Generation of Tumor Antigen-Specific iPSC-Derived Thymic Emigrants Using a 3D Thymic Culture System

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SUPPLEMENTAL INFORMATION
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DECLARATION OF INTERESTS
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SUMMARY

Induced pluripotent stem cell (iPSC)-derived T cells may provide future therapies for cancer patients, but those generated by current methods, such as the OP9/DLL1 system, have shown abnormalities that pose major barriers for clinical translation. Our data indicate that these iPSC-derived CD8 single-positive T cells are more like CD4+CD8+ double-positive T cells than mature naive T cells because they display phenotypic markers of developmental arrest and an innate-like phenotype after stimulation. We developed a 3D thymic culture system to avoid these aberrant developmental fates, generating a homogeneous subset of CD8ab- antigen-specific T cells, designated iPSC-derived thymic emigrants (iTEs). iTEs exhibit phenotypic and functional similarities to naive T cells both in vitro and in vivo, including the capacity for expansion, memory formation, and tumor suppression. These data illustrate the limitations of current methods and provide a tool to develop the next generation of iPSC-based antigen-specific immunotherapies.

Graphical abstract

**In Brief:** A barrier for clinical application of iPSC-derived CD8 T cells using OP9/DLL1 is their abnormal biology. Vizcardo et al. show that a 3D thymic culture system enables the generation of a homogeneous antigen-specific T cell subset, named iTEs, which closely mimics naive T cells and exhibits potent anti-tumor activity.

INTRODUCTION

Adoptive cell transfer (ACT) can be an effective treatment for some patients with advanced cancer, with the antitumor efficacy of transferred cells associated with in vivo persistence (Rosenberg et al., 2011). However, many patients receiving ACT-based immunotherapies do
not experience tumor regression, and transferred cells fail to establish long-term immunological memory. Preclinical data show that ACT can be limited when anti-tumor T cells are terminally differentiated effector cells exhibiting poor persistence (Gattinoni et al., 2009), and emerging data from the clinic supports these observations (Rosenberg et al., 2011; Singh et al., 2016). Thus, there is considerable interest in induced pluripotent stem cell (iPSC) technologies that enable the generation of stem cell-like naive and very early memory T cells derived from highly differentiated T cells (Crompton et al., 2014; Gattinoni et al., 2012; Takahashi and Yamanaka, 2006).

Stem cell-like CD8+ T cells have a robust ability to proliferate and persist, but the capacity for long-lived memory can wane as T cells acquire effector functions, like cytotoxicity (Roychoudhuri et al., 2015). Epigenetically, T cells silence stemness genes during the acquisition of effector gene expression (Buchholz et al., 2013; Crompton et al., 2016; Henning et al., 2018a, 2018b; Restifo and Gattinoni, 2013). Unlike effector T cells, minimally differentiated naive and memory T cells are stem cell-like and capable of robust expansion, immune reconstitution, and long-term persistence, qualities that make them of great clinical interest (Busch et al., 2016). In fact, it has been shown that such minimally differentiated T cells possess superior anti-tumor properties upon adoptive transfer in vivo and are associated with longer persistence (Gattinoni et al., 2005). This linear loss of “stemness” is characteristic of most adult cells, and both differentiation and aging of CD8+ T cells cannot be reversed under physiological conditions (Gattinoni et al., 2009, 2011). Thus, there is interest to use iPSC technology to epigenetically reprogram T cells and “turn back the clock” on aging and differentiation (Crompton et al., 2014).

Recent studies have reported the regeneration of antigen-specific cytotoxic T lymphocytes from T cell-derived human iPSCs using the OP9/DLL1 co-culture system, a method that has been used for in vitro differentiation of hematopoietic stem cells into T cells by induction of Notch signaling (Nishimura et al., 2013; Vizcardo et al., 2013). These regenerated T cells retained the same T cell receptors (TCRs) as the original T cell from which the iPSC clone was established. However, numerous factors limit the clinical potential of these cells. Cells derived by this method are reported to express the CD8αα homodimer, which functions as an ineffective co-receptor for TCR signaling, and have phenotypic similarities to innate lymphocytes and strong TCR-independent cytotoxicity (McNicol et al., 2007; Themeli et al., 2013). Therefore, T cells with an appropriate CD8αβ heterodimer are needed, and although an improved method to generate CD8αβ+ T cells has been reported, these cells exhibit an effector-like phenotype (Maeda et al., 2016). As we will show in this report, the expression of CD8αα+ by cells matured on OP9/DLL1 is emblematic of a much broader pattern of dysregulated gene expression that persists even when cells can be triggered to express the CD8αβ heterodimer. Thus, current methods to derive antigen-specific T cells from iPSCs fail to produce a homogeneous population of T cells that are phenotypically and functionally similar to endogenous naive T cells, rendering them unsuitable for therapeutic ACT applications.

Current in vitro iPSC differentiation methods employ strong TCR signaling triggered by either anti-CD3 or anti-TCR antibodies (Nishimura et al., 2013; Vizcardo et al., 2013) or by agonist peptides (Snauwaert et al., 2014). However, T cells induced by TCR stimulation or
high-affinity peptides are generally incompetent functionally or are driven into unconventional T cell lineages, including regulatory T cells, natural killer (NK) T cells, and CD8αα T cells (Takada et al., 2017; Yamagata et al., 2004). These findings indicate that iPSC-derived T cell differentiation may require the provision of additional physiologically relevant signals. Much has been learned over the past decades about how T cells develop within the thymus, and although the signals provided within the thymic microenvironment are still incompletely elucidated, they are not limited to Notch/Delta-like ligands and TCR stimulation as currently provided to iPSCs for in vitro differentiation (Etzensperger et al., 2017; Singer et al., 2008).

Thus, we hypothesized that mimicking thymic signaling in vitro may aid in the generation of iPSC-derived T cells that more closely resemble endogenous cells. We therefore developed a method that uses the thymus itself to allow T cells to mature in a more physiologically relevant in vitro environment. We describe a 3D thymic culture method based on the traditional fetal thymic organ culture (FTOC) system (Nitta et al., 2013) that involves seeding immature T cells derived from iPSCs into thymocyte-depleted fetal thymic lobes in vitro. With this approach, we generated a unique T cell product we have named iPSC-derived thymic emigrants (iTEs). We demonstrate that iTEs are a homogeneous population of tumor antigen-specific post-thymic T cells with similarities to naive T cells. The functional and phenotypic similarities of iPSC-derived iTEs to naive T cells opens further avenues in basic research as well as future therapeutic ACT applications.

RESULTS

Extrathymic T Cells Derived from iPSCs by OP9/DLL1 Displayed Markers of Immature T Lineage Cells and Innate-like Immune Cells

We sought to explore whether iPSC technology could be used to generate a homogeneous population of de novo tumor antigen-specific T cells. We employed the Pmel TCR transgenic mouse model developed in our laboratory (Overwijk et al., 2003). We generated iPSC lines both with and without pre-rearranged αβTCR genes: Pmel-iPSCs, OR-iPSCs (open repertoire-iPSC), and CD3-iPSCs. The Pmel-iPSC line was generated by reprogramming a Pmel mouse embryonic fibroblast encoding a major histocompatibility complex (MHC) class I-restricted αβTCR specific for the melanocyte antigen gp100 using non-integrating Sendai virus particles encoding Oct4, Sox2, Klf4, and c-Myc mRNA. A C57BL/6 embryonic fibroblast with an intact TCRαβ locus was used to generate the OR-iPSC line. A control CD3-iPSC line was established from a randomly selected C57BL/6 CD3αα T cell carrying a pre-rearranged αβTCR of unknown specificity. The OR-iPSC and CD3-iPSC lines controlled for the absence or presence of pre-rearranged αβTCR genes, respectively, in the Pmel model. All iPSC lines were found to be morphologically indistinguishable from murine embryonic stem cells (Figure S1 and data not shown).

To test the effect of a pre-rearranged αβTCR on the in vitro generation of functional T cells from iPSCs, we used the established OP9/DLL1 co-culture system (Figure 1A). This system, supplemented with medium containing Flt3L and IL-7, provides extrinsic Notch signaling to induce iPSC differentiation and commitment to the T cell lineage (Schmitt et al., 2004). Heterogeneous populations of non-lymphoid cells and beating cardiomyocytes appeared...
after 10 days of culture (Movie S1), indicating passage through the primitive mesoderm stage (Slukvin, 2013). By day 16, iPSC-derived cells were predominantly CD45<sup>+</sup>CD4<sup>−</sup>CD8<sup>α<sup>−</sup></sup> double-negative (DN) (Figures S2A and S2B). From day 18, cells had begun to differentiate into CD4<sup>+</sup>CD8<sup>+</sup> double-positive (DP) or CD4<sup>+</sup>CD8<sup>+</sup> single-positive (CD8αSP) T cells, and these two cell populations persisted as culture on OP9/DLL1 continued (Figure 1B). The CD8αSP T cells from days 18–26 were used in these analyses. We will refer to these CD8αSP T cells grown using established techniques as extrathymic T cells.

Cells differentiated using the OP9/DLL1 system exhibited precocious expression of TCRβ in CD3-iPSC and Pmel-iPSC-derived DN cells compared with OR-iPSC-derived DN cells (Figures S2C and S2D), likely because of the pre-rearranged nature of the αβTCR genes within CD3-iPSCs and Pmel-iPSCs relative to OR-iPSCs (Serwold et al., 2007). We observed that cells progressively lost MHC class I expression during differentiation to the DP stage (Figures S2E and S2F), whereas expression of CD62L, CD8β, and MHC class I in iPSC-derived DP T cells was comparable with that observed in endogenous thymocytes (Figures 1C and 1D). At the SP stage, Pmel-iPSC-derived extrathymic T cells demonstrated variable expression of CD8β and CD62L relative to endogenous thymocytes, and expression of the MHC class I complex was dramatically reduced (Figures 1C and 1D). Expression of the MHC class I complex marks a developmental turning point, dividing immature thymocytes from mature, proliferation-competent SP thymocytes (Xing et al., 2016). Therefore, our results indicated that extrathymic T cells differentiated using the current OP9/DLL1 system exhibit a developmental divergence from endogenous T cells during the DP-to CD8αSP-transition.

In an effort to provide activation signals to generate CD8αSP T cells in vitro, we stimulated extrathymic T cells with an agonist peptide (Chidgey and Boyd, 1997). Day 18 Pmel-iPSC-derived cells from OP9/DLL1 co-culture were stimulated with irradiated splenocytes in the presence of cognate hgp100 peptide for 8 days. As with naive CD8<sup>+</sup> T cells, Pmel-iPSC-derived T cells expanded specifically in response to cognate peptide and released tumor necrosis factor (TNF) after re-stimulation with anti-CD3/CD28 (Figures S2G and S2H), indicating that they were proliferation-competent and functional, which are markers of T cell maturity (Hogquist et al., 2015). However, most cells lost surface expression of CD8β and obtained a CD8αα<sup>+</sup> or DN phenotype during expansion (Figure 1E), similar to when immature self-reactive T cells bind strongly to self-antigens (Yamagata et al., 2004). Moreover, the cytoplasmic domain of the CD8β molecule is essential for efficient downstream signaling following TCR-MHC class I engagement (Irie et al., 1998), indicating a functional defect in extrathymic T cells following peptide stimulation. We also used an anti-TCR antibody to trigger the generation of CD8αSP T cells by TCR activation (Takahama et al., 1994). However, this resulted in expression of the innate immune marker NK1.1, which is not typical of CD8αβ<sup>+</sup> peripheral T cells (Figure S2I). These results were in accordance with a previous report in which iPSC-derived extrathymic T cells carrying a pre-determined TCR silenced CD8β expression and had an aberrant, innate-like gene signature after stimulation (Themeli et al., 2013).
To further interrogate the differences between endogenous CD8αSP T cells and those produced from iPSCs by OP9/DLL1, we performed a transcriptional analysis of naive CD8+ T cells and extrathymic T cells by RNA sequencing (RNA-seq), identifying 2,462 differentially expressed genes (Figure 1F; log2 fold change [FC] > 2, adjusted p value < 0.01). We used a systems biology approach to identify transcriptional differences in the regulation of biological pathways using the overrepresentation analysis versus the accumulated perturbation analysis (Tarca et al., 2009; Supplemental Experimental Procedures). The analysis performed on differentially expressed genes identified 21 significantly different pathway terms (Figure 1G; Table S1). We identified three pathways with clinical relevance to ACT: antigen processing and presentation, graft versus host disease, and allograft rejection. An investigation of genes within these pathways revealed that extrathymic T cells had decreased expression of classical MHC class I (H2-D1 and H2-K1) and non-classical MHC genes (H2-Q4, H2-T3, H2-T10, H2-Q7, H2-Ob, H2-DMb1, and H2-DMa) as well as increased expression of NK receptors (Klrc1, Klrc2, and Klrc3) (Figure 1H). The down-regulation of MHC class I genes as well the upregulation of Ptcra, Rag1, Rag2, and Rorc suggest that extrathymic T cells have an immature T cell phenotype similar to immature CD8SP (CD8iSP) T cells, potentially because of an inadequate positive selection, impaired TCR signaling, or an unfinished allelic exclusion program (Figure 1I; Carpenter and Bosselut, 2010; Hogquist et al., 2015; von Boehmer, 2005). However, NK receptor expression also confirms potential innate-like features among the extrathymic T cells. Thus, extrathymic T cells display an aberrant transcriptional program that diverges from those of physiologically differentiating naive CD8+ T cells. Our results indicated that extrathymic T cells generated by OP9/DLL1 were characterized by gene deviations, including high NK cell markers, low “classical” MHC class I, and persistent expression of Rag genes, suggesting that these cells lacked proper physiologic developmental signals.

### Generation of iTEs Using a 3D Thymic Culture System

We hypothesized that the restoration of physiological thymic signaling could induce in vitro differentiation of iPSC-derived immature T cells to be more consistent with endogenous thymocyte development. We considered the properties of the FTOC system as a means to provide developing T cells with more relevant growth conditions. However, the FTOC system is designed to allow analysis of intra-thymic development of T cells from DN progenitors to CD8SP thymocytes, limiting our ability to analyze whether iPSC-derived T cells could reach a post-thymic maturation stage (Nitta et al., 2013). Also, T cells grown with the FTOC system are typically harvested by mechanical disruption, which eliminates the whole thymic lobe. On the other hand, hanging drop re-aggregation allows thymic lobes to be cultured with immature T cells in suspension but with a limit of 48 hr because of the restricted volume of medium, which becomes exhausted (Anderson and Jenkinson, 2007). Thus, the conventional method supporting FTOC systems as currently practiced had several drawbacks that limited its practical and heuristic utility in the generation of T cells from iPSCs. Therefore, a redesigned thymic culture system was necessary to achieve our goal.

To develop an improved thymic culture system that did not disrupt the thymic lobes, we performed a long-term 3D FTOC system characterized by ultra-low attachment cylinders without a solid bottom in a 96-well format so that spheroids could be cultured in suspended
medium by surface tension (Figure 2A). We placed intact thymic lobes at the air-medium interface to allow maximal oxygen exchange, circumventing the need for hyperoxic devices (Watanabe and Katsura, 1993). Unlike the traditional hanging drop method, the 3D culture system was accessible to microscopy and micro-manipulation in real time. This allowed the medium to be changed several times a day without mechanical disruption and permitted the continual harvesting solely of the T cells that egressed from the lobes (Figure 2A). Thus, we sought to develop a long-term 3D organ culture method that could improve existing FTOC systems for generating more physiologically representative iPSC-derived T cells.

Combining iPSC-derived T lineage cells with the 3D thymic culture system, we sought to explore whether thymic tissue could prevent the aberrant developmental fate of iPSC-derived immature T lineage cells. Fetal thymic lobes from day 15.5 embryos were treated with the cytotoxic agent 2-deoxyguanosine to deplete endogenous lymphocytes (Nitta et al., 2013). The treated thymic lobes were seeded with non-adherent iPSC-derived T lineage cells collected from OP9/DLL1 co-culture on days 16–21, hereafter referred to as iPSC-derived immature T cells, which were predominantly DN and DP cells (Figures S2A–S2D). After overnight culture, thymic lobes were extensively washed to remove any immature T cells that were unable to migrate inside. After 4–10 days, we observed mononuclear cells migrating out of the thymic lobes, forming a halo-like pattern (see photomicrographs in Figure 2B and Movie S2). Using cytofluorometry, we could verify the presence of cells with forward and side scatter that were in a lymphocyte like-gate and that were of the TCRβ+CD8αSP T cell phenotype (Figures S3A and S3B). This homogeneous population of cells produced using the 3D thymic culture system were named iTEs. Neither the fetal thymic lobes nor the iPSC-derived immature T cells cultured alone showed any evidence of T cell maturation or proliferation (Figures 2B and S3A and S3B).

To test whether iPSC-derived T lineage cells were indeed migrating deep into the thymic lobes, we sectioned the co-cultured tissues. Histological analysis of unseeded control lobes showed a tissue architecture characterized by an astrocyte-like thymic epithelial web (Hamazaki et al., 2016) but no CD3+ cells. When seeded with iPSC-derived immature T cells, the tissue was repopulated with CD3+ mononuclear cells (Figure 2C). This confirmed the capacity of iPSC-derived T cells to migrate and repopulate thymic lobes devoid of endogenous T cells. Phenotypic examination of the cell types present in the 3D thymic culture performed by mechanical disruption of the thymic lobes showed a mix of DN, DP, and CD8α.SP T cells (Figure S3C). Importantly, most of the CD8α.SP T cells inside the lobes were CD8β+CD62L−CD69− and MHC class I+ (Figures S3C–S3E), indicating their successful passage through positive selection. A single seeding of just one thousand (10^3) iPSC-derived immature T cells into the 3D thymic culture resulted in continuous generation of a halo of cells that could be harvested every 1–2 days over a 2-week period without disrupting the thymic lobes. Notably, when lobes were seeded with OR-iPSCs, which lack a pre-rearranged αβTCR gene, cells recovered from the culture consisted of a diverse mixture of iTEs (CD4*CD8α− and CD8αβ SP T cells) and DN cells, indicating the thymic lobes’ potential to generate several types of cells, as occurs in vivo (Figure 2D). Conversely, when lobes were repopulated with Pmel-iPSCs, the cell yield was a uniform population of iTEs exhibiting a predominantly CD8α.SP phenotype (Figure 2D). These results were consistent with the hypothesis that iPSC with a pre-rearranged MHC class I-restricted TCR would only

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produce CD8+ T cells after thymic education, as occurs physiologically in Pmel-1 TCR transgenic mice (Overwijk et al., 2003).

To explore the diversity of the TCR repertoire of cells generated using the 3D co-culture method, we performed deep sequencing of TCRs in sorted CD8αSP iTEs. We found that OR-iTEs had a diverse TCRβ repertoire suggestive of normal αβ TCR recombination and selection. In contrast, Pmel-iTEs showed one dominant clonal pre-rearranged TCRβ chain (>98%) (Figure 2E). Moreover, the TCRα locus rearrangements in Pmel-iTEs were more restricted than OR-iTEs (Figure S3F). These results indicated that OR-iPSCs differentiated into CD4+ or CD8αβ+ iTEs with a broad TCR repertoire, whereas Pmel-iPSCs, having pre-rearranged αβ TCR genes, largely suppressed recombination events as they developed into iTEs.

**iTEs Phenotypically and Functionally Match Endogenous Post-thymic T Cells**

Given the thymic homing properties of iPSC-derived immature T cells and their capacity to undergo thymic education (Figures 2B and S3C–S3E), we analyzed the properties of iTEs. Specifically, we compared the phenotype, function, and global gene expression profile of iTEs with the cells co-cultured with OP9/DLL1 alone (extrathymic T cells). Flow cytometry was used to measure markers important for T cell differentiation and maturation, including MHC class I, L-selectin (CD62L), and CD69 (Figures 3A–3C). iTEs showed a downregulation of the activation marker CD69 and high expression of MHC class I and CD62L, markers associated with high proliferative competency, cytokine production, peripheral survival, and lymphoid homing (Hogquist et al., 2015; Rosen, 2004; Vivier et al., 2011). This phenotype was consistent with M2 SP thymocytes, the most mature population of SP T cells in the thymus (Hogquist et al., 2015), suggesting that iTEs have transitioned through a normal thymic development program. Moreover, when stimulated in vitro with cognate peptide, Pmel-iTEs rapidly expanded (>1,000-fold), analogous to naive CD8+ T cells, whereas extrathymic T cells lacked robust expansion after cognate peptide stimulation (Figures 3D and S4A). Importantly, Pmel-iTEs maintained CD8αβ+ expression after prolonged re-stimulation (Figures 3E and S4B). These results indicate the importance of thymic education in correcting the aberrant downregulation of key markers like MHC class I and CD8β+ expression with preservation of proliferative capacity.

Antigen-dependent release of cytokines is critical for the appropriate function of T cells, so we sought to determine the peptide-specific cytokine profile of Pmel-iTEs. In the absence of peptide stimulation or in the presence of an irrelevant nucleo-protein peptide, Pmel-iTEs did not release significant amounts of interleukin-2 (IL-2), TNF-α, or interferon γ (IFN-γ). However, stimulation with the cognate peptide for Pmel T cells (hgp100) and, to a lesser extent, with a mixture of antibodies to CD3 and CD28, triggered robust functional release of IL-2 and TNF-α and low amounts of IFN-γ (Figures 3F and S4C). Although the peptide specificity of control CD3-iTEs is unknown, these cells were also capable of releasing IL-2 and TNF-α, with lesser amounts of IFN-γ, in response to anti-CD3 and anti-CD28 (Figure S4D). Cell functionality was also compared with both primary CD8SP thymocytes and naive T cells (Figure S4E). It is perhaps notable that thymically educated iTEs had cytokine release patterns consistent with those seen in CD8+ T cells that have not yet achieved full
effector function because full-blown CD8+ T cell effector differentiation is marked by reduction or elimination of IL-2 production and acquisition of robust levels of IFN-γ (Gattinoni et al., 2011; Hogquist et al., 2015).

To interrogate the differences between endogenous CD8αSP T cells and iTEs on a whole-transcriptome level, we used RNA-seq. We identified 2,059 differentially expressed genes (Figure 3G, $\log_2$ FC > 2, adjusted $p < 0.01$). First, to examine whether iTEs execute aberrant transcriptional programs like those observed in extrathymic T cells, we analyzed the differentially expressed genes included in the three clinically relevant pathways selected previously (Figure 3H). iTEs displayed upregulation of genes associated with activation ($Gzmb$, $Ifng$, and $IL2$), which can be associated with recent thymic maturation and subsequent activation because of extrinsic IL-7 in the 3D culture system medium (Kimura et al., 2013). Additionally, genes aberrantly regulated in extrathymic T cells, including downregulation of classical MHC complex genes and upregulation of NK receptors, were rectified in iPSC-derived iTEs after thymic education. Notably, unlike extrathymic T cells (Figure 1I), iTEs showed loss of immature T cell markers ($Ptcra$, $Rag1$, $Rag2$, and $Rorc$) and upregulation of classical MHC class I post-selection markers (H2-K1 and H2-D1). Furthermore, the iTEs clustered with naive T cells (Figure 3I), indicating their completion of the positive selection stage (Carpenter and Bosselut, 2010; Hogquist et al., 2015; von Boehmer, 2005). Taken together, these results are consistent with the idea that iTEs have completed positive selection without indication of key abnormalities found in extrathymic T cells.

**Whole-Transcriptome Analysis Reveals a Shift in iTE Gene Expression toward a Naive CD8+ T Cell Program**

To further probe the transcriptional differences between iPSC-derived T lineage cells with and without thymic education (i.e. iTEs and extrathymic T cells), we compared the expression of a curated list of 102 genes known to be important for T cell ontogeny, thymocyte activation, and memory formation (Best et al., 2013; Hogquist et al., 2015; Schmitz et al., 2003; Table S2). We compared the gene expression patterns with primary naive CD8+ Pmel T cells and with DP T lineage cells differentiated using OP9/DLL1 (OP9-DP) (Figures 4A). Using hierarchical clustering and correlation of sequenced samples, we found that iTEs and naive T cells clustered together and were distinct from cells matured in the absence of thymic education. We also observed a striking similarity between DP and CD8αSP T cells derived in OP9/DLL1 (Figures 4B and 4C). To identify global transcriptional gene expression signatures, we analyzed activated signaling pathways and upstream regulators in iTEs versus extrathymic T cells using gene set enrichment analysis (GSEA) and ingenuity pathway analysis (IPA) (Figures 4D and S5A and S5B). GSEA identified enrichment of signaling pathways associated with positive selection and endogenous recent thymic emigrant (RTE) development, including the activation of IFN, nuclear factor κB (NF-κB), and TNF pathways and down-regulation of E2F signaling pathways (Figures S5A and S5A; Table S3; Carpenter and Bosselut, 2010; Xing et al., 2016). IPA showed an enrichment of similar regulatory pathways in iTEs, including activation of IFN pathway genes, as well as activation or inhibition of upstream regulators correlating to changes in activity that have been associated with late-stage thymocyte maturation (Figure
Thus, iTEs exhibited transcriptional profiles observed in late stages of thymic maturation and RTE development.

To further delineate the developmental stage of iTEs, we selected key genes marking the transition from mature CD8aSP thymocytes to the naive stage and compared the expression patterns of OP9-DP, extrathymic T cells, iTEs, and CD8aSP naive T cells (Carpenter and Bosselut, 2010; Fink, 2013; Hogquist et al., 2015; Figure S5C). We found that iTEs closely clustered with naive CD8aSP T cells in genes important for post-thymic selection and RTE peripheral maturation and displayed an expression profile of genes related to thymic egression. Collectively, RNA-seq data reveal that extra-thymic T cells have a transcriptional program similar to that of immature DP thymocytes, whereas iTEs, which have been subjected to thymic education, constitute a unique population of cells that are transcriptionally similar to naive CD8aSP T cells.

**iTEs Launch a Functional T Cell Program Analogous to that of Post-thymic Mature CD8+ T Cells**

Given the phenotypic and transcriptional similarities of iTE with naive T cells, we hypothesized that iTEs might be capable of launching a functional T cell program in vivo that matches that of endogenous T cells. To test this, we transferred small numbers (1 × 10^4) of either Pmel-iTEs or naive CD8^+ Pmel T cells into Rag−/− hosts as described previously (Gattinoni et al., 2009; Figure 5A). We analyzed the frequency and phenotype of congenically marked peripheral blood mononuclear cells (PBMCs) and splenic cells 4 weeks after their adoptive transfer. We found that transferred iTEs retained CD8β and TCRBV13 expression as their naive T cell counterparts in vivo; however, the levels of these markers were lower. Like physiologically occurring naive T cells, most iTEs expressed high levels of CD44 and low levels of CD62L. A small but significant increase in the number of T cell progeny from the central memory subset (CD44^+CD62L^+) was observed in mice receiving naive cells (Figures 5B–5D). We speculated that this relative impairment of central memory formation by iTEs could be attributed to their close similarity to RTEs, which are pre-activated because of recent positive selection events and, thus, have a lower potential to form memory cells (Friesen et al., 2016; Smith et al., 2014).

Most importantly, we observed similar numbers of progeny T cells within the spleens of mice receiving iTEs or physiologically produced naive T cells (Figure 5E). This finding indicated that 4 weeks of in vivo expansion produced nearly a two-log increase in the cells that were transferred. However, it remained unknown whether these massively expanded cells retained functionality. We measured the ability of progeny cells recovered from the spleens of recipient mice to produce cytokines upon specific stimulation with cognate peptide. We observed similar patterns of cytokine production between ex vivo expanded iTEs and naive T cells (Figure 5F). Quantitatively, many progeny cells produced IFN-γ and TNF-α and relatively fewer made IL-2. This pattern of cytokine production was consistent with that observed during physiological T cell maturation (Gattinoni et al., 2009). Notably, all recipient mice remained healthy with no evidence of weight loss (Figure S6A), autoimmunity, lymphomagenesis, or teratoma formation at a follow-up of more than 180 days (data not shown).
Given that the iT proary bore a strikingly similar function and phenotype to naive T cells after in vivo expansion, we sought to compare their transcriptional programs by whole-transcriptome analysis. We found a significant correlation between iTes and naive T cells by both RNA-seq (Figure 5G; coefficient of determination $R^2 = 0.952$) and microarray analyses (Figure S6B; $R^2 = 0.983$). Because of early concerns of slightly decreased expression of classical MHC class I in iT-derived cells compared with naive T cells (Figure 3I), we closely examined reads per kilobase of transcript per million mapped reads (RPKM) values of H2-K1 and H2-DI (Figure 5H), which were found to be transcriptionally equivalent to naive T cells. Taken together, these findings indicated that, with time, iT acquired phenotypic, functional, and gene expression patterns similar to naive T cells in vivo.

**Small Numbers of iTes Suppress Growth of Established Tumors and Prolong the Survival of Tumor-Bearing Animals**

Given our primary goal of developing in vitro-generated, less differentiated T cells suitable for cancer treatment via ACT, we tested whether iTes were effective at recognizing and treating established solid tumors (Figure 6A). To test this, we used the Pmel-1 mouse model and syngeneic wild-type mice bearing B16 tumors established for 8 days to ensure establishment of independent vasculature (Overwijk et al., 1998). In an effort to measure the therapeutic potency of the iT product, we elected to adoptively transfer a small number (5 × 10⁴) of cells (naive CD8⁺ Pmel T cells, CD3-iTes or Pmel iTes) into tumor-bearing mice (Figure 6A).

The ACT of Pmel-iTes resulted in significant suppression of tumor growth ($p = 0.028$) compared with CD3-iTes (Figure 6B). CD3-iTes lack expression of the tumor antigen-specific TCR and had no greater effect on tumor growth than non-treatment control groups. Mice treated with Pmel-iTes had significant anti-tumor activity (Figure 6B) and significantly prolonged survival (Figure 6C) ($p = 0.0018$). It should be duly noted that Pmel-iTes were not as efficient as naturally occurring T cells at this small dose, especially in the mice measured blindly 24 days after ACT (Pmel-iTes versus Pmel-naive, $p = 0.009$) (Figure 6B). Thus, iTes constitute a homogeneous population of in vitro-generated tumor antigen-specific T cells from iPSCs with a pre-rearranged TCR. iTes do not display the classical developmental aberrations observed in T cells generated using the OP9/DLL1 culture system alone and demonstrate a rejuvenated capacity to expand, persist in vivo, and mediate regression of solid tumors.

**DISCUSSION**

The success of ACT relies on the isolation and subsequent clonal expansion of tumor antigen-specific T cells from patients (Restifo et al., 2012; Rosenberg et al., 2011). In the majority of cases, the cells that are isolated are terminally differentiated or senescent, which is further exacerbated by expansion prior to their adoptive transfer (Restifo et al., 2012). These issues lead to considerable interest in using iPSC technologies for enabling the reprogramming of terminally differentiated T cells into less differentiated (naive) T cell populations with identical TCR rearrangements specific for mutant peptides expressed on
the surfaces of tumor cells (Rosenberg and Restifo, 2015). The ability to generate less
differentiated T cells from reprogrammed tumor antigen-specific T cells might provide a
significant advance in the approach to ACT (Klebanoff et al., 2012).

We and others have established that, by employing the conventional OP9/DLL1 co-culture
system, iPSC technology can be used to generate functional T cells in vitro carrying a tumor
antigen-specific pre-rearranged TCR (Lei et al., 2011; Saito et al., 2016; Vizcardo et al.,
2013). Although many aspects of regenerated T cell development using these extrathymic
methods remain unclear, numerous examples of abnormal T cell development, especially in
T cells carrying pre-rearranged TCRs, have been documented (Serwold et al., 2007; Themeli
et al., 2015). Although the OP9/DLL1 system recapitulates thymopoiesis consistently until
the DP stage, the subsequent non-physiological stimulation of extrathymic T cells leads to a
loss of CD8β expression after prolonged activation and the conversion to a CD8αα or
NK1.1+ phenotype (Figures 1E and S2I; Takada et al., 2017; Yamagata et al., 2004). The
major divergence from conventional T cell development is revealed by the lack of expression
of proteins related to activation (CD69) and self-recognition (MHC class I) in extrathymic T
cells, indicating their maturational arrest prior to positive selection (Hogquist et al., 2015;
Xing et al., 2016). This observation is reinforced by the fact that extrathymic T cells express
pre-positive selection (Ptcra, Rag1, Rag2, and Roc) and NK cell receptor genes (Klra7,
Klrc1, Klrc2, and Klrc3) (Figures 1G and 1H). These properties are native to thymocytes
that are either in a transitional developmental stage to become CD8SP (CD8iSP) or in
thymocytes that develop with inadequate positive selection signals, including activation
through alternative mechanisms, diverting them to an innate-like phenotype (Takada et al.,
2017; Yamagata et al., 2004). These results identify extrathymic T cells to be a heterogeneous
mix of cells that are developmentally derailed because of the lack of proper positive
selection signals after the DP stage. Thus, expression of conventional markers used
previously, like TCR, CD3, and CD8αβ+, is insufficient to enable characterization of a
broad pattern of dysregulated gene expression cells matured on OP9/DLL1.

Although other groups have shown that extrathymic T cells from iPSCs carrying a pre-
rearranged TCR display antitumor properties in vivo, these studies depend on the use of very
large numbers of diverse, non-homogeneous cell types in animals with a functional thymus
(Lei et al., 2011; Saito et al., 2016). Consequently, iPSC-derived aberrant cells lacking a
thymic environment could default into CD8αα or NK cells with potentially deleterious
clinical consequences (Fink, 2013; Hakim et al., 2005; Kuderer et al., 2006). Furthermore,
differentiation of T cells from a source carrying pre-rearranged αβTCRs gives rise to a
broad mix of DN, DP, and CD8 SP T cells characterized by MHC-independent lytic activity,
alopecia, osteomalacia, and spontaneous TCR-driven lymphomagenesis (Lei et al., 2011;
Maeda et al., 2016; Serwold et al., 2010). Thus, these previous reports and our own previous
data (Zhao et al., 2007) raise safety concerns regarding the use of heterogeneous and ill-
de fined cell products for future therapeutic applications. It is therefore essential that iPSC-
derived products are well characterized and differentiated into a mature lineage with a
homogeneous phenotype capable of causing regression of solid tumors without any observed
long-term abnormalities or clinical side effects, as we have demonstrated for iTEs.
Taken together, it is clear that provision of Delta-like ligands in the OP9/DLL1 system to induce Notch signaling for iPSC differentiation is insufficient to determine the fate specification to fully mature, post-thymic T cells. This observation of the insufficiency of Delta-like ligands is often obscured when investigators transfer a mixed population of cells with unknown maturational states into mice, which have a non-involuted thymus (Lei et al., 2011; Saito et al., 2016), allowing further maturation in vivo.

The influence of thymic education on iPSC-derived immature T cells is evident in the 3D thymic culture system, where iPSC-derived immature T cells are co-cultured in a thymic lobe at the air-medium interface. This system enables the culture and continuous recovery of thymically educated mature CD8SP T cells (iTEs) for extended periods of time (Figure 2A). Although the previously established FTOC methods with a cultured intact thymus yield a heterogeneous mix of immature T cells at various developmental stages, including some SP cells (Lee et al., 2001; Ueno et al., 2002), the 3D thymic culture system generates a homogeneous population of CD8SP T cells. The iTEs generated by the 3D thymic culture system retain the expression of the CD8β coreceptor and demonstrate antigen-specific proliferation and function (Figure 3). The use of congenically labeled iPSCs and donor thymocytes has helped to confirm iTEs to be a product arising from the thymic maturation of iPSC-derived immature T cells (Figure 2B). The 3D system described in this manuscript does not require the use of hyperoxia, tissue-surface contact, or mechanical disruption of thymic lobes. Moreover, the 3D thymic culture system enables easy recovery of post-thymic iPSC-derived iTes without the need for mechanical disruption of the thymic tissue, permitting the continuous generation of iTEs from one single thymic lobe. In summary, this 3D system is a thymic organ culture method that enables iPSC-derived immature T lineage cells to experience a physiological thymic micro-environment, generating iTEs, a homogeneous, physiologically relevant population of in vitro-generated post-thymic T cells.

The iTEs generated using the 3D thymic culture system are phenotypically and functionally close to naturally occurring post-thymic naïve T cells in several aspects. First, we found that iTEs express T cell markers associated with thymic education and post-thymic maturation and that they can expand long-term after prolonged stimulation without loss of CD8β expression. Furthermore, iTEs released cytokines with a profile similar to that of endogenous T cells after stimulation with cognate but not irrelevant peptide, demonstrating their antigen-specific functions. RNA-seq analysis of thymic maturation genes indicates close clustering of iTEs with naïve T cells and not with extrathymic T cells, which clustered with OP9-DP T cells. Analysis of gene signatures using GSEA shows that iTEs have signaling patterns in the IFN, E2F, NF-κB, and TNF-α pathways previously shown to be important for the last stages of thymic maturation (Xing et al., 2016). Based on whole-transcriptome analysis, iTEs also display a gene expression upstream regulator signature related to late stages of T cell thymic development, such as post-positive selection, thymic egression, and RTE peripheral maturation. More importantly, when transplanted in vivo, iTEs can form effector memory cells (CD44+CD62L+) and, at slightly reduced efficiency, central memory cells (CD44−CD62L+), indicating that these T cells followed a maturational trajectory similar to the physiologically occurring naïve T cells used as controls in the same in vivo experiment. Finally, a dosage of iTEs, approximately two logs lower than previously used for human or mouse iPSC-derived T cells (Maeda et al., 2016; Saito et al., 2016),
caused tumor regression similar to naive T cells in an ACT model of established solid
tumors. Overall, iTes demonstrate a pheno-typic and functional similarity to naturally
occurring naive T cells, with no known track of aberrant phenotype, function, or gene
expression patterns as observed in conventional iPSC-derived T cells matured in the absence
of a thymic microenvironment.

In conclusion, we have successfully developed a method to efficiently generate a
homogeneous population of CD8αβ+ SP antigen-specific naive-like T cells from iPSCs that
exhibit the capacity to expand, persist in vivo, and mediate regression of established solid
tumors. iTes are the first cells to be generated from iPSCs with such a promising naive-like
T cell phenotype. Thus, iTes might hold great potential for identifying the molecular signals
necessary for thymic selection. Understanding the naturalistic signals provided by the
thymus might facilitate the creation of culture systems that enable the generation of
clinically relevant tumor antigen-specific naive-like T cells for human therapeutic use.

EXPERIMENTAL PROCEDURES

Mice

Animal experiments were approved by the Institutional Animal Care and Use Committees of
the National Cancer Institute (NCI) or National Heart, Lung, and Blood Institute (NHLBI)
and performed in accordance with NIH guidelines. Female C57BL/6N (NCI) mice were
used as recipients in tumor experiments. C57BL/6N mice crossed with CD45.1+ Pep Boy
mice (NCI B6-Ly5.1Cr), purchased from Charles River Laboratories, were used as donors
for thymic lobes in 3D tumor culture experiments. Rag1−/− mice (B6.129S7-Rag1tm1Mom/J,
stock no. 002216) were purchased from The Jackson Laboratory. NOD Scid Gamma (NSG)
mice, also from The Jackson Laboratory (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ, stock no.
005557), were used for teratoma formation as a part of iPSC validation. In vivo
transplantation assays, such as memory formation assays and ACT tumor immunotherapy
are described in detail in the Supplemental Experimental Procedures.

Generation of iPSCs, Maintenance, and Differentiation into T Lineage Cells

iPSC lines were generated from Pmel-1 mouse embryonic fibroblast (MEF), C57BL/6 MEF,
and CD3+ T cells. Cell lines were cultured in DMEM-based medium at 37°C with 5% CO2.

iPSCs were characterized using alkaline phosphatase staining, RT-PCR analysis, fluorescent
cytochemistry, and high-resolution spectral karyotype and teratoma formation. iPSCs were
differentiated based on a previously published protocol (Schmitt et al., 2004). Further details
regarding iPSC establishment, characterization, differentiation, and in vitro assays applied
can be found in the Supplemental Experimental Procedures.

3D Thymic Culture System

CD45.1+ CD45.2+ mouse fetal thymic lobes were harvested at embryonic day 15.5 (E15.5)
and treated with 2-deoxyguanosine for 7 days at 37°C with 5% CO2. On day 7 or 8, thymic
lobes were transferred to a 3D hanging drop plate and seeded with iPSC-derived immature T
cells collected from OP9/DLL1 co-cultures on days 16–21. iPSC-derived iTes could be
harvested at day 4 after seeding. The 3D thymic culture system and iTe characterization by
confocal microscopy, flow cytometry, and TCR sequencing are described in detail in the Supplemental Experimental Procedures.

**Whole-Transcriptome and Microarray Analyses**

RNA was isolated using the QIAGEN RNeasy micro kit, and the integrity was checked by the RNA integrity number (RIN) value. Prepared libraries were processed at the NCI CCR sequencing facility. Further analysis, including sequencing data, microarray, regression, GSEA, and pathway analysis are detailed in the Supplemental Experimental Procedures.

**Quantification and Statistical Analysis**

Statistical parameters, including the exact value of n, dispersion and precision measures (mean ± SEM), and statistical significance are reported in the figures and figure legends. Data are judged to be statistically significant when p < 0.05 by two-tailed Student's t test. In the figures, asterisks denote statistical significance as calculated by Student’s t test (*p < 0.5, **p < 0.01, ***p < 0.001, ****p < 0.0001). Survival significance was determined by a log rank Mantel-Cox test. Statistical analysis was performed in GraphPad Prism 7.

**DATA AND SOFTWARE AVAILABILITY**

The accession number for the microarray data reported in this paper is NCBI GEO: GSE81639. The accession number for the RNA-seq data reported in this paper is NCBI GEO: GSE105110.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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### Highlights

- iPSC-derived CD8 single-positive T cells generated using OP9/DLL1 are abnormal
- 3D thymic culture enables generation of iPSC-derived thymic emigrants (iTEs) \textit{in vitro}
- iTEs are homogeneous and have gene expression patterns similar to naive T cells
- Tumor antigen-specific iTEs form immunologic memory and kill tumors
Figure 1. Extrathymic T Cells Derived from iPSCs by OP9/DLL1 Displayed Markers of Immature T Lineage Cells and Innate-like Immune Cells

(A) Experimental layout of iPSC differentiation in vitro.

(B) Generation of iPSC-derived CD4−CD8α+ (extrathymic) and CD4+CD8α+ (double-positive, DP) cells at day 21 and 24 (cells were gated on CD45+).

(C) Analysis of CD8β and CD62L expression in C57BL/6 thymocytes and iPSC-derived T cells at day 18. Cells were gated on CD45+.

(D) Log fold change of gene expression in Novel vs Naive T cells.

(E) % CD8β+CD62L+ (of CD45+ T cells).

(F) MHC-I 

(G) Staining of extrathymic cells with antibodies to CD8β, CD62L, and CD45R.

(H) Genes significantly changed in Novel T cells.

(I) Heatmap of gene expression in Novel vs Naive T cells.

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(D) MHC class I expression of C57BL/6 thymocytes and extrathymic T cells at day 26. Cells were pre-gated on CD4+CD8+ (DP) and CD4−CD8+ (CD8α.SP).

(E) Post-cognate peptide stimulation for 8 days of Pmel naive splenocytes and OP9/DLL1 cultures at day 18. Cells were gated on CD45+TCRβ+. The bar plot shows the percentages of CD8α+CD8β+ T cells after peptide stimulation. Values represent mean ± SEM (n = 3, ****p < 0.0001).

(F) Volcano plot showing differentially expressed genes between extrathymic and naive CD8SP T cells (log2 fold change [FC] > 2 and adjusted p < 0.01).

(G and H) Two-way pathway analysis of extrathymic versus naive T cells. (G) Plot of significantly enriched pathways (red dots), with size indicating the number of genes included in the pathway. Arrows indicate pathways determined to be the most clinically relevant with regard to ACT. (H) Log FC of genes enriched in the three pathways highlighted in (G). Bar color denotes upregulated (red) or downregulated (blue) genes in extrathymic versus naive T cells.

(I) Hierarchical clustered heatmap of genes related with positive selection. Data are representative of three to five independent experiments. See also Figures S1 and S2 and Movie S1.
Figure 2. Generation of iTEs Using a 3D Thymic Culture System
(A) Experimental layout of the 3D thymic culture system. Day 16–21 iPSC-derived immature T cells generated by OP9/DLL1 co-culture were collected and seeded in ultra-low attachment cylinders. iTEs are collected daily as they egress from the thymic lobe.
(B) Bright-field microscopy images of a 3D thymic organ culture on day 6 after seeding. Fetal thymus lobe and iPSC-derived immature T cells cultured alone are shown as controls. Shown is an enlargement of iTEs egressing from the lobe (red rectangle). Representative images of 12 independent experiments are shown. Scale bars, 200 mm.
(C) Left: H&E staining of a thymic lobe with and without seeding of iPSC-derived immature T cells. Right: confocal images of the sectioned lobes stained with DAPI (nucleus) and CD3 (T cell). Images are representative of four independent experiments. Scale bars, 100 mm.

(D) Analysis of iTEs 6 days post-seeding. Data shown are representative of 12 independent experiments.

(E) TCR repertoire analysis on CD8α.SP iTEs from OR-iPSCs and Pmel-iPSCs by RNA deep sequencing. Shown are skyscraper plots of the TRBV (x axis) versus the TRBJ gene (y axis). The z axis shows the frequency of that specific TRBVJ recombination. (n = 2/group). See also Figure S3 and Movie S2.
Figure 3. iTEs Phenotypically and Functionally Match Endogenous Post-thymic T Cells

(A) Analysis of Pmel-iTE versus thymocytes and extrathymic T cells. CD8α.SP gated populations were further analyzed for CD69, MHC class I, CD8β, and CD62L. Cells were pre-gated on CD45+NK1.1−TCRγδ−CD44−CD25−.

(B) Percentage of MHC class I+ CD69+ on CD8α.SP gated populations shown in (B). Values represent mean ± SEM (n = 3 for thymocytes and extrathymic T cells, n = 6 for iTEs, ***p < 0.001, NS indicates no significant difference).
(C) Percentage of CD8β+CD62L+ on CD8α.SP gated populations shown in (B). Values represent mean ± SEM (n = 5 for thymocytes and extrathymic T cells, n = 8 for iTes, ***p < 0.001). Data are representative of three independent experiments. (D and E) Cognate peptide stimulation of CD8αβ+ Pmel naive, extrathymic, and iTE cells.
(D) FC.
(E) Analysis of CD8β CD62L expression. Data are representative of two independent experiments.
(F) Percentage of Pmel-iTEs expressing IL-2, IFN-γ, and TNF-α after cognate (hgp100) or irrelevant (nucleoprotein) peptide stimulation. The boxplot represents mean ± SEM (n = 3). Data are representative of three independent experiments.
(G) Volcano plot showing differentially expressed genes between iTE and naive CD8SP T cells (log2 FC > 2 and adjusted p < 0.01).
(H) Log FC of differentially expressed genes between iTE and CD8SP T cells after enrichment in the three pathways identified in extrathymic T cells (Figure 1H). Bar color denotes upregulated (red) or downregulated (blue) genes in iTes versus naive T cells.
(I) Hierarchical clustered heatmap of genes related with positive selection. See also Figure S4.
Figure 4. Whole-Transcriptome Analysis Reveals a Shift in iTE Gene Expression toward a Naive CD8⁺ T Cell Program

(A) Schematic outline of the cell groups used for whole-transcriptome RNA-seq. (B and C) Correlation plot (B) and hierarchical clustering (C) of naive, DP, extrathymic, and iTE cells based on a curated gene list of 102 genes important for T cell ontogeny, thymocyte activation, and memory formation.

(D) Representative GSEA plots of significantly enriched gene sets in iTEs versus extrathymic T cells corresponding to IFN, TNF, NF-κB, and E2F signaling. The table summarizes all significantly enriched gene sets (|normalized enrichment score [NES]| > 2.00, false discovery rate [FDR] q-val < 0.001) from the Hallmark C2 collections corresponding to these 4 pathways (bold, upregulated in iTEs; not bold, downregulated in iTEs). The weighted NES average was calculated as described in the Supplemental Experimental Procedures.

See also Figure S5.
Figure 5. iTEs Launch a Functional T Cell Program Analogous to that of Post-thymic Mature CD8+ T Cells

(A) Schematic outline to evaluate the in vivo reconstitution potency of iTEs.

(B) Analysis of CD8β versus TCRBV13 and CD44 versus CD62L expression in congenically marked CD45+CD8α+ peripheral blood T cells 4 weeks after transfer.

(C and D) Percentages of (C) CD44+CD62L− (effector memory) and (D) CD44+CD62L+ (central memory) cells analyzed in (B). Values represent mean ± SD (n = 3, *p < 0.05). Data are representative of two independent experiments.

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(E) Total number of transferred Pmel naive T cells and iTEs, as measured by CD45+CD8α+TCRBV13+ T cells in spleens. Values represent mean ± SD from two pooled experiments (n = 6). Data are representative of two independent experiments.

(F) Cytokine production of transferred Pmel naive T cells and iTEs enriched from the spleens 4 weeks after transfer after ex vivo stimulation with cognate antigen (hgp100), irrelevant antigen (nucleoprotein), or anti-CD3/CD28. Cells were gated on CD45+CD8α+. Values represent mean ± SEM (n = 3). Data are representative of two independent experiments.

(G) Transcriptional correlation of RNA-seq data from naive Pmel T cells versus iTEs, sorted from the spleen 4 weeks post-transfer (n = 3/group, R² = 0.9520879).

(H) Bar plots showing the RPKM expression values ± SD/SEM. See also Figure S6.
Figure 6. Small Numbers of iTEs Suppress Growth of Established Tumors and Prolong the Survival of Tumor-Bearing Animals

(A) Representative layout of T cell preparation for use in the treatment of tumors established for 8 days in C57/BL6 mice.

(B) Tumor growth curve over time after ACT of (5 × 10^4) Pmel iTEs, CD3-iTEs, naive T cells, or vehicle. Values represent mean ± SEM (n = 5/group, *p < 0.05, Wilcoxon rank-sum test). Tumor size was measured in a blinded fashion approximately every 3 days after cell transfer. Tumor areas were calculated as the product of the perpendicular diameters and are presented as mean ± SEM. Data are representative of two independent experiments.

(C) Survival data of mice represented as Kaplan-Meier curves. Mice were euthanized when tumors reached 400 mm². Data are representative of two independent experiments (n = 5, *****p < 0.0021) using a Mantel-Cox test.