



Published in final edited form as:

Cancer Immunol Immunother. 2015 May ; 64(5): 539–549. doi:10.1007/s00262-015-1655-y.

Interleukin-12 enhances the function and anti-tumor activity in murine and human CD8⁺ T cells

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Abstract

Mouse CD8⁺ T cells conditioned with Interleukin (IL)-12 *ex vivo* mediate the potent regression of established melanoma when transferred into lymphodepleted mice. However, the quantitative and qualitative changes induced by IL-12 in the responding mouse CD8⁺ T cells have not been well defined. Moreover, the mechanisms by which IL-12-conditioning impacts human CD8⁺ T cells, and how such cells might be expanded prior to infusion into patients is not known. We found that *ex vivo* IL-12-conditioning of mouse CD8⁺ T cells led to a 10- to 100-fold increase in persistence and anti-tumor efficacy upon adoptive transfer into lymphodepleted mice. The enhancing effect of IL-12 was associated with maintenance of functional avidity. Importantly, in the context of ongoing ACT clinical trials, human CD8⁺ T cells genetically modified with a tyrosinase-specific T-cell receptor exhibited significantly enhanced functional activity when conditioned with IL-12 as indicated by heightened granzyme B expression and elevated peptide-specific CD107a degranulation. This effect was sustainable despite the 20 days of *in vitro* cellular expansion required to expand cells over 1,000-fold allowing adequate cell numbers for administration to cancer patients. Overall, these findings support the efficacy and feasibility of *ex vivo* IL-12-conditioning of TCR-modified human CD8⁺ T cells for adoptive transfer and cancer therapy.

Keywords

IL-12; CD8⁺ T cells; ACT; adoptive T cell therapy; Tc1; cancer

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Conflict of interest:

The authors have no conflicts of interest to declare.

Introduction

Adoptive cell therapy (ACT) has shown great potential for inducing curative responses in patients with advanced metastatic cancer. Recent findings provide additional optimism as it is apparent that tumor-reactive T cells can be rapidly and reliably generated by the transfer of tumor-reactive T-cell receptor (TCR) and chimeric antigen receptor (CAR) genes [1-4]. Furthermore, it is becoming increasingly clear in murine studies that the conditioning of T cells prior to ACT is also a critical parameter affecting *in vivo* efficacy. For CD8⁺ T cells, polarization with IL-12, IL-4, or TGF β /IL-6 leads to the generation of Tc1, Tc2, and Tc17 subsets, respectively [5-7]. These subsets each have unique functional properties and have demonstrated improved anti-tumor immunity when compared to unpolarized T cells (Tc0). Although there is a growing body of preclinical data demonstrating that polarized T cell subsets have improved durability and greater anti-tumor efficacy, current ACT clinical trials still use unpolarized T cells.

Perhaps the most therapeutically promising of the CD8⁺ T cell subsets are Tc1 cells. Early studies demonstrated that human CD8⁺ T cells activated and cultured with IL-12 were skewed toward a phenotype associated with enhanced function and cytotoxicity, as indicated by their ability to secrete IFN γ and degranulate upon antigen recognition [8-11]. These results were expanded upon using mouse T cells, leading to the formal identification of Tc1 cells [12-14]. Then, in a series of mouse studies, Dutton and colleagues showed Tc1 cells can mediate effective anti-tumor immunity in mice in a variety of settings and in a manner unique to that of other polarized CD8⁺ T cell subsets [15-19]. Importantly, in most models, Tc1 mediate much more effective anti-tumor immunity than Tc2 or Tc17 cells [20,19,18,21].

While most studies have focused on a comparison of Tc1 cells with other effector cell subsets, Mescher and colleagues have more precisely defined the role of IL-12 in this process [22]. Using mouse T cells, they demonstrated that the presence of IL-12 during activation dramatically enhanced the functionality of CD8⁺ T cells as indicated by their ability to produce IFN γ , to be cytotoxic, to persist *in vivo*, and to mediate anti-tumor immunity. Chang et al. demonstrated similar findings to Mescher and colleagues, and also showed using a mixture of wildtype and IL-12R β 1^{-/-} T cells that IL-12 acts directly on CD8⁺ T cells [23]. Interestingly, in all these studies, control CD8⁺ T cells cultured without IL-12 also produced IFN γ upon antigen stimulation, albeit less than with the inclusion of IL-12. These results demonstrate that IL-12 can not only promote a Tc1 phenotype, but IL-12 can fundamentally improve the functional quality of an activated CD8⁺ T cells already producing IFN γ .

In our previous work [24], we used an approach similar to Mescher and colleagues to assess the impact of *ex vivo* IL-12-conditioning on tumor-reactive CD8⁺ T cells from pmel-1 TCR transgenic mice. Pmel-1 CD8⁺ T cells express a TCR that recognizes the H-2D^b-restricted gp100₂₅₋₃₃ epitope, an endogenous B16 tumor antigen [25]. Using peptide stimulation, we activated pmel-1 CD8⁺ T cells with (pmel^{IL-12}) or without (pmel^{sham}) IL-12-conditioning. We found that pmel^{IL-12} CD8⁺ T cells did not merely exhibit improved function *in vitro*, but also mediated robust regression of established melanoma when infused into mice

lymphodepleted with cyclophosphamide (CTX) [24]. Thus, the combination of both *ex vivo* IL-12 conditioning of donor CD8⁺ T cells and host lymphodepletion led to synergistically enhanced anti-tumor immunity.

Here, we expand upon our previous findings by mechanistically defining how IL-12-conditioning augments the function and anti-tumor activity of CD8⁺ T cells. Further, we demonstrate the ability to generate an IL-12-conditioned cellular product in support of a clinical trial platform. First, using mouse pmel-1 CD8⁺ T cells, we find that IL-12-conditioning improves persistence and anti-tumor efficacy 10-100-fold. The enhanced effectiveness of IL-12-conditioning was associated with a maintenance in functional avidity. In studies with human CD8⁺ T cells, we genetically modified T cells with a tyrosinase-reactive T-cell receptor (TCR), TIL1383i, which recognizes the HLA-A2-restricted tyrosinase₃₆₈₋₃₇₆ epitope, an antigen expressed on a high frequency of melanoma tumors [26,27]. (This TIL 1383I TCR is being used in an ongoing ACT clinical trial (NCT01586403) at Loyola Medical Center in Chicago^(coauthor GS).) Using TIL 1383I-modified CD8⁺ T cells, we found that IL-12-conditioning led to enhanced functional activity, including elevated expression of granzyme B and ability to degranulate, as indicated by surface CD107a expression in response to relevant antigen. Importantly, this enhanced functional ability was maintained during the three-week period of expansion required for the CD8⁺ T cells to reach numbers adequate for patient administration.

Materials and Methods

Mice

C57BL/6 (B6), B6.PL (Thy1.1), pmel-1 TCR transgenic [25], IFN γ ^{-/-}, HLA-A2 transgenic, and NSG mice were obtained from Jackson Laboratory (Bar Harbor, ME). We have described the generation of h3T TCR transgenic mice previously [28]. Pmel-1 mice were maintained by crossing a pmel-1 (male) to a Thy1.1 (female) generating hemizygous offspring. We generated pmel-1/IFN γ ^{-/-} mice in our colony. All animals were housed under specific pathogen-free conditions in accordance with institutional and federal guidelines at the Medical University of South Carolina.

Cell cultures

B16-F1 tumor cells were obtained from ATCC (Manassas, VA) and cultured as previously described [24]. T2-A2 cells are a TAP-deficient hybridoma expressing HLA-A2. For generation of mouse gp100-reactive T cells, pmel-1 TCR transgenic splenocytes (1.5×10⁶ cells/well in 1.5ml) were stimulated with 1 μ g/ml H-2D^b-restricted human gp100₂₅₋₃₃ peptide (KVPRNQDWL, American Peptide Company) for 3 days with or without mIL-12 (10ng/ml, Shenandoah Biotechnology, Warwick, PA) to generate pmel^{IL-12} or pmel^{sham} T cells, respectively. In some experiments we generated pmel^{IL-2} cells by substituting hIL-2 (200ng/ml) for IL-12 during the 3 day culture. For generation of mouse tyrosinase-reactive T cells, h3T TCR transgenic splenocytes (1.5×10⁶ cells/well in 1.5ml) were cultured with irradiated HLA-A2 transgenic splenocytes (3.8×10⁶ cells/well) and stimulated with 1 μ g/ml HLA-A2-restricted human tyrosinase₃₆₈₋₃₇₆ (hTyr) peptide (YMDGTMSQV, American Peptide Company) for 3 days with or without mIL-12 (10ng/ml) to generate h3T^{IL-12} or

h3T^{sham} T cells, respectively. For analysis of functional avidity, pme1^{IL-12}, pme1^{sham}, h3T^{IL-12}, or h3T^{sham} were restimulated with the indicated concentration of relevant peptide for 6 hours and assessed for IFN γ expression. For pme1-1 experiments, 10⁵ T cells were cocultured with 10⁵ irradiated B6 splenocytes. For h3T experiments, 10⁵ T cells were cocultured with 10⁵ irradiated T2-A2 cells.

Human T cells were obtained from Research Blood Components (Boston, MA) and cultured using one of the following two protocols. For generation of TCR-modified human T cells, we used a modification of a previously described protocol [29-31]. On day 1, human PBMC were stimulated with soluble anti-CD3 mAb (OKT3, NCI preclinical repository) for 48 hours. Except during the spinoculation step, cells were cultured with hIL-2 (30 IU/ml) and hIL-15 (100ng/ml), and maintained between 1-2 \times 10⁶ cells/ml. On day 3, activated T cells were transduced by co-culture with 50% retroviral supernatant from PG13 packaging cells transfected with the TIL1383ITCR/CD34t construct. Transduction was done with retronectin-coated plates and spinoculation (2000g for 2 hours at 32°C). On day 8, cells underwent a rapid expansion protocol by incubation in an upright T175 flask of 1 \times 10⁶ transduced T cells with 2 \times 10⁸ irradiated (5000rad) allogeneic feeder cells from human donors. Soluble anti-CD3 mAb (OKT3, 30ng/ml) was also added to the cultures. On day ~20, cultures were harvested and analyzed by flow cytometry or in functional assays. For assessment of CD107a degranulation, human T cells (10⁵) were mixed with T2-A2 cells (10⁵) with or without 1 μ g/ml hTyr peptide. As control, we added PMA/ionomycin. Antibody against CD107a was added to the culture during the last 5 hours of the 6 hour culture. For expansion of non-genetically modified T cells for transfer into NSG mice, on day 1 PBMCs were stimulated with anti-CD3 mAb (OKT3) for 48 hours. Beginning on day 3, cells were cultured in hIL-2 and hIL-15 and maintained between 1-2 \times 10⁶ cells/ml. Throughout the culture, cells were grown with or without hIL-12 (10ng/ml). Cells were phenotyped and injected on day 7 of culture.

Flow cytometry

For flow cytometry, cells were analyzed as previously described [32]. For intracellular flow cytometry, cells were stained using the BD Cytotfix/Cytoperm with GolgiStop kit (BD Bioscience, San Jose, CA). Flow cytometry was performed on a BD LSRII or a BD Accuri and data was analyzed using Flowjo software (TreeStar, Ashland OR) and CFLOW software (BD Bioscience).

RNA isolation and Real-time PCR

Total cellular RNA was isolated from pme1^{IL-12} and pme1^{sham} CD8⁺ T cells using Trizol (Invitrogen). cDNA was generated from total RNA (1 μ g per sample) using the High-Capacity cDNA Reverse Synthesis Kit (Bio-Rad, Hercules, CA). The resulting cDNA was amplified by PCR using primer pairs for the BCL3 (forward 5' - CCGGAGGCCCTTACTACC-3'; reverse 5' - GAGTAGGCAGGTCAGCAGC-3'), 18S (forward 5' -CCAGAGCGAAAGCATTTGCCAAGA-3'; reverse 5' - TCGGCATCGTTTATGGCTGGA-3'), and the beta-actin (forward 5' - ACGTAGCCATCCAGGCTGGTG-3'; reverse 5' -TGGCGTGAGGGAGAGCAT-3') using 2X Sso advance SYBR green on CFX96 Touch real-time PCR detection system (Bio-Rad).

The levels of Bcl-3 cDNA in each sample were normalized to beta-actin and 18S cDNA. The final relative expression of mRNA species was calculated using the comparative Ct method.

Tumor challenge, cyclophosphamide (CTX) preconditioning, and adoptive T cell transfer

For tumor experiments, B6 mice were injected (s.c.) with 2.5×10^5 B16-F1 tumor cells. Tumor growth was measured blindly by caliper twice per week and tumor surface area (mm^2) was calculated by length \times width. For CTX injections, mice were given 4mg by i.p. injection. For adoptive transfer experiments, T cells were injected i.v. at the doses indicated. For antibody mediated depletion of $\text{IFN}\gamma$, mice received 150 μg anti- $\text{IFN}\gamma$ mAb (XMG1.2 clone, Bioxcell) on days 0, 2, 4, and 6, after adoptive transfer.

Statistical Methods

Data from all experiments were graphically displayed to identify the need for transformation. When analyzing number or % donor cells, a squareroot transformation was used to adhere to assumptions of Anova and other regression models (i.e. assumption of constant variance of residuals). Although conditions were compared using transformed endpoints, data is displayed on the original scale. Data shown in figure 1A-1B were analyzed using ANOVA. Data in figures 1C and 1D were analyzed using random effects linear regression where random intercepts were included per mouse and main effects of time, group and their interactions were included. To adequately represent the non-linear pattern over time, both time and time^2 were included in the models. Likelihood ratio tests were used to evaluate significance of conditions. Time to sacrifice data are graphically displayed using Kaplan-Meier curves (Suppl. Fig 1) and groups were compared using a Gehan-Wilcoxon test (the modified Peto-Peto version). Conditions in supplemental figure 3A were compared using a random effects model where random intercepts were included for experiment and a fixed effect of IL-12 (vs. sham). $p < 0.05$ considered significant for all experiments.

Results

Ex vivo IL-12-conditioning improves T cell persistence

To better characterize how *ex vivo* IL-12-conditioning of T cells improves the anti-tumor responses upon transfer into mice after host lymphodepletion, we initially assessed T cell persistence in non-tumor bearing mice. Mice were pre-treated with or without cyclophosphamide (CTX, 4mg, i.p.) and then adoptively transferred with 3×10^6 $\text{pme}^{\text{IL-12}}$ or pme^{sham} CD8^+ T cells. Only the combination of IL-12 conditioning and lymphodepletion led to a significantly enhanced donor CD8^+ T cell persistence (**Fig. 1A**). Importantly, this improved persistence was systemic in nature as it was also observed in the liver and lymph nodes (**Fig. 1B**). Titration of adoptively transferred cells demonstrated that the IL-12-conditioned CD8^+ T cells ($\text{pme}^{\text{IL-12}}$) in the peripheral blood persisted at least 100-fold better than control CD8^+ T cells (pme^{sham}) (**Fig. 1C**). Finally, the enhanced persistence induced by IL-12-conditioning was not seen when the T cells were cultured with a high dose of IL-2 ($\text{pme}^{\text{IL-2}}$) for 72 hours (**Fig. 1D**) indicating that IL-12 imparts a unique signal.

Ex vivo IL-12-conditioning improves anti-tumor efficacy

To determine if improved T cell persistence correlated with enhanced anti-tumor efficacy, we treated mice bearing subcutaneous B16 tumor cells with CTX and titrated numbers of adoptively transferred pmel^{IL-12} or pmel^{sham} cells. Pmel^{IL-12} cells exhibited at least a 10-fold improvement in efficacy compared to pmel^{sham} cells as indicated by reduced tumor growth (**Fig. 2**) and enhanced survival (**Sup. Fig. 1**). As we have observed previously, anti-tumor immunity was dependent on both *ex vivo* IL-12 conditioning and lymphodepletion [24].

To understand the mechanism by which pmel^{IL-12} cells mediated anti-tumor immunity after adoptive transfer into a lymphodepleted host environment, we assessed the role of donor cell IFN γ . In some tumor models, Tc1 cells require IFN γ [33,16] while in other models IFN γ is dispensable [15,34]. As our experiments are unique in that donor pmel^{IL-12} were transferred into a lymphopenic environment, we assessed the role of IFN γ in our model and generated pmel^{IL-12} T cells from wildtype or IFN $\gamma^{-/-}$ mice. As shown in **figure 3**, effective anti-tumor immunity was dependent on the ability of donor T cells to produce IFN γ , as pmel^{IL-12} lacking IFN γ exhibited markedly inferior anti-tumor immunity.

We also assessed whether IL-2 could substitute for IL-12 during the priming of pmel-1 CD8⁺ T cells. Similar to our findings in figure 1, pmel^{IL-2} cells failed to mediate effective anti-tumor immunity compared with pmel^{IL-12} cells (**Fig. 3**). Pmel^{IL-2} and pmel^{sham} cells were not distinguishable. Thus IL-12 induces a qualitatively unique signal in these CD8 T cells that cannot be achieved by increasing the level of IL-2 signaling.

IL-12-mediated STAT4 phosphorylation requires T cell activation

The ability of IL-12 to improve the function of tumor-reactive T cells is thought to be most efficient on activated T cells, as activation (or TCR engagement) leads to upregulation of the IL-12 receptor (IL-12R) subunits [35-37]. To validate that activated T cells are more responsive to IL-12 compared with resting T cells on a per cell basis, we used flow cytometry to assay for STAT4 phosphorylation, which is immediately downstream of the IL-12R [38,39]. Resting mouse or human CD8⁺ T cells cultured with IL-12 failed to induce the phosphorylation of STAT4 (**Sup. Fig. 2, left**), which is consistent with resting T cells not expressing a functional IL-12R. In contrast, IL-12 culture of activated mouse or human CD8⁺ T cells induced significant phosphorylation of STAT4 (**Sup. Fig. 2, right**). These results are consistent with T cell responsiveness to IL-12 that is dependent on T cell activation.

IL-12-conditioning improves T cell survival and avidity

To better understand the qualitative changes induced by IL-12 conditioning, we compared the survival of pmel^{IL-12} and pmel^{sham} CD8⁺ T cells in the absence of exogenous growth factors such as IL-2 or IL-15. Pmel^{IL-12} exhibited better short-term viability as compared with pmel^{sham} after washing and overnight culture without addition of cytokines (**Sup. Fig. 3A**). In comparison, culture with a low dose of IL-15 (1ng/ml) improved the viability of both pmel^{sham} and pmel^{IL-12} cells (**Sup. Fig. 3B**). These results suggest that the improved functional efficacy of pmel^{IL-12} cells may be due to their ability to survive or function at

least initially in the absence of critical T cell growth factors in the host. These results are consistent with previous findings and the enhanced survival may be mediated by IL-12-induced upregulation of Bcl-3 [40,23]. In fact, we observed elevated Bcl-3 in pmel^{IL12} versus pmel^{sham} cells (**Sup. Fig. 3C**).

A property critical to optimal anti-tumor immunity is the ability to recognize even nominal amounts of antigen on a tumor cell. As IL-12-conditioning was reported to reduce the ability of T cells to bind relevant tetramer [23], it was important to determine if IL-12-conditioning impacted the ability to functionally recognize limiting amounts of antigen as indicated by the production of IFN γ . Pmel^{IL-12} showed a slightly enhanced ability to produce IFN γ in response to low amounts of antigen as compared to pmel^{sham} cells (**Fig. 4A**). We also found that pmel^{IL-12} cells bound relevant tetramer comparably to pmel^{sham} cells (**Fig. 4B**) and this was not temperature dependent (**Fig 4C**). Using a second TCR, from h3T TCR transgenic mice (which expresses a murine version of TIL 1383I [28]), we observed a similar trend toward enhancement of IFN γ production in response to low amounts of antigen (**Fig 4D, E**). Cumulatively, our results demonstrate that conditioning of T cells with IL-12 does not impair their ability to recognize the limiting amounts of antigen that may be present on tumor cells.

IL-12 improves the functional qualities of human T cells genetically modified with a tyrosinase-reactive TCR and expanded using a clinically relevant protocol

As a next step in translating our approach, we defined the functional parameters of human T cells cultured with or without IL-12 using conditions that would facilitate translation to a clinical trial for cancer patients. As part of this protocol, we genetically modified human T cells with the TIL 1383I TCR, generating tumor-reactive T cells reactive against the melanoma tumor antigen, tyrosinase. We used a modified version of a T cell expansion protocol that has been successfully used to generate sufficient numbers of receptor-modified cells for patients in a variety of trials [29-31] including an ongoing trial at Loyola Medical Center in Chicago using the TIL 1383I TCR platform. However, in contrast to the ongoing Loyola trial using lentiviral vectors, we used retroviral vectors encoding the same three gene products, which include the TIL 1383I TCR α and β , and a truncated CD34 gene [41]. Expression of the truncated CD34 gene enables the enrichment and *in vivo* detection of genetically modified cells. Using this established clinical protocol as a starting point, we cultured human T cells with or without IL-12 for ~3 weeks and monitored the cellular expansion. At the end of the culture, we assayed cells phenotypically and by functional readouts.

As shown in **Figure 5A**, addition of IL-12 to the expansion protocol did not compromise our ability to generate sufficient numbers of cells for patient infusion, although there was a trend towards lower cellular expansion with IL-12 in the culture. There were high frequencies of genetically modified T cells with or without IL-12 culture (**Fig. 5B**). Similar to mouse cells, we observed higher levels of granzyme B in the presence of IL-12 (**Fig. 5B**). In response to relevant peptide, IL-12-conditioned CD34^{hi} cells degranulated to a greater extent than CD34^{hi} unconditioned counterparts (**Fig 5C**). Cumulatively, our results indicate that the addition of IL-12 did not impede the generation of sufficient numbers of genetically

modified T cells needed for patient infusion, and furthermore, IL-12-conditioned human T cells have the characteristics consistent with improved *in vivo* functional activity.

IL-12-conditioned human T cells persist in immunodeficient NSG mice

In an attempt to understand how IL-12-conditioned cells would engraft in cancer patients, we next sought to determine how IL-12-conditioning impacts the ability of human T cells to persist in immunodeficient mice. In addition to functional capability, T cell persistence correlates with the therapeutic effectiveness of ACT therapy [42,5]. Thus, we used a simplified version of our clinical protocol and activated human T cells with anti-CD3 mAb for two days followed by culture for 1 week with hIL-2 and hIL-15. Human T cells were cultured with or without hIL-12 during the entire length of the culture, and then harvested for phenotypic analysis and subsequently injected *in vivo* into immunodeficient NSG mice [43]. As observed in our clinical T cell transduction culture protocol, culture with IL-12 enhanced granzyme B expression (**Sup. Fig. 4A**). Human donor cells exhibited efficient engraftment (**Sup. Fig. 4B**), and furthermore, IL-12 conditioned cells appeared to preferentially persist. However, in addition to the initial engraftment, there appeared to be a 2nd rapid expansion phase beginning about 2-3 weeks post injection, possibly suggesting graft versus host disease. Despite the potential of graft versus host disease, we noted that the *ex vivo* IL-12-conditioned donor T cells exhibited a trend toward elevated frequencies of donor cells as compared to non-conditioned cells when transferred into NSG hosts.

Discussion

The field of adoptive T cell therapy is developing the ability to reliably generate T-cell receptor (TCR) and chimeric antigen receptor (CAR) gene transfer platforms with an increasing capability of inducing curative responses in patients with advanced metastatic cancer. It is becoming clear, however, that the conditioning of T cells with cytokines capable of affecting the functional and phenotypic fingerprint of transferred T cells prior to ACT is also a critical means of improving *in vivo* efficacy and durability. Despite this growing body of pre-clinical data, current ACT therapies have yet to leverage this dimension by incorporating these potentially more sophisticated T cells into phase I studies. While it would seem that translation of these concepts into human trials would be relatively straightforward; there are significant conceptual and logistical barriers that must be addressed. These hurdles include identifying a cytokine-conditioning methodology that can have a durable impact *in vivo*. Furthermore, it will be critical to develop the methodology for reliably and efficiently integrating the application of cytokine pre-conditioning into the complex methodology of T cell transduction that needs to be considered in order to reliably deploy a TCR transduction clinical protocol that meets FDA standards

Amongst several therapeutically promising phenotypes, including Tc2 and Tc17 cells, we have focused on Tc1 cells because early studies by several investigators have clearly demonstrated that CD8⁺ T cells cultured with IL-12, and skewed to a Tc1 phenotype, had enhanced functional ability and anti-tumor efficacy [8-11,15-21,24,22,23]. The *ex vivo* dimension of these findings are especially cogent for IL-12 where significant *in vivo* clinical toxicity [44,45] has limited the utilization of this promising cytokine and led investigators to

look for non-systemic approaches. With the goal of translating these observations into a safe and effective therapy for cancer patients, we have characterized the impact of *ex vivo* IL-12-conditioning on both mouse and human T cells. We found that IL-12-conditioned mouse T cells exhibit 10-100-fold improvement in persistence and anti-tumor immunity when transferred into lymphopenic hosts. Furthermore, IL-12-conditioned cells demonstrate improved survival in the absence of T cell growth factors and also retention of T cell avidity. Importantly, when applied to a clinical expansion protocol, we found that IL-12-cultured and TCR-modified human T cells are capable of being expanding to the numbers necessary for patient administration. Furthermore, IL-12-conditioning of these TCR-modified human T cells improved their functionality as indicated by granzyme B and antigen-specific CD107a degranulation.

One important aspect of our study was the direct titration of cell numbers transferred and the benefits of IL-12-conditioning on the persistence and function of mouse T cells. IL-12-conditioned mouse T cells exhibited at least a 10-fold increase in anti-tumor activity versus control cells. Similarly, we observed that the ability of these IL-12-conditioned CD8⁺ T cells to persist *in vivo* was improved approximately 100-fold by IL-12 conditioning. These beneficial effects were dependent on cyclophosphamide preconditioning, suggesting that the *in vivo* environment induced by lymphodepletion is critical for the optimal function of IL-12-conditioned T cells. This result suggests the existence of host factors that may selectively support the functional ability of IL-12-conditioned cells during lymphodepletion. Although not directly addressed in this body of work, the discernment of the mechanisms involved with this synergistic partnership will be important to understand as we work to thoughtfully build on our current ACT protocols.

In direct support of the potential of *ex vivo* IL-12-conditioning in patient care, we utilized a modification of a currently used clinical protocol for the expansion and genetic modification of human TIL 1383I-modified T cells (NCT01586403). Variations of this protocol have been used by several groups to induce efficient engraftment of TCR- and CAR- modified T cells, and in some cases anti-tumor responses [29-31]. However, in these prior studies, IL-12 was not incorporated into the culture methodology. Our findings show that addition of IL-12 to the three weeks of culture did not impair our ability to generate sufficient numbers of T cells for patient administration. Furthermore, the T cells generated exhibited functional qualities which would predict enhanced anti-tumor efficacy *in vivo* including higher levels of granzyme B, and the improved ability to mobilize CD107a in response to relevant antigen.

To assess the impact of IL-12-conditioning on persistence of human T cells *in vivo*, we used the NSG immunodeficient mouse model [43]. These mice, which are on a NOD, SCID, and IL-2R $\gamma^{-/-}$ background, are thought to represent one of the best models for assaying the function of human immune cells in mice. We cultured human T cells using a simplified ~9 day protocol involving soluble anti-CD3 mAb stimulation with or without continual IL-12. Unfortunately, engraftment of the human cells into NSG mice was only successful in 1 of 3 experiments independent of IL-12-conditioning. In the one experiment where engraftment could be assessed (**Sup. Fig. 4**), we observed a trend of enhanced persistence in cells cultured with IL-12. Thus, at a minimum, IL-12 conditioning was not detrimental to T cell

persistence. Despite the limitations of our model, these results provide a basis for future experiments involving the transfer of human T cells into NSG mice and identification of the factors important for achieving stable engraftment. Moving forward in the NSG mouse model, our goal is to determine whether IL-12-conditioned human T cells genetically modified with the TIL 1383I TCR and adoptively transferred will not only persist longer but mediate enhanced anti-tumor immunity.

It is relevant to note that one other group has reported the transfer of IL-12-conditioned human T cells into immunodeficient mice [10]. Using a three week culture protocol but no rapid expansion protocol (REP) as in our experiments, the authors demonstrated that IL-12-conditioned human T cells genetically modified with a CAR recognizing CEA led to enhanced anti-tumor immunity compared with human T cells cultured without IL-12. The authors did not assess the persistence of the human T cells in the immunodeficient mice, but demonstrated *in vitro* a functional improvement in human T cells following culture with IL-12. Our findings build on these earlier observations and demonstrate the ability to generate adequate numbers of cells for patient administration.

One alternative IL-12-based approach for augmenting T cell-mediated immunity warrants review. Several groups have recently reported that genetically modifying tumor-reactive CD8⁺ T cells to express IL-12 significantly improves anti-tumor immunity [46-49]. While these results are exciting and the approach is being evaluated clinically, we note the mechanism is likely distinct from that described in our study. Thus, Kerkar et al. found that CD8⁺ T cells deficient in functional IL-12 receptor still exhibited improved anti-tumor ability when genetically modified to express IL-12 [46]. These authors concluded that IL-12 was not acting on the donor T cells and instead IL-12 was modifying the myeloid cell compartment to facilitate the function of the donor T cells. In contrast, in our studies the impact of IL-12 is only on the donor T cells and IL-12 is removed prior to T cell infusion. Given the clinical toxicities that have been reported with IL-12, this methodological distinction is important.

In summary, we find that IL-12-conditioning of murine and human tumor-reactive CD8⁺ T cells can lead to a significant enhancement in function and anti-tumor immunity. We also demonstrate for the first time that TCR-modified human T cells can be effectively expanded with IL-12-conditioning to numbers sufficient for patient administration using a clinically relevant protocol. Our findings demonstrate a pathway for the safe clinical application of IL-12 which could have application in tumor immunology and other domains of clinical immunology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Daniel Neitzke for critical review of this manuscript. Grant funding for this project was provided by the following grants from the National Institutes of Health and the National Cancer Institute: P01CA154778-01, R01CA138930, R01CA133503, and R01CA175061. This work was also supported in part by the Cell Evaluation & Therapy Shared Resource, Hollings Cancer Center, Medical University of South Carolina (P30CA138313).

Abbreviations

ACT	adoptive cell therapy
CAR	chimeric antigen receptor
CTX	cyclophosphamide
IL-12	Interleukin 12
REP	rapid expansion protocol
Tc0	CD8 ⁺ T cells not polarized
Tc1	CD8 ⁺ T cells polarized with IL-12
TCR	T-cell receptor

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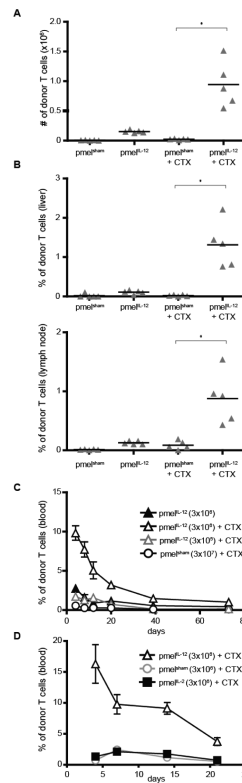


Figure 1. IL-12 conditioning improves CD8⁺ T cell persistence in lymphodepleted hosts at least 100-fold

(A) B6 mice were treated with or without cyclophosphamide (CTX), and one day later adoptively transferred with 3×10^6 pmel^{IL-12} or pmel^{sham} T cells. On day 10 after adoptive transfer, spleens were harvested and the number of donor T cells (CD8⁺Thy1.1⁺) was determined. Each triangle represents an individual mouse and the bar indicates the mean. (B) As in 'A', except liver (top) and lymph node (bottom) cells were harvested and the frequency of donor T cells was determined. In panels A and B, pmel^{sham} + CTX and pmel^{IL-12} + CTX conditions (denoted with a “*”) were significantly different ($p < 0.0001$). (C) As in 'A', except titrated numbers of pmel^{IL-12} or pmel^{sham} were injected. Blood was harvested at the indicated time points. Each point represents the mean of 3-6 mice. The % donor T cells differed significantly across time for all three conditions with CTX ($p < 0.0001$). (D) Similar to 'C' with the transfer of pmel^{IL-2} cells. Each point represents the mean of 3-5 mice. There was a significant difference ($p < 0.0001$) between pmel^{IL-12} and pmel^{IL-2}. There was no difference between pmel^{sham} and pmel^{IL-2} ($p = 0.40$). Data are representative of at least 2 independent experiments.

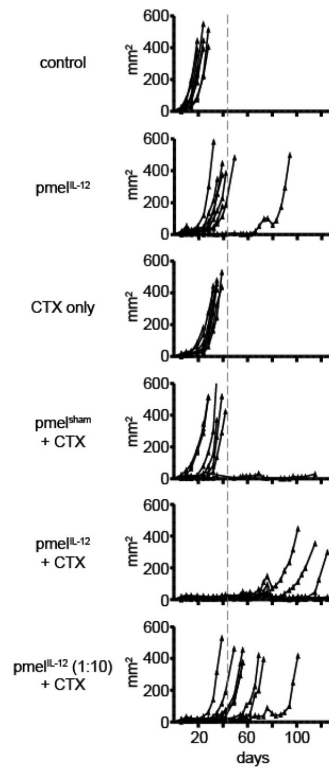


Figure 2. IL-12 conditioning improves CD8⁺ T cell anti-tumor immunity by least 10-fold
 Mice with s.c. B16 tumors were treated on day 7 with cyclophosphamide (CTX). On day 8, mice were adoptively transferred with pmeI^{IL-12} or pmeI^{sham} cells. In mice receiving T cells, 4×10^6 T cells were injected except 4×10^5 T cells in the condition designated (1:10). Tumors were measured blindly twice per week and each line represents one mouse (n=8-10/group). Similar results were obtained in an independent experiment shown as a survival curve in supplemental figure 1. Using a Gehan-Wilcoxon test, time to sacrifice (mice were sacrificed when tumors reached 400mm^2) was compared across groups using data from this figure and supplemental figure 1. There were significant differences between certain groups, and notably between pmeI^{sham} + CTX and pmeI^{IL-12} (1:10) + CTX (p=0.004).

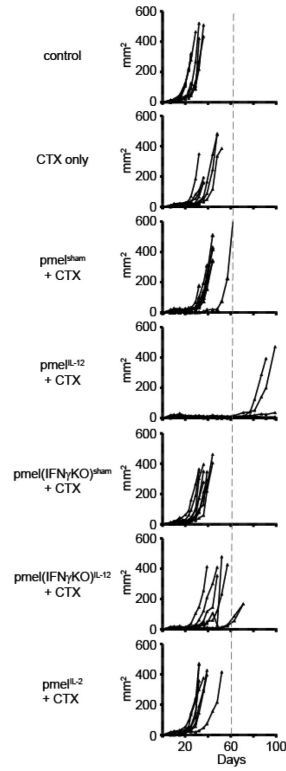


Figure 3. IL-12-conditioned CD8⁺ T cells depend on IFN γ for optimal anti-tumor immunity
 Mice with s.c. B16 tumors were treated on day 7 with cyclophosphamide (CTX). On day 8, mice were adoptively transferred with 3×10^6 pmel^{IL-12}, pmel^{IL-2}, or pmel^{sham} cells from wildtype or IFN $\gamma^{-/-}$ pmel-1 mice. Tumors were measured blindly twice per week and each line represents one mouse (n=8-10/group). A similar requirement for IFN γ was also observed in B16-tumor-bearing mice given 6 Gy (in lieu of CTX), adoptively transferred with pmel^{IL-12}, and injected with or without 150ug of anti-IFN γ mAb (clone XMG1.2) on days 0, 2, 4, and 6.

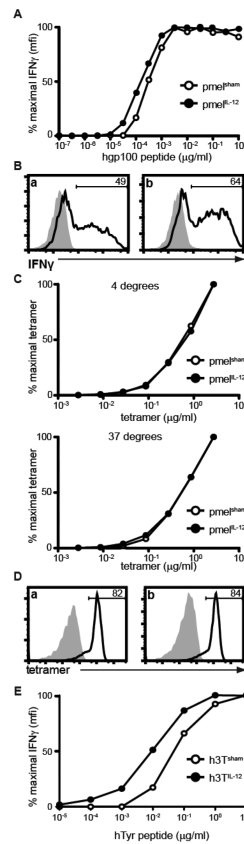
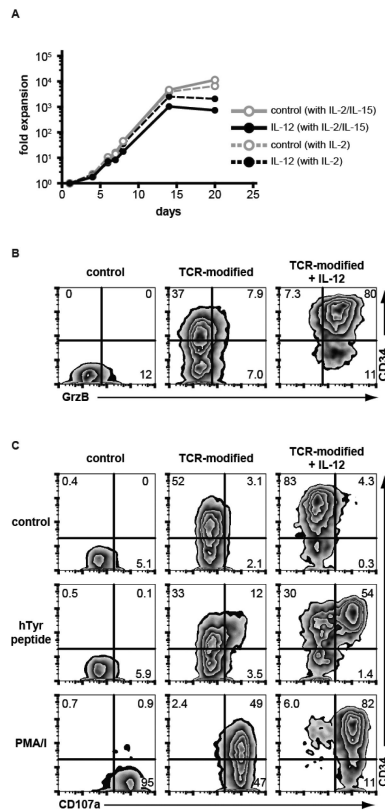


Figure 4. IL-12 conditioning maintains the functional avidity of activated CD8⁺ T cells
(A) PmeI^{IL-12} or pmeI^{sham} cells were co-cultured for 6 hours at a 1:1 ratio with naïve B6 splenocytes and titrated amounts of peptide, and then intracellular IFN γ was assessed by flow cytometry. Shown is the % maximal IFN γ as a percentage based on maximal MFI. **(B)** Representative staining for ‘A’ at the maximal peptide concentration. The shaded region shows staining of cells not stimulated with peptide. **(C)** PmeI^{IL-12} or pmeI^{sham} cells were incubated with titrated tetramer at either 4°C (top) or 37°C (bottom). **(D)** Representative tetramer staining at the maximum concentration (2.5 μ g/ml). The shaded region shows control staining without the addition of tetramer. **(E)** As in ‘A’, except we compared h3T^{IL-12} versus h3T^{sham} T cells.



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Figure 5. IL-12 conditioning enhances the functional ability of human T cells genetically modified with the TIL 1383I, tyrosinase-reactive T-cell receptor
(A) Human PBMCs were stimulated with anti-CD3 mAb and retrovirally modified with the TIL 1383I TCR/CD34t construct encoding TIL 1383I α and β , and CD34t. T cells then underwent a rapid expansion protocol (REP) prior to phenotyping. As indicated, cells were maintained in either IL-2 or IL-2/IL-15, and also with or without hIL-12 throughout the culture. **(B)** Cells from 'A' maintained in IL-2/IL-15 were analyzed by flow cytometry after conclusion of the REP. Shown are cells gated on CD8⁺ expression, and depicting CD34 and granzyme B expression. **(C)** Cells in 'B' were stimulated for 6 hours with peptide, PMA/ionomycin(I), or control. Shown are cells gated on CD8⁺ expression and depicting CD34 expression and CD107a binding during culture. Results are representative of at least 2 independent experiments.