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## Tumor-specific CD8<sup>+</sup> T cells expressing IL-12 eradicate established cancers in lymphodepleted hosts

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### Abstract

T-cell based immunotherapies can be effective in the treatment of large vascularized tumors, but they rely on adoptive transfer of substantial numbers (~20 million) of tumor-specific T cells administered together with vaccination and high-dose IL-2. In this study, we report that ~10,000 T cells gene-engineered to express a single-chain IL-12 molecule can be therapeutically effective against established tumors in the absence of exogenous IL-2 and vaccine. Although IL-12 engineered cells did not persist long-term in hosts, they exhibited enhanced functionality and were detected in higher numbers intra-tumorally along with increased numbers of endogenous NK and CD8<sup>+</sup> T cells just prior to regression. Importantly, transferred T cells isolated from tumors stably overproduced supra-physiological amounts of IL-12 and the therapeutic impact of IL-12 produced within the tumor microenvironment could not be mimicked with high doses of exogenously provided IL-12. Furthermore, anti-tumor effects could be recapitulated by engineering wild-type open-repertoire splenocytes to express both the single-chain IL-12 and a recombinant tumor-specific T-cell receptor (TCR), but only when individual cells expressed both the TCR and IL-12, indicating that arrested migration of T cells at the tumor site was required for their activities. Successful tumor eradication was dependent on a lymphodepleting pre-conditioning regimen that reduced the number of intra-tumoral CD4<sup>+</sup> Foxp3<sup>+</sup> T regulatory cells. Our findings reveal an approach to genetically modify T cells to reduce the cell number needed, eliminate the need for vaccines or systemic IL-2, and improve immunotherapy efficacy based on adoptive transfer of gene-engineered T cells.

### Keywords

Adoptive transfer; IL-12; tumor microenvironment

### Introduction

CD8<sup>+</sup> T cells migrate through tumor with high instantaneous velocity but arrest upon contact with cognate antigen (1,2). As a result, we reasoned that T cells might be able to overproduce an anti-tumor agent directly into a suppressive microenvironment that includes negative

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regulatory factors such as myeloid-derived suppressor cells and regulatory T cells (3-10). We chose to genetically engineer tumor antigen-specific CD8<sup>+</sup> T cells to secrete IL-12, a potent pro-inflammatory and anti-angiogenic cytokine capable of activating multiple aspects of innate and adaptive anti-tumor immunity (11-20). The mechanisms of anti-tumor action are complex, as IL-12 can directly augment the functionality of multiple end effectors such as CD4<sup>+</sup> (type 1), CD8<sup>+</sup>, and natural killer (NK) cells (12,21-25).

In clinical trials, attempts to give IL-12 systemically have been restricted by toxicities, limiting the benefits seen in pre-clinical models (12,20,21,24,26,27). Due to systemic dosing restrictions, the ability to reach therapeutic levels of IL-12 at the tumor site likely represents a major barrier for treatment of solid tumors. Several preclinical and clinical studies have demonstrated successful tumor regression by direct injection of IL-12 into lesions or genetic manipulations to induce tumor cells to produce IL-12 in situ (27-30). However, the majority of patients with metastatic cancer present with widespread disease not accessible to direct application at all sites and even if lesions are approachable, uniform distribution remains problematic. Several studies have engineered tumor cells, dendritic cells, and fibroblasts to overproduce a single chain functional IL-12, but achieving high local concentrations through a systemic approach remains elusive (26,30-34).

In this study, we examined the effects of delivering IL-12 directly to the tumor site by adoptively transferring tumor-specific gene-engineered CD8<sup>+</sup> T cells. We found that transferring small numbers of IL-12 engineered T cells into lymphodepleted hosts resulted in the destruction of large vascularized tumors without the need for vaccine or IL-2. We show that T cells engineered to produce IL-12 infiltrate tumors in higher numbers than non-transduced cells and continue to secrete supra-physiologic levels of IL-12 directly at the tumor site just prior to regression. T-cell receptor specificity was critical for tumor regression, suggesting that arrested migration of T cells at the tumor site was essential for successful treatments. These results describe a systemic approach to achieve high local concentrations of IL-12, leading to dramatic improvements in adoptive cell therapies.

## Materials and Methods

### Mice and Tumor Lines

Female thy1.1<sup>+</sup> pmel-1 TCR transgenic (Tg) mice were generated in our laboratory (35) and made available at www.jax.org. Female C57BL/6 mice and C57BL/6 TCR- $\alpha^{-/-}$  mice (obtained from Jackson Laboratories) bearing B16, an H-2<sup>b+</sup>/gp100<sup>+</sup> murine melanoma, established and maintained as previously described (36), were used as recipients for adoptive immunotherapies. Experiments were conducted with the approval of the National Cancer Institute Animal Use and Care Committee.

### Retroviral Production and Transduction

**Single Chain IL-12 Vector**—The cDNA for the p40 (*Il12b*) and p35 (*Il12a*) genes for IL-12 were linked by a sequence encoding a 15 amino acid (Gly<sub>4</sub>Ser)<sub>3</sub> flexible linker and inserted into a MSGV-1 based retroviral vector with a *woodchuck hepatitis virus* post-transcriptional regulatory element (WPRE) to help increase transgene expression. **Pmel-1 Vector**: The codon optimized pmel-1 TCR- $\alpha$  (*Tcra*) and TCR- $\beta$  (*Tcrb*) cDNA genes were linked by a sequence encoding the foot and mouth disease picornavirus 2A “self-cleaving” linker and inserted into a MSGV-1 vector (37). **Retroviral Production**: Platinum Eco 293 based cells (Cell Bio Labs) were plated on poly-d-lysine coated 100mm plates (BD Biosciences) and transfected with 6  $\mu$ g of pCL-Eco helper plasmid (Imgenex) and 9.3  $\mu$ g of the MSGV-1<sup>IL-12</sup> or the MSGV-1 pmel-1 TCR vector with lipofectamine 2000 (Invitrogen) overnight in antibiotic-free CM. Viral supernatants were harvested 36-48 hrs post transfection. **Retroviral transductions**: Pmel-1

splenocytes were cultured in the presence of 1  $\mu$ M hgp100<sub>25-33</sub> and CM containing 60 International Units (IU)/mL of recombinant human (rh) IL-2 (Chiron). For transduction of C57BL/6 splenocytes, 1  $\mu$ g/mL of soluble anti-CD3 (BD Biosciences), and 1  $\mu$ g/mL of soluble anti-CD28 (BD Biosciences) were used to stimulate bulk splenocytes. Two days later, splenocytes were collected and resuspended in retroviral supernatant with 60 IU/mL rhIL-2 and 10  $\mu$ g/mL protamine sulfate (Abraxis Pharmaceutical Products), and spun at 1000g at 37°C for 90 minutes in 24 well plates. Cultured cells were adoptively transferred 3-5 days post transduction (> 90% CD8<sup>+</sup> T cells).

### Adoptive Cell Transfer

Six to twelve week old mice (n=5 for all groups) were injected subcutaneously with  $5 \times 10^5$  B16 melanoma cells. Ten to fourteen days later, they were irradiated with 5 Gy total body irradiation (TBI) and given IL-12 transduced pmel-1 CD8<sup>+</sup> T cells or pmel-1 TCR and IL-12 double transduced C57BL/6 by tail vein. Tumors were measured using digital calipers and the tumor area was calculated as the product of perpendicular diameter by investigators in a blinded manner. All experiments were performed independently at least twice with similar results and all tumor curve data is shown as mean  $\pm$  standard error of the mean.

### Analysis of Adoptively Transferred Cells: Flow Cytometry, Cell Enumeration, Cytokine Release, Histology, and Real Time PCR

Prior to transfer, cells were characterized by flow cytometry for CD8 $\alpha$ , CD62L, CD44, IL-7R $\alpha$ , IL-2R $\alpha$  and Sca-1 (BD Biosciences). IL-12, IFN- $\gamma$ , TNF- $\alpha$  and IL-2 were analyzed using intracellular staining kits (BD Biosciences) with or without a 4 hour stimulation with phorbolmyristate acetate, 50 ng/mL (PMA) and ionomycin, 1  $\mu$ g/mL (Sigma). Following transfer, tumor samples were harvested and lymphocytes were isolated using lymphocyte cell separation media (Cedarlane Laboratories) and enumerated by flow cytometry. Transferred pmel-thy1.1<sup>+</sup> cells were analyzed by flow cytometry for thy 1.1 (CD90.1), NK1.1, CD4, CD8 $\alpha$  and IL-12 expression. Hematoxylin and Eosin staining was performed on paraffin fixed tumor samples and analyzed at 100X magnification by an Olympus IX-FLA microscope. Real-time reverse transcription PCR were performed as previously described (38).

### Statistical Analysis

Tumor growth slopes were compared using Wilcoxon rank sum test. One-way ANOVA and student t-tests were used to test for significant differences in enumeration assays.  $P < 0.05$  was considered significant.

### Results

To assess the ability of tumor-specific T cells to over-produce IL-12, we constructed a retrovirus based on a derivative of the murine stem cell virus, MSGV-1 (39), encoding a single-chain, bioactive IL-12 obtained by fusing the p35 and p40 subunits with a flexible (Gly<sub>4</sub>Ser)<sub>3</sub> linker (40-42) and designated the construct as MSGV-1<sup>IL-12</sup> (Fig. 1A left panel). We transduced pmel-1 CD8<sup>+</sup> T cells, which express a transgenic T-cell receptor specific for the melanoma-associated antigen, gp100, with this vector to express high levels of IL-12 (designated as pmel-1<sup>IL-12-TD</sup>; Fig. 1A right panel). We next examined the phenotype of pmel-1<sup>IL-12-TD</sup> T cells used for adoptive transfer experiments and showed several distinct characteristics compared to mock-transduced cells (Supplementary Fig. 1). IL-12 engineered cells also expressed higher levels of IFN- $\gamma$  (Fig. 1B, C) and TNF- $\alpha$  (Supplementary Fig. 2A) upon secondary stimulation, but expressed less IL-2 (Supplementary Fig. 2B). Two critical T-box transcription factors for CD8<sup>+</sup> T cells were altered with increased relative T-bet expression and down-regulation of eomesodermin (Supplementary Fig. 3) (43). On the other hand, IL-12 engineered cells underwent apoptosis when prolonged in culture beyond 7 days, failed to

proliferate upon secondary stimulation (Supplementary Fig. 4) and exhibited a small decrease in cytotoxic ability (Supplementary Fig. 5).

To assess the *in vivo* efficacy and toxicity of pmel-1<sup>IL-12-TD</sup> cells, we adoptively transferred decreasing doses of the gene-modified T cells cultured *in vitro* 5-7 days into sublethally-irradiated mice (5 Gy total body irradiation; TBI) bearing large vascularized B16 tumors established for 14 days. Surprisingly, we found that as few as 10,000 pmel-1<sup>IL-12-TD</sup> cells mediated significant tumor destruction (Fig. 2A; left panel). This potent anti-tumor response was observed in the absence of high-dose recombinant IL-2 (rIL-2) and a gp100 vaccine, which we previously found were required for effective anti-tumor immunity (35). It is noteworthy that we observed tumor destruction without significant weight loss (Fig. 2A; right panel) or pathological end-organ damage (Supplementary Fig. 6). Although all the experiments in this study were completed without observable toxicities, administration of doses greater than 500,000 pmel-1<sup>IL-12-TD</sup> cells given alone or in concert with IL-2 and vaccination produced weight loss and decreased survival (Supplementary Fig. 7). These toxicities were clearly related to the increased number of cells transferred and hence, likely correlated to the amount of IL-12 produced systemically, a well described clinical phenomenon (44). Experiments in mice treated with less than 500,000 cells did not show any clear signs of systemic side effects. Thus, small numbers of pmel-1 CD8<sup>+</sup> T cells engineered to secrete IL-12 safely enabled the destruction of large established tumors.

Due to pmel-1<sup>IL-12-TD</sup> cells undergoing apoptosis after one week in culture, we were surprised by the potency of these cells and next assessed the *in vivo* persistence of transferred cells, a characteristic classically associated with improved tumor regression (38). We adoptively transferred thy1.1 gene-marked, pmel-1<sup>IL-12-TD</sup> cells into sublethally-irradiated (5 Gy) tumor-bearing mice and detected increased engraftment of pmel-1<sup>IL-12-TD</sup> cells compared to non-transduced cells on day 3 and 7, but found that the persistence of IL-12 engineered cells dramatically dropped on day 14 (Fig. 2B).

We were surprised by the degree of tumor destruction despite the lack of persistence and therefore hypothesized that there maybe differences at the tumor site contributing to the marked enhancement in response. Similar to previous experiments, we adoptively transferred thy1.1 gene-marked, pmel-1<sup>IL-12-TD</sup> cells into sublethally-irradiated tumor-bearing mice, harvested tumors one week after transfer and witnessed an increase in cellular infiltration in conjunction with higher amounts of necrosis within tumors of mice treated with pmel-1<sup>IL-12-TD</sup> cells (Fig. 3A). We next quantified the characteristics and numbers of tumor infiltrating cells and found that mice treated with pmel-1<sup>IL-12-TD</sup> cells had a significant increase in the number of CD8<sup>+</sup> thy 1.1<sup>+</sup> adoptively transferred T cells (Fig. 3B; left panel). Importantly, the transgene expression of IL-12 in transferred cells remained robust (Fig. 3B; right panel), indicating the ability of these cells to secrete supraphysiologic amounts of IL-12 directly into the tumor microenvironment just prior to regression. We next assessed infiltration of tumors by endogenous cells (thy1.1<sup>-</sup>) and observed a significant increase in NK cell infiltration and an increased trend in endogenous CD8<sup>+</sup> T cell infiltration (Fig. 3C). On the other hand, we did not observe any differences in the number of endogenous CD4<sup>+</sup> T cells infiltrating the tumors (data not shown). It is important to note that all mice received a 5 Gy TBI lymphodepleting regimen just prior to adoptive transfer, indicating that tumors were infiltrated by reconstituting endogenous cells. Thus, pmel-1<sup>IL-12-TD</sup> cells were capable of delivering IL-12 at therapeutic levels directly into the tumor microenvironment, leading to an increase in infiltration of transferred cells along with endogenous NK and CD8<sup>+</sup> T cells.

Based on the results from cells infiltrating the tumor, we next assessed the ability of non-transduced pmel-1 CD8<sup>+</sup> T cells (mock) to assist in tumor eradication. We hypothesized that co-transferring non-transduced pmel-1 CD8<sup>+</sup> T cells in combination with pmel-1<sup>IL-12-TD</sup> cells

may impact anti-tumor responses. We adoptively transferred pmel-1<sup>IL-12-TD</sup> cells alone or in combination with mock transduced pmel-1 CD8<sup>+</sup> T cells into sublethally-irradiated tumor-bearing mice. We did not observe any significant differences in anti-tumor responses by adding non-transduced pmel-1 CD8<sup>+</sup> T cells, most likely due to the potency of pmel-1<sup>IL-12-TD</sup> cells alone (Fig. 4A). To further understand the nature of these anti-tumor responses, we tested the importance of local versus systemic IL-12. We sought to ascertain whether we could replicate the successful treatments using exogenously provided IL-12. We compared pmel-1<sup>IL-12-TD</sup> cells to mock-transduced pmel-1 CD8<sup>+</sup> T cells adoptively transferred into sublethally-irradiated tumor-bearing mice in combination with increasing doses of exogenous recombinant murine IL-12 (rIL-12) given at doses up to 2 µg/day. Unlike pmel-1<sup>IL-12-TD</sup>, exogenously provided rIL-12 given together with pmel-1 CD8<sup>+</sup> T cells alone did not induce major tumor regression (Fig. 4B). We also compared pmel-1 CD8<sup>+</sup> T cells exposed to rIL-12 *ex vivo* (Tc1) to pmel-1 CD8<sup>+</sup> T cells engineered to secrete IL-12 and found that pmel-1 Tc1 cells failed to exhibit anti-tumor responses similar to pmel-1<sup>IL-12-TD</sup> cells when transferred in similar low numbers (Fig. 4B). Thus, IL-12 produced by the transferred T cells appeared to be more effective than that provided exogenously.

The reason for the selective retention and efficacy of IL-12 producing tumor-specific T cells was unclear, but the requirement of host immunodepletion was unambiguous in this model (Fig. 5A). Therapy failed when endogenous cells were not depleted by a preparative dose of 5 Gy TBI. We hypothesized that host immunodepletion functioned to create a “*niche*” that facilitated engraftment of adoptively transferred cells, but we did not measure significantly greater numbers of pmel-1<sup>IL-12-TD</sup> cells in the tumors or the spleens of mice receiving 5 Gy TBI (Fig. 5B; left panel). Instead, we observed that lymphodepleted mice had significantly lower numbers of intra-tumoral CD4<sup>+</sup> T cells (Fig. 5B; right panel) and that these remaining T cells from mice receiving 5 Gy TBI had lower expression of Foxp3, the transcription factor marking regulatory T cells (Fig. 5B; right panel). To further test the negative functional impact of endogenous T cells, we transferred pmel-1<sup>IL-12-TD</sup> cells into non-ablated wild type versus TCRα<sup>-/-</sup> (no endogenous T cells) tumor bearing mice (Fig. 5C). We observed a potent anti-tumor response in non-ablated TCRα<sup>-/-</sup> mice, indicating that endogenous T cells were indeed imparting a negative or regulatory impact on the adoptively transferred pmel-1<sup>IL-12-TD</sup> cells. Several studies by our laboratory and others have described the importance of a preparative ablative regimen for improving adoptive T cell transfer experiments and once again we show a similar requirement in this model (36,45-47).

The one aspect of the therapeutic response that still remained unclear was the importance for a tumor antigen specific TCR. To address the question of specificity using “open-repertoire” CD8<sup>+</sup> T cells, we developed a retrovirus that encoded the α/β pmel-1 TCR. We created three distinct cell types from a common precursor pool of CD8<sup>+</sup> splenocytes expressing: a) the pmel-1 TCR alone; b) IL-12 alone; or c) both the pmel-1 TCR and IL-12 (Fig. 6A). We found that the amount of IL-12 produced by single- and double-transduced cells was similar (Fig. 6A). Interestingly, open-repertoire CD8<sup>+</sup> T cells expressing either the single-chain IL-12 or the pmel-1 TCR failed to induce tumor regression in sublethally-irradiated tumor-bearing mice (Fig. 6B). The treatment was also ineffective when mice were given a 1:1 mixture of CD8<sup>+</sup> T cells expressing the pmel-1 TCR mixed with cells expressing the single chain IL-12. Only double-transduced CD8<sup>+</sup> T cells expressing both the pmel-1 TCR and IL-12 on the same cell were able to induce potent anti-tumor responses (Fig. 6B). We also found that CD8<sup>+</sup> T cells possessing both the pmel-1 TCR and IL-12, but not either component alone, accumulated at the tumor site in significantly higher numbers (Fig. 6C). These results indicated that IL-12 must be produced by T cells with specificity for the tumor in order to increase local infiltration and induce anti-tumor responses. Furthermore, our experiments revealed that enhanced engraftment of the gene-engineered T cells possessing both the pmel-1 TCR and IL-12 occurred significantly at the tumor site but not within the spleens or lymph nodes of the same recipient

mice (Fig. 6C). These results were consistent with the observations seen previously with transgenic pmel-1 CD8<sup>+</sup> T cells transduced with IL-12.

## Discussion

The adoptive transfer of autologous tumor-infiltrating lymphocytes (TIL) or TCR redirected peripheral blood lymphocytes (PBL) is a promising treatment for patients with metastatic cancer (48,49). The experiments described in this report demonstrate that small numbers of tumor-antigen-specific CD8<sup>+</sup> T cells engineered to produce high levels of IL-12 can lead to the regression of large vascularized tumors without the need for exogenous IL-2 or vaccine in lymphodepleted hosts. The marked improvement in treatment was associated with an increase in tumor infiltration by adoptively transferred T cells, along with an increase in endogenous NK cells and endogenous CD8<sup>+</sup> T cells from the reconstituting host. Based on the delayed kinetics of tumor destruction, modulation of endogenous host immune cells likely plays an important role in facilitating tumor destruction, but the impact of host NK cells and CD8<sup>+</sup> T cells remains unclear.

Nevertheless, one of our key findings in this study included isolating gene engineered T cells from tumors one week following adoptive transfer and observing the continued stable expression of high levels of IL-12. At the same time point, we also observed an increase in the degree of necrosis within tumors treated with pmel-1<sup>IL-12-TD</sup> T cells, an observation not surprising given the overall decrease in tumor size. We found that the expression of a tumor-specific TCR was critical for the ability of T cells to deliver IL-12 to the tumor microenvironment, suggesting that the arrested migration of T cells upon recognizing cognate antigen played a mechanistic role in the local delivery of IL-12.

Interestingly, a preparative lymphodepleting regimen, known to deplete suppressive factors such as regulatory T cells was necessary for successful tumor treatments. We were surprised by the requirement for lymphodepletion, largely due to the observation that the transfer of IL-12 engineered T cells into a lymphodepleted host resulted in an increase in endogenous NK and CD8<sup>+</sup> cells within the tumor. Our laboratory and others have described the ability of host cells to function as cytokine sinks, limiting the ability for homeostatic activation of adoptively transferred cells (36). Lymphodepletion likely removes endogenous regulatory T cells, such as CD4<sup>+</sup> Foxp3<sup>+</sup> cells, along with other T cells that function as suppressors and 'sinks', allowing for transferred IL-12 engineered T cells to arrest within the tumor upon recognizing cognate antigen. Once the host immune system reconstitutes, the changes within the tumor microenvironment induced by IL-12 likely trigger multiple downstream factors contributing to the profound changes observed. Thus, the reconstituting endogenous immune response to IL-12 secretion within the tumor may play a significant role in the anti-tumor effects described in our experiments.

In regard to treatment related toxicities, none of the mice in this study had observable toxicities attributed to the transferred T cells. However, during initial pilot experiments, we observed decreased survival when we transferred greater than 500,000 pmel<sup>IL-12-TD</sup> cells. This observation likely correlates directly to the level of systemic IL-12 produced by the constitutive expression of IL-12 in retrovirally transduced T cells. When we transferred 500,000 cells or less, thus decreasing the amount of systemic IL-12, we did not observe the previously witnessed toxicities. While TCR mispairing leading to off-target recognition has been raised as a theoretical concern, we did not observe any graft-versus-host-like toxicities in experiments following double transduction of open repertoire CD8<sup>+</sup> T cells with the pmel TCR and IL-12 (50). The inability of IL-12 engineered cells to persist long-term likely contributed to mitigating any toxicities related to TCR redirection. In regard to efforts to translate our findings into human clinical trials, a careful phase I dose escalation trial will likely be necessary to obtain

a safe therapeutic treatment cell number. Our group has taken additional measures to reduce the level of systemic IL-12 toxicity by developing retroviral vectors driven by an NFAT (nuclear-factor of activated T cells) promoter, capable of producing the single chain functional IL-12 only upon TCR stimulation (manuscript in preparation).

In conclusion, engineering tumor infiltrating lymphocytes (TIL) or antigen-specific peripheral blood lymphocytes (PBL) with IL-12 may dramatically improve cellular therapies against metastatic cancer. Furthermore, decreasing the number of cells required for adoptive transfer and eliminating the need for systemic IL-2 may help facilitate the widespread acceptance of a promising treatment modality. We recently completed the construction and amplification of a gammaretrovirus encoding a single-chain human IL-12 using “good manufacturing process” (GMP) conditions. This retrovirus will be used for the transduction of naturally-occurring and receptor-engineered T cells in the treatment of patients with advanced cancers. In the future, we may be able to engineer tumor-specific lymphocytes to locally deliver a wide array of immunomodulating payloads.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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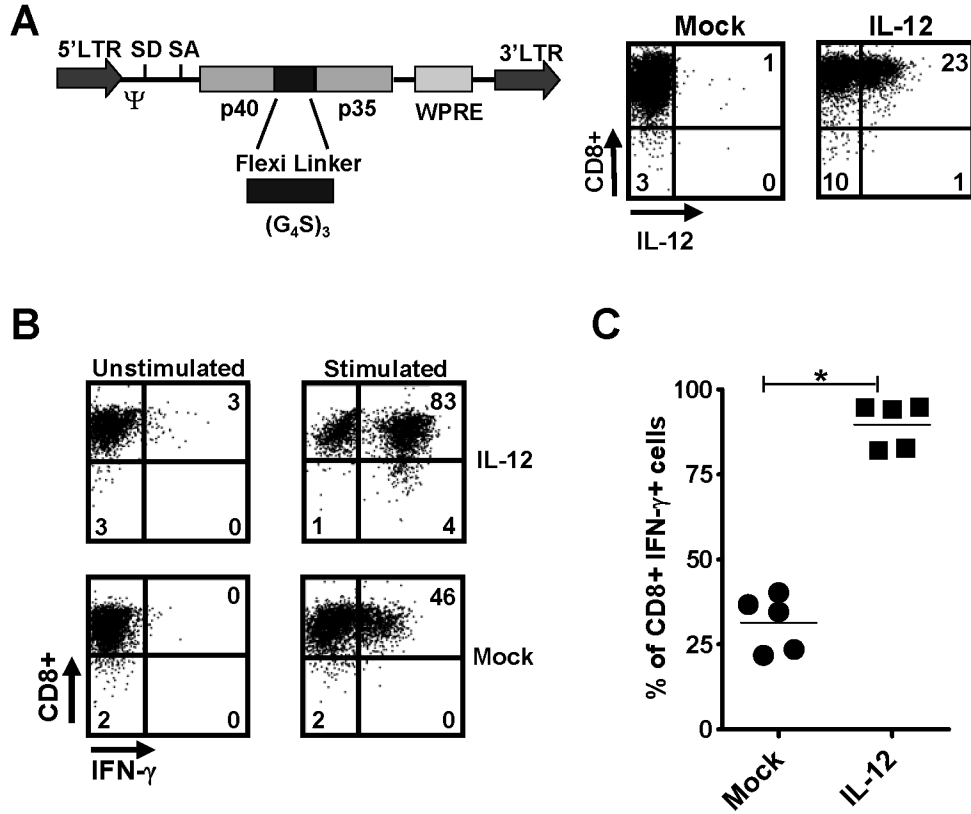
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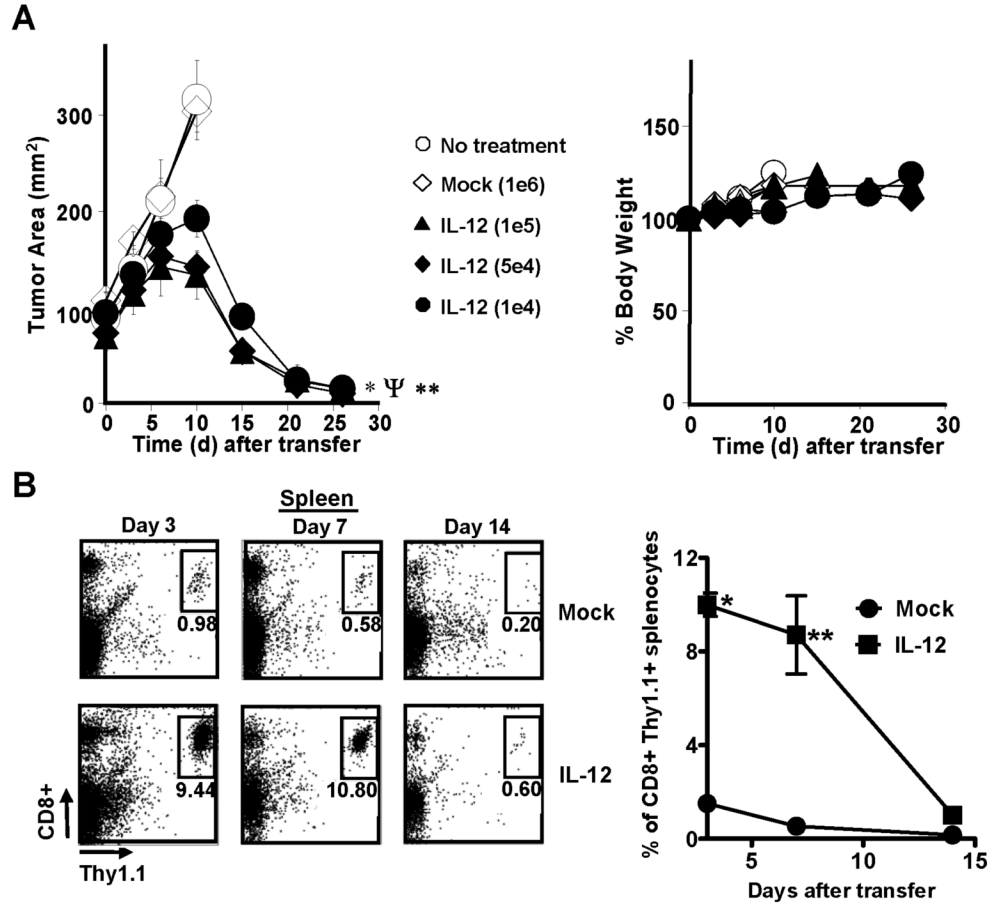


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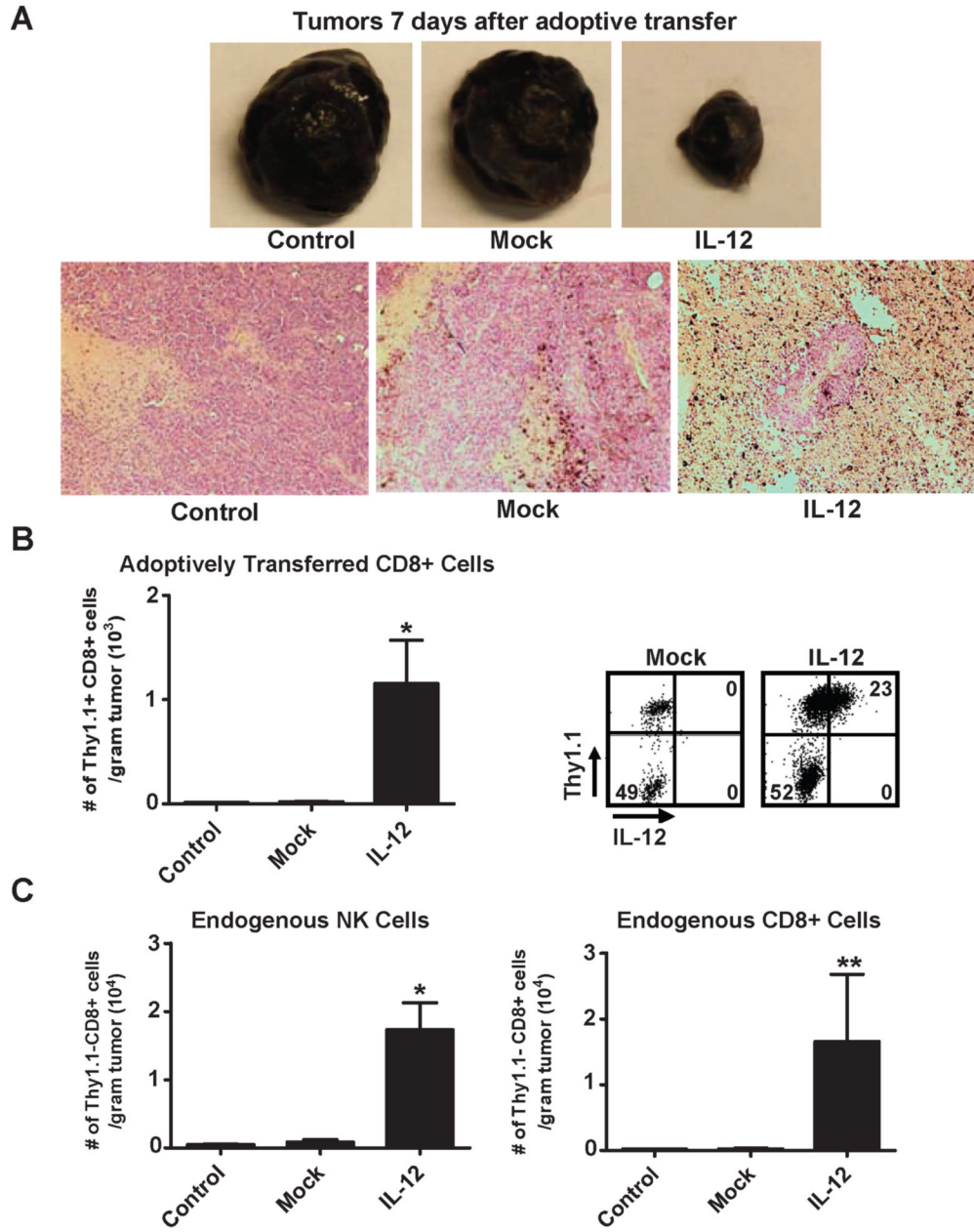
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**Figure 1.** Pmel-1 CD8<sup>+</sup> T cells engineered to overproduce IL-12 possess a distinct phenotype and enhanced functionality. A, The MSGV-1<sup>IL-12</sup> retroviral vector (left panel), which encodes the p40 and p35 subunits of IL-12 linked by (Gly<sub>4</sub>Ser)<sub>3</sub> flexible linker. SD, splice done; SA, splice acceptor; LTR, long terminal repeat; Ψ, packaging sequence. Pmel splenocytes were stimulated with 1 μM hgp100<sub>25-33</sub> for 2 days, transduced with the MSGV-1<sup>IL-12</sup> retrovirus, and analyzed by flow cytometry 3 days after for IL-12 expression (right panel). B, IL-12 transduced pmel-1 CD8<sup>+</sup> cells from A were analyzed by intracellular staining without or with a 4hr secondary stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin. C, The percentage of CD8<sup>+</sup> IFN-γ<sup>+</sup> cells following secondary stimulation in mock versus IL-12 transduced pmel cells was quantified (n=5; \* p < 0.05). Experiments in A, B, and C are representative of at least 2 independent experiments.

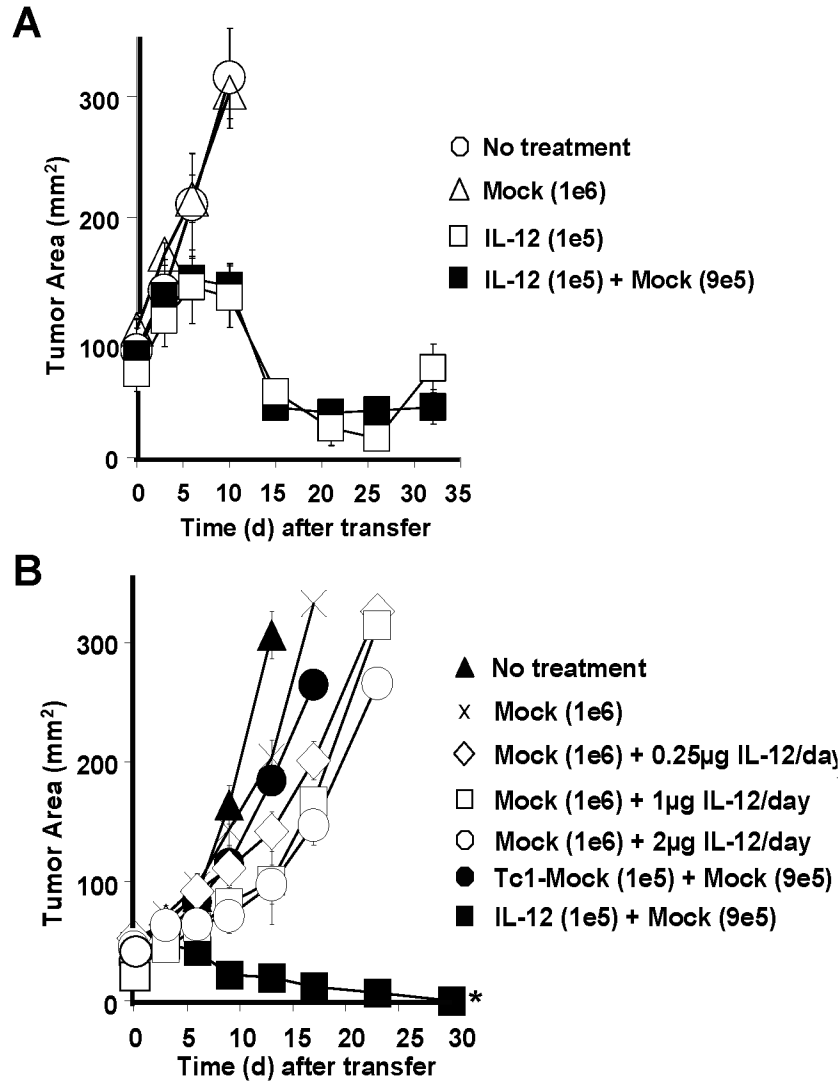


**Figure 2.** Adoptive transfer of IL-12 engineered pmel-1 CD8<sup>+</sup> T cells induces the regression of large, established tumors without exogenous IL-2 and vaccine. A, Anti-tumor activity following the adoptive transfer of  $1 \times 10^5$ \*,  $5 \times 10^4$  $\Psi$ , or  $1 \times 10^4$ \*\* pmel-1<sup>IL-12</sup>-TD cells into sublethally-irradiated (5 Gy) mice bearing B16 tumors ( $n=5$ ) established for 14 days (\*,  $\Psi$ , \*\*,  $p < 0.05$  compared no treatment) with no evidence of weight loss (right panel). B,  $2.5 \times 10^5$  mock versus IL-12 transduced pmel-1 thy1.1<sup>+</sup> CD8<sup>+</sup> T cells were transferred into sublethally-irradiated (5 Gy) mice ( $n=3$ ) and spleens were harvested on days 3, 7, and 14 after transfer and analyzed by flow cytometry for the percentage of transferred CD8<sup>+</sup> thy1.1<sup>+</sup> cells (left panel). B (right panel), Quantification of the percentage of CD8<sup>+</sup> thy1.1<sup>+</sup> cells in spleens (\*, \*\*  $p < 0.05$  compared to mock). Experiments of A, and B are representative of at least 2 independent experiments.

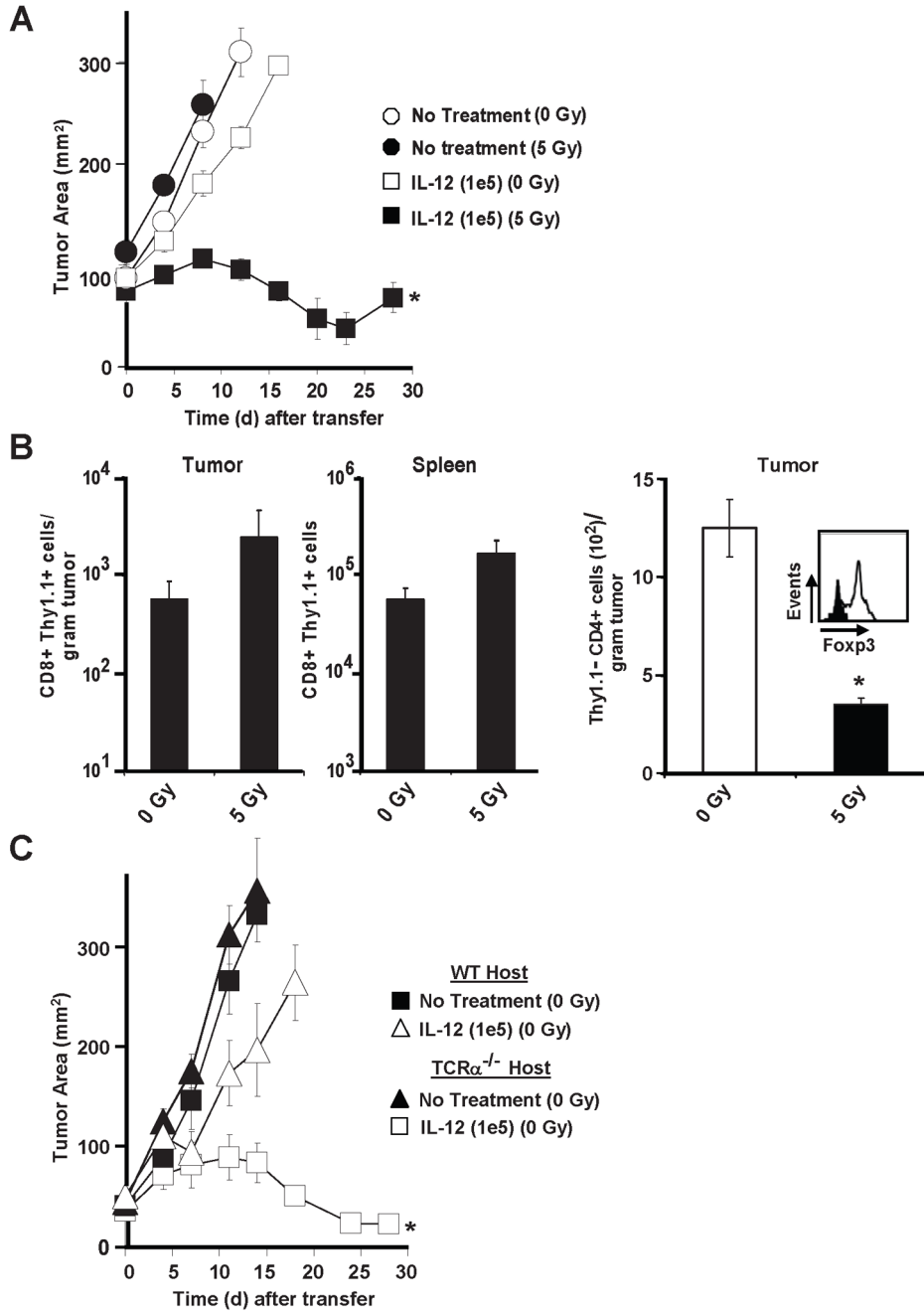


**Figure 3.** Treatment with IL-12 engineered CD8<sup>+</sup> T cells leads to increased tumor infiltration of adoptively transferred cells that stably expressing IL-12, and increased tumor infiltration by endogenous NK and CD8<sup>+</sup> T cells. A, Representative subcutaneous tumor samples (top panel) excised 7 days (n=5) following the transfer of  $5 \times 10^5$  mock or IL-12 transduced pmel-1 CD8<sup>+</sup> T cells into sublethally-irradiated (5 Gy) mice bearing B16 tumors established for 14 days. Corresponding H and E stains (bottom panel; 100X magnification) for tumors in the top panel. B,  $5 \times 10^5$  mock versus IL-12 transduced pmel-1 thy1.1<sup>+</sup> CD8<sup>+</sup> T cells were transferred into sublethally-irradiated (5Gy) mice (n=3), and tumors were excised 7 days after transfer, mechanically disrupted, and enumerated by flow cytometry for infiltration of adoptively transferred thy1.1<sup>+</sup> CD8<sup>+</sup> cells per gram of tumor (left panel; \* p < 0.05 compared to control).

B (right panel), IL-12 expression in tumor infiltrating thy1.1<sup>+</sup> CD8<sup>+</sup> cells (gated on the CD8<sup>+</sup> population). C, Tumor samples from B, were also analyzed by flow cytometry for the number of endogenous thy1.1<sup>-</sup>, NK1.1<sup>+</sup> cells (left panel; \* p < 0.05 compared to control) and thy1.1<sup>-</sup>, CD8<sup>+</sup> cells (right panel; \*\* p < 0.10 compared to control) per gram of tumor. All flow cytometry plots gated on live propidium iodide (PI)<sup>-</sup> populations. Experiments in A, B, and C representative of at least 2 independent experiments.



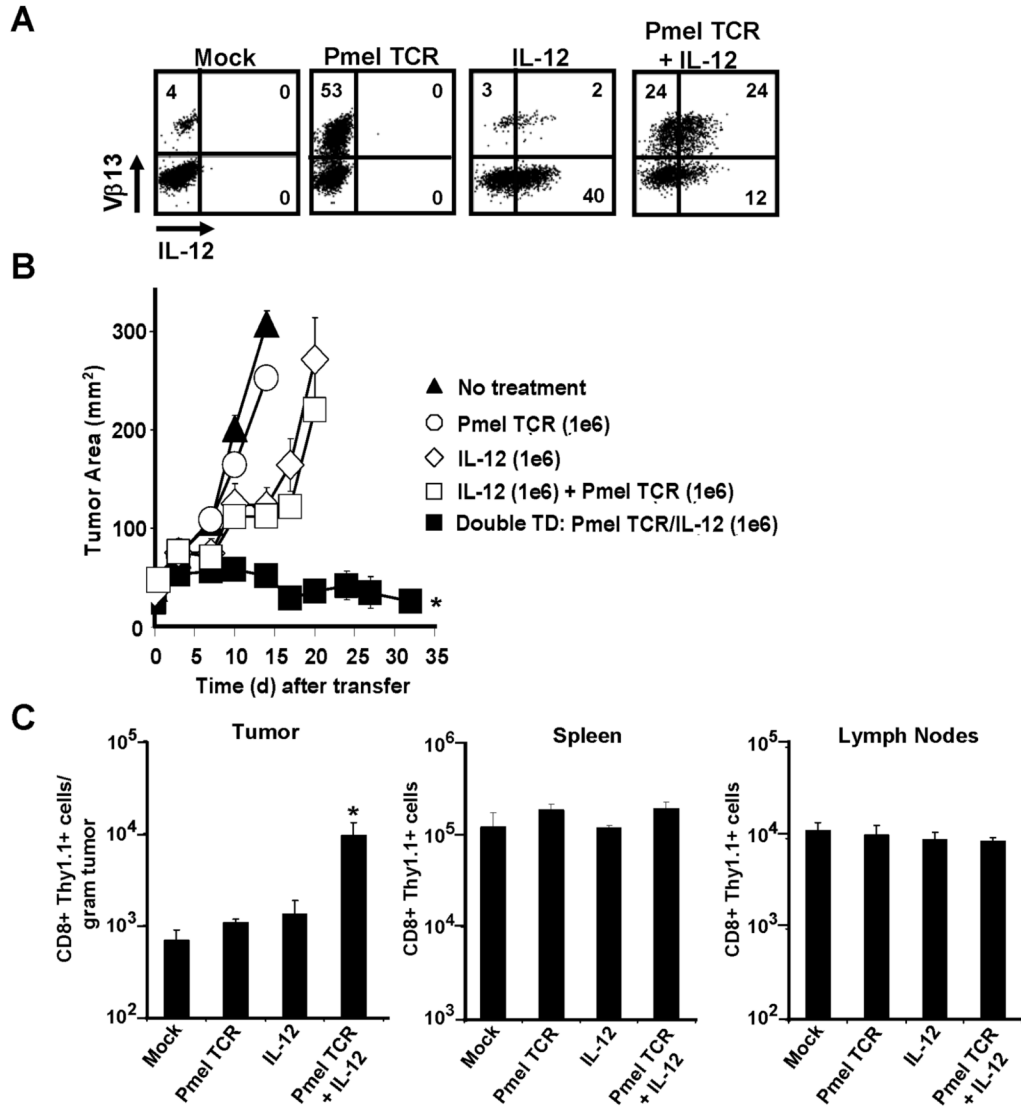
**Figure 4.** IL-12 engineered pmel-1 CD8<sup>+</sup> T cells display enhanced anti-tumor responses compared to rIL-12 administered exogenously. A, Anti-tumor activity following the adoptive transfer of  $1 \times 10^5$  pmel-1<sup>IL-12-TD</sup> cells alone or in combination with  $9 \times 10^5$  mock transduced pmel-1 CD8<sup>+</sup> T cells into sublethally-irradiated (5Gy) mice bearing B16 tumors ( $n=5$ ) established for 14 days. B, Treatment responses in sublethally-irradiated (5 Gy) tumor bearing mice ( $n=5$ ) following the transfer of  $1 \times 10^6$  mock-transduced pmel-1 cells with exogenous rIL-12 daily administered intraperitoneally for 3 days (0.25  $\mu$ g, 1  $\mu$ g, or 2  $\mu$ g),  $1 \times 10^5$  mock-transduced Tc1 cells (polarized with 3.33 ng ml<sup>-1</sup> IL-12 *ex vivo*) or  $1 \times 10^5$  pmel-1<sup>IL-12-TD</sup> cells co-transferred with  $9 \times 10^5$  mock-transduced cells (\*  $p < 0.05$  compared to all other treatment groups). Experiments in A and B are representative of at least 2 independent experiments.



**Figure 5.** Host irradiation (5 Gy) is required for anti-tumor immunity of adoptively transferred pmel-1<sup>IL-12-TD</sup>. A, Tumor treatment of sublethally-irradiated (5 Gy) or non-irradiated (0 Gy) WT B16 tumor bearing mice (n=5) treated with 1×10<sup>5</sup> pmel-1<sup>IL-12-TD</sup> cells. B, Enumeration for thy1.1<sup>+</sup> CD8<sup>+</sup> T cells from isolated tumor samples and spleens (n=4) 6 days after the adoptive transfer of 5×10<sup>5</sup> thy1.1<sup>+</sup>-marked pmel-1<sup>IL-12-TD</sup> into 0 Gy or 5 Gy treated hosts (left panel). Enumeration (n=5) for thy1.1<sup>-</sup> CD4<sup>+</sup> T cells in tumor samples (right panel; \* p<0.05 compared to 0 Gy treatment). Flow-cytometry of isolated thy1.1<sup>-</sup> CD4<sup>+</sup> T cells for Foxp3 expression (right panel; gated on thy1.1<sup>-</sup>, CD4<sup>+</sup> population). All flow cytometry samples gated on live propidium iodide (PI<sup>-</sup>) populations. C. Anti-tumor responses following the treatment of



$1 \times 10^5$  pmel-1<sup>IL-12-TD</sup> cells into non-ablated (0 Gy) WT or TCR $\alpha^{-/-}$  B16 tumor bearing mice (n=5). Experiments in A and B are representative of at least 2 independent experiments.



**Figure 6.**

Tumor antigen-specific T-cell receptors are critical for the therapeutic responses of IL-12 engineered T cells. A, Cytofluorometric analysis of open-repertoire (WT) CD8<sup>+</sup> T cells expressing IL-12 or pmel-1 TCR (Vβ-13<sup>+</sup> staining) individually or on the same cell following retroviral transduction. All plots gated on CD8<sup>+</sup> cells. B, Anti-tumor responses in sublethally-irradiated (5 Gy) tumor-bearing WT mice (n=5) treated with 1×10<sup>6</sup> single-transduced cells (expressing either the pmel-1 TCR or IL-12), a combination of both single transduced populations (2×10<sup>6</sup> cells), or 1×10<sup>6</sup> CD8<sup>+</sup> T cells co-expressing both the pmel-1 TCR and IL-12 (\* p < 0.05 compared to all other treatments). C, Enumeration of tumor, spleen and draining lymph nodes (n=5) for adoptively transferred open-repertoire CD8<sup>+</sup> thy1.1<sup>+</sup> T cells engineered to express IL-12 and/or the pmel-1 TCR as in panel (A). \* p < 0.05, compared to mock. All flow cytometry samples gated on live propidium iodide (PI)<sup>-</sup> populations. Experiments in A, B, and C are representative of at least 2 independent experiments.