy compared with control-modified T_{CM}. Mechanistically, we conclude that the dominant contributor of the enhanced in vivo anti-tumor efficacy we found using Fas DNR-modified T cells was attributable to the disruption of cell death and not the infusion of less differentiated cells. These findings are also consistent with recent papers from Zhu et al., Horton et al., and Lakins et al. demonstrating that FasL-induced apoptosis of tumor infiltrating lymphocytes limits the efficacy of immune checkpoint inhibitors(19, 71, 72).

While our analyses indicated that *FASLG* expression is enriched within the TMEs of many human tumors, they do not define which specific cell type is expressing the ligand. Using immunohistochemical protein staining, previous studies have identified that FasL can be expressed directly on the surface of many of the solid cancers identified in our pan-cancer analysis. This includes cancers of the breast, colon, brain, kidney, and cervix(73, 74). Additionally, recent studies have also identified that FasL is expressed along the luminal surface of the neovasculature surrounding human ovarian and brain cancers, creating a tumor endothelial death barrier limiting T cell infiltration(73, 75). Further, it is possible that FasL can be expressed within the TME by cells of both the innate and adaptive immune system. This possibility has previously been shown by others(19) and is further suggested by our own analysis demonstrating a high degree of correlation between *FASLG* and many immune-related genes. Finally, our functional data demonstrate that Fas DNR modification also affords protection from other apoptosis-inducing stimuli a T cell might experience following adoptive cell transfer into a tumor-bearing host. These include AICD, cytokine withdrawal, and proximity to antigen-expressing tumor cells. Collectively, these data suggest that the Fas DNR can enhance T cell

survival to a broad range of potential FasL sources, including tumor cells, non-transformed cells in the TME, as well as T cells themselves.

Fas DNR now joins a list of other candidate DNRs with which a T cell might be modified to intrinsically disrupt signaling by immune-suppressive factors present within the TME, including TGF β (76) and PD1(77). Disruption of Fas using a short hairpin RNA approach has been reported in human T cells in vitro(78); however, due to the relatively poor efficiency of Fas knockdown, this approach required lengthy in vitro selection. Furthermore, the in vivo anti-tumor capacity of these cells was not tested. Germline loss of function in Fas signaling can result in an autoimmune lymphoproliferative disease in both mice and humans, a potential safety consideration for the Fas DNR approach. Despite augmented survival of Fas^{ADD}-modified T cells, we found no evidence of uncontrolled lymphoaccumulation, formation of an aberrant CD3⁺B220⁺ lymphocyte population, or evidence of autoimmunity using two different mouse strains. This included performing adoptive transfer of a polyclonal T cell population into the ALPS-prone MRL-Mp strain. Based on these data, we conclude that infusion of mature T cells impaired in Fas signaling is unlikely to result in an acquired lymphoproliferation syndrome.

Although Fas is a critical mediator for initiating the extrinsic apoptotic signaling cascade, intrinsic apoptotic pathways remain intact in our cells. Thus, competition for homeostatic cytokines, neglect due to an absence of antigen, and T cell exhaustion may all contribute to regulating the homeostasis of Fas DNR cells in vivo. Despite these reassuring safety data in mice, refinement of this approach for clinical application will include the introduction of a suicide mechanism, such as a truncated EGFR upstream of the Fas DNR(79, 80) or an inducible caspase 9 variant(81).

In conclusion, we have discovered that the FasL/Fas pathway is poised to be activated in many patients receiving adoptive immunotherapy for the treatment of human cancers. We have

developed novel dominant negative receptors which intrinsically abrogate the apoptosis-inducing functions of this pathway in primary mouse and human T cells. This engineered resistance to Fas-mediated apoptosis in turn led to enhanced in vivo cellular persistence and augmented anti-tumor efficacy in two different syngeneic models for the treatment of a B16 melanoma and a B-ALL using TCR- and CAR-modified T cells, respectively. These data lay the groundwork for a potential universal strategy to enhance the potency of adoptive immunotherapies against both solid and hematologic cancers.

METHODS:

The Cancer Genome Atlas (TCGA) pan-cancer bioinformatics analysis: RNA-sequencing (RNA-seq) data from 26 human cancers from the TCGA dataset and matched normal tissues from the GTEx dataset were collected and analyzed by UCSC Xena(82) in the form of normalized RNA-seq by Expectation-Maximization (RSEM) values. *FASLG* gene expression as normalized RSEM counts was analyzed in each. Statistics were corrected by Mann-Whitney *t* test. To identify genes positively correlated to *FASLG* expression, we ran a pre-ranked gene set enrichment against all KEGG pathways in the mSigDB database. Pearson's correlation was performed on the top 1000 genes positively correlated to *FASLG* expression averaged across 26 TCGA histologies.

Human Specimens: PBMC were obtained from melanoma and diffuse large B cell lymphoma patients enrolled on an adoptive immunotherapy clinical protocol, or from age- and sex-matched healthy donors.

Mice: Adult 6-12-week-old male or female C57BL/6 NCR (B6; Ly5.2⁺) mice were purchased from Charles River Laboratories at NCI Frederick. B6.SJL-*Ptprc*^a *Pepc*^b/BoyJ (Ly5.1⁺), B6.129S7-*Rag1*^l*l*^m/*Mom*/J (Rag), B6.MRL-*Fas*^{lpr}/J (B6-lpr), B6.Cg-*Thy1*^a/Cy Tg(*TcrαTcrβ*)8Rest/J (pmel-1(83)), MRL/MpJ (MRL-Mp), and MRL/MpJ-*Fas*^{lpr}/J (MRL-lpr) mice were purchased from Jackson Laboratory. Where indicated, pmel-1 mice were crossed to a Ly5.1 or Rag background. All mice were maintained under specific pathogen-free conditions.

Retroviral vectors and transduction of murine and human CD8⁺ T cells: Murine and human Fas cDNA sequences were synthesized and separately cloned (Genscript) into the MSGV

retroviral plasmid preceding a T2A ‘self-cleavage’ sequence and selectable marker Thy1.1. Murine T cell transductions were performed as previously described(84). Briefly, Platinum-E ecotropic packaging cells (Cell BioLabs) were plated on BioCoat 10cm dishes (Corning) overnight. The following day, 24 µg of retroviral plasmid DNA encoding MSGV-Thy1.1 (Empty), MSGV-WT-mFas-Thy1.1 (mWT), MSGV-I246N-mFas-Thy1.1 (Fas^{I246N}), MSGV-ΔDD-mFas-Thy1.1 (Fas^{ΔDD}), or MSGV-1D3-28Z (anti-CD19 CAR(55)) were separately mixed with 6 µg of pCL-Eco plasmid DNA along with 60 µL of Lipofectamine 2000 (ThermoFisher) in OptiMEM, then applied to the Platinum-E cells for 7h in antibiotic-free 10% FBS-containing medium. Medium was replaced after 7h; viral supernatant was collected from the cells after 48h and centrifuged to remove debris. Retroviral supernatants were spun for 2h at 2000xg 32°C on non-TC-treated 24-well plates that had been coated overnight in 20 µg mL⁻¹ Retronectin (Takara Bio). T cells activated for 24h were added to plates that had all but 100 uL of viral supernatant removed, spun for 5m at 1500 rpm at 32°C, then incubated overnight. The transduction was repeated a second time the next day. For human T cell transduction, 293T cells (ATCC)(85) and RD114 were used in place of Platinum-E cells and pCL-Eco, respectively, and proceeded as during the murine virus production described above.

T cell culture and Fas death assay: Human PBMC from healthy donors or patients were obtained either by leukapheresis or venipuncture and centrifuged over Ficoll-Hypaque (Lonza) gradient to remove red blood cells, then washed twice with PBS containing 1 mM EDTA, stained with fixable cell viability dye (ThermoFisher) in PBS, then washed twice with PBS supplemented with 2% FBS and 1 mM EDTA (FACS buffer). Untouched human CD8α⁺ T cells were isolated using a human CD8 isolation kit (Stem Cell Technologies). Murine and human T cells and E2a-PBX leukemia cells(57) were maintained in RPMI 1640 (Gibco) with 10% heat-

inactivated fetal bovine serum (FBS), 1% penicillin/streptomycin (100 U mL^{-1} and $100 \mu\text{g mL}^{-1}$, respectively; Gibco), gentamicin ($10 \mu\text{g mL}^{-1}$), MEM non-essential amino acids (Gibco), sodium pyruvate (1 mM , Gibco), GlutaMAX (2 mM , Gibco), 2-mercaptoethanol (0.011 mM , Gibco) and amphotericin B (250 ng mL^{-1} , Gibco). B16-mhgp100 tumor cells (a kind gift from K. Hanada, Surgery Branch, NCI, Bethesda, MD), Platinum-E cells and 293T cells were maintained in DMEM (Gibco) supplemented with 10% FBS and the above-mentioned additives.

Untouched murine $\text{CD8}\alpha^+$ T cells were isolated from splenocytes using a MACS CD8^+ negative selection kit (Miltenyi Biotec) and stimulated in TC-treated 24-well plates with plate-bound anti-CD3 ($2 \mu\text{g mL}^{-1}$, clone 145-2C11, BD Biosciences), soluble anti-CD28 ($1 \mu\text{g mL}^{-1}$, clone 37-51, BD Biosciences) and rhIL-2 (5 ng mL^{-1} , Promethues). Pmel-1 T cells were stimulated in whole splenocyte cultures with $1 \mu\text{g mL}^{-1}$ human gp100₍₂₅₋₃₃₎ peptide and IL-2 (5 ng mL^{-1}). Human PBMC or $\text{CD8}\alpha^+$ T cells were stimulated with plate-bound anti-CD3 ($1 \mu\text{g mL}^{-1}$, clone OKT3, BD Biosciences), soluble anti-CD28 ($1 \mu\text{g mL}^{-1}$, clone CD28.2, BD Biosciences) for 2d, then given IL-2 (20 ng mL^{-1}) during the remainder of culture. Cells were stimulated for 24h before transduction with viral supernatant on days 1 and 2 of culture. On day 3 cells were removed from Retronectin-coated plates and returned to tissue-culture treated 24-well plates or flasks. Where noted cells were grown either with vehicle or the indicated concentrations of lz-FasL, a recombinant form of oligomerized FasL(45, 59). Five to six days (for murine cells) or 10-11d (for human cells) after stimulation, T cells were washed twice, plated at $1\text{-}2 \times 10^5$ cells/well in a 96-well plate with the indicated concentrations of lz-FasL and incubated at 37°C with 5% CO_2 for 6h or 24h. Cells were then washed twice and stained with either Annexin V and PI or Live/Dead Fixable Dye (ThermoFisher) as well as $\text{CD8}\alpha$ (clone 53-6.7, BD Biosciences) and Thy1.1 (clone HIS51, eBioscience).

Flow cytometry, intracellular cytokine staining and phosphoflow: Cells were stained with fixable cell viability dye in PBS, then washed twice with FACS buffer. Human cells were stained with the following fluorochrome-conjugated antibodies: CD3 (UCHT1), CCR7 (3D12), CD45RA (HI100), CD45RO (UCHL1), CD28 (CD28.2), CD95 (DX2) (BD Biosciences); and CD27 (M-T271), CD62L (DREG-56), CD8 α (SK1), CD4 (OKT4) (BioLegend). Murine T cells, bone marrow and splenocytes were stained with the following antibodies: CD3 (145-2C11), CD8 α (53-6.7), Vb13 (MR12-3), Ly5.1 (A20), Ly5.2 (104), CD62L (MEL-14), CD95 (Jo2), B220 (RA3-6B2), (BD Biosciences); CD44 (IM7), CD19 (6D5), CD93 (AA4.1) (BioLegend); Thy1.1 (HIS51, eBioscience). For anti-CD19 CAR detection(67) Biotin-Protein L (Genscript) was utilized.

For phosphoflow, cells were fixed and permeabilized using the BD Phosflow reagents and following the manufacturer's protocol. After permeabilization cells were stained with pAkt (S473) (D9E) and pS6 (S235/236) (D57.2.2E) from Cell Signaling. For intracellular cytokine staining, cells were stained for surface antibodies in FACS buffer, then fixed and permeabilized (BD Biosciences) and stained for IFN γ (XMG1.2, BD Biosciences) and IL-2 (JES6-5H4, BioLegend). For FasL staining of tumor cells, tumor cells were incubated with vehicle (PBS) or murine IFN γ (100 ng mL⁻¹, BioLegend) for 24h, then stained with FasL (Kay-10) and H-2Db (KH95) (BD Biosciences). All flow cytometric data were acquired using a BD Fortessa flow cytometer (Becton Dickinson) and analyzed using FlowJo v. 9.9 software (TreeStar).

Sanger sequencing analysis: Genomic DNA from Thy1.1-enriched empty- or Fas^{I246N}-transduced cells was extracted using the AllPrep DNR/RNA Mini Kit (Qiagen). Primers (IDT) were designed such that the forward primer was located in Fas upstream of the Fas^{I246N} point

mutation and the reverse primer located in the Thy1.1 reporter. After PCR amplification (Invitrogen) Sanger sequencing was performed by the NCI Sequencing Core Facility.

Adoptive cell transfer, T cell enumeration, and tumor treatment: For analysis of in vivo persistence, female B6 mice aged 6-12 weeks received 6 Gy total body irradiation. One day later, were injected by tail vein injection with 5×10^5 congenically-marked pmel-1 T cells transduced with a Thy1.1-containing reporter construct. Mice were sacrificed on indicated days and splenocytes were analyzed for homeostatic expansion of pmel-1 T cells.

For tumor treatment experiments, male or female B6 mice aged 6-12 weeks were injected with 5×10^5 cells of a previously described B16 melanoma line(86) which overexpresses chimeric human/mouse gp100 antigen KVPRNQDWL (aa25-33), or 1×10^6 CD19⁺ E2a-PBX leukemia cells. Tumor-bearing mice received 5-6 Gy total body irradiation on indicated days. Mice were left untreated as controls or received by tail vein injection indicated doses of congenically marked pmel-1 or anti-CD19 CAR-transduced T cells modified with a Thy1.1-containing reporter construct. To analyze anti-CD19 CAR-transduced T cell persistence and leukemia burden, mice were sacrificed after 14 days and cellular analysis performed on the spleen and bone marrow.

For experiments with MRL-Mp mice, female mice aged 8 weeks received 6 Gy total body irradiation. One day later, mice were injected with 3×10^6 of anti-CD19 CAR-transduced CD8 α ⁺ T cells also transduced with a Thy1.1-containing reporter construct. Age-matched MRL-lpr female mice were left untouched.

All transduced T cells were bead-enriched to >92% purity using anti-Thy1.1 magnetic microbeads prior to infusion (Miltenyi Biotec). All treated mice received once daily injections of

12 µg of IL-2 i.p. for 3 days. All tumor measurements were performed in a blinded fashion by an independent investigator.

T cell and tumor cell co-culture assay: After 5-6d in culture, T cells were washed twice in PBS and plated in IL-2-free T cell media at 5×10^4 cells per well in a 96-well round bottom plate. T cells were incubated either alone, with plate-bound anti-CD3/CD28 ($2 \mu\text{g mL}^{-1}$, each), with 1.5×10^5 B16-mhgp100 or E2a-PBX cells per well for an E:T of 1:3, or with 50-100 ng mL⁻¹ of lz-FasL. Cells were cultured together for 6 or 24h before being washed and stained for cell viability.

ELISA assay: Analysis of serum anti-nuclear and anti-dsDNA antibodies was performed on serum diluted 1:5; ELISA performed according to manufacturer's instructions (Alpha Diagnostic International).

Histopathology: Lung tissues were fixed in buffered 10% formalin and stained with haematoxylin and eosin (H&E). Tissue sections were scored in a blinded manner by an interpreting pathologist. Scoring: 0 = no specific findings, 1 = mild infiltrates, 2 = minimal infiltrates, 3 = moderate infiltrates, 4 = severe infiltrates.

Statistical Analysis: 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 Mantel Cox test. 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 using a 1-

or 2-way ANOVA, as indicated. In all cases, *P* values of less than 0.05 were considered significant. Statistics were calculated using Prism 7 GraphPad software (GraphPad Software Inc.).

Study Approval: All anonymous NIH Blood Bank donors and cancer patients providing PBMC 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. Animal experiments were approved by the Institutional Animal Care and Use Committees of the NCI and performed in accordance with NIH guidelines.

Data and Materials Availability: All data reported in the paper are recorded in the manuscript, are available in the supplemental materials, or through the publicly available databases TCGA (<https://cancergenome.nih.gov/>) and UCSC Xena (<http://xena.ucsc.edu>).

AUTHOR CONTRIBUTIONS AND ACKNOWLEDGMENTS:

Author Contributions: T.N.Y., N.P.R. and C.A.K. designed and conceived of all experiments and wrote and edited the manuscript. T.N.Y. and P.-H.L. performed all tissue culture, retroviral transduction, and immune-phenotyping experiments. S.K.V., D.G. and R.J.K. assisted in RNA-seq analysis and experimental design. R.L.E. assisted in the design and generation of Fas-DNR engineered T cells. J.F. and L.G. assisted in experimental design and performing in vivo experiments. J.N.K. and T.J.F. provided in vivo reagents and helpful discussion on performing in vivo experiments. T.N.Y., Z.Y., and A.E. performed in vivo experiments and all animal husbandry and colony management. A.C.C. and R.M.S. provided the Iz-FasL reagent and assisted with death assays and experimental design. B.A.A. and J.E.H. assisted with all bioinformatic analyses. All co-authors edited and commented on the final manuscript.

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Conflict of interest statement: C.A.K., T.N.Y. and N.P.R. have submitted a provisional patent related to the Fas DNR technology described in this manuscript.

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FIGURES AND FIGURE LEGENDS:

Figure 1:

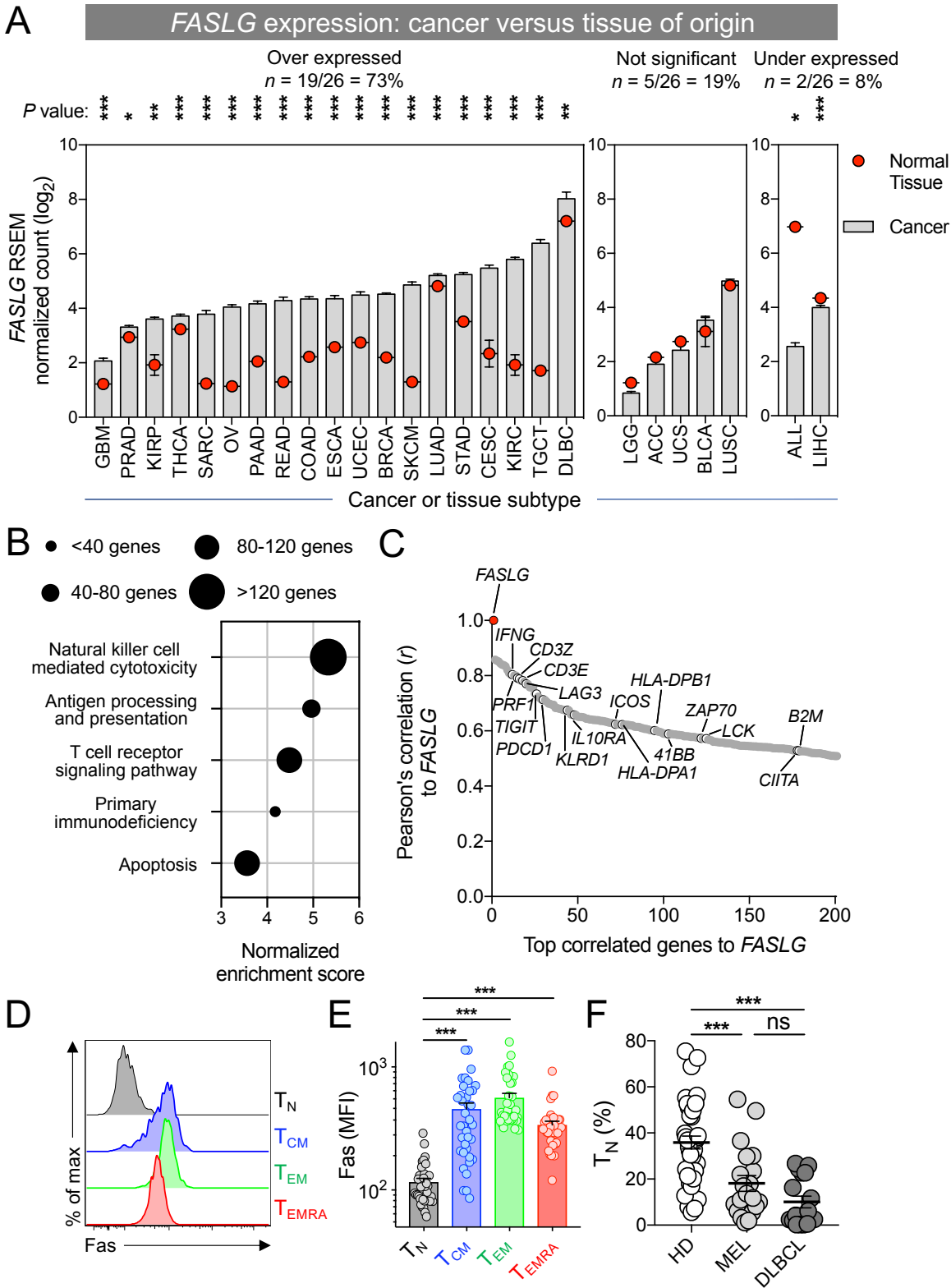


Figure 1. Human tumor microenvironments overexpress the death-inducing ligand *FASLG*. (A) A pan-cancer analysis of *FASLG* expression within the microenvironments of 26 different tumor types relative to matched normal tissues of origin. RNA-sequencing (RNA-seq) data from 9,330 human cancers and matched normal tissues was extracted from the TCGA and GTEx datasets. Statistical comparisons of expression between tumors and normal tissues were made using a Mann-Whitney *t* test with Bonferroni correction; ****P*<0.001, ***P*<0.01, **P*<0.05. (B) Selected, pre-ranked gene set enrichment analyses (GSEAs) against all KEGG pathways of genes positively correlated to *FASLG* expression averaged across 26 TCGA histologies. Circle diameters reflect the number of genes identified within the GSEA signature sets. The nominal *P*-value and FDR *q* value for all displayed GSEAs were <0.001. (C) Pearson's correlation of the top 200 correlated genes to *FASLG* gene expression across 26 human cancer types in the TCGA database. Selected immune-related genes associated with the GSEA signature sets listed in panel (B) are identified. (D, E) Representative histogram (D) and summary plot of Fas MFI (E) on phenotypically defined CD8 α^+ T cell subsets. Data shown are from peripheral blood T cells from 47 patients and HDs. CD8 α^+ T cell subsets in panels (D) and (E) were defined as follows: T_N cells, CD8 α^+ CD45RA⁺CD45RO⁻CCR7⁺CD62L⁺CD27⁺CD28⁺Fas⁻; T_{CM}, CD8 α^+ CD45RO⁺CD45RA⁻CCR7⁺CD62L⁺; T_{EM}, CD8 α^+ CD45RO⁺CD45RA⁻CCR7⁻CD62L⁻; T_{EMRA}, CD8 α^+ CD45RA⁺CCR7⁻CD62L⁻. ****P*<0.001, (one-way ANOVA, corrected with Tukey's multiple comparisons). (F) The fraction of T_N among all CD8 α^+ T cells in the circulation of age-matched healthy donors (HD; n=39; left), and patients with melanoma (MEL; n=20; middle) and diffuse large B cell lymphoma (DLBCL; n=17; right) at the time of enrollment to an adoptive immunotherapy clinical trial. ****P*<0.001, ns = not significant (one-way ANOVA, corrected with Tukey's multiple comparisons).

Figure 2:

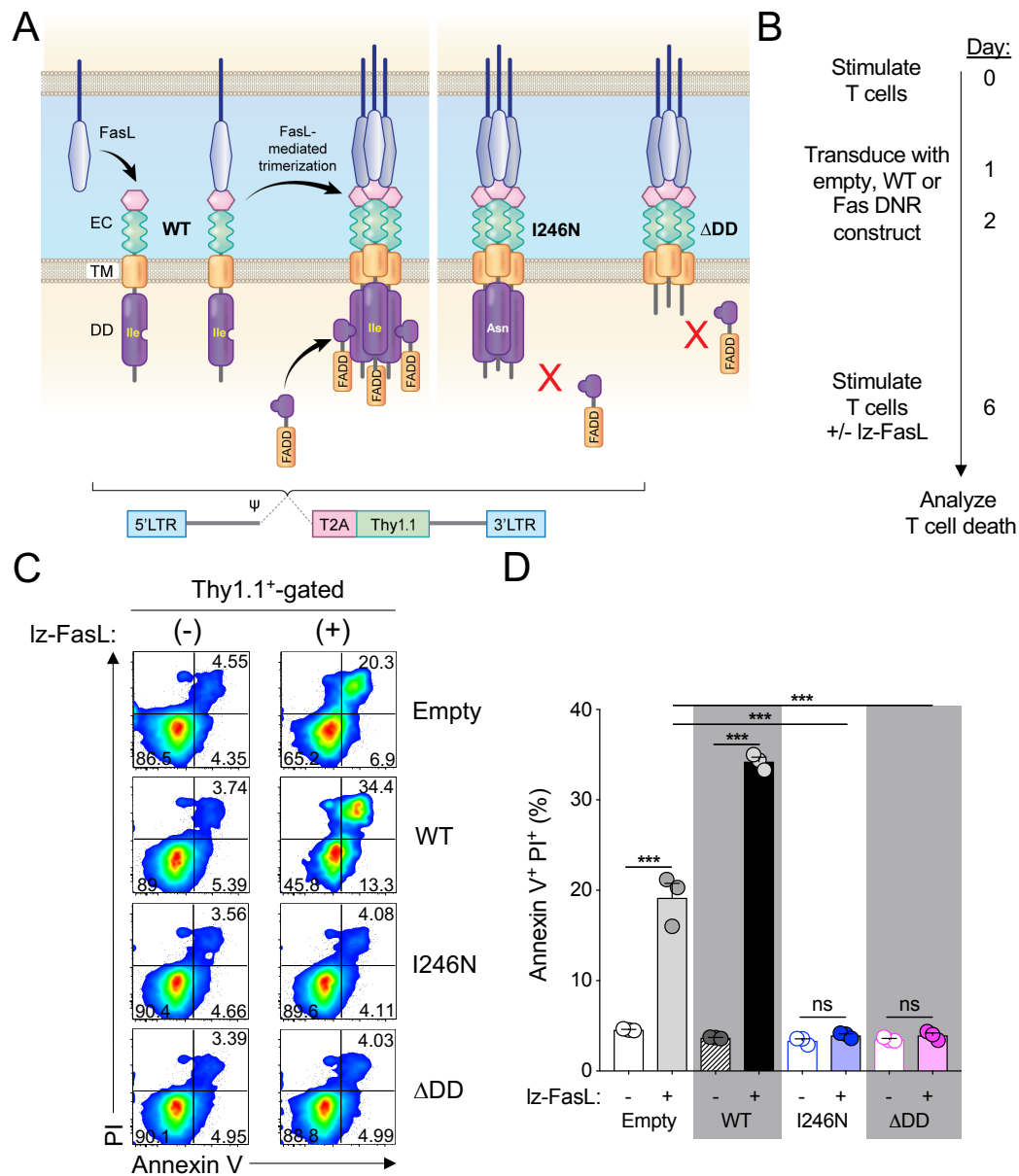


Figure 2. T cells engineered with Fas DNRs prevent FasL-mediated apoptosis. (A) Schematic representation of physiologic Fas signaling and design of two murine Fas dominant negative receptors (DNRs). Fas DNRs were designed to prevent recruitment of Fas-associated protein with death domain (FADD) either by (i) substitution of an asparagine for an isoleucine residue at position 246 of the death domain (DD; Fas^{I246N}), or (ii) truncation of the majority of the intracellular death domain (Fas^{ΔDD}). Wildtype Fas (Fas^{WT}) and an empty vector were used as controls. Receptors were cloned into a retroviral bicistronic vector containing a Thy1.1 reporter. EC, extracellular domain; TM, transmembrane domain; T2A, thosea asigna virus 2A self-cleaving peptide. (B) Experimental timeline for the stimulation, retroviral transduction, and testing of Iz-FasL-mediated apoptosis of CD8 α ⁺ T cells modified with Fas constructs or an empty vector control. (C) Representative FACS plots and (D) summary bar graph showing the frequency of apoptotic Annexin V⁺ PI⁺ transduced T cells at rest and 6h following exposure to Iz-FasL (50 ng mL⁻¹). Results are shown after gating on transduced Thy1.1⁺ cells. Data shown is representative of 6 independently performed experiments and is displayed as mean \pm SEM with *n* = 3 per condition. ****P* < 0.001, ns = not significant (two-way ANOVA).

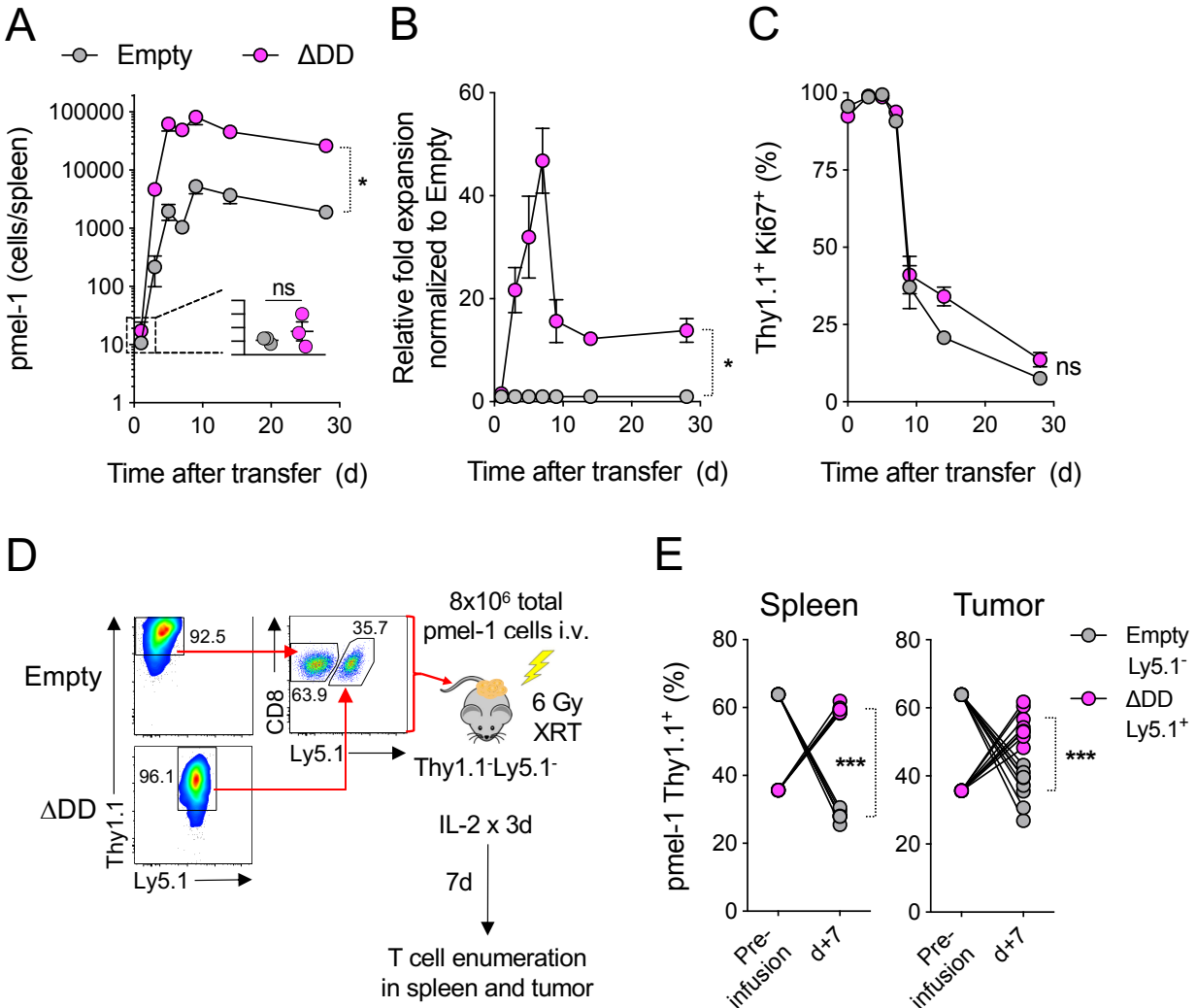
Figure 3:

Figure 3. Enhanced persistence and survivability of Fas DNR-engineered T cells in vivo. Transduced Ly5.1⁺ d11 pmel-1 CD8 α ⁺ T cells were generated as described in Fig. 2B, then 5x10⁵ cells from each group were injected into sublethally irradiated (6 Gy), Ly5.2⁺ B6 mice. Mice were sacrificed on indicated days and splenocytes were analyzed via flow cytometry for homeostatic expansion of Ly5.1⁺ pmel-1CD8 α ⁺ T cells. **(A)** Total number of live, Ly5.1⁺CD8 α ⁺Vb13⁺ cells transduced with the Empty or Fas ^{ΔDD} constructs. **(B)** Relative fold expansion of Fas ^{ΔDD} normalized to Empty construct found in spleen on indicated days. **(C)** Percentage of live, Ly5.1⁺CD8 α ⁺Vb13⁺ cells expressing Ki67 for each condition. Representative plots from two independent experiments. Data is displayed as mean \pm SEM with $n = 3$ per condition displayed and is representative of 3 independent experiments. * $P < 0.05$, ns = not significant (Wilcoxon rank sum test). **(D)** Experimental schema for the generation and co-infusion of pmel-1 T cells engineered with Fas ^{ΔDD} DNR (Ly5.1⁺Thy1.1⁺) or an empty vector control (Ly5.1⁺Thy1.1⁺). Thy1.1⁺ T cells were enriched prior to recombination in a ~1:1 mixture and a total of 8x10⁶ T cells were infused i.v. into sublethally irradiated (6 Gy XRT) Thy1.1⁺Ly5.1⁻ mice bearing 10d established B16 melanoma tumors. Spleens and tumors were harvested for analysis on d7. **(E)** Relative persistence of Fas ^{ΔDD} DNR-modified to empty vector-modified T cells in the spleens and tumors of recipient mice. Results displayed after gating on live, CD8 α ⁺Thy1.1⁺ lymphocytes and are representative of two independent experiments, each with $n = 5-8$ mice. *** $P < 0.001$ (unpaired 2-tailed Student's t-test).

Figure 4:

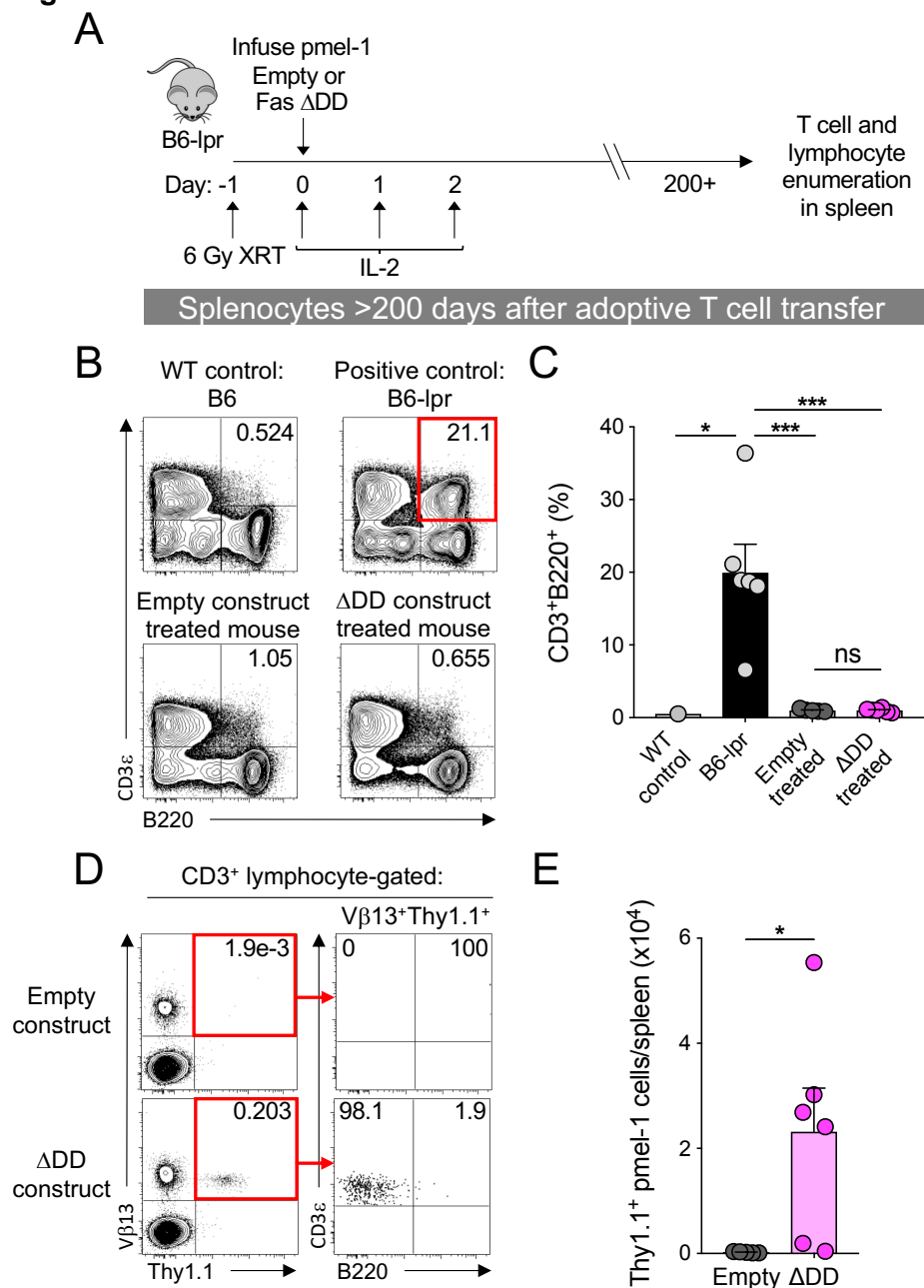


Figure 4. Transfer of Fas DNR-modified T cells does not result in acquired ALPS. (A) Experimental design to analyze long-term persistence of WT pmel-1 CD8 α^+ T cells modified with Fas $^{\Delta DD}$ or empty vector control in B6 mice. **(B)** Representative FACS plots and **(C)** summary bar graph of the frequency of CD3 $^+$ B220 $^+$ CD4 $^-$ CD8 α^- lymphocytes in the spleens of sublethally irradiated WT mice who received 5×10^5 bead-purified Thy1.1 $^+$ pmel-1 T cells modified with Fas $^{\Delta DD}$ DNR or an empty vector control. Age-matched WT mice and Fas-deficient B6-lpr mice served as negative and positive controls, respectively. *** $P < 0.001$, * $P < 0.05$, ns = not significant (two-way ANOVA). **(D)** Representative FACS plots **(E)** and summary bar graph demonstrating the persistence and surface phenotype of transferred pmel-1 Thy1.1 $^+$ T cells modified with Fas $^{\Delta DD}$ DNR or an empty vector control. All data shown is representative of 5 independent experiments, each with $n = 5-8$ mice per cohort. *** $P < 0.001$, * $P < 0.05$, ns = not significant (one-way ANOVA).

Figure 5:

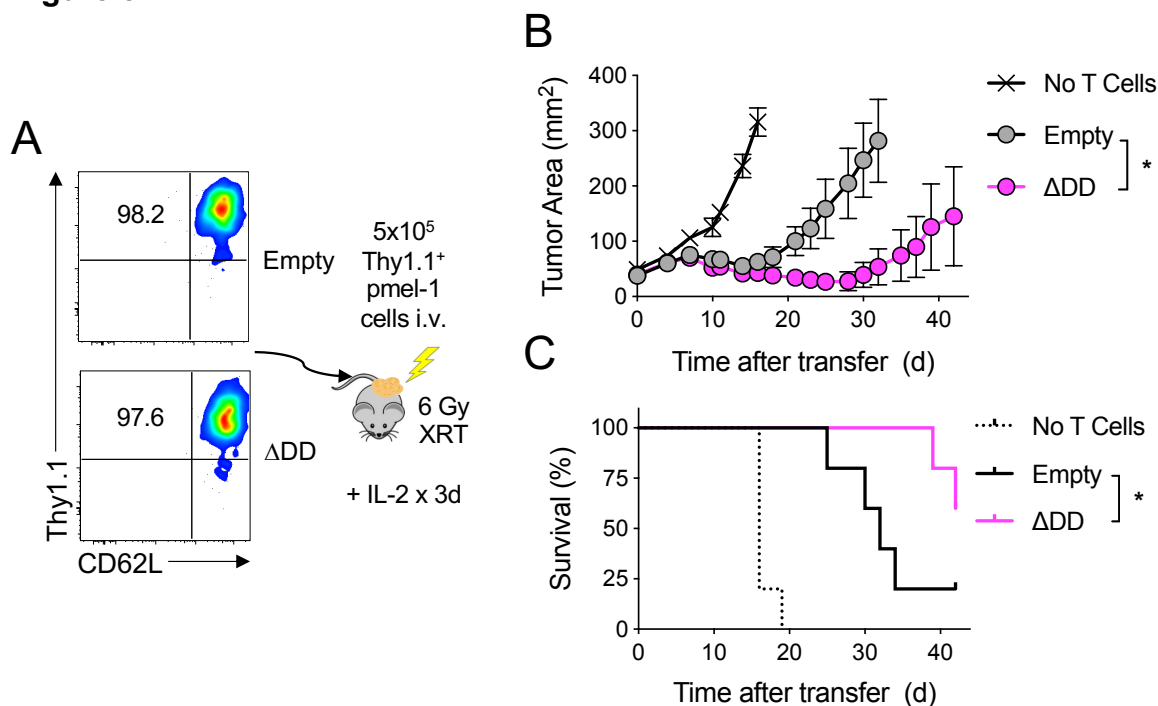


Figure 5. Adoptive transfer of Fas DNR-modified T cells enhances anti-tumor efficacy independently of T cell differentiation status. (A) Representative FACS plots demonstrating the purity of sorted CD62L⁺CD44⁺Thy1.1⁺ T_{CM}-like pmel-1 T cells modified with Fas^{ΔDD} or empty vector control prior to infusion. The percentage of gated Thy1.1⁺CD62L⁺ cells is shown in FACS plot. (B) Tumor regression and (C) survival of mice bearing 10d established B16 melanoma tumors who were untreated or received 5x10⁵ of sort-purified T_{CM}-like Thy1.1⁺ modified cells. Representative results from two independent experiments are shown as mean ± SEM using *n*=5-8 mice/cohort. Statistical comparisons performed using Wilcoxon rank sum test (B) or the Log-rank Mantel Cox test (C), **P*< 0.05.

Figure 6:

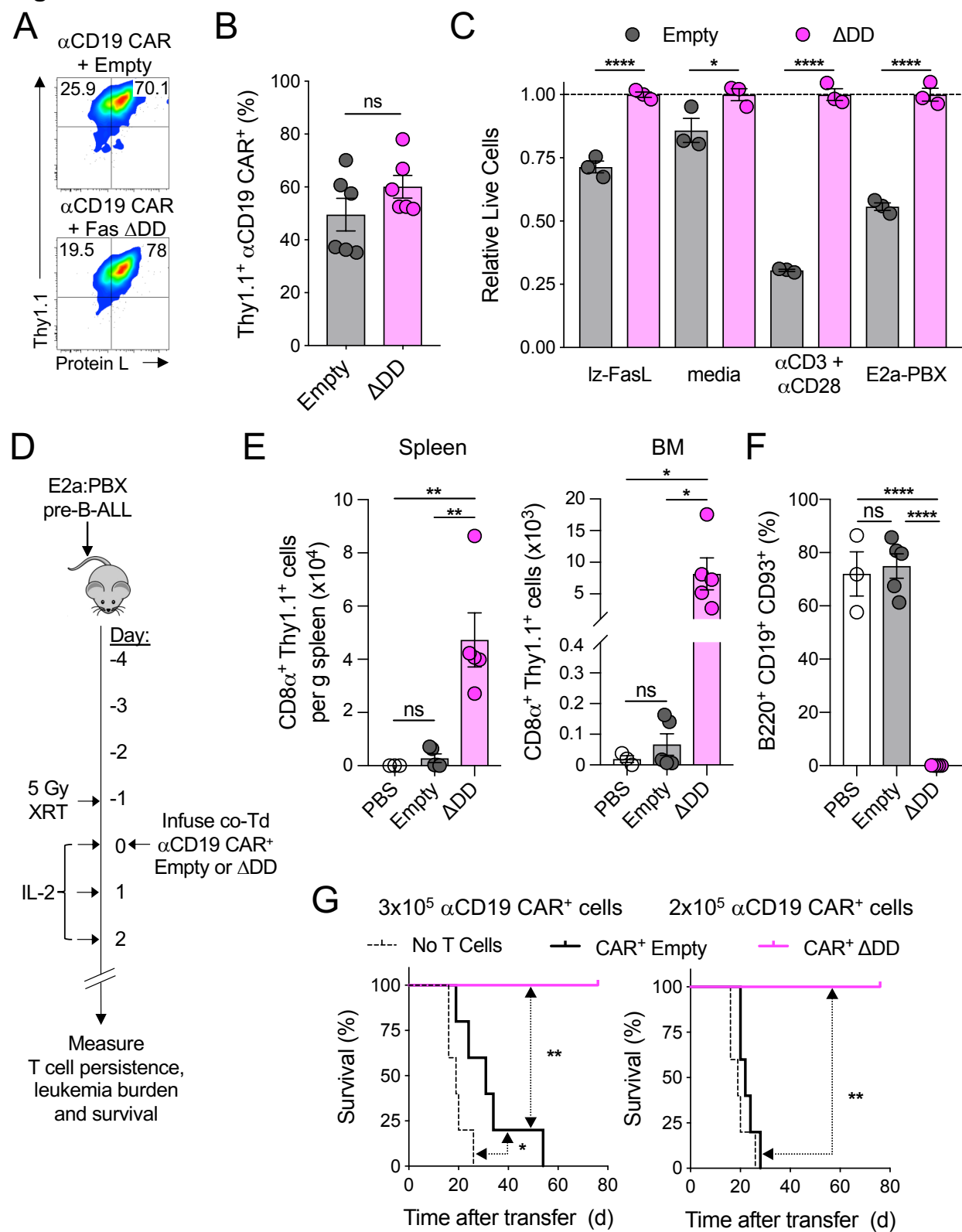


Figure 6: Expression of Fas DNR enhances anti-apoptotic functions and in vivo persistence in anti-CD19 CAR model. (A) Representative flow plots and (B) summary data of double transduction of B6 CD8 α^+ T cells with retroviral constructs encoding anti-CD19 CAR and Empty or Fas DNR. Analysis performed on d11 after Thy1.1 bead-enrichment on d6. (C) Summary bar graph of relative T cell viability (to Fas^{ADD}) following overnight culture in cytokine-free media alone, with Iz-FasL (100 ng mL⁻¹), 2 μ g mL⁻¹ each of anti-CD3/CD28, or E2a-PBX. Data shown after gating on Thy1.1⁺ lymphocytes, is representative of 3 independently performed experiments, and displayed as mean \pm SEM with $n = 3$ per condition. *** $P < 0.001$, ns = not significant (two-way ANOVA). (D) Experimental schema for the generation and infusion of WT CD8 α^+ T cells engineered to express anti-CD19-CAR⁺ along with Fas^{ADD} DNR or an empty vector control. Transduced T cells were Thy1.1-bead enriched prior to injection and T cells were infused i.v. into sublethally irradiated (5 Gy) mice bearing 4d established E2a-PBX leukemia. Spleens and bone marrow (BM) were harvested for analysis on d14. (E) Summary data of numbers of live, CD8 α^+ Thy1.1⁺ lymphocytes in spleens and BM of recipient mice. (F) Summary data of the frequency of E2a-PBX leukemia in the bone marrow of recipient mice. (F, E) Results are representative of two independent experiments, each with $n=5$ mice. * $P < 0.05$ ** $P < 0.01$ **** $P < 0.0001$, ns = not significant (one-way ANOVA, corrected with Tukey's multiple comparisons). (G) Survival of mice bearing 4d established E2a-PBX leukemia who were untreated or received 3×10^5 (left) or 2×10^5 (right) of anti-CD19 CAR⁺ Thy1.1⁺ modified cells. Representative results from four independent experiments are shown as mean \pm SEM using $n=5$ mice/cohort. Statistical comparisons performed using the Log-rank Mantel Cox test (C), * $P < 0.05$ ** $P < 0.01$.

Figure 7:

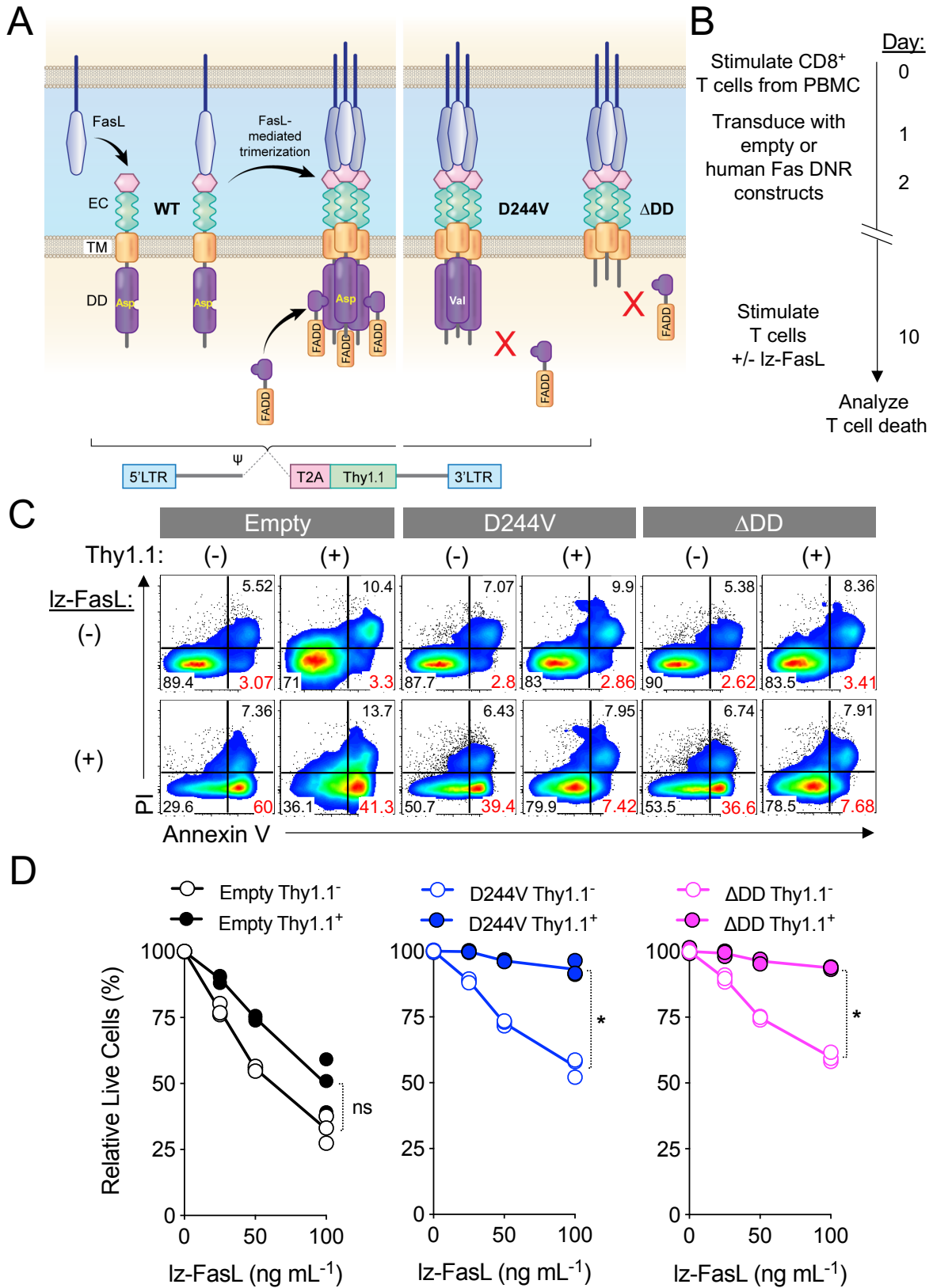


Figure 7. Genetic engineering with Fas DNR protects human T cells from FasL-induced apoptosis.

(A) Schematic representation of physiologic Fas signaling and the design of two human Fas DNRs. Retroviral-encoded human Fas DNRs were designed to prevent recruitment of Fas-associated protein with death domain (FADD) either by (i) substitution of a valine for an aspartic acid residue at position 244 of the death domain (DD; hFas^{D244V}), or (ii) truncation of the majority of the human intracellular death domain (hFas^{ΔDD}). An empty vector was used as a negative control. Receptors were cloned into a bicistronic vector containing a Thy1.1 reporter. EC, extracellular domain; TM, transmembrane domain; T2A, thosea asigna virus 2A self-cleaving peptide. (B) Experimental timeline for the stimulation, retroviral transduction, and testing of lz-FasL-mediated apoptosis of human CD8⁺ T cells modified with Fas^{D244V}, Fas^{ΔDD}, or an empty vector control. (C) Representative FACS plots (0 and 100 ng mL⁻¹ lz-FasL) and (D) summary graph showing the frequency of apoptotic Annexin V⁺ T cells at rest and 6h following exposure to titrated concentrations of lz-FasL. Results shown after gating on transduced (Thy1.1⁺) or untransduced (Thy1.1⁻) T cells. Data is displayed as mean ± SEM with *n* = 3 per condition displayed and is representative of 3 independent experiments. **P* < 0.05, ns = not significant (Wilcoxon rank sum test).