Heterodimeric IL15 Treatment Enhances Tumor Infiltration, Persistence, and Effector Functions of Adoptively Transferred Tumor-specific T Cells in the Absence of Lymphodepletion

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Abstract

Purpose: Adoptive cell transfer (ACT) is a promising immunotherapeutic approach for cancer. Host lymphodepletion is associated with favorable ACT therapy outcomes, but it may cause detrimental effects in humans. We tested the hypothesis that IL15 administration enhances ACT in the absence of lymphodepletion. We previously showed that bioactive IL15 in vivo comprises a stable complex of the IL15 chain with the IL15 receptor alpha chain (IL15Rα), termed heterodimeric IL15 (hetIL15).

Experimental Design: We evaluated the effects of the combination regimen ACT + hetIL15 in the absence of lymphodepletion by transferring melanoma-specific Pmel-1 T cells into B16 melanoma-bearing mice.

Results: hetIL15 treatment delayed tumor growth by promoting infiltration and persistence of both adoptively transferred Pmel-1 cells and endogenous CD8⁺ T cells into the tumor. In contrast, persistence of Pmel-1 cells was severely reduced following irradiation in comparison with mice treated with hetIL15. Importantly, we found that hetIL15 treatment led to the preferential enrichment of Pmel-1 cells in B16 tumor sites in an antigen-dependent manner. Upon hetIL15 administration, tumor-infiltrating Pmel-1 cells showed a "nonexhausted" effector phenotype, characterized by increased IFNγ secretion, proliferation, and cytotoxic potential and low level of PD-1. hetIL15 treatment also resulted in an improved ratio of Pmel-1 to Treg in the tumor.

Conclusions: hetIL15 administration improves the outcome of ACT in lymphoreplete hosts, a finding with significant implications for improving cell-based cancer immunotherapy strategies.

Introduction

Adoptive immunotherapy with tumor-specific T cells either isolated from tumor tissue or engineered to recognize tumor-associated antigens is a promising approach for cancer immunotherapy (1–4). Studies in mice and humans have shown that the effectiveness of adoptive cell transfer (ACT) therapy can be improved by lymphodepleting the host prior to cell infusion (5, 6). Several mechanisms have been proposed for this beneficial effect. Previous studies showed that lymphodepletion removes endogenous lymphocytes functioning as cellular sinks for homeostatic cytokines and allows free cytokines to induce survival and proliferation of adoptively transferred cells (7). In line with these findings, increased plasma levels of IL7 and IL15 were measured in humans undergoing lymphodepleting regimens (6). Host preconditioning also results in depletion of Tregs and myeloid-derived suppressor cells (MDSC), cellular subsets associated with immune suppression, and tolerance (4). However, in humans, T-cell recovery after lymphodepletion treatment is delayed and frequently incomplete (8, 9), resulting in potentially severe and prolonged immune dysfunction and significant morbidity and mortality from opportunistic and recurrent infections (10). Delays in immune reconstitution can also contribute to the relapse of malignant disease. Therefore, although lymphopenia creates a modified immune environment that can favor the effectiveness of adoptive immunotherapy, the negative consequences of T-cell depletion could offset the benefits.

ACT therapy benefits from the administration of γ-chain cytokines that play a pivotal role in promoting differentiation, proliferation, and survival of the adoptively transferred T cells (11). Several studies have identified IL15 as a key factor for the homeostatic proliferation of CD8⁺ T cells (12, 13) and evaluated its role in supporting ACT therapies. Klebanoff and colleagues (14) demonstrated that IL15 is superior to IL2 in inducing T cells with greater proliferative and cytokine secretion potential as well as effectiveness in inducing regression of established melanoma upon adoptive cell transfer in mice. IL15 is also essential for the in vivo persistence of the transferred T cells (14). Similar results were obtained in a macaque model in which autologous...
**Translational Relevance**

Heterodimeric IL15 (hetIL15) is a lymphocyte growth and activation factor presently in clinical trials for immunotherapy of metastatic cancers. This study shows that hetIL15 is able to replace the need for lymphodepletion prior to adoptive cell transfer (ACT) for cancer therapy. hetIL15 induces lymphocyte entry into tumor sites and activation of intratumoral CD8+ T cells. In contrast to other protocols, heterodimeric IL15 induces an enrichment of antigen-specific lymphocytes in the tumor over time and greatly increases CD8+ T cell ratio. Application of heterodimeric IL15 to ACT will provide new tools and techniques for cancer immunotherapy protocols. Elimination of the need for lymphodepletion will make more patients eligible for cell transfer protocols. In addition, IL15 could be a general method to place T cells into tumors, increasing the success rate of other immunotherapy interventions.

CMV-specific CD8+ T-cell clones generated in the presence of IL15 acquired a central memory phenotype rather than terminally differentiated effector phenotype and displayed superior persistence (15). Additional findings also demonstrated a role of IL15 in breaking tolerance and in rescuing tolerant CD8+ T cells for use in adoptive immunotherapy of established tumors (16, 17) and in augmenting antigen-specific CD8+ T cells response upon vaccination (18).

We have previously shown that IL15 is produced and functions as a heterodimeric complex of two polypeptide chains, IL15 and IL15 receptor alpha (IL15Rx; ref. 19). The two polypeptide chains are coproduced and form a complex in the endoplasmic reticulum, before they get fully glycosylated and traffic through the Golgi to the plasma membrane (20, 21). The membrane-embedded IL15Rx is responsible for IL15 retention on the cell surface, where it is trans-presented to adjacent responding cells expressing the IL2/IL15 receptor beta (22). In addition, after a specific proteolytic cleavage of the IL15Rx, a soluble heterodimeric form of IL15 is released, circulates in the blood, and is biologically active (19, 20, 23). These data suggest that IL15Rx is not a receptor for the IL15 polypeptide chain, but the other half of heterodimeric IL15 (hetIL15; ref. 24).

In this report, we exploit the potential of hetIL15 in modifying the lymphoid milieu at tumor sites to enhance the effectiveness of adoptively transferred cells in the absence of lymphodepletion. We show that in a lymphoreplete host, hetIL15 promotes targeted tumor infiltration, proliferation, and effector functions of adoptively transferred tumor-specific T cells, resulting in inhibition of tumor growth.

**Materials and Methods**

**Mice**

Female C57BL/6-pmel-1-Thy1.1 transgenic mice (25) were kindly provided by Drs. C. Mackall and O. Rimas, National Cancer Institute (Bethesda, MD). C57BL/6 mice were obtained from Charles River Laboratory. IL15 knockout (KO) mice were purchased from Taconic. The study was approved by the National Cancer Institute-Frederick Animal Care and Use Committee and were maintained in accordance with the ALIC guidelines and the NIH Guide for the Care and Use of Laboratory Animals.

**Subcutaneous mouse tumor model**

B16F10 melanoma cells were maintained in DMEM supplemented with 10% heat-inactivated FBS (Thermo Fisher Scientific) and 1× penicillin/streptomycin. Seven-week-old wild-type C57BL/6 animals were injected with 4 × 10^3 tumor cells subcutaneously in the flank. MC38 colon carcinoma cells were maintained in DMEM supplemented with 10% heat-inactivated FBS (Thermo Fisher Scientific), 1× penicillin/streptomycin, 1× essential amino acids, and 1× HEPES. In some experiments, C57BL/6 animals were injected with 4 × 10^3 B16 melanoma cells subcutaneously into one flank and with 3 × 10^3 MC38 colon carcinoma cells subcutaneously into the other flank. Tumor area (length × width) was measured every 2–3 days in a blinded fashion.

**Immunotherapy of B16 melanoma-bearing mice**

Five days after inoculation of B16 cells, tumor-bearing mice were randomized into three groups receiving ACT, ACT+IL2, or ACT+hetIL15. In some experiments, mice received ACT+IL2. Splens from pmel-1 TCR/Thy1.1 transgenic mice were harvested and splenocytes were used as source of melanoma antigen (hgp10025–33)-specific T cells (Pmel-1 T cells) for ACT. Splenocytes were activated in vitro using plates coated with anti-CD3 antibody (145-2C11, BD Biosciences) and soluble no azide/low endotoxin (NA/LE) anti-CD28 antibody at 1 μg/mL (37, 51, BD Biosciences). Human IL2 (12.5 ng/mL, PeproTech) was provided on day 2 and cells were harvested on day 5. Cells (1–5 × 10^6; in 100 μL PBS) of in vitro–activated Pmel-1 T cells were injected intravenously in mice, in the absence of vaccination. For lymphodepletion preconditioning, mice were subjected to whole-body irradiation (5 Gy; X-ray source, 1.29 Gy/minute, 137-cesium chloride irradiator) one day before ACT. For hetIL15 treatment, mice received intraperitoneal injection of 3 μg (molar mass of IL15) of hetIL15 (Admune Therapeutic LLC; ref. 24) 3 times/week for 8 total injections. For the IL2 treatment, mice received intraperitoneal injection of 3 or 9 μg of human IL2 (Teceluekin, Hoffman-Roche) 3 times/week for 8 total injections. For the analysis of tumor-infiltrating lymphocytes, two independent experiments were performed using 5 × 10^6 Pmel-1 cells per mouse for ACT. One experiment was performed using 1 × 10^6 Pmel-1 cells for ACT, leading to similar conclusions.

**Isolation of lymphocytes from tumor and lymphoid organs**

Excised tumors and lungs were cut into small pieces and digested by collagenase IV (200 U/mL, Sigma-Aldrich) and DNase I (30 U/mL, Roche Diagnostic GmbH) at 37°C for 1 hour. Tumor cells suspensions were layered on 3-mL histopaque 1116 medium (Sigma-Aldrich). Spleens and inguinal lymph nodes were dissociated using a 100-μm cell strainer and washed to remove any remaining organ stroma. Recovered cells were subsequently washed and stained with the fixable viability dye (Thermo Fisher Scientific) for 30 minutes at 4°C, before surface and intracellular staining for flow cytometry analysis.

**Intracellular cytokine production assay by TILs and splenocytes**

Single-cell suspensions from tumor and inguinal lymph nodes were cultured in medium only or in the presence of hgp10025–33 peptide (KPVPRQDWL, 10 μmol/L, NeoScientific) at 37°C for...
Flow cytometry analysis

Surface staining was performed using antibodies for the following markers: CD3 (145-2C11), CD4 (RM4-5), CD8 (53-6.7), CD45 (30-F11), CD90.1 (OX-7), PD-1 (RMP1-30; BD Biosciences; ebioscience, Inc.; Biolegend). Adoptively transferred Pmel-1 T cells were identified as CD3^+ CD8^+ Thy1.1^+ cells, while endogenous CD8^+ T cells were identified as CD3^+ CD8^+ Thy1.1^- for intracellular staining, cells were fixed and permeabilized using the Foxp3 staining buffer (ebioscience, Inc.), following manufacturer’s instructions. Samples were stained with Ki-67 (Mib-1) and deparaf

Immunohistochemistry

IHC of murine T-cell populations within the tumor was performed as reported previously (26). Tumors were harvested and fixed for 24 hours at room temperature in Zinc-Fixation Buffer (0.5 g calcium acetate, 5 g zinc acetate, and 5 g zinc chloride in one liter 0.1 mol/L Tris pH 7.4, final pH 6.5–7.0). Tumor sections were paraffin embedded using Tissue-Tek automated tissue processor (sakura) and embedded with Leica tissue embedder. Sections (4.5-μm thick) were cut on microtome and floated onto plus-slides (Cardinal ColorFrost) in a tissue fixation bath set at 40°C. Slides were allowed to dry at room temperature overnight.

Slides were placed onto staining rack in the Leica autostainer, and deparaffinization protocol was run (xylene, 4 minutes; 100% ethanol, 2 minutes; 95% ethanol, 1 minute; 70% ethanol, 1 minute; water). Slides were treated with PeroxAbolish (Biocare Medical) for 20 minutes to reduce endogenous peroxidase activity. Slides were rinsed with H2O and TBS-T and blocked with goat serum (Vector Laboratories) for 20 minutes. Rabbit anti-CD3 antibody (SP7, Spring Bioscience, M3074) was diluted 1:100 in Renaissance antibody diluent (Biocare Medical), added to the slide and incubated for 45 minutes on an orbital shaker at room temperature. After washes in TBS-T, anti-rabbit HRP secondary antibody (Life Technologies, 87-0900-81, 1:50) antibody, followed by the secondary anti-rat HRP (Vector Laboratories, MP-7444-15) antibody. Slides were washed with TBS-T and H2O followed by antibody stripping using the antibody-stripping buffer [0.1 mol/L glycine (Sigma, G2879), pH10 using NaOH (Fisher Chemical, SS267), 0.5% Tween] for 10 minutes at room temperature. The same cycles were performed for the rat anti-CD4 (RM4-5, BD Biosciences, 550280, 1:100) antibody, followed by the anti-rat HRP (Vector Laboratories, MP-7444-15) secondary antibody, and for the rat anti-CD90.1 PE (H1551, ebioscience, 12-0900-81, 1:50) antibody, followed by the anti-PE HRP (KPL, 04-40-02, 1:50). After wash in TBS-T, DAPI (Life Technologies, D1306, 1 mg/mL stock, 1:500 in PBS) was added to slides for 5 minutes at room temperature. Slides were rinsed with TBS-T and H2O and cover-slipped with VectaShield Hard Mount (Vector Laboratories). Slides were imaged at both 4×, and 20× using Vectra imaging software (PerkinElmer), and the number of cells were enumerated from fifteen 20× fields using InForm analysis software (PerkinElmer).

Statistical analysis

Differences among groups were evaluated by one-way ANOVA or unpaired Student t test. The P values were corrected for multiple comparisons using Holm–Sidak test. Tumor growth over time was analyzed using repeated measures ANOVA after appropriate transformation of raw tumor area values to be consistent with the assumptions of the method. Prism 6.0c software package (GraphPad Software, Inc.) was used for analysis.

Results

Adoptively transferred Pmel-1 cells infiltrate and persist in tumor sites upon hetIL15 administration

Previous studies have shown that the increased availability of the homeostatic cytokine IL15 following host lymphodepletion sustains the proliferation of adoptively transferred cells and results in significantly improved ACT therapy outcomes for cancer (7). Prompted by these studies, we analyzed the behavior of CFSE-labeled transferred CD8^+ T cells in wild-type as well as in IL15 KO mice upon irradiation. We confirmed that lack of IL15 decreased the proliferation of adoptively transferred cells, suggesting that IL15 is a nonredundant factor participating in this process (Supplementary Fig. S1). Given IL15’s role, we tested the hypothesis that hetIL15 administration could overcome endogenous cellular “sinks,” normally competing for access to IL15 (7), to sustain ACT in the absence of lymphodepletion. For this purpose, we selected the B16 melanoma mouse model expressing the melanoma cell–associated antigen gp100. For ACT therapy, C57BL/6/6-pmel-1-Thy1.1 transgenic mice were used as a source of Pmel-1 cells, whose T-cell receptor specifically recognizes the gp100\(53,54\)-peptide (25). Pmel-1 cells were transferred into B16 melanoma-bearing C57BL/6 mice and lymphocyte tumor infiltration and persistence was compared using 3 strategies (Fig. 1A): (i) cell transfer without lymphodepletion (ACT alone), (ii) cell transfer into irradiated mice (ACT+XRT), and (iii) cell transfer plus exogenous hetIL15 administration (ACT+hetIL15) in nonirradiated mice. We measured tumor infiltration of adoptively transferred Pmel-1 cells as well as endogenous CD8^+ T cells over time. Tumors were isolated at specified time points, and the tumor-infiltrating lymphocytes (TIL) were analyzed by flow cytometry (Supplementary Fig. S2). Tumor-infiltrating Pmel-1 cells (green gate) were distinguished from endogenous CD8^+ T cells (purple gate) by the expression of the congeneric marker CD90.1. In the ACT alone group, approximately 300 Pmel-1 cells per million cells were present at the tumor site at day 5 after cell transfer (Fig. 1B). At the same time point, a trend toward a modest increase (2- to 3-fold) in the proportion of Pmel-1 cells in the tumor was detected both in mice pretreated with XRT and in mice receiving hetIL15,
Figure 1. hetIL15 promotes tumor infiltration and persistence of adoptively transferred Pmel-1 and endogenous CD8+ T cells in the absence of lymphodepletion. A, ACT therapy in B16 melanoma-bearing mice. Pmel-1 cells (5 × 10^6) were adoptively transferred comparing three treatment protocols: (i) cell transfer without lymphodepletion (ACT, gray symbols), (ii) cell transfer in irradiated host (ACT+XRT, red symbols), and (iii) cell transfer plus IP hetIL15 administration (ACT+hetIL15, blue symbols). Mice were sacrificed at day 5, 7, and 12 for tumor and spleen analysis. (Continued on the following page.)
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Although this difference did not achieve statistical significance (one-way ANOVA; Fig. 1B). Importantly, in both the ACT and ACT+XRT groups, we observed a progressive decline in the frequency of Pmel-1 cells in the tumor; the number of Pmel-1 cells per million cells in the tumor decreased by approximately 60% between day 5 and day 12 after cell transfer (Fig. 1B and C). In contrast, in tumor-bearing mice that received hetIL15, Pmel-1 cells were still present at relatively high numbers (~2,000 Pmel-1 cells per million cells in the tumor) at day 12 after cell transfer (Fig. 1B and C). Therefore, administration of hetIL15 in the absence of lymphodepletion favors the infiltration and persistence of antigen-specific transferred cells into the tumor.

We further investigated the effects of hetIL15 treatment on tumor infiltration by endogenous CD8+ T cells. As with adoptively transferred Pmel-1 cells, hetIL15 administration also significantly increased the frequency of endogenous CD8+ T cells in the tumor in comparison with both ACT and ACT+XRT groups at day 12 after ACT (Fig. 1D). In contrast, there was no difference in the number of tumor-resident endogenous CD8+ T cells in the ACT and ACT+XRT (Fig. 1D).

To confirm that the lymphocytes isolated upon in vitro digestion of the tumor were of intratumoral origin rather than peripherally associated with excised tumors, we examined the tumor infiltration of T cells by fluorescence IHC. Staining of tumor sections at day 13 after cell transfer was performed using antibodies against CD3, CD4, CD8, and CD90.1 (Fig. 1E and F). These data confirmed that, in comparison with the other groups, treatment with hetIL15 resulted in an increased accumulation of both tumor-specific Pmel-1 cells (green) and endogenous CD8+ T cells (merigold) that were widespread throughout the tumor area. Quantification of Pmel-1 cells and endogenous CD8+ T cells/mm2 by fluorescence IHC showed a significant increase in the ACT+hetIL15 group (Fig. 1G). Taken together, these data suggest that IL15 promotes the infiltration of tumor sites by both adoptively transferred antigen-specific T cells and endogenous CD8+ T cells and favors their in situ persistence in the absence of lymphodepletion.

The body weight and hematologic parameters of treated animals were compared to assess the toxicity of the three protocols. No significant changes in body weight were observed over time in any of the treatment groups (data not shown). Hematologic parameters were determined at day 5, 7, and 12 after ACT (Supplementary Table S1). As expected, irradiation resulted in a severe but transient reduction in WBC and lymphocyte counts at day 5 and 7 after ACT. WBCs and lymphocytes returned to normal health risk for the treated host.

hetIL15 administration promotes preferential enrichment of Pmel-1 cells in tumors in an antigen-dependent manner

We investigated whether hetIL15 treatment differentially affects tumor-specific Pmel-1 cells and endogenous CD8+ T cells in the B16 tumor compared with other tissues lacking gp100, that is, spleen, lung, and a gp100-negative tumor (MC38 colon carcinoma). Similar to the findings in the tumor, hetIL15 administration in the absence of lymphodepletion resulted in a significant increase in the total count of both Pmel-1 cells (Supplementary Fig. S3A) and endogenous CD8+ T cells (Supplementary Fig. S3B) in spleen, compared with both ACT and ACT+XRT. Interestingly, in comparison with ACT alone, hetIL15 administration resulted in a proportionally greater enrichment of Pmel-1 cells than endogenous CD8+ T cells in tumor, as revealed by analysis by both flow cytometry (Fig. 2A left, day 12 after cell transfer) and IHC (day 13 after cell transfer, data not shown) in contrast, hetIL15 treatment induced similar changes in Pmel-1 cells and endogenous CD8+ T cells in the spleen. Whereas the hetIL15-dependent expansion of CD8+ T cell was comparable between tumor and spleen, hetIL15 preferentially increased Pmel-1 cells infiltrating the tumor relative to Pmel-1 cells in the spleen (Fig. 2A, left). In mice pretreated with XRT, Pmel-1 cells were also significantly enriched in comparison with endogenous CD8+ T cells in the tumor, but Pmel-1 cells were equally affected by the treatment in both tumor and spleen (Fig. 2A, right). These results suggest that, upon hetIL15 treatment, the tumor accumulation of Pmel-1 cells was not just a consequence of the global IL15-driven effects on the whole CD8+ population but, rather, that continuous presence of hetIL15 resulted in a tumor enrichment of TILs and adoptively transferred cells in an antigen-specific manner.

Prompted by these results, we also investigated the frequency of Pmel-1 cells within the CD8+ T-cell population as well as the Pmel-1/CD8+ T-cell ratio in different organs. In mice that received ACT+hetIL15, approximately 10%–15% of CD8+ T cells infiltrating the tumor were Pmel-1 cells in comparison with approximately 2% in spleen (Fig. 2B), resulting in an approximately 10-fold increase in the Pmel-1/CD8+ T-cell ratio in B16 tumor in comparison with spleen (Fig. 2C). To determine whether
increased Pmel-1/CD8\(^{+}\) ratio in tumors was just the result of a global IL15-dependent mobilization of transferred cells to peripheral nonlymphoid sites, we also evaluated the effect of hetIL15 on both Pmel-1 and endogenous CD8\(^{+}\) T cells in the lung. Upon hetIL15 administration, only approximately 5% of CD8\(^{+}\) T cells infiltrating the lung were Pmel-1 cells (Fig. 2B), and the Pmel-1/CD8\(^{+}\) T cells ratio in lung was similar to the one observed in spleen (Fig. 2C).

Next, we examined whether infiltration and persistence of Pmel-1 cells in tumor areas depend on gp100 antigen. Mice were implanted with gp100-positive B16 melanoma cells and gp100-negative MC38 colon carcinoma cells on opposite flanks. The
The Pmel-1/CD8$^+$ T cells ratio was determined in both the B16 and MC38 tumors as well as the spleen. Interestingly, Pmel-1 disproportionately accumulated in B16 tumors, while the proportion of Pmel-1 cells in MC38 tumors was only marginally greater than that of the spleen and lung (Fig. 2D). hetIL15 induced similar CD8$^+$ T-cell accumulation in both tumors (not shown). Taken together, these data suggest that hetIL15 administration promotes an antigen-dependent enrichment of transferred tumor-specific T cells into tumor sites compared with lymphoid and nonlymphoid tissues.

hetIL15 administration supports effector functions of transferred tumor-infiltrating Pmel-1 cells

We next evaluated whether tumor-resident Pmel-1 were functionally competent. All Pmel-1 cells in infiltrating the tumor, regardless of the treatment, were characterized by low expression of CD62L, indicative of an effector phenotype (Fig. 3A). The frequency of GzmB$^+$ Pmel-1 cells in tumors is expressed as the percentage of total Pmel-1 cells (left) and number of GzmB$^+$ Pmel-1 cells normalized per million of cells present in the tumor suspension (right); mean values ±SEM are shown for the three groups. Data collected from day 7 and day 12 after ACT were combined. * P < 0.05; ** P < 0.01. D, The frequency of IFN$\gamma$-producing Pmel-1 cells (left) and endogenous CD8$^+$ T cells (middle) in tumor and of Pmel-1 cells in inguinal lymph nodes (right) was determined within 6 hours (tumor) or 12 hours (lymph node) ex vivo cultures in medium only or in the presence of the hgp10025–33 peptide. Analysis was performed at day 7 after ACT. ACT: n = 3; ACT+XRT: n = 5; and ACT+hetIL15: n = 5. * P < 0.05; ** P < 0.01.
IL15 has been reported to play a pivotal role in stimulating the killing activity of lymphocytes through the upregulation of the cytotoxic molecule granzyme B (GzmB; ref. 27). Intracellular staining followed by flow cytometry was used to evaluate the frequency of GzmB-expressing Pmel-1 cells infiltrating the tumor (Fig. 3B and C). Irradiation preconditioning resulted in a significant increase in the percentage of tumor-resident GzmB⁺ Pmel-1 cells in comparison with the ACT alone regimen, suggesting that irradiation generated an environment that supports the killing activity of transferred cells. Moreover, providing hetIL15 in the absence of lymphodepletion resulted in the highest proportion of tumor-infiltrating GzmB⁺ Pmel-1 cells (Fig. 3B and C). This led to tumor accumulation of GzmB⁺ Pmel-1 cells upon hetIL15 administration (Fig. 3C, right), which was significantly superior to the other treatments.

We also investigated the production of IFNγ by adoptively transferred Pmel-1 cells upon ex vivo culture after tumor excision and dissociation. In all three treatment groups, B16-tumor resident Pmel-1 cells were able to secrete IFNγ in the absence of peptide stimulation. Under these conditions, significantly more...
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IFNγ⁺ Pmel-1 cells were found in the ACT+hetIL15 group (Fig. 3D, left), suggesting that hetIL15 increases the frequency of adoptively transferred cells able to produce IFNγ in the tumor. In contrast, ex vivo cultures of the gp100-negative dissociated MC38 tumors did not result in any IFNγ release by tumor-infiltrating Pmel-1 cells without exogenous peptide stimulation (data not shown). These results are consistent with the hypothesis that Pmel-1 cells are induced to produce IFNγ (data not shown). These results are consistent with the hypothesis that Pmel-1 cells are induced to produce IFNγ in the tumor. Upon stimulation with hgp10025 peptide, all three treatment groups showed an increase in the frequency of Pmel-1 cells producing IFNγ and no statistical difference was found among the groups (Fig. 3D, left). As control, we also evaluated the proportion of endogenous CD8⁺ T cells producing IFNγ in ex vivo 6-hour cultures of dissociated tumors. In all the groups, less than 10% of endogenous CD8⁺ T cells secrete IFNγ and, as expected, this frequency did not change upon hgp10025 peptide stimulation (Fig. 3D, middle). A similar analysis was also performed on total lymphocytes isolated from lymph nodes of treated mice. In the absence of stimulation, Pmel-1 cells did not secrete IFNγ, suggesting that the tumor lymphocyte response described above in the absence of peptide stimulation is antigen specific. Stimulation of lymph node–derived lymphocytes with the hgp10025 peptide induced an IFNγ response in all groups. Mice receiving ACT+hetIL15 treatment showed a significantly higher frequency of IFNγ⁺ Pmel-1 cells in comparison with the other treatments (Fig. 3D, right). Overall, these data suggest that hetIL15 treatment sustains the cytotoxic potential and the ability to produce IFNγ of adoptively transferred cells in the absence of lymphodepletion.

hetIL15 administration sustains proliferation and cytotoxic functions of PD-1low Pmel-1 cells

The tumor microenvironment is immunosuppressive and can be characterized by high levels of negative regulators, such as PD-1/PD-L1 and Treg cells (28). In comparison with ACT alone, both the ACT+XRT and ACT+hetIL15 treatments increased the frequency of tumor-infiltrating Pmel-1 cells expressing the proliferation marker Ki-67, with hetIL15 administration resulting in a higher frequency of proliferating tumor infiltrating Pmel-1 cells (Fig. 4A, Supplementary Fig. S4). In the ACT+XRT regimen group, tumor-proliferating Pmel-1 cells were characterized by higher level of PD-1, suggesting an “exhausted” phenotype (Fig. 4B). In contrast, treatment with hetIL15 resulted in a significantly increased tumor accumulation of a population of proliferating Pmel-1 cells with a lower level of PD-1 expression (Fig. 4B). These cells were also the main producers of GzmB and represented approximately 15% of the whole Pmel-1 population resident in the tumor (versus approximately 5% in the ACT alone and ACT+XRT groups, Fig. 4C, left). hetIL15 administration also resulted in a significantly reduced tumor frequency of Pmel-1 cells with the exhaustion-like phenotype GzmB⁻ Ki-67⁺ PD-1high, supporting a role for IL15 in rescuing cells from exhaustion (Fig. 4C, right). Similar results were also obtained for tumor-infiltrating endogenous CD8⁺ T cells. The proportion of the different subsets of tumor-infiltrating Pmel-1 and endogenous CD8⁺ T cells analyzed for the expression of Ki-67, GzmB, and PD-1 is depicted in Supplementary Figs. S4 and S5, respectively. These data suggest that hetIL15 treatment promotes proliferation and cytotoxic functions of adoptively transferred T cells.

hetIL15 increases the ratio of Pmel-1 to Treg cells in tumors

We also evaluated the effects of hetIL15 administration on tumor-resident CD4⁺ Foxp3⁺ Treg cells. Analysis at day 12 after ACT showed no difference in the number of Tregs per million cells present at the tumor sites among the three groups (Fig. 4D, left), suggesting that hetIL15 does not significantly impact the frequency of Tregs in the tumor. Favorable cancer immunotherapy treatments have been previously linked to the ratio of CD8⁺ T cells to Tregs (29, 30). For this purpose, we determined the Pmel-1/Treg ratio within the tumor after ACT. Tumors of mice that received either ACT alone or ACT+XRT were characterized by a Pmel-1/Treg ratio of approximately 0.2, showing that Treg cells largely outnumber tumor-specific adoptively transferred cells. Because of its positive effect on the persistence of Pmel-1 cells, hetIL15

![Figure 5.](image_url)

hetIL15 and ACT promote tumor control in the absence of lymphodepletion. Mice were implanted with 5 × 10⁶ B16 cells SC at day −5. Mice were randomized in different treatment groups: PBS administration (black, n = 10), ACT alone (gray, n = 7), hetIL15 alone (green, n = 7), and ACT+hetIL15 (blue, n = 8) for A, and ACT alone (gray, n = 5), XRT alone (black, n = 7), ACT+XRT (red, n = 9), and ACT+hetIL15 (blue, n = 10) for B. Splenic-derived Pmel-1 cells (1 × 10⁶/mouse) were administered at day 0. Injections of hetIL15 were performed 3 times per week for a total of 8 doses (3 µg/dose/mouse). Tumor measurements were performed every 2 to 3 days. Mean ± SEM for each time point are shown. One of three similar experiments is shown. Statistical significance was calculated using repeated measures one-way ANOVA. The P values were corrected for multiple comparisons using Holm–Sidak test (*, *P < 0.05; **, *P < 0.01).
HetIL15 promotes tumor control after ACT

Given the effects of hetIL15 administration on transferred tumor-specific T cells, we tested the ability of the hetIL15+ACT treatment to control tumor growth. Monotherapy with IL15 has been reported to promote tumor control in several murine cancer models (31–33). Indeed, in B16-melanoma-bearing mice, administration of hetIL15 resulted in a significant delay in tumor growth in comparison with PBS-treated mice (Fig. 5A). We also investigated the antitumor potential of ACT alone in comparison with ACT+hetIL15. In the absence of lymphodepletion preconditioning and vaccination postinjection (which was reported as the most effective treatment protocol in this model; refs. 7, 34), ACT alone had only a marginal effect on tumor growth control that did not reach statistical significance, while addition of hetIL15 to ACT resulted in a significant improvement in tumor control in comparison with both ACT only- and IL15 only-treated animals (Fig. 5A). All animals that received either PBS or ACT alone were sacrificed within 5 weeks after tumor injection due to a large tumor mass. However, at the same time point, the survival rate in the hetIL15+ACT group was 60% (data not shown). We also compared the outcome of the hetIL15+ACT treatment with the antitumor effects achieved by XRT+ACT. Adoptive transfer of Pmel-1 cells has been previously shown to induce B16 tumor regression in lymphopenic mice, in combination with IL2 and vaccination (7). Even in the absence of vaccination, we found that irradiation prior to T-cell transfer resulted in a tumor growth rate comparable with the one obtained in lymphoreplete mice treated with hetIL15+ACT (Fig. 5B). Importantly, under our experimental condition, XRT alone also indicated a trend toward tumor control. These data suggest that administration of hetIL15 can improve treatment outcomes of ACT without the use of potentially toxic host immune depletion prior to cell infusion.

IL2+ACT regimen sustained tumor accumulation of both Pmel-1 and Treg cells

We wanted to compare the effects of IL15 to IL2 in combination with ACT in absence of lymphodepletion. Like IL15, IL2 is a member of the γ-chain family of cytokines, and it is presently the clinically available cytokine for growing lymphocytes (6, 35). To this purpose, B16-bearing mice were randomized in 3 groups receiving the following treatments: ACT alone, ACT+hetIL15, and ACT+IL2. Despite the toxicity reported in clinical studies (36), we verified that treatment with IL2 is well tolerated in mice. A trend toward an increase in WBC and lymphocyte counts comparable with the ones induced by hetIL15 was observed at day 12 after ACT, and no other hematologic changes were observed (Supplementary Table S1).

Tumors were isolated at day 10 after ACT and TILs were analyzed by flow cytometry. In agreement with the results presented in Fig. 1B, hetIL15 induced an approximately 10× increase in the accumulation of Pmel-1 cells at tumor sites, in comparison with mice that received ACT alone. Administration of IL2 in the absence of irradiation resulted in a similar accumulation of tumor-infiltrating Pmel-1 cells (Fig. 6A). Functional analysis of tumor-infiltrating Pmel-1 cells showed that both cytokines induced a similar frequency of proliferating Ki67+ Pmel-1 that was significantly higher than animals receiving ACT alone (Fig. 6B).

IL2 is the main growth factor for Treg in vivo (37). Upon IL2 administration, the frequency of Treg within the tumor increased significantly in comparison with both ACT and ACT+hetIL15 (Fig. 6C). We also determined the Pmel-1/Treg ratio within the tumor for the three treatment regimens. The positive effects of IL2 on the tumor accumulation of both Pmel-1 cells and Tregs resulted in a Pmel-1/Treg ratio of approximately 0.3, similar to the one observed in animals that received ACT alone (Fig. 6D). In contrast, hetIL15 resulted in an increased Pmel-1/Treg ratio (~1; Fig. 6D), as also concluded above (Fig. 4D).

We also investigated how these regimens compared in the control of tumor growth. Both cytokines were effective in inducing a significant delay in tumor growth, in comparison with untreated animals (Fig. 6E). In comparison with IL2, hetIL15 showed a trend toward better tumor control.

In conclusion, these data suggest that the γ-chain family of cytokines IL2 and hetIL15 can be beneficial in supporting ACT without irradiation. While both cytokines favor the accumulation of adoptively transferred tumor-specific T cells, hetIL15 has the additional advantage to prevent Treg accumulation.

Discussion

This work provides proof-of-concept that hetIL15 administration in combination with ACT can enhance antitumor treatment efficacy in the absence of lymphodepletion. This cancer immunotherapy protocol aims to replicate the advantages of lymphodepletion preconditioning of the host for successful ACT while avoiding potential adverse effects associated with lymphodepletion, including bacterial and opportunistic infections, need for transfusions, and renal insufficiency. In our mouse experiments, irradiation preconditioning was associated with both systemic lymphopenia and with a transient reduction in platelet counts. The transient decline in platelets counts upon irradiation can increase the risk of internal and external bleeding. In addition, platelets are important source of cytokines and growth factors involved in several processes, such as wound healing and blood clotting. The decline in platelet count observed upon irradiation suggested an increased risk of impairment in these processes.

The nonredundant role of IL15 in the survival, proliferation, and cytotoxic activity of lymphocytes is well established (38, 39). Because of its functions, IL15 has promising applications in cancer immunotherapy, as several experiments in mice have suggested (31–33). IL15, either as single-chain molecule produced in E. coli (40) or as mammalian-derived heterodimer (NCT01885897, NCT02452268; ref. 24) is currently being evaluated in phase I clinical trials in cancer patients. The first results of these studies suggest that IL15 is well tolerated and characterized by an acceptable toxicity profile in humans (40). In our study, we used hetIL15 that is similar to the natural and stable form of the cytokine (19). No significant toxicities were observed in mice upon effective doses of hetIL15 in combination with ACT for cancer therapy.

Determinants for effective ACT therapy resulting in tumor rejection have been previously identified (34). Successful ACT therapies depend on the transfer of a high number of tumor-specific lymphocytes that are previously capable of infiltrating the tumor, persisting and proliferating in vivo (41–43). In addition,
antitumor T cells must maintain specific effector properties, such as the production of cytokines IFNγ (43), IL2 (44), and cytotoxic molecules (43). Several lines of evidence have linked the stemness phenotype of T cells with a greater degree of ACT therapy success (45). In addition, successful ACT outcomes also require manipulation of the host. Host lymphodepletion by irradiation or chemotherapy has been incorporated into clinical protocols. However, these interventions pose serious risks to humans. It is hypothesized that, in lymphodepleted hosts, endogenous IL15 plays an important role in supporting the proliferation of transferred cells, suggesting that administration of exogenous IL15 can overcome the “cytokine sink” of a normal lymphocyte number in a lymphoreplete host. Indeed, our ACT+hetIL15 regimen resulted in increased infiltration and persistence of adoptively transferred cells in the tumor. Importantly, transferred T cells proliferated in situ and exhibited a cytotoxic phenotype, resulting in slower tumor growth. Overall, our study confirmed previous findings that lymphopenia is not a prerequisite for effective ACT (46). Several other approaches to improve ACT outcomes in the absence of irradiation and chemotherapy have been recently explored, including the use of antibodies for specific cell-type depletion (46), genetically engineered tumor-specific T cells (47), Toll-like receptor (TLR) ligands (48), and other γ-chain cytokines (46, 49). One foreseeable advantage of the regimen proposed in

**Figure 6.** IL2 coadministration with ACT results in tumor accumulation and proliferation of Pmel-1 cells similar to hetIL15, but significantly increases the frequency of tumor-associated Tregs (A). Pmel-1 cells (5 × 10⁶) were adoptively transferred comparing 3 treatment protocols: cell transfer without lymphodepletion (ACT, gray symbols), cell transfer plus IP hetIL15 administration (ACT+hetIL15, blue symbols), and cell transfer plus IP IL2 administration (9 μg/dose, green symbols). Mice were sacrificed at day 10 for tumor analysis. The frequency of tumor-infiltrating Pmel-1 cells was determined by flow cytometry for each treatment group. The number of Pmel-1 cells in each tumor was normalized per million of cells present in the tumor suspension. Bars represent mean ± SEM. B, Percentage of Pmel-1 cells in tumor expressing the proliferation marker Ki-67 for the mice in each of the three treatment groups at day 10 after ACT. Bars represent mean ± SEM. C, The frequency of tumor-infiltrating Tregs was determined by flow cytometry at day 10 after ACT for each treatment group. The number of Tregs in each tumor was normalized per million of cells present in the tumor suspension. Bars represent mean ± SEM (left). D, The Pmel-1/Treg ratio was determined in tumor for each treatment group at day 10 after ACT. Bars represent mean ± SEM. E, Mice were implanted with 5 × 10⁶ B16 cells subcutaneously at day 5. Three treatment groups were compared: no treatment (gray, n = 10), ACT+hetIL15 (blue, n = 10), and ACT+IL2 (green, n = 10). Splenic-derived Pmel-1 cells (1 × 10⁶/mouse) were administered at day 0. Intraperitoneal injections of hetIL15 and IL2 were performed 3 times per week for a total of 8 doses (3 μg/dose/mouse). Tumor measurements were performed every 2 to 3 days. Mean ± SEM for each time point is shown. *, P < 0.05; **, P < 0.01.
this study is the absence of CD4+ T-cell ablation. Several studies have underlined the important contribution of these cells in tumor control, via both exerting direct cytotoxic effector functions and providing help to CD8+ T cells (50).

A major obstacle to successful cancer immunotherapy is overcoming the immunosuppressive environment of the tumor. Two major categories of immune resistance within the tumor microenvironment have been proposed: lack of tumor-infiltrating cytotoxic T cells and immune inhibitor pathways (51, 52). In this study, we addressed the effect of hetIL15 treatment in overcoming tumor immune resistance by acting on both mechanisms.

Tumor infiltration by CD8+ T cells is a favorable prognostic marker in many malignancies. Evaluation of lymphocyte infiltration has gained increasing interest with the approval of new immunotherapy agents, including anti–CTLA-4, –PD-1, and –PD-L1 antibodies, which can modify the tumor microenvironment by blocking immune suppression. The success of these therapies presumably depends on tumor T-cell infiltration, and IL15 could be a general method to place cells into the tumor in combination with other immunotherapy interventions. Our results showed that hetIL15 administration promotes infiltration and proliferation of adoptively transferred cells specifically in the tumor, in an antigen-specific way. Under this regimen, the frequency of Pmel-1 cells within the CD8+ T cells population and the Pmel-1/CD8+ T cells ratio was higher in B16 melanoma (gp100+ tumor), in comparison with another type of tumor (MC38 colon carcinoma) in the same mouse, or in comparison with lymphoid and nonlymphoid organs (spleen and lung) lacking gp100 expression. It has previously been shown that, upon irradiation, adoptively transferred cells are characterized by a ubiquitous distribution in the body regardless of the expression of the specific antigen, while retaining their cytotoxic activity only against tumor cells (53). Our results agree with this conclusion as our measurements show that irradiation increased Pmel-1 cells in tumor and spleen to similar levels. We hypothesize that the superior result obtained by hetIL15 is due to longer availability of hetIL15 for Pmel-1 expansion and maintenance, whereas lymphodepletion results in lower and transient levels of cytokine available to the adoptively transferred cells. This highlights one advantage of the hetIL15 administration over lymphodepletion protocols, in addition to the possibility that hetIL15 administration would be a less toxic intervention compared with lymphodepletion.

Several studies suggest that an important metric to predict the success of cancer immunotherapies is the ratio of CD8+ Treg cells within the tumor (29, 30). An advantage of hetIL15 administration is the absence of Treg increase in the tumor. The IL2 cytokine, used for the maintenance of transferred cells in many experiments (7, 25) and in the clinic (6), favors the proliferation, maintenance, and function of Treg. Treg cells inhibit antitumor effector cell proliferation and function and are a barrier to tumor immunotherapy (54). In contrast, our results show that hetIL15 has minimal effects on the number of Treg in the tumor. This together with the great increase of CD8+ T cells and tumor-specific Pmel-1 cells in the tumor results in a greatly increased CD8+ Treg ratio.

Although hetIL15 administration resulted in superior persistence and effector functions of Pmel-1 cells in comparison with irradiation, the effects of the two treatments on tumor growth over time were similar. Irradiation as single treatment resulted in a slight delay of tumor growth. This result reinforces the notion that irradiation provides further benefits, in addition to supporting growth of adoptively transferred cells. It has been shown that irradiation results in direct killing of tumor cells and in decreased frequency and function of regulatory cells within the tumor, such as Tregs and MDCs that can have a negative impact on immunotherapeutic approaches (4). In addition, irradiation has been reported to activate multiple innate immune mechanisms, including the translocation of LPS across the damaged gut epithelium or alarmin release by dying cancer cells (55). It is anticipated that hetIL15 treatment has several advantages and can be further optimized using combination protocols. Protocol optimization using hetIL15 in combination with vaccination, TLR ligands, and checkpoint inhibitors warrant further investigation.

In conclusion, our study identified several benefits of hetIL15 in combination with ACT for cancer immunotherapy. hetIL15 administration improves the outcome of ACT providing a clear advantage over protocols using host lymphodepletion.

**Disclosure of Potential Conflicts of Interest**

B.A. Fox is a consultant/advisory board member for PerkinElmer, B.K. Felber, C. Bergamaschi, and G.N. Pavlakis are listed as coinventors on U.S. Government-owned patents and patent applications related to IL15 and gene expression optimization. No potential conflicts of interest were disclosed by the other authors.

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Other (contributed to the multispectral immunohistochemical evaluation of the tumors): B.A. Fox

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