Original Article
Synergy of brief activation of CD8 T-cells in the presence of IL-12 and adoptive transfer into lymphopenic hosts promotes tumor clearance and anti-tumor memory

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Abstract: Adoptive T-cell therapy holds great promise for the treatment of metastatic melanoma. However, prohibitive costs associated with current technology required for culture and expansion of tumor-reactive T-cells, the need for intense preconditioning regimens to induce lymphopenia, and the unpredictable anti-tumor effect of adoptively transferred T-cells remain significant impediments for its clinical implementation. Here we report a simplified combinatorial approach that involves short activation of CD8+ T cells in the presence of IL-12 followed by adoptive transfer into tumor bearing animals after a single injection of cyclophosphamide. This approach resulted in complete eradication of B16 melanoma, and the establishment of long term immunological memory capable of fully protecting mice after a second B16 melanoma challenge. The activated donor cells were unique because they simultaneously exhibited traits for cytotoxic effector function, central memory-like, homing, and senescence. After tumor eradication and within three months after transfer, CD8+ cells exhibited a conventional memory CTL phenotype. Moreover, these memory CTLs acquired functional attributes characteristic of memory stem cells, including the ability to resist chemotherapy-induced toxicity. Our results suggest that short-term T-cell receptor signaling in the presence of IL-12 promotes promiscuous qualities in naïve CTL which – upon transfer into lymphopenic hosts– are sufficient to eradicate tumors and generate life-long tumor-specific memory.

Keywords: Pmel, melanoma, IL-12, ACT, T cell therapy, memory, CD8

Introduction

While the prognosis of localized melanoma is favorable with surgical resection alone, stage 4 metastatic melanoma has a very poor prognosis with a median overall survival of approximately 11 months with chemotherapy and 5-year survival of only 5% [1]. The recently FDA approved CTLA-4 monoclonal antibody, ipilimumab, when combined as first line therapy in patients with metastatic melanoma with chemotherapy was able to prolong overall survival by only 2 months (11.2 vs 9.1 months; hazard ratio [HR] for death, 0.72; *P* < .001),and 3 year survival by only 8% (20.8% vs 12.2%) [2]. Melanoma tumors often overexpress tissue-specific developmental antigens that can be recognized by the immune system [3]. Therefore, immunologists have devised and begun to refine three major experimental therapies. They include using therapeutic vaccinations that use modified tumor cells and dendritic cells, lowering the threshold of immune activation by reducing negative controls exercised by CTLA-4 and regulatory T-cells (TReg) [3], and employing adoptive cell therapy (ACT) directed against the tumor. The major conceptual hurdles and goals of ACT employing CD8+ CTL against melanoma and other tumors are two-fold. First, tumor-specific CTL should be primed and expanded *in vitro* in such a manner that they will act as effector CTL *in vivo* by seeking out tumor cells and killing them upon recognition. Second, tumor-specific memory CTL that can self renew and, if needed,

reacquire effector function should develop *in vivo* for life-long protection of the host against reccurrence of the tumor.

Rather elegant *in vivo* immunological studies have shown that the division of a single naïve CTL is sufficient to defeat a viral infection by give rise to effector CTL and memory CTL [4, 5]. In some circumstances, the acquisition of the effector CTL stage by naïve CTL may be required prior to their further development into memory CTL [6, 7]. Because ACT with naïve CTL usually fails to eradicate tumors, we begin this report with the investigation of the tumoricidal activity of the progeny of CTL primed *in vitro* under distinct culture conditions as well as in the context of prior conditioning of the host which is known to improve ACT for several reasons [8-15]. Our results indicate that CTL priming and expansion protocols for ACT must take into consideration that the activation of CTL results in developmentally different fates [16, 17] which will determine their response to lymphopenic environments, their ability to lyse the tumor, and the development of anti-tumor immunity. These outcomes were preferentially endorsed by the quality and quantity or length of the exogenous signals [18-21]. Our report specifically explores the activation and control of naïve CTL by T-cell receptor signals and exogenous cytokine at the time of the initial *in vitro* activation. We found that a brief antigen-dependent activation of naïve CD8+ T cells in the presence of IL-12 followed by exposure to a lymphopenic environment results in a novel differentiation program that clears B16 melanoma tumors and generates cells that will protect against a tumor rechallenge.

Materials and methods

Mice

C57BL/6 (Thy1.1-) and Pmel-1 transgenic [22] (Thy1.1+Vβ13+) mice were purchased from Jackson Laboratory (Bar Harbor, ME). All animals were housed under specific pathogen-free conditions in accordance with Institutional and Federal guidelines at the University of Miami and the experiments were approved by the local IACUC.

Antibodies and flow cytometry

All antibodies were purchased from BD Biosci-

ences. They were used at concentrations recommended by their manufacturers and the stained cell samples were examined on an LSR-II (BD Biosciences). The analysis was performed with FlowJo (Treestar, Ashland, OR). To assess the efflux capability of the cells, they were incubated with 2.5 µM daunorubicin (Sigma) for 20 min at 37°C prior to staining with APC anti-mouse CD8 PE anti-mouse Thy1.1 mAbs. Daunorubicin fluorescence was detected at 610/15 nm.

Melanoma culture and tumor growth

B16-F10 cells, derived from a gp100+ spontaneous murine melanoma cell line, were obtained from American Type Culture Collection (ATCC) (Manassas, VA). The cells were cultured in RPMI 1640 containing 10% FBS, 0.1% penicillin/streptomycin, 0.2% L-glutamine, 0.05% 2-mercaptoethanol, 0.01% sodium pyruvate, 0.1% HEPES and 0.1% nonessential amino acids. Melanoma tumors were established by subcutaneous (s.c.) injection of 2.5×10^5 B16-F10 cells in the right flank. Tumors were measured using calipers every other day. Mice with tumors larger than 400mm^2 were euthanized.

Ex vivo CD8+T cell activation and adoptive cell transfer

Cell suspensions from spleen and lymph nodes of Pmel-1 mice were adjusted to 1×10^6 cells/ml in complete RPMI and activated with 1 µg/ml of cognate peptide (KVPRNQDWL) (American Peptide; CA). Where indicated, IL-12 (R&D Systems) was added at the time of priming to provide 10 ng/ml. Three days after activation, so-called "early" Pmel cells were harvested. To generate so-called "late" Pmel cells, early Pmel cells were recultured for four more days in the presence of 10ng/ml of IL-2 (R&D Systems). One day prior to ACT, wild type C57BL6 mice bearing 7-day-old B16-F10 tumors were conditioned by a single intraperitoneal injection of 4 mg CTX (Sigma). 24 hours later, adoptive immunotherapy (ACT) was performed by intravenous injection of 5×10^6 Pmel cells that had been primed under various conditions. To determine their persistence, blood, bone marrow, spleen, and lymph node suspensions were stained with mAb against Thy1.1, CD8 and and donor Pmel cells were identified as CD8+ Thy1.1+ cells by flow cytometry and compared to the endogenous CD8+Thy1.1- CTL.

Gene expression analysis

Early Pmel[sham] and early Pmel[12] were harvested from tissue culture flasks. Memory Pmel cells were sorted (CD8^{+} Thy1.1^{+}) from spleen suspensions from recipients of early Pmel[12] cells 90 days after adoptive transfer. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and cDNA was synthesized from 1 mg of RNA using the Omniscript RT Kit (Qiagen), as per manufacturer's instructions. Gene expression was measured using quantitative real-time PCR and TaqMan probes (Applied Biosystems, Foster City, CA, USA) in a final reaction volume of 20 ml. Ribosomal 18s RNA was used as the internal standard. RT-PCR was performed on a StepOne real-time PCR system (Applied Biosystems). The relative quantification of the target transcripts normalized to the endogenous control was determined by the comparative Ct method. Relative changes in gene expression between samples were analyzed using the 2^{-ddCt} method.

Statistical analyses

p values were calculated using student t test and a significant difference among experimental groups was defined as a p value of <0.05. Cumulative survival was calculated using a Kaplan-Meier curve. The relationship between donor Pmel cells and tumor size was examined by scatter plot analysis and descriptive statistics as well as by fitting a regression model.

Results

Anti-melanoma activity of early Pmel[12] cells synergizes with preconditioning of the host

The most frequently used syngeneic mouse melanoma model is the B16 tumor. This tumor expresses several melanoma associated antigens including gp100 that is recognized by the transgenic Pmel-1 T-cell receptor in C57BL/6 mice [22]. The striking biological and molecular similarities of this model to human melanoma have been recently discussed [23]. Because this model is also known to be resistant to consistent cures by various immunotherapeutical strategies it was chosen in the following studies. Initially, we noticed that ACT with naïve Pmel cells activated for three days in the presence of IL-12 (early Pmel[12]) significantly delayed the tumor growth of established B16 melanoma tumors (see **Figure 1A**) as compared to other

culture conditions (**Figure 1B**). However, eventually all tumors progressed and the animals succumbed to the disease.

Next, we decided to augment the efficacy of ACT by preconditioning the host [11]. Tumor bearing animals received a single intraperitoneal injection of 4 mg of cyclophosphamide (CTX). This treatment has been shown to induce a transient lymphopenia that lasts for three days and is completely resolved after 14 days [24]. 24 hours after CTX injection, early Pmel[sham], early Pmel[12], late Pmel[sham] or late Pmel[12] cells were adoptively transferred into the mice (**Figure 1A**) and tumor progression was monitored (**Figure 1C**). CTX treatment on its own delayed tumor growth and it was further delayed by the transfer of late Pmel[sham] and, more impressively, by late Pmel[12] or early Pmel[sham]. In sharp contrast, when early Pmel[12] were transferred into CTX treated mice the tumor was quickly eradicated and the animals survived (>70 days after ACT; p=<0.001 as compared to CTX and p=0.003 as compared to both PBS and CTX) (**Figure 1D**).

Because of the long-term survival of the experimental group receiving CTX and early Pmel[12] (individual mice were kept alive for more than 120 days) we assessed whether the animals had developed immunity against the tumor. Indeed, these animals resisted a second challenge with the B16 melanoma 50 days after adoptive therapy (**Figure 1D**). These results suggested that only naïve tumor-specific CD8^{+} T cells primed for three days in the presence of IL-12 were optimally differentiated to respond to the host environment 24 hours post conditioning with CTX.

Circulation of transferred cells after ACT correlates with tumor regression

In patients receiving ACT for melanoma the persistence of adoptively transferred CTL has been suggested to correlate with their anti-tumor activity [25]. To this end, we collected blood samples from mice with distinctly different tumor burdens, ranging from palpable – but not measurable – to >400 mm[2]. When we compared the tumor size to the number of circulating Pmel cells, lower percentages of donor cells seemed to coincide with larger tumors (**Figure 2A**). To expand this observation to a scatter plot, blood and tumors from 15 independent mice were assessed on various days post ACT (**Figure 2B**). Despite considerable variation in the tumor
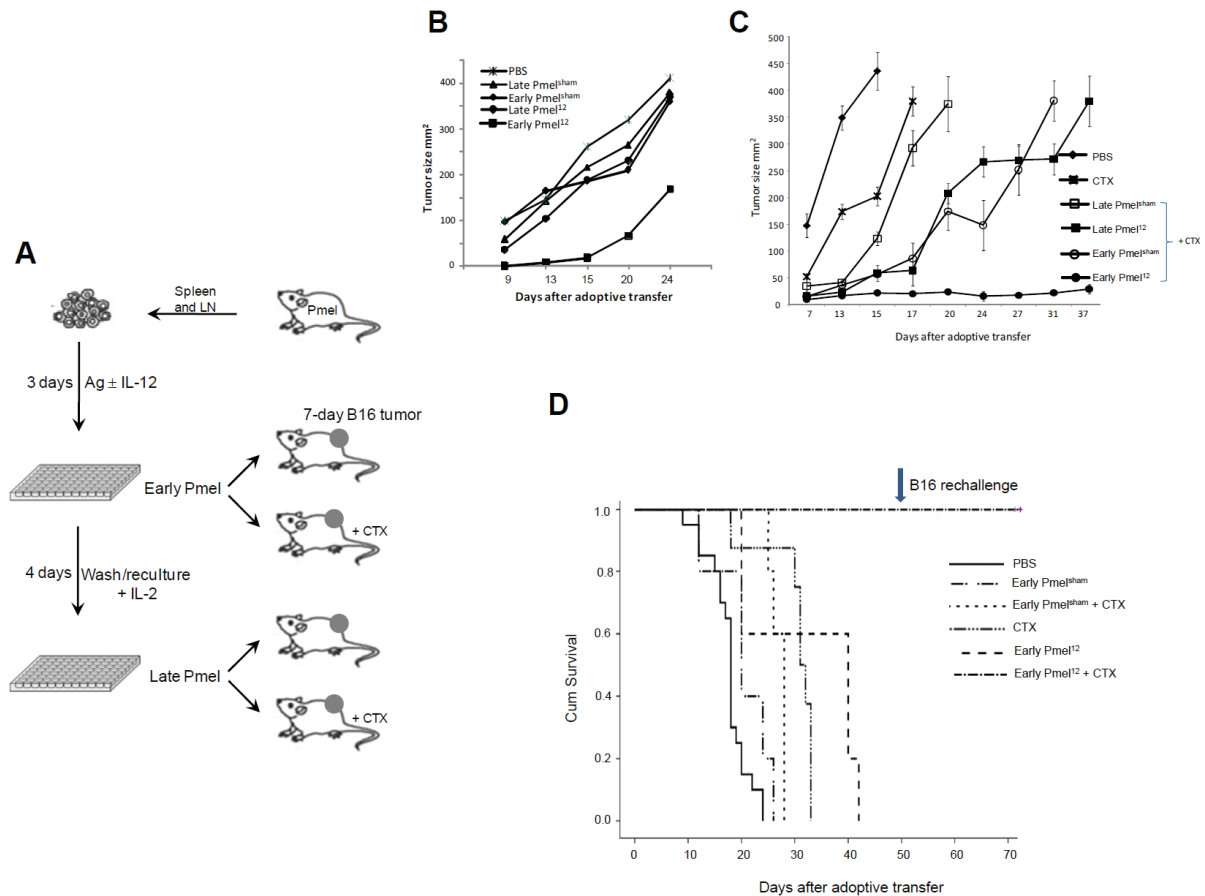
Figure 1. Antitumor activity of CTL primed in the presence of IL-12 synergizes with host preconditioning.   (**A**) Naïve Pmel cells were activated in *in vitro* as indicated. Tumor progression was monitored in mice receiving primed cells as indicated (**B**) and in mice pre-treated with a single dose of CTX (**C**). Experiments shown in B and C represent a minimum of three individual observations with 5 animals per group. (**D**) Cumulative survival of mice from indicated groups.

sizes (mean 160.04, std. dev. 190.51; median 75.33, IQR: 0 to 310.00) their inverse correlation to the percentages of circulating Pmel cells (mean 2.68, std. dev. 2.51; median 1.74, IQR: 0.30 to 4.80) seemed significant. Using a linear regression without intercept to the reciprocal of the donor Pmel percentage (using the 15 independent observations from days 39 to 45), the resulting model explained approximately 81% of the variation in tumor size (R^2=0.809, p<0.001 model fit) and supported the idea that the persistence of donor cells is linked to tumor regression.

Next, we examined the impact of the preconditioning of the host on the persistence of Pmel primed under different conditions. The number of circulating early Pmel[12] was significantly elevated in lymphopenic hosts (**Figure 2C**) and it

paralleled the superior anti-tumor activity shown in **Figure 1C**. The donor cells were detectable in circulation as early as day 4 and remained readily detectable beyond 3 weeks post ACT (approximately 4% of all circulating lymphocytes). When early Pmel[12] were transferred into lymphocomplete hosts, however, their persistence was significantly reduced and they reached the detection limit three weeks post ACT. The comparison of these conditions suggests a synergistic interplay between *in vitro* priming conditions and factor(s) available in preconditioned hosts.

Progeny of early Pmel[12] are dynamically established in preconditioned hosts

Long-term persistence of the donor cells and immunological anti-tumor memory (**Figure 3** and

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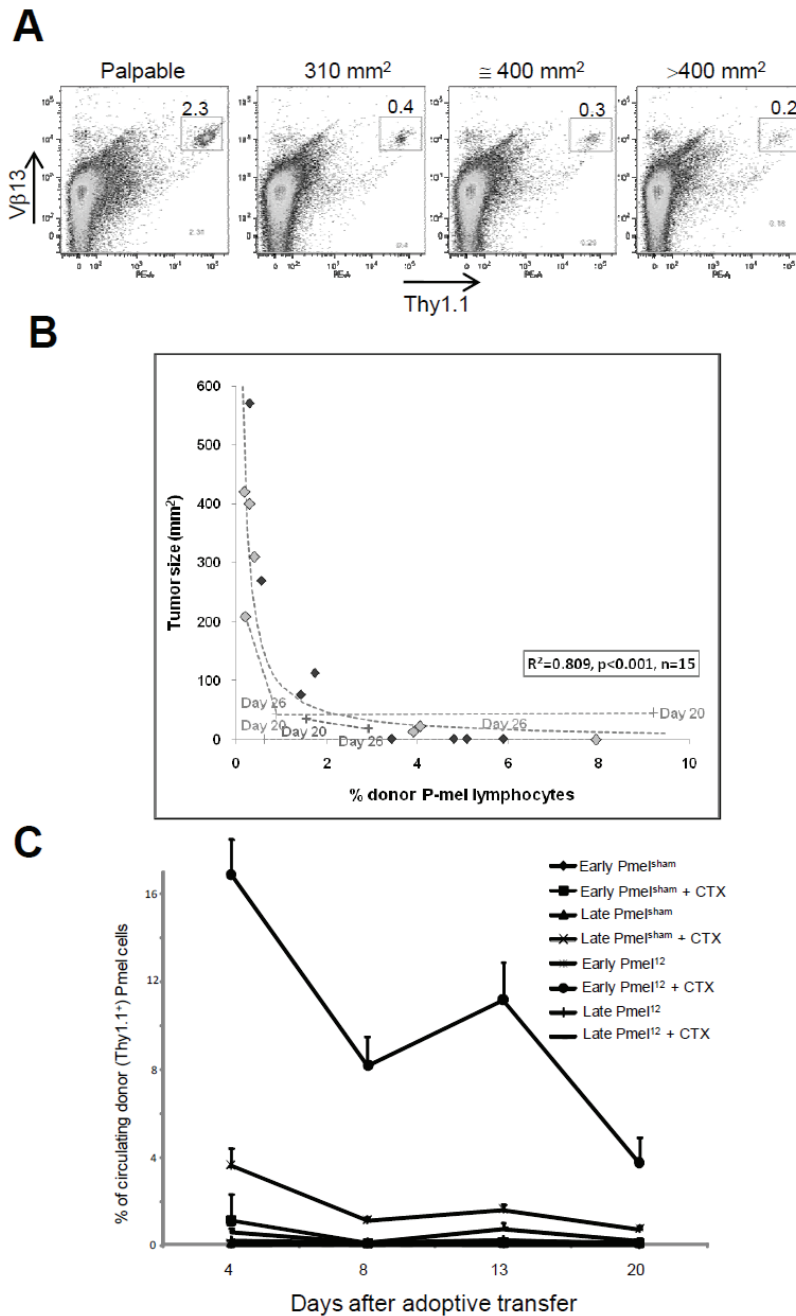

B

C

Days after adoptive transfer

Figure 2. Tumor regression and eradication correlate with persistence of donor cells in circulation after ACT. Lymphodepletion enhances the survival of early Pmel[12] which inversely correlates with tumor burden. (**A**). Representative scatter plot of frequency of donor Pmel cells (Vβ13+Thy1.1+) in mice with the indicated tumor burden. (**B**) Scatter plot analysis of the relationship between tumor burden and frequency of donor Pmel cells in 15 mice. (**C**) Frequency of donor cells in mice treated as indicated. Results shown are the average of 5 independent measurements ± SDEV, per group. Adoptively transferred mice were bled at the indicated time points and the percentage of circulating donor cells (Thy1.1+) was determined by flow cytometry.

majority of the donor cells in contrast to the host cells (**Figure 4**). Interestingly, their relative organ distribution changed with time. Initially, they equivalently accumulated in spleen, blood and lymph nodes but after four weeks they had preferentially seeded the lymph nodes and bone marrow, organs known to support self-renewing memory CTL [28]. These findings suggested that the offspring of early Pmel[12] undergoes extensive changes for at least one month in lymphopenic hosts.

Early Pmel[12] exhibit unusual traits

Figure 1D) suggested that some donor cells had acquired characteristics associated with self-renewing memory CTL, such as a CD44^{lo}/Sca-1^{hi} phenotype [26, 27]. To that end, we determined the number of donor-derived CTL and their anatomical location after ACT of preconditioned hosts (**Figure 3**). Progeny of the transferred early Pmel[12] were present in the examined lymphoid compartments for more than 31 days and CD44^{lo}/Sca-1^{hi} cells comprised the

Having identified a hereto unreported immunotherapy approach that cures 100% of established B16 melanomas, we were interested in characterizing underlying reasons for the effectiveness of the ACT. Based on the potent *in vivo* activity of early Pmel[12] (in contrast to early Pmel[sham], late Pmel[sham], and late Pmel[12]; **Figure 1** and data not shown) it became relevant to characterize the apparently unique activation of early Pmel[12]. Based on flow cytometry, the cell
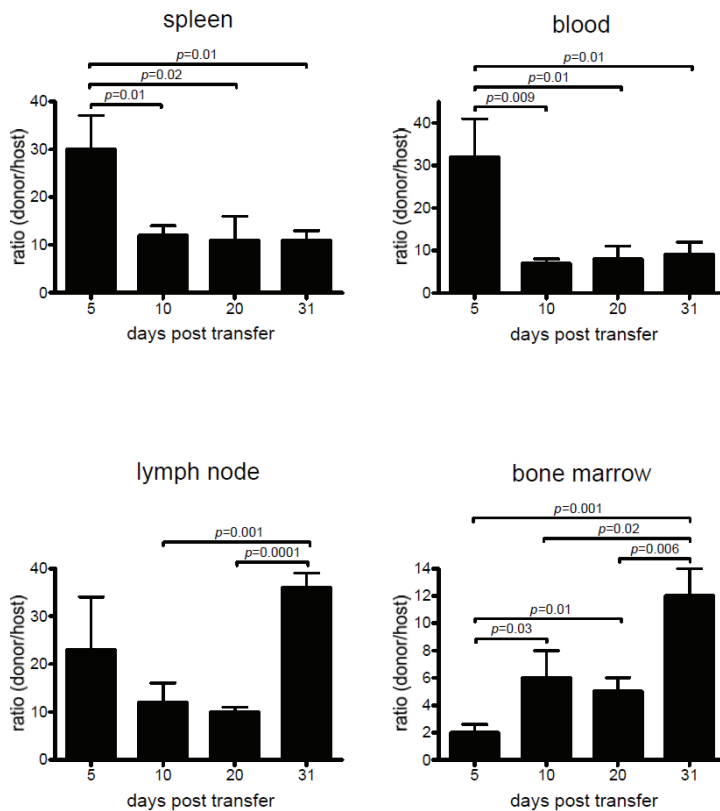
Figure 3. Donor cells dynamically engraft the host after ACT. Wild type mice adoptively transferred with early Pmel[12] cells were sacrificed at the indicated time points, and cell suspensions from spleen, lymph nodes, bone marrow, and peripheral blood were prepared. Cell suspensions were stained with anti-thy1.1, CD8, CD44 and Sca-1 mAbs. Expression of CD44 and Sca-1 was determined on both host (Thy1.1^{-}) and donor (Thy1.1^{+}) CD8^{+} populations. Frequency of donor Pmel cells is shown as the average donor:host ratio±SDEV (n=3). P values were calculated using a two sided Student t test. The experiment shown is representative of three repeats.

population seemed to represent central memory CTL, i.e., CD62L+, CD44+ CTL as we and others reported previously [29, 30]. On the other hand, the cells also expressed high levels of the IL-2Rα (CD25) as well as the cytokine IFN-g and the cytotoxic granule molecule granzyme B (**Figure 5A**) suggesting that they also had acquired traits of active effector CTL. To shed light on this apparent discrepancy, we used real time RT-PCR to compare the expression of several molecules in early Pmel1[12] with early Pmel[sham] representing conventional effectors (**Figure 5B**). We included probes for a) CTL effector molecules (perforin and granzyme B); b) transcription factors critical for the various fates of activated CTL (Runx3, Eomes, T-bet, prdm1, bcl-6 and TCF-1 [31-39]; c) members of the gc-cytokine receptor cytokine receptor family (CD25, CD127 and CD132); d) a master regulator of mitochondrial biogenesis (ppargc1a and ppargc1b) to interrogate energy metabolism [40]; e) the multidrug efflux molecule abcb1b to monitor the recently described chemotherapy resistance of memory CTL [41]; and f) p19 arf because of its negative control of self-renewing ability and promotion of senescence [42] as well as Klrg1 whose

increased expression correlates with the progressive differentiation of CTL into short-lived effector cells [43]. We found that some genes were underexpressed while others were overexpressed (left versus right of **Figure 5B**). Here we focus only on statistically significant differences. It was interesting that the t-box transcription factor ratio between eomes and tbet was inverted because t-bet has been proposed to divert central–memory CTL into effector and effector–memory CTL consistent with their expression of IFN-g and granzyme B (**Figure 5A**). We also found that the CTL memory transcription factor tcf7 was strongly repressed while the expression of the effector function transcription factor prdm1 was elevated. Also elevated were the terminal differentiation marker Klrg1 and even much more so p19arf. Taken together, these markers pointed to the generation of a late effector stage CTL by IL-12 at the time of transfer, which seemed to contradict conventional wisdom that effector cells are less suitable for ACT[44]. Also, these cells expressed lymph node homing receptor CD62L used by naïve and memory CTL as we have previously shown [29]. Taken all together, Pmel[12] seemed
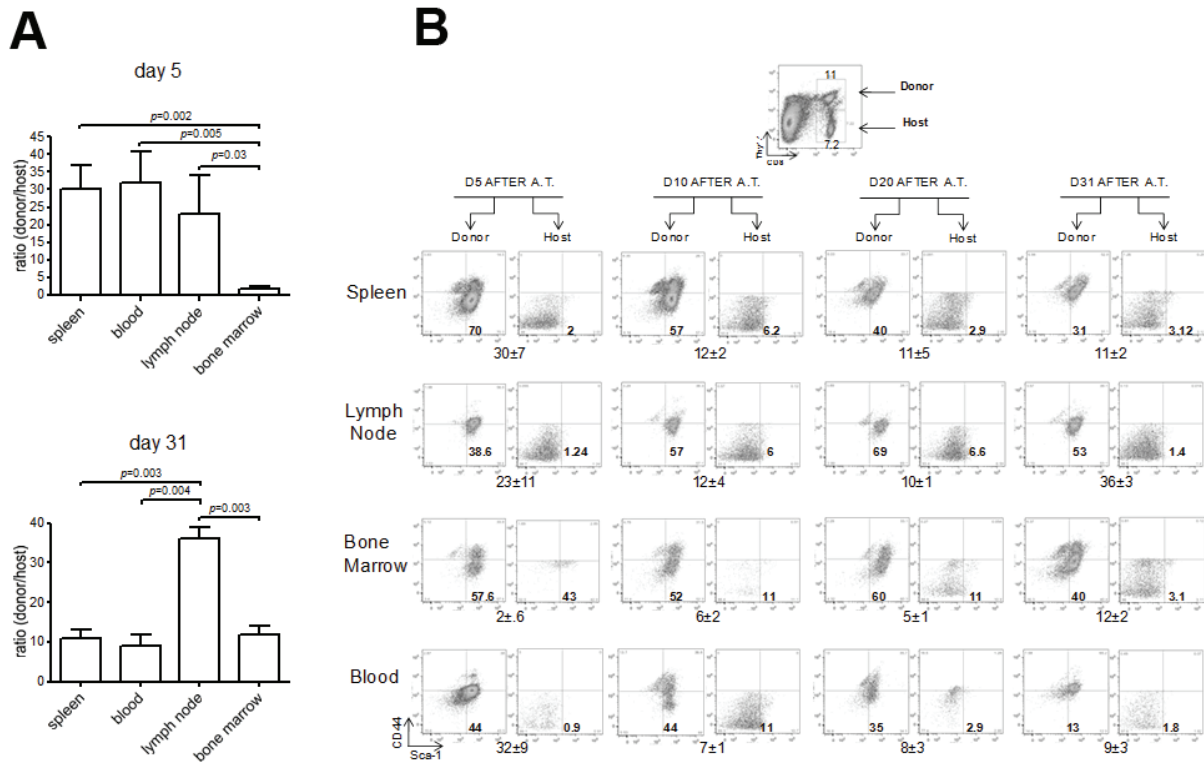
Figure 4. Distribution of CD44^{lo}Sca-1^{hi} donor Pmel cells. Wild type mice adoptively transferred with early Pmel^{12} cells were sacrificed, and cell suspensions from spleen, lymph nodes, bone marrow, and peripheral blood were prepared. Cell suspensions were stained with anti-thy1.1, CD8, CD44 and Sca-1 mAbs. Expression of CD44 and Sca-1 was determined on both host (Thy1.1$^-$) and donor (Thy1.1$^+$) CD8$^+$ populations. Frequency of donor Pmel cells is shown as the average donor:host ratio±SDEV (n=3) 5 and 31 days after adoptive transfer (**A**). Gating strategy to determine the distribution of CD44^{lo}Sca-1^{hi} host and donor cells (**B**).

to be uniformly made up of promiscuous CTL traits.

Adoptively transferred early Pmel^{12} eventually convert into conventional memory CTL

Next, we wanted to know whether the unusually programmed donor cells and their progeny would continue to maintain unusual properties after ACT. If not, they should have differentiated into resting memory CTL by the time of the analysis (1-3 months after transfer). In support of the latter possibility, the eomes/t-bet ratio was reversed, and CD25, granzyme B as well as p19 arf expression were abolished. Moreover, the IL-7R (CD127) and tcf7 were upregulated. Yet, mRNA expression of the abcb1b transporter that contributes to the survival of long-lived memory CD8$^+$ T cells [45] was not significantly upregulated (**Figure 6A**). Therefore, we also investigated its functional activity by evaluating

the cells' ability to efflux a fluorescent anthracycline (daunorubicin; [41]). After allowing the cells to efflux for 30 min (**Figure 6B**), a significant percentage of the early Pmel^{12} progeny had removed daunorubicin in contrast to early Pmel^{12} prior to ACT (20% versus 1.7%). These differences were not intrinsic to the transgenic cells because the efflux ability of naïve Pmel cells and the host's own cells compared favorably to each other (7.7% versus 8.6%). This functional assessment together with the gene expression results and the dynamic homing of the transferred CTL suggested that critical memory characteristics were not acquired *in vitro* prior to ACT but later during their further differentiation *in vivo*.

Engrafted early Pmel^{12} are capable of immune reconstitution

Based on the accumulating evidence that early
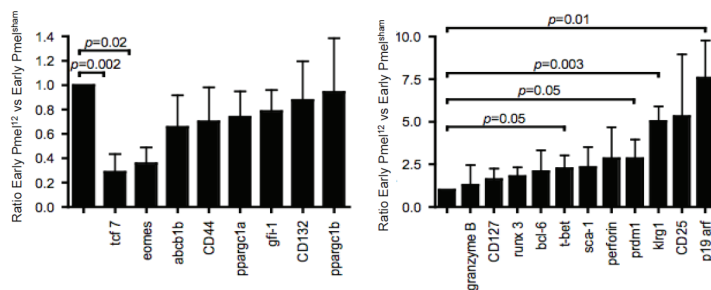
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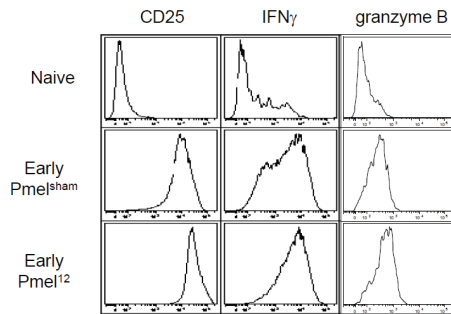
B

Figure 5. Promiscuous traits of early Pmel[12] cells at the time of ACT. (**A**) Naïve, early Pmel[sham] and Pmel[12] cells were incubated with mAbs against the indicated parameters. Cells were analysed by flow cytometry and representative histograms are shown. (**B**) Gene expression analysis by real time rtPCR was performed on mRNA extracted from early early Pmel[sham] and Pmel[12] cells. Expression is depicted as the ratio of Pmel[12] to Pmel[sham]. Data shown is the average of three independent measurements ± SDEV. P values were calculated using a two sided Student t test.

Pmel[12] acquired conventional memory CTL traits *in vivo*, we hypothesized that the cells also may have acquired the ability to self renew because self renewal is an intrinsic characteristic of memory CTL providing life-long protection [41]. To that end we assessed their ability to re-establish themselves after a second CTX treatment of the animals. Seven and 10 days later (day 47 and day 50), we determined the percentage of donor CTL progeny (Thy1.1+) and host CTL progeny (Thy1.1-) by flow cytometry (**Figure 7A**, fourth row) as compared to their steady state levels without the second CTX treatment. While on both days the total CTL remained reduced to approximately 40% of their numbers prior the second CTX treatment (day 47: 1.76 + 6.15 / 5.45 +7.85; day 50: 2.40 + 5.07 15 / 5.45 +7.85) progeny of early Pmel[12] began to preferentially recover from 22% to 32% of the total CTL. This fast recovery is consistent with their direct development from memory CTL rather than a thymus-dependent recovery that is expected to be responsible for the recovery of naïve host cells.

We also compared the antigen-dependent replicative potentials of the progeny of donor CTL in

CTX conditioned mice with mice that received a second CTX treatment (**Figure 7B**). To that end, Pmel[12] recipient mice were challenged with the Pmel antigen (i.p. injection of 100 µg of gp100 peptide). Antigen-dependent expansions were approximately 5-fold irrespective of the second CTX treatment. Similarly, and exactly as expected for normal CTL responses [46], the cells contracted equally well to slightly elevated numbers. These results suggest that memory CTL derived from Pmel[12] are capable of immune reconstitution of functional CTL.

The possibility that Pmel progeny recovered from contaminating transgenic stem cells present in the cultures used for ACT was excluded because the *in vitro* cultures required antigen receptor stimulation to detect any progeny *in vivo* (**Figure 8A**) and to effectively control de progression of tumors (**Figure 8B**).

Discussion

CD8+ T cells optimally suited for anti-tumor ACT should be able to travel to the site of the tumor, recognize the malignant cells, and erradicate them. In addition, some of the transferred CTL
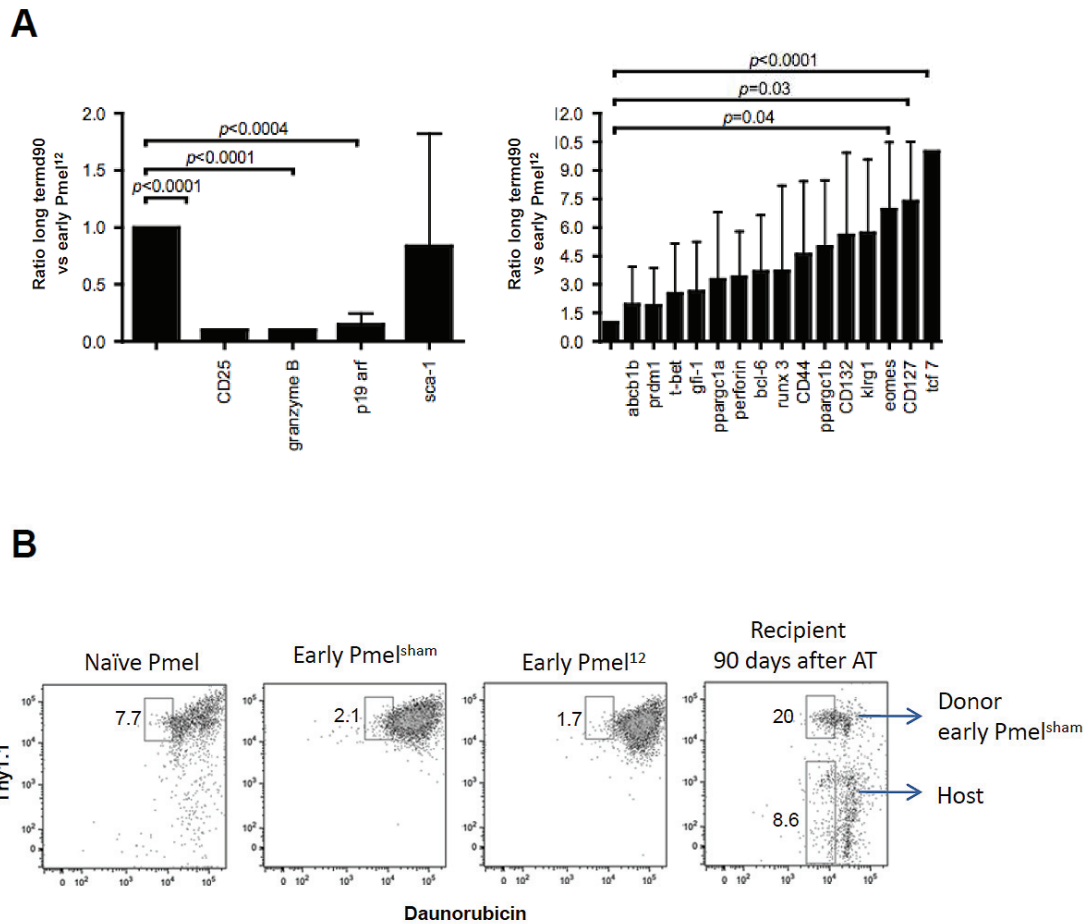
A

B

Figure 6. Long term Pmel[12] survivors display late effector/memory-like phenotype and resist daunorubicin induced toxicity. (**A**) Gene expression analysis of memory associated markers was performed on early Pmel[12] and sorted long term Pmel survivors. Expression is depicted as the ratio of long term Pmel[12] survivors to early Pmel[12]. Data shown is the average of three independent experiments ± SDEV. P values were calculated using the Student t test. (**B**) Cell suspensions of naïve, early Pmel[sham], early Pmel[12], and splenocytes from wt recipient of early Pmel[12] cells 90 days after adoptive transfer, were incubated in the presence of daunorubicin and stained with mAbs against CD8^{+} and Thy1.1. The subset of CD8^{+} positive cells that effluxed daunorubicin is shown in the gate on each panel . Data represents three separate experiments.

should persist as memory CTL lifelong to ensure anti-tumor immunity. Here, we described a simple strategy for *in vitro* CTL priming and host conditioning that fulfills these demands based on its reproducible tumor eradication and generation of immunity in a melanoma mouse model known to be fairly resistant to immunotherapy.

Our study investigated in detail the role of the specific stage of CTL activation/differentiation at the time of ACT and their transition into long-term anti-tumor immunity *in vivo*. It is worthwhile mentioning that our differentiation strat-

egy employed naïve CTL, rather than tumor infiltrating lymphocytes (TIL). The former, but not the latter, are easily obtained and may provide more reproducible results because TIL of late stage tumors may be dysregulated by multiple mechanisms [47]. On the other hand, the choice of naïve T-cells requires redirecting their specificity to known tumor antigens. Experiments addressing the latter strategy are underway in several laboratories, including ours.

Our investigations demonstrated that an early stage of CTL activation (day 3) can be differentially programmed by cytokine to benefit from
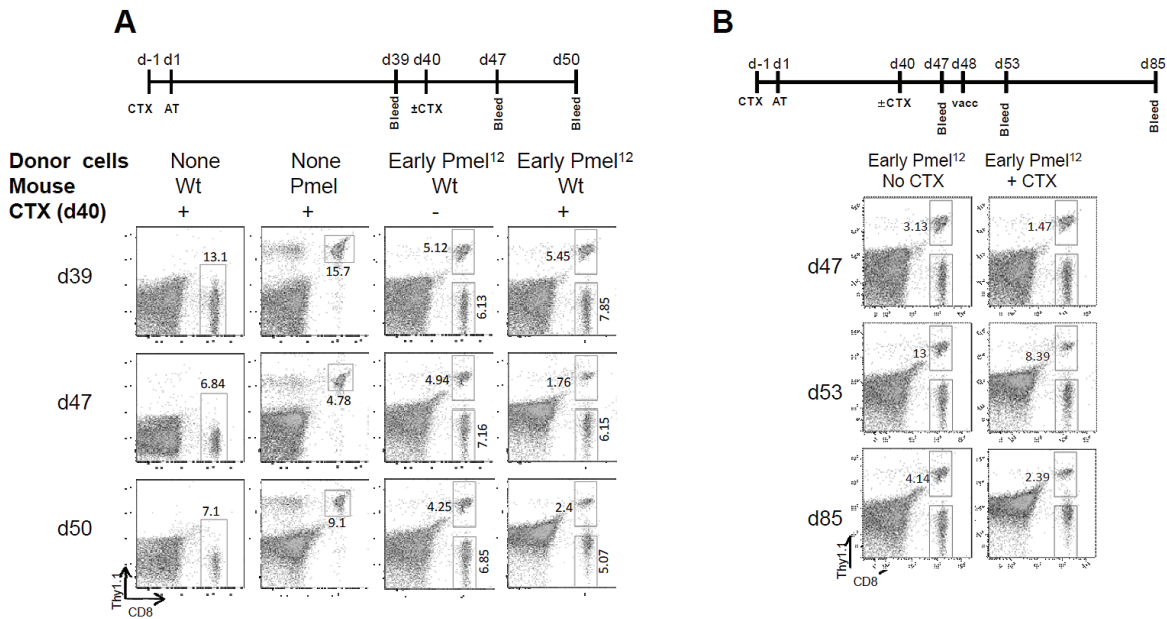
Figure 7. Long term Pmel[12] cells are capable of peripheral CTL reconstitution followed chemotherapy induced lymphodepletion. (**A**) Wild type recipients of early Pmel[12] received 4 mg of CTX i.p. 40 days after adoptive transfer or were left untreated. Frequencies of donor and CD8+ T cells on peripheral blood were determined by flow cytometry at the indicated time points. (**B**) Unmanipulated (no CTX) or reconstituted (+ CTX) wt recipients were vaccinated with 100 μg of gp100 peptide i.p. Mice were bled 3 and 45 days after vaccination (d53 and d85 after adoptive transfer, respectively) and the frequency of donor Pmel cells was assayed by flow cytometry. Dot blots shown are representative of at least 5 independent observations.

the environment of CTX treated hosts. Only cells primed in the presence of IL-12, but not IL-2 (**Figure 1** and data not shown), achieved the therapy goal. Likewise, extending the *in vitro* culture to a total of seven days generated a different cell stage (MD-M, MP and MGL; unpublished observation) and failed to eradicate the tumor (**Figure 1**).

Over the past decade many elegant studies have shed molecular light on how activation of CTL proceeds *in vivo* upon viral infection. An early stage has been defined as memory precursors that are IL-7R high and KLRG1 low [43, 48]. During further activation and differentiation *in vivo* most of them may acquire cytotoxic activity and eventually become terminal effectors before they die or exhaust [46, 49, 50] while few will survive to give rise to long-term memory CTL. Unexpectedly, we found that the cells most effective in the adoptive transfer acquired an unusual gene expression profile if they represented an identical stage at the time of their analysis by real-time RT-PCR rather than a combination of distinct populations. The for-

mer, but not the latter, is supported by the results from the flow cytometry because the histograms primarily displayed single populations (**Figure 5A** and data not shown). Transcription factor expression, such as prdm1, and surface molecule expression such as CD25 (**Figure 5**), corresponded to strongly activated effector CTL while klrg1 and p19arf accumulation suggested they were terminally differentiating into senescent and/or exhausted cells [46, 49, 50]. Similarly perplexing, they expressed CD62L suggesting that they are homing to lymph nodes thought to be reserved for quiescent naïve and central memory CTL. Interestingly, the high expression of t-bet by these cells has been associated previously not only with the effector stages of T-cells but specifically also to their homing to tumors *in situ* [51]. Taken all together, our *in vitro* activation of CTL seems unlikely to proceed analogously to their physiological activation *in vivo*. Perhaps it should be expected that CTL capable of efficiently seeding CTX treated hosts are distinct from CTL stages defined according to anti-viral CTL responses. Additional correlative experiments in our laboratories point to a
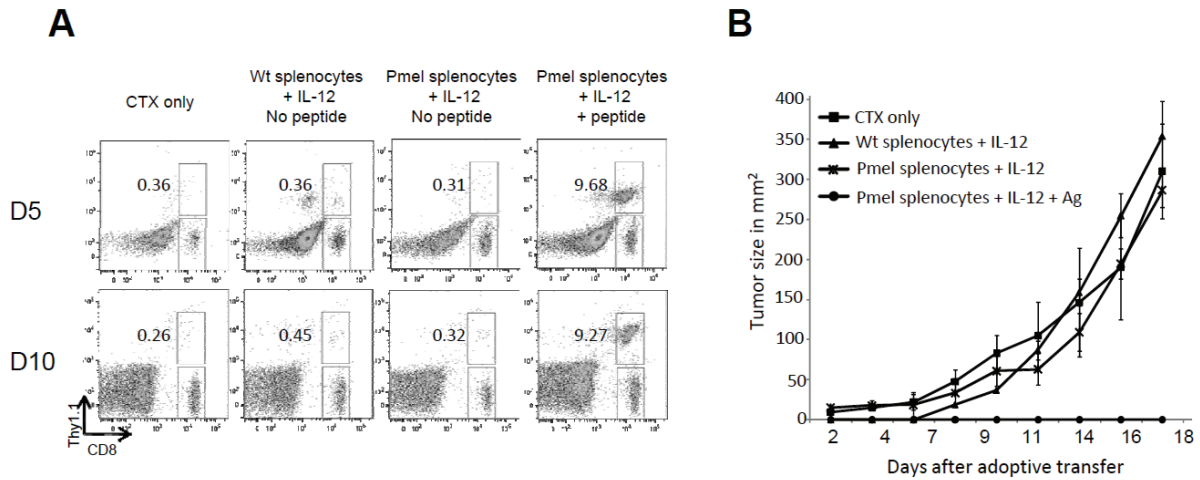
Figure 8. Long term Pmel[12] cells are not the progeny of a stem cell progenitor present in the spleen. Splenocytes from wt C57BL/6 (Thy1.1-/Vb13-) and Pmel (Thy1.1+/Vb13+) were cultured in the presence of IL-12 for 3 days. Early Pmel[12] were also generated as positive control. Wt recipient mice bearing 7 day-old B16 melanoma tumors were injected i.p with 4 mg of CTX and 24 hrs later received 5x10^{6} of the cells described above. Frequency of donor cells (Thy1.1+/CD8+) was determined by flow cytometry, and data is shown from one representative mouse per group (**A**). Tumor progression was followed in the same mice. Each data point represents the average of 5 independent measurements ± SDEV (**B**).

unique role of the tumor suppressor p19arf for the IL-12 dependent *in vitro* activation/ differentiation (M.D.-M., M.P. and M.G.L. unpublished observation).

The success of the ACT strategy was contingent on a synergy between the use of IL-12 to prime naïve CTL and the conditioning of the host with CTX. Notably, a lymphodepletion regimen of a single bolus of CTX without a second drug or total body irradiation was sufficient to convert the limited anti-tumor activity of transferred early Pmel[12] into their capability of complete tumor rejection and, ultimately, anti-tumor immunity. This finding could have important clinical implications because it is often suggested that the efficacy of adoptively transferred T cells is proportional to the intensity of lymphodepletion [52, 53] while intensification comes at the expense of higher toxicities. We are currently addressing whether the IL-12 primed CD8+ T cells are "supraoptimally fit" and thus require a less rigorous conditioning regimen. Our current working hypothesis is that the synergistic action is due to the *in vitro* generation of a transient CTL stage that is optimally responsive *in vivo* to the cytokine milieu in the CTX treated host [54]. Based on the known pleiotropic effects of CTX, other explanations may include its induction of lymphopenia [55], its reduction of Tregs [56]

and its systematic activation of DC or even its induction of a highly immunogenic form of tumor cell lysis [57]. These explanations are not exclusive of each other and individual mechanism may become critical at different time points after the adoptive transfer.

Our results show a strong correlation between the levels of circulating donor cells and tumor regression with ineffective populations disappearing by day 8 after the ACT (**Figure 2**). Similar findings have been described in patients receiving ACT for melanoma. In these patients, transferred cells with long-term persistence in circulation expressed high levels of CD127 (IL-7R), which suggested an IL-7-dependent survival mechanism [58]. The cells we used for ACT did not significantly upregulate the IL-7R (**Figure 5B**) and it was upregulated only among their long-term surviving progeny (**Figure 6A**). Similarly, the transcription factor TCF-7 that is known to promote long-term memory and longevity of memory CTL [38], was down regulated in the donor cells, but it became the most highly induced gene in their long-term surviving progeny **Figure 6A**. The high expression of TCF-7 and IL-7R by the Pmel cells remaining in the animals for at least nine months after cure (data not shown) led us to investigate whether long-term survival of the adoptively transferred

CTL became associated with the acquisition of memory stem-cell like traits that were reported to reside within a CD44^{low}/Sca-1^{high} population [26]. Indeed, we found that a significantly larger population of CD44^{low}/Sca-1^{high} cells existed among the donor population compared to the endogenous CTL (**Figure 4**). These cells also preferentially homed to the lymph node and bone marrow (**Figure 3**), the two organs in which memory CTL are preferentially maintained [28]. Functionally these cells were even able to efflux daunorubicin and to propagate themselves upon lymphopenia-inducing cytotoxic chemotherapy, a trait they share with hematopoietic stem cells [59]. This cumulative evidence suggests that the donor derived CTL evolving after ACT had acquired stem-cell like features necessary to maintain long-term memory *in vivo*. Finally, these cells were readily responsive to a second antigen and tumor challenges (**Figure 7** and **Figure 1**).

In conclusion, this report describes a simple and extremely potent strategy for ACT of CD8+ T -cells in a pre-clinical mouse melanoma model. The two key components entail the brief activation of naïve CTL in the presence of IL-12 *in vitro* and their transfer into tumor-bearing animals treated with a single dose of CTX. The key results include that an apparently novel differentiation stage of the transferred CTL promotes both tumor eradication and the generation of self-renewing memory CTL. Our results also suggest that transcription factors driving CTL activation and differentiation may become useful biomarkers to predict the efficacy of cells for ACT prior to their infusion and that a threshold of donor cells needs to be detectable in the blood circulation within the first week long term tumor eradication.

Acknowledgments

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