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Heterodimeric IL-15 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 IL-15 administration enhances ACT in the absence of lymphodepletion. We previously showed that bioactive IL-15 *in vivo* comprises a stable complex of the IL-15 chain with the IL-15 receptor alpha chain (IL-15R α), termed heterodimeric IL-15 (hetIL-15).

Experimental Design—We evaluated the effects of the combination regimen ACT+hetIL-15 in the absence of lymphodepletion by transferring melanoma-specific Pmel-1 T cells into B16 melanoma-bearing mice.

Results—hetIL-15 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 to mice treated with hetIL-15. Importantly, we found that hetIL-15 treatment led to the preferential enrichment of Pmel-1 cells in B16 tumor sites in an antigen-dependent manner. Upon hetIL-15 administration, tumor-infiltrating Pmel-1 cells showed a “non-exhausted” effector phenotype,

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Disclosure of Potential Conflicts of Interest

B.K. Felber, C. Bergamaschi, and G.N. Pavlakis are inventors on US Government-owned patents and patent applications related to vaccines, IL-15 and gene expression optimization. No potential conflicts of interest were disclosed by the other authors.

Author's Contributions

Performed experiments and analyzed data: S. Ng, C. Bergamaschi, S.M. Jensen

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characterized by increased IFN- γ secretion, proliferation and cytotoxic potential and low level of PD-1. hetIL-15 treatment also resulted in an improved Pmel-1 to Treg ratio in the tumor.

Conclusions—hetIL-15 administration improves the outcome of ACT in lymphoreplete hosts, a finding with significant implications for improving cell-based cancer immunotherapy strategies.

Keywords

Heterodimeric IL-15; adoptive cell transfer; lymphodepletion; cancer immunotherapy; tumor-specific T cells; tumor-infiltrating lymphocytes

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 IL-7 and IL-15 were measured in humans undergoing lymphodepleting regimens (6). Host pre-conditioning 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 IL-15 as a key factor for the homeostatic proliferation of CD8⁺ T cells (12,13) and evaluated its role in supporting ACT therapies. Klebanoff et al. (14) demonstrated that IL-15 is superior to IL-2 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. IL-15 is also essential for the *in vivo* persistence of the transferred T cells (14). Similar results were obtained in a macaque model where autologous CMV-specific CD8⁺ T cell clones generated in the presence of IL-15 acquired a central-memory phenotype rather than terminally differentiated effector phenotype and displayed superior persistence (15). Additional findings also demonstrated a role of IL-15 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 IL-15 is produced and functions as a heterodimeric complex of two polypeptide chains, IL-15 and IL-15 Receptor alpha (IL-15R α) (19). The two polypeptide chains are co-produced 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 IL-15R α is responsible for IL-15 retention on the cell surface, where it is trans-presented to adjacent responding cells expressing the IL-2/IL-15 receptor $\beta\gamma$ (22). In addition, after a specific proteolytic cleavage of the IL-15R α , a soluble heterodimeric form of IL-15 is released, circulates in the blood and is biologically active (19,20,23). These data suggest that IL-15R α is not a receptor for the IL-15 polypeptide chain, but the other half of heterodimeric IL-15 (hetIL-15) (24).

In this report, we exploit the potential of hetIL-15 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, hetIL-15 promotes targeted tumor infiltration, proliferation and effector functions of adoptively transferred tumor-specific T cells, resulting in inhibition of tumor growth.

Material 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 (Frederick, MD). IL-15 KO mice were purchased from Taconic. The study was approved by the National Cancer Institute-Frederick Animal Care and Use Committee and were conducted in accordance with the ACUC 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 (FBS, ThermoFisher, Waltham, NY) and 1x penicillin/streptomycin. Seven-week old wild type C57BL/6 animals were injected with 4×10^5 tumor cells subcutaneously (SC) in the flank. MC38 colon carcinoma cells were maintained in DMEM supplemented with 10% heat-inactivated FBS (FBS, ThermoFisher, Waltham, NY), 1x penicillin/streptomycin, 1x essential amino acids and 1x HEPES. In some experiments, C57BL/6 animals were injected with 4×10^5 B16 melanoma cells SC into one flank and with 3×10^5 MC38 colon carcinoma cells SC into the other flank. Tumor area (length $\times$ 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+XRT or ACT+hetIL-15. In some experiments, mice received ACT+IL-2. Spleens from pmel-1 TCR/Thy1.1 transgenic mice were harvested and splenocytes were used as source of melanoma antigen (hgp100_{25–33})-specific T cells (Pmel-1 T cells) for ACT. Splenocytes were activated *in vitro* using plates coated with anti-CD3 antibody (145-2C11, BD Bioscience, Franklin Lakes, NJ) and soluble no azide/low

endotoxin (NA/LE) anti-CD28 antibody at 1 µg/ml (37.51, BD Bioscience). Human IL-2 (12.5 ng/ml, Peprotech, Rocky Hill, NJ) was provided on day 2 and cells were harvested on day 5. $1-5 \times 10^6$ (in 100 µl PBS) of *in vitro* activated Pmel-1 T cells were injected intravenously (IV) 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/min, 137-cesium chloride irradiator) one day before ACT. For hetIL-15 treatment, mice received intraperitoneal injection of 3 µg (molar mass of IL-15) of hetIL-15 (Admune Therapeutic LLC, Danvers, MA) (24) 3 times/week for 8 total injections. For the IL-2 treatment, mice received intraperitoneal injection of 3 or 9 µg of human IL-2 (Teceleukin, 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, St. Louis, MO) and DNase I (30 unit/ml, Roche Diagnostic GmbH, Mannheim, Germany) at 37 °C for 1 hour. Tumor cells suspensions were layered on 3 ml histopaque 1116 (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 (ThermoFisher) for 30 min 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 (KVPRNQDWL, 10 µM, NeoScientific, Woburn, MA) at 37 °C for 6 hours (tumor) or 12 hours (lymph nodes). Anti-CD107a antibody (1D4B, 1:50) and GolgiStop (BD Bioscience) were added during culture. Cells were then harvested and stained for surface and intracellular markers.

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 Bioscience; eBioscience, Inc., San Diego, CA; Biolegend, San Diego, CA). 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 (SOLA15), Foxp3 (FJK-16s) and granzyme B (GB11). For intracellular cytokine staining, cells were fixed with BD fixation and permeabilization buffer (BD Bioscience). Samples were stained with the anti-interferon-γ (IFN-γ) antibody (XMG1.2) and analyzed using LSRII and Fortessa flow cytometers (BD Bioscience). Data analysis was performed using FlowJo software (Tree Star, Inc, Ashland, OR).

Immunohistochemistry

Immunohistochemistry of murine T cell populations within the tumor was performed as previously reported (26). Tumors were harvested and fixed for 24 hours at room temperature (RT) in Zinc-Fixation Buffer (0.5g calcium acetate, 5g zinc acetate and 5g zinc chloride in one liter 0.1M 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. 4.5 μ m thick sections were cut on microtome and floated onto plus-slides (Cardinal ColorFrost) in a tissue floatation bath set at 40°C. Slides were allowed to dry at RT overnight.

Slides were placed onto staining rack in the Leica autostainer, and deparaffinization protocol was run (xylene – 4 min; 100% ethanol – 2 min; 95% ethanol – 1 min; 70% ethanol – 1 min; water). Slides were treated with PeroxAbolish (Biocare Medical) for 20 min to reduce endogenous peroxidase activity. Slides were rinsed with H₂O and TBS-T and blocked with goat serum (Vector Labs) for 20 min. 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 min on an orbital shaker at RT. After washes in TBS-T, anti-rabbit HRP secondary antibody (Life Technologies, 87-9623) was added for 10 min RT, and subsequently washed with TBS-T. Slides were incubated with tyramide-fluorophore reagent (PerkinElmer, NEL791001KT; Life Technologies, T20950) at 1:100 dilution in Amplification plus buffer (PerkinElmer, NEL791001KT) for 10 min at RT and washed in TBS-T and H₂O. Slides were treated with PeroxAbolish for 20 min to eliminate peroxidase activity. The same cycle was repeated for the rat anti-CD8 (53–6.7, BD Biosciences, 550281, 1:100) antibody, followed by the secondary anti-rat HRP (Vector Labs, MP-7444-15) antibody. Slides were washed with TBS-T and H₂O followed by antibody stripping using the antibody-stripping buffer (0.1M glycine (Sigma, G2879), pH10 using NaOH (Fisher Chemical, SS267), 0.5% Tween) for 10 min at RT. 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 Labs, MP-7444-15) secondary antibody, and for the rat anti-CD90.1 PE (HIS51, 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, 1mg/mL stock, 1:500 in PBS) was added to slides for 5 min at RT. Slides were rinsed with TBS-T and H₂O and cover-slipped with VectaShield Hard Mount (Vector). Slides were imaged at both 4x, and 20x using Vectra imaging software (PerkinElmer), and the number of cells were enumerated from fifteen 20X fields using inForm analysis software (PerkinElmer).

Statistical Methods

Differences among groups were evaluated by One-Way analysis of variance ANOVA or unpaired student's 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., La Jolla, CA) was used for analysis.

Results

Adoptively transferred Pmel-1 cells infiltrate and persist in tumor sites upon hetIL-15 administration

Previous studies have shown that the increased availability of the homeostatic cytokine IL-15 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 IL-15 KO mice upon irradiation. We confirmed that lack of IL-15 decreased the proliferation of adoptively transferred cells, suggesting that IL-15 is a non-redundant factor participating in this process (Supplementary Figure 1). Given IL-15's role, we tested the hypothesis that hetIL-15 administration could overcome endogenous cellular "sinks", normally competing for access to IL-15 (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-*pmel-1*-Thy1.1 transgenic mice were used as a source of Pmel-1 cells, whose T-cell receptor specifically recognizes the gp100₂₅₋₃₃ 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 (Figure 1A): (i) cell transfer without lymphodepletion (ACT alone), (ii) cell transfer into irradiated mice (ACT+XRT) and (iii) cell transfer plus exogenous hetIL-15 administration (ACT+hetIL-15) in non-irradiated 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 (TILs) were analyzed by flow cytometry (Supplementary Figure 2). Tumor-infiltrating Pmel-1 cells (green gate) were distinguished from endogenous CD8⁺ T cells (purple gate) by the expression of the congenic marker CD90.1. In the ACT alone group, ~300 Pmel-1 cells per million cells were present at the tumor site at day 5 after cell transfer (Figure 1B). At the same time point, a trend towards a modest increase (~2–3 fold) in the proportion of Pmel-1 cells in the tumor was detected both in mice pre-treated with XRT and in mice receiving hetIL-15, although this difference did not achieve statistical significance (one-way ANOVA) (Figure 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 ~60% between day 5 and day 12 after cell transfer (Figure 1B&C). In contrast, in tumor-bearing mice that received hetIL-15, Pmel-1 cells were still present at relatively high numbers (~2000 Pmel-1 cells per million cells in the tumor) at day 12 after cell transfer (Figures 1B&C). Therefore, administration of hetIL-15 in the absence of lymphodepletion favors the infiltration and persistence of antigen-specific transferred cells into the tumor.

We further investigated the effects of hetIL-15 treatment on tumor infiltration by endogenous CD8⁺ T cells. As with adoptively transferred Pmel-1 cells, hetIL-15 administration also significantly increased the frequency of endogenous CD8⁺ T cells in the tumor in comparison to both ACT and ACT+XRT groups at day 12 after ACT (Figure 1D). In contrast, there was no difference in the number of tumor-resident endogenous CD8⁺ T cells in the ACT and ACT+XRT (Figure 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 immunohistochemistry. Staining of tumor sections at day 13 after cell transfer was performed using antibodies against CD3, CD4, CD8 and CD90.1 (Figure 1E&F). These data confirmed that, in comparison to the other groups, treatment with hetIL-15 resulted in an increased accumulation of both tumor specific Pmel-1 cells (green) and endogenous CD8⁺ T cells (magenta) that were spread throughout the tumor area. Quantification of Pmel-1 cells and endogenous CD8⁺ T cells/mm² by fluorescence immunohistochemistry showed a significant increase in the ACT+hetIL-15 group (Figure 1G). Taken together, these data suggest that IL-15 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 hematological parameters of treated animals were compared to assess the toxicity of the three protocols. No significant changes in body weight were observed overtime in any of the treatment groups (data not shown). Hematological parameters were determined at day 5, 7 and 12 after ACT (Supplementary Table 1). As expected, irradiation resulted in a severe but transient reduction in WBC and lymphocyte counts at day 5 and 7 after ACT. WBC and lymphocytes returned to normal levels by day 12. In contrast, hetIL-15 administration resulted in a significant increase in WBC and lymphocyte counts, that persisted at day 12 after ACT. Additionally, we found that the platelet counts significantly decreased at day 7 in the XRT-treated animals, in comparison to mice that received ACT alone or ACT+hetIL-15. The platelet count returned to normal levels by day 12 after ACT. Overall, these data confirmed that irradiation pre-conditioning results in reduction in blood cell counts, that may be a potential health risk for the treated host.

hetIL-15 administration promotes preferential enrichment of Pmel-1 cells in tumors in an antigen-dependent manner

We investigated whether hetIL-15 treatment differentially affects tumor-specific Pmel-1 cells and endogenous CD8⁺ T cells in the B16 tumor compared to other tissues lacking gp100, i.e., spleen, lung and a gp100-negative tumor (MC38 colon carcinoma). Similar to the findings in the tumor, hetIL-15 administration in the absence of lymphodepletion resulted in a significant increase in the total count of both Pmel-1 cells (Supplementary Figure 3A) and endogenous CD8⁺ T cells (Supplementary Figure 3B) in spleen, compared to both ACT and ACT+XRT. Interestingly, in comparison to ACT alone, hetIL-15 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 (Figure 2A left panel, day 12 after cell transfer) and immunohistochemistry (day 13 after cell transfer, data not shown). In contrast, hetIL-15 treatment induced similar changes in Pmel-1 cells and endogenous CD8⁺ T cells in the spleen. Whereas the hetIL-15-dependent expansion of CD8⁺ T cell was comparable between tumor and spleen, hetIL-15 preferentially increased Pmel-1 cells infiltrating the tumor relative to Pmel-1 cells in the spleen (Figure 2A, left panel). In mice pre-treated with XRT, Pmel-1 cells were also significantly enriched in comparison to endogenous CD8⁺ T cells in the tumor, but Pmel-1 cells were equally affected by the treatment in both tumor and spleen (Figure 2A, right panel). These results suggest that, upon hetIL-15 treatment, the

tumor accumulation of Pmel-1 cells was not just a consequence of the global IL-15-driven effects on the whole CD8⁺ population but, rather, that continuous presence of hetIL-15 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+hetIL-15, ~10–15% of CD8⁺ T cells infiltrating the tumor were Pmel-1 cells in comparison to ~2% in spleen (Figure 2B), resulting in a ~10-fold increase in the Pmel-1/CD8⁺ T cells ratio in B16 tumor in comparison to spleen (Figure 2C). To determine whether increased Pmel-1/CD8⁺ ratio in tumors was just the result of a global IL-15-dependent mobilization of transferred cells to peripheral non-lymphoid sites, we also evaluated the effect of hetIL-15 on both Pmel-1 and endogenous CD8⁺ T cells in the lung. Upon hetIL-15 administration, only ~5% of CD8⁺ T cells infiltrating the lung were Pmel-1 cells (Figure 2B), and the Pmel-1/CD8⁺ T cells ratio in lung was similar to the one observed in spleen (Figure 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 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 (Figure 2D). hetIL-15 induced similar CD8⁺ T cell accumulation in both tumors (not shown). Taken together, these data suggest that hetIL-15 administration promotes an antigen-dependent enrichment of transferred tumor-specific T cells into tumor sites compared to lymphoid and non-lymphoid tissues.

hetIL-15 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 infiltrating the tumor, regardless of the treatment, were characterized by low expression of CD62L, indicative of an effector phenotype (Figure 3A). Approximately 50% of the tumor-resident Pmel-1 cells were CD62L^{low}KLRG1^{high} and represented the most differentiated effector subpopulation, while the other 50% Pmel-1 cells had the T-effector memory-like phenotype CD62L^{low}KLRG1^{low}.

IL-15 has been reported to play a pivotal role in stimulating the killing activity of lymphocytes through the up-regulation of the cytotoxic molecule granzyme B (GzmB) (27). Intracellular staining followed by flow cytometry was used to evaluate the frequency of GzmB-expressing Pmel-1 cells infiltrating the tumor (Figure 3B&C). Irradiation pre-conditioning resulted in a significant increase in the percentage of tumor-resident GzmB⁺ Pmel-1 cells in comparison to the ACT alone regimen, suggesting that irradiation generated an environment that supports the killing activity of transferred cells. Moreover, providing hetIL-15 in the absence of lymphodepletion resulted in the highest proportion of tumor-infiltrating GzmB⁺ Pmel-1 cells (Figure 3B&C). This led to tumor accumulation of GzmB⁺

Pmel-1 cells upon hetIL-15 administration (Figure 3C, right panel), which was significantly superior to the other treatments.

We also investigated the production of interferon- γ (IFN- γ) 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 IFN- γ in the absence of peptide stimulation. Under these conditions, significantly more IFN- γ^+ Pmel-1 cells were found in the ACT+hetIL-15 group (Figure 3D, left panel), suggesting that hetIL-15 increases the frequency of adoptively transferred cells able to produce IFN- γ in the tumor. In contrast, *ex vivo* cultures of the gp100-negative dissociated MC38 tumors did not result in any IFN- γ 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 IFN- γ by tumor cells expressing the gp100 antigen present in the cell suspension. Upon stimulation with hgp100₂₅₋₃₃ peptide, all three treatment groups showed an increase in the frequency of Pmel-1 cells producing IFN- γ and no statistical difference was found among the groups (Figure 3D, left panel). As control, we also evaluated the proportion of endogenous CD8⁺ T cells producing IFN- γ in *ex vivo* 6-hour cultures of dissociated tumors. In all the groups, less than 10% of endogenous CD8⁺ T cells secrete IFN- γ , and, as expected, this frequency did not change upon hgp100₂₅₋₃₃ peptide stimulation (Figure 3D, middle panel). 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 IFN- γ , 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 hgp100₂₅₋₃₃ peptide induced an IFN- γ response in all groups. Mice receiving ACT +hetIL-15 treatment showed a significantly higher frequency of IFN- γ^+ Pmel-1 cells in comparison to the other treatments (Figure 3D, right panel). Overall, these data suggest that hetIL-15 treatment sustains the cytotoxic potential and the ability to produce IFN- γ of adoptively transferred cells in the absence of lymphodepletion.

hetIL-15 administration sustains proliferation and cytotoxic functions of PD-1^{low} 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 to ACT alone, both the ACT+XRT and ACT+hetIL-15 treatments increased the frequency of tumor-infiltrating Pmel-1 cells expressing the proliferation marker Ki-67, with hetIL-15 administration resulting in a higher frequency of proliferating tumor infiltrating Pmel-1 cells (Figure 4A and Supplementary Figure 4). In the ACT+XRT regimen group, tumor proliferating Pmel-1 cells were characterized by higher level of PD-1, suggesting an “exhausted” phenotype (Figure 4B). In contrast, treatment with hetIL-15 resulted in a significant increased tumor accumulation of a population of proliferating Pmel-1 cells with a lower level of PD-1 expression (Figure 4B). These cells were also the main producers of GzmB and represented ~15% of the whole Pmel-1 population resident in the tumor (versus ~5% in the ACT alone and ACT+XRT groups, Figure 4C, left panel). hetIL-15 administration also resulted in a significantly reduced tumor frequency of Pmel-1 cells with the exhaustion-like phenotype GzmB⁻Ki-67⁻PD-1^{high}, supporting a role for IL-15 in

rescuing cells from exhaustion (Figure 4C, right panel). 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 Figures 4 and 5, respectively. These data suggest that hetIL-15 treatment promotes proliferation and cytotoxic functions of adoptively transferred T cells.

hetIL-15 increases the ratio of Pmel-1 cells to Treg in tumors

We also evaluated the effects of hetIL-15 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 (Figure 4D, left panel), suggesting that hetIL-15 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 ~0.2, showing that Treg cells largely outnumber tumor-specific adoptively transferred cells. Due to its positive effect on the persistence of Pmel-1 cells, hetIL-15 administration resulted in a ~10x increase in the Pmel-1/Treg ratio within the tumor, in comparison to both ACT and ACT+XRT (Figure 4D, right panel). Overall, these data suggest that hetIL-15 does not significantly affect the frequency of tumor-resident Treg and promotes an increased Pmel-1/Treg ratio within the tumor.

hetIL-15 promotes tumor control after ACT

Given the effects of hetIL-15 administration on transferred tumor-specific T cells, we tested the ability of the hetIL-15+ACT treatment to control tumor growth. Monotherapy with IL-15 has been reported to promote tumor control in several murine cancer models (31–33). Indeed, in B16-melanoma bearing mice, administration of hetIL-15 resulted in a significant delay in tumor growth in comparison to PBS-treated mice (Figure 5A). We also investigated the anti-tumor potential of ACT alone in comparison to ACT+hetIL-15. In the absence of lymphodepletion pre-conditioning and vaccination post-injection (which was reported as the most effective treatment protocol in this model (7,34)), ACT alone had only a marginal effect on tumor growth control that did not reach statistical significance, while addition of hetIL-15 to ACT resulted in a significant improvement in tumor control in comparison to both ACT only- and IL-15 only-treated animals (Figure 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 hetIL-15+ACT group was 60% (data not shown). We also compared the outcome of the hetIL-15+ACT treatment with the anti-tumor 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 IL-2 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 to the one obtained in lymphoreplete mice treated with hetIL-15+ACT (Figure 5B). Importantly, under our experimental condition, XRT alone also indicated a trend towards tumor control. These data suggest that administration of hetIL-15 can improve treatment outcomes of ACT without the use of potentially toxic host immune depletion prior to cell infusion.

IL-2+ACT regimen sustained tumor accumulation of both Pmel-1 and Treg cells

We wanted to compare the effects of IL-15 to IL-2 in combination with ACT in absence of lymphodepletion. Like IL-15, IL-2 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+hetIL-15 and ACT+IL-2. Despite the toxicity reported in clinical studies (36), we verified that treatment with IL-2 is well tolerated in mice. A trend toward an increase in WBC and lymphocyte counts comparable to the ones induced by hetIL-15 was observed at day 12 after ACT, and no other hematologic changes were observed (Supplementary Table 1).

Tumors were isolated at day 10 after ACT and TILs were analyzed by flow cytometry. In agreement with the results presented in Figure 1B, hetIL-15 induced a ~10x increase in the accumulation of Pmel-1 cells at tumor sites, in comparison to mice that received ACT alone. Administration of IL-2 in the absence of irradiation resulted in a similar accumulation of tumor-infiltrating Pmel-1 cells (Figure 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 (Figure 6B).

IL-2 is the main growth factor for Treg *in vivo* (37). Upon IL-2 administration, the frequency of Treg within the tumor increased significantly in comparison to both ACT and ACT+hetIL-15 (Figure 6C). We also determined the Pmel-1/Treg ratio within the tumor for the three treatment regimens. The positive effects of IL-2 on the tumor accumulation of both Pmel-1 cells and Tregs resulted in a Pmel-1/Tregs ratio ~ 0.3, similar to the one observed in animals that received ACT alone (Figure 6D). In contrast, hetIL-15 resulted in an increased Pmel-1/Treg ratio (~1) (Figure 6D), as also concluded above (Figure 5D).

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 to untreated animals (Figure 6E). In comparison to IL-2, hetIL-15 showed a trend towards better tumor control.

In conclusion, these data suggest that the γ -chain family of cytokines IL-2 and hetIL-15 can be beneficial in supporting ACT without irradiation. While both cytokines favor the accumulation of adoptively transferred tumor-specific T cells, hetIL-15 has the additional advantage to prevent Treg accumulation.

Discussion

This work provides proof-of-concept that hetIL-15 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 pre-conditioning 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. Additionally, 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 non-redundant role of IL-15 in the survival, proliferation and cytotoxic activity of lymphocytes is well established (38,39). Due to its functions, IL-15 has promising applications in cancer immunotherapy, as several experiments in mice have suggested (31–33). IL-15, either as single-chain molecule produced in *E. coli* (40) or as mammalian-derived heterodimer (NCT01885897, NCT02452268, (24)) is currently being evaluated in Phase I clinical trials in cancer patients. The first results of these studies suggest that IL-15 is well tolerated and characterized by an acceptable toxicity profile in humans (40). In our study, we used hetIL-15 that is similar to the natural and stable form of the cytokine (19). No significant toxicities were observed in mice upon effective doses of hetIL-15 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 capable of infiltrating the tumor, persisting and proliferating *in vivo* (41–43). Additionally, anti-tumor T cells must maintain specific effector properties, such as the production of cytokines IFN- γ (43), IL-2 (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 IL-15 plays an important role in supporting the proliferation of transferred cells, suggesting that administration of exogenous IL-15 can overcome the “cytokine sink” of a normal lymphocyte number in a lymphoreplete host. Indeed, our ACT+hetIL-15 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 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 hetIL-15 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-CTLA4, -PD1, 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 IL-15 could be a general method to place cells into the tumor in combination with other immunotherapy interventions. Our results showed that hetIL-15 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 to another type of tumor (MC38 colon carcinoma) in the same mouse, or in comparison to lymphoid and non-lymphoid 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 since our measurements show that irradiation increased Pmel-1 cells in tumor and spleen to similar levels. We hypothesize that the superior result obtained by hetIL-15 is due to longer availability of hetIL-15 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 hetIL-15 administration over lymphodepletion protocols, in addition to the possibility that hetIL-15 administration would be a less toxic intervention compared to 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 hetIL-15 administration is the absence of Treg increase in the tumor. The IL-2 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 anti-tumor effector cell proliferation and function and are a barrier to tumor immunotherapy (54). In contrast, our results show that hetIL-15 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 hetIL-15 administration resulted in superior persistence and effector functions of Pmel-1 cells in comparison to irradiation, the effects of the two treatments on tumor growth overtime 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 Treg and MSDC that can have a negative impact on immunotherapeutic approaches (4). Additionally, 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 hetIL-15

treatment has several advantages and can be further optimized using combination protocols. Protocol optimization using hetIL-15 in combination with vaccination, TLR ligands and checkpoint inhibitors warrant further investigation.

In conclusion, our study identified several benefits of hetIL-15 in combination with ACT for cancer immunotherapy. hetIL-15 administration improves the outcome of ACT providing a clear advantage over protocols using host lymphodepletion.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational Relevance

Heterodimeric IL-15 is a lymphocyte growth and activation factor presently in clinical trials for immunotherapy of metastatic cancers. This study shows that heterodimeric IL-15 (hetIL-15) is able to replace the need for lymphodepletion prior to Adoptive Cell Transfer (ACT) for cancer therapy. hetIL-15 induces lymphocyte entry into tumor sites and activation of intratumoral CD8⁺ T cells. In contrast to other protocols, heterodimeric IL-15 induces an enrichment of antigen-specific lymphocytes in the tumor over time and greatly increases CD8⁺/Treg cell ratio. Application of heterodimeric IL-15 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, IL-15 could be a general method to place T cells into tumors, increasing the success rate of other immunotherapy interventions.

Figure 1.

hetIL-15 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. 5×10^6 Pmel-1 cells were adoptively transferred comparing 3 treatment protocols: (i) cell transfer without lymphodepletion (ACT, grey symbols), (ii) cell transfer in irradiated host (ACT+XRT, red symbols) and (iii) cell transfer plus IP hetIL-15 administration (ACT+hetIL-15, blue symbols). Mice were sacrificed at day 5, 7 and 12 for tumor and spleen analysis. (B) The frequency of tumor-infiltrating Pmel-1 cells was

determined by flow cytometry at the indicated time points after ACT 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 $\pm$ SEM. Data of two independent experiments were combined. * $p < 0.05$, ** $p < 0.01$. (C) The proportion of Pmel-1 cells present in the tumors overtime was calculated as percentage of the mean value at day 5 after ACT for each treatment group. Mean values $\pm$ SEM are shown. For each treatment group, r^2 and significant deviation from zero were calculated by linear regression. Comparisons of the different treatment groups were performed using Two-Way ANOVA. **, $p < 0.01$; ns, non-significant. (D) The frequency of tumor-infiltrating endogenous CD8⁺ T cells was determined by flow cytometry at the indicated time points after ACT for each treatment group. The number of endogenous CD8⁺ T cells in each tumor was normalized per million of cells present in the tumor suspension. Individual animal values and mean $\pm$ SEM are shown from data of two independent combined experiments. ** $p < 0.01$. (E) Tumor sections from mice in the ACT (top panel), ACT+XRT (middle panel) and ACT+hetIL-15 (bottom panel) treatment groups. TILs were identified by immunohistochemistry staining using antibodies specific for CD3⁺ (white), CD4⁺ (orange), CD8⁺ (magenta) and CD90.1⁺ (green, staining Pmel-1 cells). A representative image (20x magnification) from one mouse/group is shown. (F) Higher magnification of the selected region in ACT+hetIL-15 group (white square) shows cells identified as T cells (CD3⁺, labeled ^), CD4⁺ T cells (CD3⁺ CD4⁺, labeled +), endogenous CD8⁺ T cells (CD3⁺CD8⁺, labeled #), and Pmel-1 T cells (CD3⁺CD8⁺ CD90.1⁺, labeled *). (G) The mean values of the Pmel-1 cell (top panel) and endogenous CD8⁺ T cell (bottom panel) counts from 9–15 tumor images are shown. Five to six tumors in each treatment group were analyzed. InForm software was used to enumerate each cell type. *, $p < 0.05$; **, $p < 0.01$.

Figure 2.

Tumor-resident Pmel-1 cells are preferentially targeted by hetIL-15. (A) Fold difference in Pmel-1 and endogenous CD8⁺ T cell counts in tumors and spleens for mice in the ACT +hetIL-15 (left panel, blue) and in the ACT+XRT (right panel, red) groups normalized to ACT alone. Bars represent mean fold change ($\pm$ SEM) compared to the mean level of the animals in the ACT group (set as 1). Data were combined from three independent experiments (day 12 after ACT). **, $p < 0.01$. (B) The percentage of Pmel-1 cells (defined by the expression of CD90.1) within the CD8⁺ T cell population was assessed by flow cytometry in tumors (left panels), spleens (middle panels) and lungs (right panel) at day 5

and 12. Representative mice from the ACT+hetIL-15 group are shown. (C) The ratio of Pmel-1 cells to endogenous CD8⁺ T cells in tumor, spleen, and lung of mice receiving ACT +hetIL-15 treatment was determined. Values from individual animals (combining data from day 5 and day 12 after ACT) and mean $\pm$ SEM are shown. Data were combined from two independent experiments. *, $p < 0.05$; **, $p < 0.01$. (D) Mice implanted with B16 melanoma cells and MC38 colon carcinoma cells on opposite flanks underwent ACT+hetIL-15 treatment. Fold increase in Pmel-1/CD8⁺ T cell ratio was calculated for B16 tumor and MC38 tumor in comparison to spleen (set as 1) for each mouse. Analysis was performed at day 9 after ACT. **, $p < 0.01$.

Figure 3. hetIL-15 increases cytotoxic potential and IFN- γ production of adoptively transferred Pmel-1 cells in the tumor. (A) Tumor-resident Pmel-1 cells were analyzed for the expression of CD62L and KLRG1 markers. A representative animal for each treatment group is shown. (B) The frequency of GzmB⁺ Pmel-1 cells in the tumor (% of total Pmel-1 cells) was determined by intracellular staining followed by flow cytometry. A representative animal for each treatment group is shown. (C) The frequency of GzmB⁺ Pmel-1 cell in tumors is expressed as the percentage of total Pmel-1 cells (left panel) and number of GzmB⁺ Pmel-1

cells normalized per million of cells present in the tumor suspension (right panel); mean values $\pm$ 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- γ producing Pmel-1 cells (left) and endogenous CD8⁺ T cells (middle panel) in tumor and of Pmel-1 cells in inguinal lymph nodes (right) was determined upon 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+hetIL-15: n=5. *, $p < 0.05$; **, $p < 0.01$.

Figure 4. hetIL-15 treatment sustains tumor-resident PD-1^{low}GzmB⁺Ki-67⁺ Pmel-1 cells and increases tumor Pmel-1/Treg ratio. (A) 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 12 after ACT. Bars represent mean $\pm$ SEM. Data from two independent experiments were combined. **, $p < 0.01$. (B) Pmel-1 cells infiltrating the tumor were analyzed for the expression of PD-1, Ki-67, and GzmB by flow cytometry. The GzmB⁺ Pmel-1 cells (red dots) were overlaid on the total Pmel-1 cell population (black contour). Representative animals from the ACT (left panel), ACT+XRT (middle panel) and ACT+hetIL-15 (right panel) treatment groups at day 12 after ACT are shown. (C) The percentage of proliferating and cytotoxic Pmel-1 cells characterized by low expression of PD-1 (PD-1^{low}GzmB⁺Ki-67⁺) was determined in tumors at day 12 after ACT (left panel). The percentage of Pmel-1 cells with a

phenotype consistent with exhaustion (PD-1^{high}GzmB⁻Ki-67⁻) was also determined in the tumor at day 12 after ACT (right panel). The values from individual animal and mean $\pm$ SEM are shown. **, $p < 0.01$. (D). The frequency of tumor-infiltrating Treg cells was determined by flow cytometry at day 12 after ACT for each treatment group. The number of Treg cells in each tumor was normalized per million of cells present in the tumor suspension. Bars represent mean $\pm$ SEM (left panel). The Pmel-1/Treg ratio was determined in tumor at day 12 after ACT for each treatment group. Bars represent mean $\pm$ SEM. **, $p < 0.01$ (right panel).

Figure 5.

hetIL-15 and ACT promote tumor control in the absence of lymphodepletion. Mice were implanted with 5×10^5 B16 cells SC at day -5. Mice were randomized in different treatment groups: PBS administration (black, $n=10$), ACT alone (grey, $n=7$), hetIL-15 alone (green, $n=7$) and ACT+hetIL-15 (blue, $n=8$) for (A), and ACT alone (grey, $n=5$), XRT alone (black, $n=7$), ACT+XRT (red, $n=9$) and ACT+hetIL-15 (blue, $n=10$) for (B). Splenic derived Pmel-1 cells (1×10^6 /mouse) were administered at day 0. Injections of hetIL-15 were performed 3 times per week for a total of 8 doses ($3 \mu\text{g}/\text{dose}/\text{mouse}$). Tumor measurements were performed every 2 to 3 days. Mean $\pm$ 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 (* $p < 0.05$; ** $p < 0.01$).

Figure 6.

IL-2 co-administration with ACT results in tumor accumulation and proliferation of Pmel-1 cells similar to hetIL-15, but significantly increases the frequency of tumor-associated Tregs (A). 5×10^6 Pmel-1 cells were adoptively transferred comparing 3 treatment protocols: cell transfer without lymphodepletion (ACT, grey symbols), cell transfer plus IP hetIL-15 administration (ACT+hetIL-15, blue symbols), and cell transfer plus IP IL-2 administration (9 μ g/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 $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$. (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 $\pm$ SEM. ** $p < 0.01$. (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 $\pm$ SEM (left panel). * $p < 0.05$. (D) The Pmel-1/Treg ratio was determined in tumor for each treatment group at day 10 after ACT. Bars represent mean $\pm$ SEM. ** $p < 0.01$. (E) Mice were implanted with 5×10^5 B16 cells SC at day -5. Three treatment groups were compared: No treatment (grey, n=10), ACT+hetIL-15 (blue, n=10) and ACT+IL-2 (green, n=10). Splenic derived Pmel-1 cells (1×10^6 /mouse) were administered at day 0. IP injections of hetIL-15 and IL-2 were performed 3 times per week for a total of 8 doses (3 μ g/dose/mouse). Tumor measurements were performed every 2 to 3 days. Mean $\pm$ SEM for each time points are shown.