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Tumor-specific CD4+ T cells maintain effector and memory tumor-specific CD8+ T cells

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Summary

Immunotherapies that augment anti-tumor T cells have had recent success for treating patients with cancer. Here we examined whether tumor-specific $CD4^+$ T cells enhance $CD8^+$ T-cell adoptive immunotherapy in a lymphopenic environment. Our model employed physiological doses of TRP-1-$CD4^+$ T cells and pmel-$CD8^+$ T cells that when transferred individually were subtherapeutic; however, when transferred together provided significant ($p \leq 0.001$) therapeutic efficacy. Therapeutic efficacy correlated with increased numbers of effector and memory $CD8^+$ T cells with tumor-specific cytokine expression. When combined with $CD4^+$ T cells, transfer of total (naïve and effector) or effector $CD8^+$ T cells were highly effective, suggesting $CD4^+$ T cells can help mediate therapeutic effects by maintaining function of activated $CD8^+$ T cells. In addition,, $CD4^+$ T cells had a pronounced effect in the early post-transfer period, as their elimination within the first 3-days significantly ($p<0.001$) reduced therapeutic efficacy. The $CD8^+$ T cells recovered from mice treated with both $CD8^+$ and $CD4^+$ T cells had decreased expression of PD-1 and PD-1-blockade enhanced the therapeutic efficacy of pmel-CD8 alone, suggesting that $CD4^+$ T cells help reduce $CD8^+$ T cell exhaustion. These data support combining immunotherapies that elicit both tumor-specific $CD4^+$ and $CD8^+$ T cells for treatment of patients with cancer.

Keywords

T cell; cancer immunotherapy; CD4 T-cell help; metastatic melanoma

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Introduction

Metastatic melanoma is a devastating disease with a poor overall survival rate. Two immunotherapies, IL-2 and anti-CTLA-4, provide objective clinical responses in approximately 15% of patients and are approved drugs [1],[2]. Recently, the combination of anti-CTLA-4 and anti-PD-1 was shown to provide profound and rapid anti-cancer activity in approximately half of the patients receiving treatment [3]. Pre-clinical models have identified T-cell dependent mechanisms as being the primary mediators of these anti-tumor effects [4]. Adoptive T cell immunotherapy in patients made lymphopenic by nonmyeloablative chemotherapy has also provided significant success against this disease [5],[6]. Many of these studies utilize T cells expanded from tumor infiltrating lymphocyte (TIL) cultures, however the use of human T cells transduced to express tumor-reactive TCRs or chimeric antigen receptors (CAR) provides a practical alternative with the potential to be broadly applied to patients with almost any type of cancer [7],[8]. Furthermore studies with CAR have shown long-term tumor regression and tumor-specific T cell persistence for over 6 months [7].

Both clinical trials and murine models, studying adoptive immunotherapy using tumor-specific TIL or TCR transgenic (Tg) T cells have shown that immunotherapy is more effective in a lymphopenic than in a lymphoreplete environment [5],[6]. A lymphopenic environment can be established in a number of ways including chemotherapy (cyclophosphamide), radiation, a combination of both or by using mice that lack endogenous T and B cells ($RAG^{-/-}$ deficient mice) [9],[10]. Each lymphopenia inducing method provides a different combination of mechanisms that enhances immunotherapy. In addition to the direct killing of tumor cells, by radiation or chemotherapy, mechanisms that augment T cell expansion and effector function include elimination or absence of suppressive cells as well as cells that serve as cytokine sinks, increased access to antigen-presenting cells (APC) and access to proinflammatory signals that may activate APCs or aid in overcoming T cell exhaustion [11]. Additionally, combination adoptive immunotherapy that included induction of lymphopenia prior to adoptive transfer and IL-2 administration, has resulted in persistence of memory and circulating $CD8^+$ T cells and correlated with successful clinical outcome [12]-[14]. This supports the concept that $CD8^+$ T cells play a dominant role in tumor elimination by directly killing tumor cells or by the secretion of cytokine or chemokines that have anti-tumor effects and recruit other adaptive or innate immune components. Thus combinations that maintain or increase tumor-specific $CD8^+$ T cells would be expected to improve therapeutic efficacy [15]. The importance of $CD4^+$ T-cell help for both priming and maintenance of memory $CD8^+$ T-cell immunity has long been appreciated [16],[17]. Our lab and others have shown that immunotherapy with tumor-specific $CD8^+$ T cells in CD4-deficient MHC class II−/− mice resulted in regression of pulmonary metastases, but did not result in long-term anti-tumor immunity and tumors eventually recurred [18],[19]. In contrast, multiple studies have shown that partial or transient CD4-depletion can enhance anti-tumor responses, but since these models do not eliminate $CD4^+$ T cells completely there may be a small population of $CD4^+$ T cells programming or maintaining $CD8^+$ T cell function [20]-[22]. Moreover induction of lymphopenia is thought to abrogate the need for $CD4^+$ help since it increases $CD8^+$ T cell

exposure to homeostatic cytokines, IL-7 and IL-15, driving memory T cell formation and enhancing anti-tumor immune responses [23],[24].

Although it has not been directly examined, it is possible that low objective response rates in some studies are due to the transfer of tumor-specific CD8 T cells in the absence of tumor-specific $CD4^+$ T cells. Two studies utilizing MART-1 and/or gp100-specific HLA class I restricted TCR gene transfer for treatment of metastatic melanoma resulted in objective clinical response rates of 13% (2/15) and 30% (6/20), which were lower than the response rates achieved using bulk CD4+ and CD8+ TIL (51–71%). While these are small studies, one possible explanation for the low response rate is the absence of tumor-specific $CD4^+$ T cells. However, attempts to identify tumor-specific $CD4^+$ T cells in the peripheral blood of patients with cancer who experienced an objective clinical response following nonmyeloablative chemotherapy and adoptive immunotherapy with TIL have been difficult [25]. Furthermore CD4 T cells alone have been expanded and adoptively transferred with high-dose IL-2 and have resulted in few clinical responses [26]. Given the prior studies underscoring the significant contribution $CD4^+$ T cells play in supporting the therapeutic efficacy of tumor-specific $CD8^+$ T cells and a number of clinical studies suggesting that neither $CD8^+$ or $CD4^+$ T cells are effective alone, in this paper we investigated the importance of tumor-specific $CD4^+$ T-cell help in augmenting anti-tumor immunity of $CD8^+$ T cells under conditions of lymphopenia-driven homeostasis.

Results

Immunotherapy with both pmel and TRP-1 Tg cells augments therapeutic efficacy

Since adoptive transfer of large numbers ($5 \times 10^4 – 2 \times 10^5$) of TRP-1 cytotoxic $CD4^+$ Tg T cells alone can induce regression of established B16-F10 melanoma [27],[28] we sought, to generate a model where therapeutic efficacy was dependent on both $CD8^+$ and $CD4^+$ T cells. Thus, we needed to identify a dose of TRP-1-$CD4^+$ Tg T cells that was not therapeutic on their own. To do this three-day experimental pulmonary metastases were established by i.v. injection of the poorly immunogenic D5 in tyrosinase-related protein (tyrp-1)$^{bw}RAG1^{-/-}$ ($RAG1^{-/-}$ tyrp-1 protein-deficient [29]) female mice, which lack endogenous T and B cells. Mice received adoptive transfer of CD4-enriched TRP-1 Tg cells at doses ranging from 1×10^6 to 50 cells. TRP-1-$CD4^+$ T cells failed to fully eliminate metastases regardless of their number, but animals treated with 5000 or less $CD4^+$ T cells had greater than 50 metastases (Figure S1). Consequently a dose of 1000 CD4-enriched TRP-1 cells, a physiologically relevant number, were used for subsequent experiments. For all experiments, pmel-$CD8^+$ T cells were activated by αCD3/IL-2 expansion. $RAG1^{-/-}$ tyrp1 protein-deficient lymphopenic mice with 3-day D5 experimental pulmonary metastases were treated with 10^6 αCD3/IL-2-expanded pmel and 1000 naïve CD4-enriched TRP-1 (Pmel + TRP-1), 10^6 αCD3/IL-2-expanded pmel alone, 1000 TRP-1 alone or no treatment (Figure 1A). All mice received 90,000 IU IL-2 i.p. daily for 3 days. Ten and 20 days after adoptive transfer mice treated with both pmel and TRP-1 cells had significantly less tumor burden than mice treated with either pmel or TRP-1 alone (Figure 1B). Mice treated with both pmel and TRP-1 showed no evidence of tumor after 40 days, while pmel or TRP-1 alone groups succumbed to tumor burden before 27 days (Figure 1C).

Treatment with both pmel and TRP-1 significantly increased the number of $CD8^+$ T cells in the blood and the spleen 10 and 20 days following adoptive transfer, compared to treatment with pmel alone (Figure 1C). This is notable because persistence of anti-tumor $CD8^+$ T cell has been correlated with long-term tumor control [2],[30],[31]. Treatment with pmel and TRP-1 also resulted in increased $CD4^+$ T cells in the blood compared to TRP-1 treatment alone, however there were decreased $CD4^+$ T cells in the spleen (Figure 1C).

The addition of TRP-1 T cells enhances the number and function of pmel-$CD8^+$ T cells

$CD4^+$ T cells are known to be important for maintenance of effector (T_{Eff}) and memory (T_{memory}) $CD8^+$ T cells [5], therefore we determined whether adoptive transfer of both pmel and TRP-1 T cells increased the number and frequency of T_{memory} and T_{Eff} pmel-$CD8^+$ T cells compared to adoptive transfer with pmel alone. Mice receiving combined therapy exhibited an increased total number of T_{Eff} ($CD44^+CD62L^{lo}CD127^{lo}$) pmel-$CD8^+$ T cells in the blood and spleen (Figure 2A). There was also an increase in total T_{memory} ($CD44^+CD127^+$) pmel-$CD8^+$ T cells in the blood and effector memory (T_{EM}) ($CD44^+CD127^+CCR7^{lo}$) pmel-$CD8^+$ T cells in the spleen.

The composition of naïve ($CD62L^+CD44^{lo}$), T_{Eff} ($CD44^+CD127^{lo}CCR7^{lo}$), T_{EM} ($CD44^+CD127^+CCR7^{lo}$) and central memory (T_{CM}) ($CD44^+CD127^+CCR7^+$) pmel-$CD8^+$ T cells 10 and 20 days after adoptive transfer indicates a much smaller proportion of T_{EM} phenotype pmel-$CD8^+$ T cells in mice treated with pmel T cells alone (Figure 2B). The presence of tumor-specific TRP-1-$CD4^+$ T cells increased the number and frequency of pmel T_{Eff} $CD8^+$ T cells that eliminated the tumor and also increased the number of long-lived memory T cells (Figure 2A–B). Analysis of $CD8^+$ T cell function by intracellular cytokine staining 10 days after transfer, showed that pmel-$CD8^+$ T cells from pmel and TRP-1 treated mice had a significantly ($p<0.001$) higher frequency of D5-specific IFN-γ expressing cells compared to stimulation with the syngeneic but unrelated MCA-310 sarcoma. However, $CD8^+$ T cells from pmel only treated mice also exhibited an increased percentage of IFN-γ positive pmel-$CD8^+$ T cells, but this difference did not reach statistical significance (Figure 2C). Pmel and TRP-1 treated mice also had a higher frequency of $CD8^+$ T cells exhibiting polyfunctional cytokine expression (TNF-α, IFN-γ, Granzyme B and IL-2), which has been associated with long-lived T cells (Figure 2D) [8],[32]-[34]. We analyzed the phenotype of TRP-1-$CD4^+$ T cells in blood and spleen and found increased numbers of T_{Eff} and T_{EM} in TRP-1 only treated mice compared to pmel and TRP-1 treated mice (Figure 2A). There were significantly more T_{Eff} TRP-1-$CD4^+$ T cells in the blood of pmel and TRP-1 treated mice, however this was only at the day 20 time point and did not translate to a proportional difference in the entire population (naive, T_{Eff}, T_{EM}, T_{CM}) as we observed in the $CD8^+$ T cells (Figure 2A–B). Mice treated with either pmel and TRP-1 or TRP-1 only had an equal percentage of tumor-specific IFN-γ producing $CD4^+$ T cells (Figure 2C). Since previous studies using the D5 experimental metastases model documented that $CD8^+$ T cells were the dominant mechanism for eliminating tumor when endogenous tumor vaccine-primed T cells were used for adoptive immunotherapy, we focused on the effect $CD4^+$ T cells had on the $CD8^+$ T cells [7],[35].

TRP-1 T cells help maintain pmel-CD8$^+$ T cells

We found that following adoptive transfer of tumor-specific Tg CD4$^+$ and CD8$^+$ T cells, tumor had not recurred by 40 days and most animals were apparently cured of their disease (Figure 1B and data not shown). We hypothesized that CD4$^+$ T cells could be helping to prime a small number of $T_{naïve}$ CD8$^+$ T cells that still remain after αCD3/IL-2 expansion. Therefore, we phenotyped αCD3/IL-2-expanded pmel at the time of adoptive immunotherapy (Day 0). This analysis revealed a large population (15–20%) of phenotypically "naïve" (CD44^{lo}CD62L$^+$) CD8$^+$ T cells (referred to as αCD3/IL-2 expanded CD44^{lo}CD62L$^+$) (Figure 2B). This observation surprised us, so we examined whether CD4$^+$ T cells needed this αCD3/IL-2-expanded CD44^{lo}CD62L$^+$ population to help prime CD8$^+$ T cells or whether they were maintaining activated T_{Eff} phenotype CD8$^+$ T cells. To test this we eliminated the CD44^{lo}CD62L$^+$ CD8$^+$ T cells from the αCD3/IL-2 expanded population by sorting on T_{Eff} phenotype pmel (CD44$^+$CD62L^{lo}). We then compared treatment with 5×10^5 sorted effector CD44$^+$CD62L^{lo} pmel and 1000 TRP-1 T cells (sort Pmel+ TRP-1), 5×10^5 sorted T_{Eff} CD44$^+$CD62L^{lo} pmel alone (sort Pmel), 5×10^5 total αCD3/IL-2-expanded pmel and TRP-1 (total Pmel + TRP-1) or 5×10^5 total pmel alone (total Pmel) (Figure S2B).

Immunotherapy with sorted T_{Eff} pmel or total pmel, combined with TRP-1 T cells had significantly less tumor growth at 10 and 20 days following treatment compared to mice treated with either pmel population alone (Figure 3A). The majority of mice treated with both pmel and TRP-1, either sorted or total, survived longer than 40 days with no symptoms of tumor progression (Figure 3B). Furthermore, while mice treated with sorted pmel and TRP-1 had fewer splenic CD8$^+$ T cells than mice receiving total pmel and TRP-1, their numbers were still increased compared to mice treated with only total or sorted pmel T cells 10 days after transfer (Figure 3C). Since elimination of the αCD3/IL-2 expanded CD44^{lo}CD62L$^+$ did not diminish the antitumor effects in vivo it suggests that tumor-specific TRP-1-CD4$^+$ T cells are able to maintain activated pmel-CD8$^+$ T cells. Futhermore comparison of proliferation and function of *ex vivo* αCD3/IL-2-expanded CD44^{lo}CD62L$^+$, αCD3/IL-2-expanded T_{Eff} CD44$^+$CD62L^{lo} cells and unstimulated pmel splenocytes showed that αCD3/IL-2 expanded CD44^{lo}CD62L$^+$ and T_{Eff} CD44$^+$CD62L^{lo} pmel cells proliferated (>5x) and showed comparable functionality (IFN-γ$^+$TNFα$^+$) whereas unstimulated pmel splenocytes did not (Figure S3). These data support that tumor-specific CD4$^+$ T cells can act in conjunction with T_{EM} CD8$^+$ T cells to increase their numbers, tumor-specific function and efficacy, even in the absence of naïve T cell priming.

TRP-1 help occurs early after adoptive immunotherapy

We attempted to determine when, in relation to adoptive transfer, tumor-specific CD4$^+$ T cells were needed to maintain anti-tumor immunity. According to the model described in Figure 1A, CD4$^+$ cells were depleted one-day prior, 3 and 10 days following adoptive immunotherapy with pmel and TRP-1 cells. Anti-CD4 antibody was administered one-day prior to adoptive transfer even though RAG1$^{-/-}$ mice have no T cells to ensure CD4$^+$ cells were immediately eliminated upon transfer. We expected this to replicate adoptive transfer with pmel alone. Indeed, depletion of CD4$^+$ cells one-day prior to adoptive immunotherapy resulted in a large tumor burden that was similar to pmel treatment alone. CD4-depletion 10 days after transfer resulted in less tumor burden, similar to undepleted mice, depletion at day

3 gave results that were intermediate (Figure 4A). This suggests that in this model tumor-specific $CD4^+$ T cells exert their effects during the first 10 days following adoptive transfer. We examined whether CD4-depletion changed the number of $CD8^+$ T cells in the blood and spleen 17/18 days after immunotherapy, CD4-depleted groups showed a substantial decrease in the number of pmel-$CD8^+$ T cells (Figure 4B and data not shown). We also looked at expression of the exhaustion marker PD-1 (J43 antibody) on splenocytes 17/18 days after adoptive transfer. PD-1 expression on pmel-$CD8^+$ T cells was significantly higher among $CD8^+$ cells in all non-therapeutic CD4-depleted groups (Day −1, 3 and pmel alone) than in $CD8^+$ cells from undepleted mice (Figure 4C). Depletion of $CD4^+$ cells one-day before or 3 days after adoptive transfer also correlated with decreased expression of IFN-γ, IL-2, TNF-α and Granzyme B measured at day 17/18, compared to deletion at day 10 or undepleted mice (Figure 4D). However, long-term anti-tumor immunity was still compromised in mice depleted of $CD4^+$ T cells 10 days after transfer compared to undepleted mice (Figure 4E). Interestingly, mice depleted of $CD4^+$ cells 10 days after transfer often developed tumors at metastatic sites, such as the skin (Figure 4E). This suggests that $CD4^+$ T cells continue to maintain pmel-$CD8^+$ T cells or potentially act to support trafficking of $CD8^+$ T cells in order to control tumor metastases [36].

PD-1-blockade augments the therapeutic efficacy of pmel-CD8 T cells

We next examined whether blocking PD-1 could restore the therapeutic efficacy of pmel T cells alone by treating mice with a PD-1 blocking antibody. PD-1-blockade augmented therapeutic efficacy of pmel T cells alone, significantly ($P<0.01$) reducing the number of metastases compared to pmel T cells alone (31±5 verses 82±16 mean±SEM) (Figure 5A). Since PD-1 treatment did not result in complete elimination of metastases, we examined other mechanisms of $CD4^+$ T-cell help including CD40-CD40L, increasing survival or decreasing apoptosis [37] [38],[39]. There was no difference in overall survival or in the total number and function of T cells with CD40-CD40L blockade (Figure S4) suggesting that in our model enhancement of $CD8^+$ T cells was not dependent on CD40-CD40L interactions. We also examined expression of the survival factor Bcl-2 and the apoptotic factor TRAIL in $CD8^+$ T cells from pmel and TRP-1 or pmel alone treated mice. There was no difference in Bcl-2 expression; however, there was a substantial decrease in TRAIL expression when mice were treated with both pmel and TRP-1 T cells (Figure 5B), implying the addition of tumor-specific $CD4^+$ T cells increases $CD8^+$ T cell persistence by reducing exhaustion by the PD-1 pathway and decreasing TRAIL-induced apoptosis.

Discussion

We recently found that tumor-vaccine specific $CD4^+$ T cells augmented therapeutic efficacy of immunotherapy with tumor-specific $CD8^+$ T cells in the $RAG1^{-/-}$ lymphopenic environment (Friedman, manuscript in preparation). Here we take advantage of the tumor-specific TRP-1 MHC class II-restricted TCR Tg $CD4^+$ T cells to examine the role of tumor-specific $CD4^+$ T cells in the lymphopenic environment. Our results suggest that tumor-specific $CD4^+$ T cells in combination with tumor-specific $CD8^+$ T cells augment therapeutic efficacy, maintain long-term tumor control and increase total survival and function of $CD8^+$ T cells. Additionally, we show that physiological doses of tumor-specific $CD4^+$ T cells

could significantly ($p<0.001$) augment therapeutic efficacy of immunotherapy with tumor-specific $CD8^+$ T cells (Figure 1B). Treatment with both pmel and TRP-1 increased tumor-specific function, polyfunctionality and total number of effector and memory $CD8^+$ T cells compared to pmel alone (Figure 1C and 2). This suggests tumor-specific $CD4^+$ T cells enhance memory, survival and effector function of tumor-specific $CD8^+$ T cells. Adoptive immunotherapy with low doses of TRP-1-$CD4^+$ cells used here did not result in significant therapeutic efficacy (Figure 1B). However, the TRP-1 only treated mice had significantly more $CD4^+$ T cells in the spleen than pmel and TRP-1 treated mice 10 and 20 days after transfer (Figure 1C and 2A). This suggests that even though there are more $CD4^+$ T cells in the spleen with TRP-1 treatment alone, the $CD4^+$ T cells are less effective at eliminating tumor, possibly because they are trafficking to the spleen rather than the tumor.

Our data also suggest that tumor-specific $CD4^+$ T cells can support therapeutic efficacy by maintaining effector $CD8^+$ T cells even in the absence of phenotypically naïve $CD8^+$ T cells (Figure 3 and S3). Ten days after transfer mice given TRP-1 and $CD44^+CD62L^-$ pmel had less total $CD8^+$ T cells than those treated with TRP-1 and total pmel, however this number was considerably more than either group treated with pmel alone (Figure 3C) and TRP-1 and $CD44^+CD62L^-$ pmel treated mice did not develop tumor after more than 70 days (Figure 3A–B). This ability of $CD4^+$ and $CD8^+$ T cells to cure mice of systemic tumor burden in the absence of a source of naive $CD8^+$ T cells is strong evidence that $CD4^+$ T cells are maintaining $CD8^+$ effector T cells in this model. Characterizing αCD3/IL-2 *ex vivo* expanded pmel showed that αCD3/IL-2expanded $CD44^{lo}CD62L^+$ cells were expressing some TNFα and IFNγ and proliferating similarly to the T phenotype cells (Figure S3). One explanation for this apparent disconnect between phenotype and production of effector cytokines could be that CD62L and CD44 are being upregulated and downregulated during the αCD3/IL-2 causing them to display an atypical phenotype, including a population of $CD44^{lo}CD62L^{lo}$ cells that are neither a $T_{naïve}$ ($CD44^{lo}CD62L^+$) or T_{EM} ($CD44^+CD62L^{lo}$) phenotype (data not shown). If this is the case it further supports that tumor-specific TRP-1 $CD4^+$ T cells are acting to maintain activated $CD8^+$ T cells in the lymphopenic environment.

Tumor-specific TRP-1-$CD4^+$ T cells were particularly important early following adoptive transfer, as elimination within 3 days, but not 10 days (Figure 4A) resulted in partial loss of therapeutic efficacy and correlated with an increase in the exhaustion marker PD-1 on $CD8^+$ T cells at day 18. The increase of PD-1 was most significant for the group depleted of $CD4^+$ cells 3 days after transfer (Figure 4C), suggesting that the early time points may be particularly important for $CD4^+$ help. These findings are consistent with reports showing that antigen-specific $CD4^+$ T-cell help can decrease PD-1 expression on $CD8^+$ T cells [20], [40][41][42].. Elimination of $CD4^+$ cells 10 days after adoptive transfer did not reduce therapeutic efficacy, measured by enumeration of pulmonary metastases at day 18 or result in increased PD-1 expression on $CD8^+$ T cells compared to undepleted mice. However, we did see a decrease in total number of pmel-$CD8^+$ T cells in the day 10 depleted group. One possible explanation is that by day 10, when the $CD4^+$ T cells are depleted, the majority of tumor has been eliminated and there is less antigen-driven proliferation of $CD8^+$ T cells. Both undepleted mice and day 10 depleted mice also had polyfunctional (IFN-γ, TNF-α, Granzyme B, IL-2) $CD8^+$ T cells, 17/18 days after adoptive transfer, which may be

responsible for the enhanced anti-tumor efficacy at this time point (Figure 4D). Interestingly, eliminating $CD4^+$ T cells 10 days after adoptive transfer resulted in late onset distant metastases (skin, ovaries) 40 days after transfer (Figure 4E). This development of distant metastases is consistent with one previous study using adoptive immunotherapy with $CD8^+$ T cells in MHC class II-deficient mice [18]. Distant tumor metastases were not observed in mice that received both pmel and TRP-1 (undepleted), even 200 days after immunotherapy and might be explained by the role of $CD4^+$ T-cell help in $CD8^+$ T cell trafficking, which has been observed in other models [36],[43] or that $CD4^+$ help is maintaining memory $CD8^+$ T cells, which are important for tumor immune surveillance [44].

Investigating the mechanism of this CD4 support, we identified that PD-1 interactions play a role in limiting the antitumor effects of immunotherapy with non-helped pmel-$CD8^+$ T cells, as PD-1 blockade significantly ($p<0.01$) improved therapeutic efficacy (Figure 5A). In contrast, blockade of CD40-CD40L interactions in animals treated with both tumor-specific CD4 and CD8 T cells did not decrease therapeutic efficacy, suggesting that these interactions are not essential (Figure S4). Such an independence of CD40-CD40L interactions has been described in a number of other preclinical cancer immunotherapy models [35]. Further, phenotypic characterization of T cells at times following adoptive transfer suggest tumor-specific TRP-1-$CD4^+$ T cells maintain pmel-$CD8^+$ T cells, in the lymphopenic environment, by decreasing TRAIL expression (Figure 5B). Together these data suggest that tumor-specific $CD4^+$ T cells are important to reduce PD-1 mediated exhaustion and potentially TRAIL-mediated apoptosis during initial tumor elimination.

Approximately 33% of all pmel and TRP-1 treated mice developed amelanotic tumors, 100–200 days after adoptive transfer, at the primary metastatic site in the pleural cavity (data not shown). This frequency of amelanotic tumor recurrence in mice treated with pmel and TRP-1 T cells is consistent with a recent report from Jensen and colleagues evaluating recurrence of experimental subcutaneous melanoma [47]. In our study, mice that recurred with amelanotic tumor had a decreased total number and function of $CD4^+$ and $CD8^+$ T cells, and tumor cells had reduced expression of gp100 and TRP-1 protein (data not shown). This indicates antigen loss is a potential problem and antigen-specific $CD4^+$ and $CD8^+$ T cells may need additional combination therapy to prevent tumor recurrence or should consider targeting antigens that are critical to tumors survival. Furthermore lymphoreplete mice made lymphopenic by radiation and given immunotherapy with the same number of TRP-1 and pmel that were therapeutic in Rag1−/− mice, did not experience a significant reduction in tumor burden (Figure S5).. These findings underscore the complexities of the immune response and the preclinical model systems and identify opportunities for studies that may uncover novel mechanisms that limit anti-tumor immunity. Given our findings of the important role tumor-specific CD4 T cells play in augmenting therapy, combining $CD8^+$ T cell adoptive immunotherapy with vaccination that includes targets with $CD4^+$ epitopes may be a good way to induce endogenous tumor-specific $CD4^+$ helper T cell responses. This approach has the theoretical advantage of developing a broad range of $CD4^+$ T cells by targeting multiple tumor-antigens or by eliciting epitope-spreading of endogenous $CD4^+$ T cells [45],[46]. This broad $CD4^+$ T cell repertoire might reduce the significance of tumor antigen-loss variants, which are seen in multiple preclinical models where a single antigen is

targeted [47]. Together our data strongly argues that tumor-specific $CD4^+$ T cells play an important role in maintaining long-term systemic anti-tumor immunity and suggest that investigators designing clinical immunotherapy trials should consider a range of vaccine or adoptive immunotherapy options that include or promote both tumor-specific $CD4^+$ and CD8+ T cells T cells in order to optimize clinical outcome of patients on these trials.

Materials and Methods

Tumor cell lines and Metastases

We used the poorly immunogenic subclone, B16BL6-D5 (D5) isolated from the spontaneously arisen B16BL6 melanoma. This tumor cell line is defined as poorly immunogenic as vaccination with irradiated D5 fails to protect mice from a subsequent challenge with viable D5 [48]. MCA-310 or D5-G6, a D5 clone stability transduced to secrete GM-CSF were used to generate MCA-310 or D5-specific $CD4^+$ T cells, as previously described [18]. T-cell stimulation assays were done using D5 CIITA and the unrelated syngeneic sarcoma MCA-310 CIITA; both were modified to express CIITA [6]. D5, D5-G6, D5 CIITA and MCA-310 CIITA were propagated using 10% FBS RPMI 1640 supplemented with 2 mmol/L L-glutamine, 0.1 mmol/L non-essential amino acids, 1 mmol/L sodium pyruvate, 5 μg/ml gentamicin-sulfate (Lonza) and 50 μM/L β-mercaptoethanol (Sigma). All tumor cell lines were propagated for less then 6-weeks. Three-day established pulmonary metastases were generated by injecting 2×10^5 D5 cells i.v.

Mice and Adoptive Immunotherapy

TRP-1 TCR x tyrp-$1^{bw}RAG^{-/-}$ ($RAG1^{-/-}$ tyrp1 protein-deficient MHC Class II-restricted TCR Tg) male mice were used to isolate tumor-specific TRP-1-$CD4^+$ splenocytes (gift from Dr. Restifo and The Jackson Laboratory). TRP-1-$CD4^+$ T cells are specific for the murine tyrosinase-related protein 1 peptide (36). Tyrp-1 protein is expressed by the B16BL6-D5 and TRP-1-$CD4^+$ T cells secrete IFN-γ in response to stimulation with class II^+ D5 tumor cells. Female $RAG1^{-/-}$ tyrp1-protein-deficient littermates, which lack the TCR transgene or $RAG1^{-/-}$ were used as hosts. $RAG1^{-/-}$ pmel-1 (MHC Class I-restricted TCR Tg) mice were generated by breeding $RAG1^{-/-}$ (The Jackson Laboratory) with pmel-1 mice (gift of Dr. Restifo), and used to isolate pmel tumor-specific $CD8^+$ T cells from male mice.

Recognized principles of laboratory animal care were followed (Guide for the Care and Use of Laboratory Animals, National Research Council, 1996) and all animal protocols were approved by the EACRI animal care and use committee.

Single cell suspensions of pmel splenocytes were incubated for 2 days on 5 μg/ml anti-CD3 (2C11) in a 24-well plate followed by 3 days with 60 IU/ml IL-2 (Chiron) in a lifecell tissue culture bag (Baxter) (referred to as αCD3-IL-2-expansion). TRP-1-$CD4^+$ splenocyte suspensions were enriched using a pan T cell isolation kit (Miltenyi). Intravenous injections used 10^6 αCD3/IL-2-expanded pmel and/or 1000 enriched TRP-1 cells, unless otherwise noted. Mice also received i.p. injections of 90,000 IU IL-2 (Chiron) given daily for 3 days. Mouse lungs were resected and stored in Feketes solution. Metastases were enumerated by

counting black nodules on the lung surface. Maximum tumor burden was recorded as 200 metastases.

Flow Cytometry

Spleens were disrupted using a 3-ml syringe in a 6-well plate and filtered to single cell suspensions. Red blood cells were lysed using ACK buffer (Lonza). Cells were stained for phenotyping and sorting with combinations of the following antibodies CD4-Qdot605 (Invitrogen), CD8-PE-Cy7, CD3-Percp-eFluor710, CD62L-Pacific blue/eFluor450, CD127-PE, CD127-APC-eFluor780, PD-1-FITC, CCR7-APC, CD44-AF700, PD-1-PE, TRAIL-PE, Bcl-2-FITC, FOXP3-eFluor450 (eBioscience), CD4-APC-Cy7, CD4-APC-H7 (Becton Dickinson). PD-1 staining was done on splenocytes harvested 17/18 days after adoptive transfer using the anti-PD-1 (J43) antibody. Intracellular staining was performed using the eBioscience fix-perm kit. The gating strategy for memory T cells is shown in Figure S2A.

Blood counts were calculated using Flow-Count Fluorospheres (Beckman Coulter). For intracellular cytokine staining splenocytes were incubated 18–24 hours adding 5 μg/ml Brefeldin A (Sigma) after two hours. Cells were stained with LIVE/DEAD fixable yellow stain (Invitrogen-Molecular Probes), CD8-V500 and CD4-APC-H7 (Becton Dickinson). Cells were fixed and permeablized (Becton Dickinson) then stained with IFN-γ-PE (Becton Dickinson), TNFα-FITC, Granzyme-B-PE-Cy7 and/or IL-2-eFluor450/Pacific Blue (eBioscience) or IL-2-Brilliant Violet 421 (Biolegend). ICS cells were gated on live-singlet lymphocytes negative for live-dead dye, followed by $CD4^+$ or $CD8^+$ and individual cytokines. Proliferation was detected using CFSE (Invitrogen-Molecular Probes) as previously reported [49]. Gates were based on fluorescence minus one (FMO) controls. All samples were run on a BD LSRII or BD Aria and analyzed using FlowJo (Treestar), Pestle and SPICE (Courtesy of Mario Roederer).

Depletion and blocking antibodies

CD4- and CD8-depleting antibodies were made from 2.43 or GK1.5 hybridomas (ATCC), respectively by purifying ascites using Biosephra MEP Hypercel (Ciphergen) as described previously [35] or purchased (BioXcell). CD40L-blocking antibody (MR1), PD-1 (RMP1-14) and hamster IgG were purchased (BioXcell). Rat IgG control antibody was purchased (Sigma).

Statistics

Unpaired or paired student t tests were done for analysis of cell numbers and phenotype using Prism (Graphpad). Mantel-Cox log rank tests were used to analyze survival curves (Prism, Graphpad). A p value of <0.05 was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

TIL	tumor-infiltrating lymphocytes
Tg	transgenic
tyrp-1	tyrosinase-related protein 1
CAR	chimeric antigen receptor
TRP-1	tyrosinase-related protein 1-specific $CD4^+$ transgenic T cells
pmel	gp100-specific $CD8^+$ transgenic T cells
D5	poorly immunogenic subclone, B16BL6-D5 melanoma

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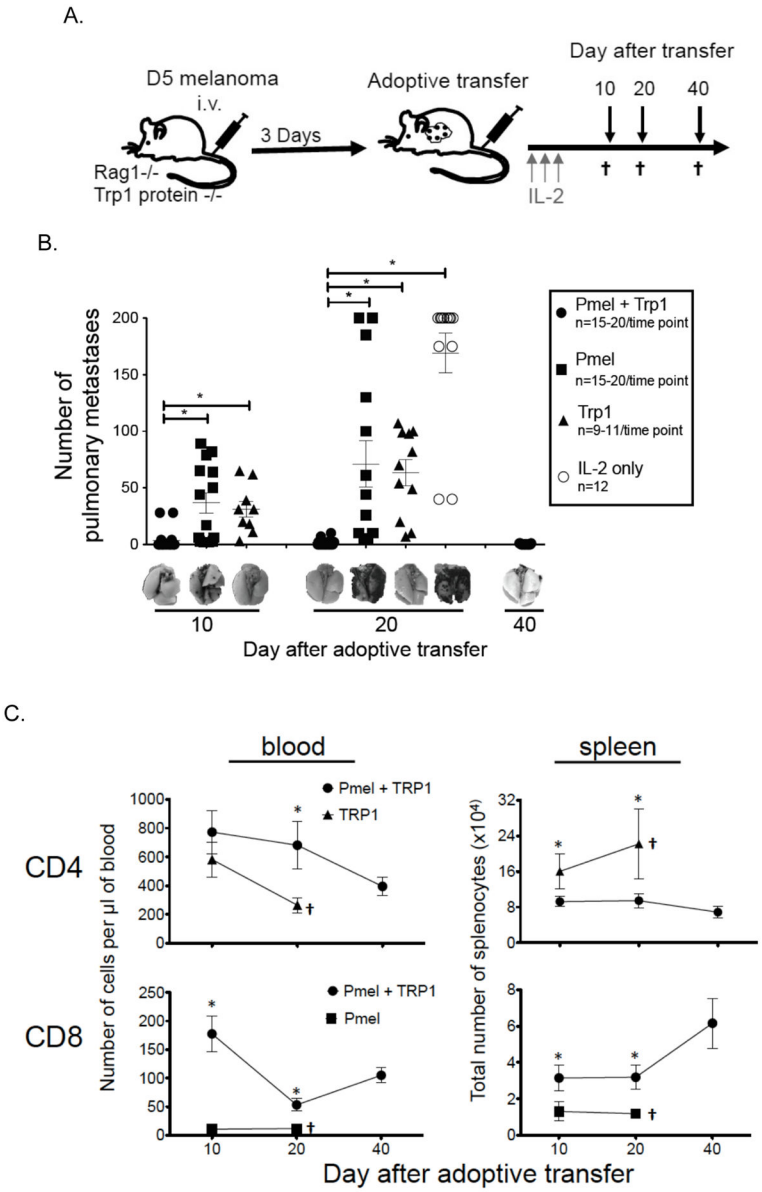

Figure 1. Treatment with tumor-specific pmel and TRP-1 T cells of lymphopenic tumor-bearing RAG1$^{-/-}$ mice eliminates tumor and increases pmel T cells in the blood and spleen

A) Overview of RAG1$^{-/-}$ lymphopenic experimental pulmonary metastases model. RAG1$^{-/-}$ lymphopenic tyrp1 protein-deficient mice were treated with 2×10^5 D5 melanoma cells i.v. After 3 days the same mice were treated with 1×10^6 αCD3/IL-2 stimulated pmel-CD8$^+$ Tg cells and 1000 CD4-enriched TRP-1 Tg cells (Pmel + TRP-1), pmel alone (Pmel), TRP-1 alone (TRP-1) or no cells (IL-2 only). All mice also received 3 doses of 90,000 IU IL-2 i.p. daily. Mice were euthanized (†) for analysis 10, 20 and 40 days after adoptive immunotherapy. B) Number of pulmonary metastases at each time point with representative resected lungs. C) Total number of CD3$^+$CD4$^+$ or CD3$^+$CD8$^+$ cells in the blood or spleen at day 10, 20 and 40 following adoptive immunotherapy. Combination of 4 experiments, n=9–20 per time point, as indicated. *p<0.001. Bars are mean±SEM.

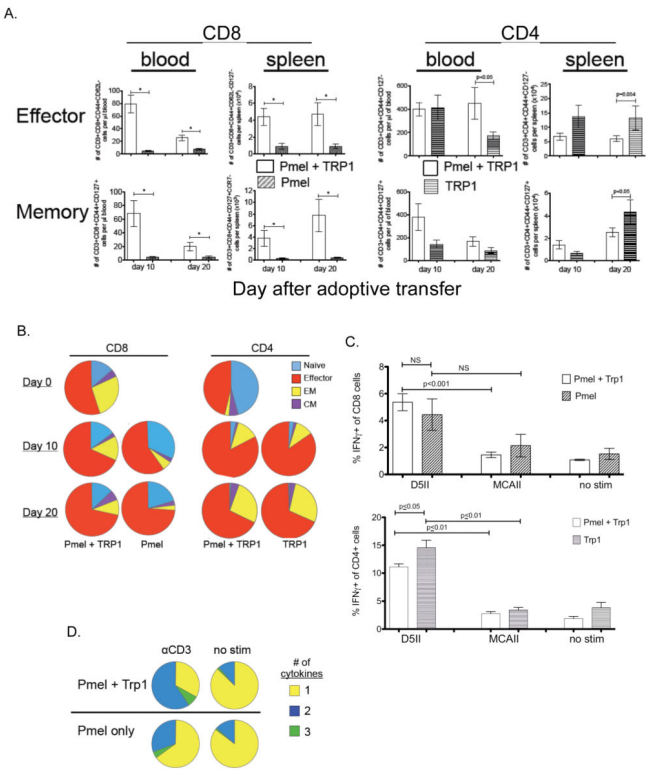

Figure 2. Treatment of tumor-bearing RAG1$^{-/-}$ mice with tumor-specific pmel and TRP-1 T cells increases survival and function of T_{Eff} and T_{memory} pmel T cells

A) T_{Eff} ($CD44^+CD62L^{lo}CD127^{lo}$) and T_{memory} ($CD44^+CD127^+$) $CD8^+$ or $CD4^+$ T cells in the blood and T_{Eff} and T_{EM} ($CD44^+CD62L^{lo}CD127^+CCR7^{lo}$) in the spleen 10 and 20 days after adoptive immunotherapy with both pmel and TRP-1 compared to treatment with pmel alone or TRP-1 alone. B) Distribution of $T_{naïve}$, T_{Eff}, T_{EM} (EM) and T_{CM} (CM) phenotype $CD8^+$ or $CD4^+$ T cells in the spleen at the day of transfer (day 0), 10 or 20 days after adoptive transfer. C) Percent of tumor-specific IFN-γ expressing $CD8^+$ or $CD4^+$ T cells stimulated with specific D5 CIITA (D5II), non-specific syngeneic MCA-310 CIITA (MCAII) or unstimulated (no stim) 20 days after adoptive immunotherapy. AC) Combination of 4 experiments, n=9–20 per time point. D) ICS of $CD8^+$ gated cells using splenocytes from pmel + TRP-1 or pmel treated mice stimulated with αCD3 or unstimulated. Representative pies from one experiment. *p<0.001 Bars are mean±SEM.

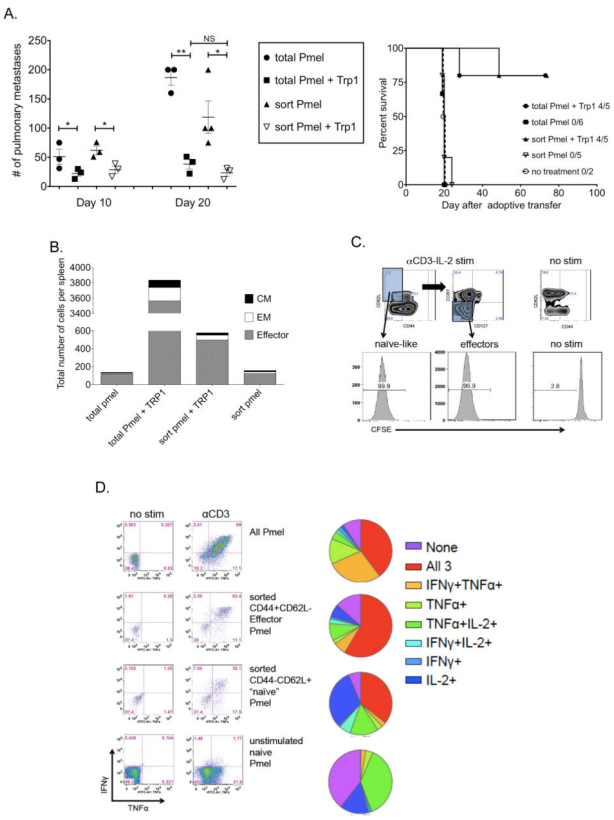

Figure 3. Tumor-specific TRP-1 T cells are important for maintaining activated pmel in the lymphopenic environment

A–B) Mice were treated with 5×10^5 αCD3/IL-2-expanded sorted T_{Eff} ($CD44^+CD62L^{lo}$) pmel and 1000 TRP-1 cells (sort pmel + TRP-1), 5×10^5 total CD3/IL-2-expanded pmel and 1000 TRP-1 (total pmel + TRP-1), sort pmel alone or total pmel alone. A) Number of pulmonary metastases 10 and 20 days after adoptive transfer n=3/group for one representative experiment. Bars are mean±SEM (*$p<0.05$, **$p<0.01$). B) Survival for a combination of 2 experiments. Numbers are mice that are tumor free of total. C) Total number of $CD3^+CD8^+$ T cells with distribution of T_{Eff}, T_{EM} and T_{CM} phenotypes in the spleen 10 days after immunotherapy.

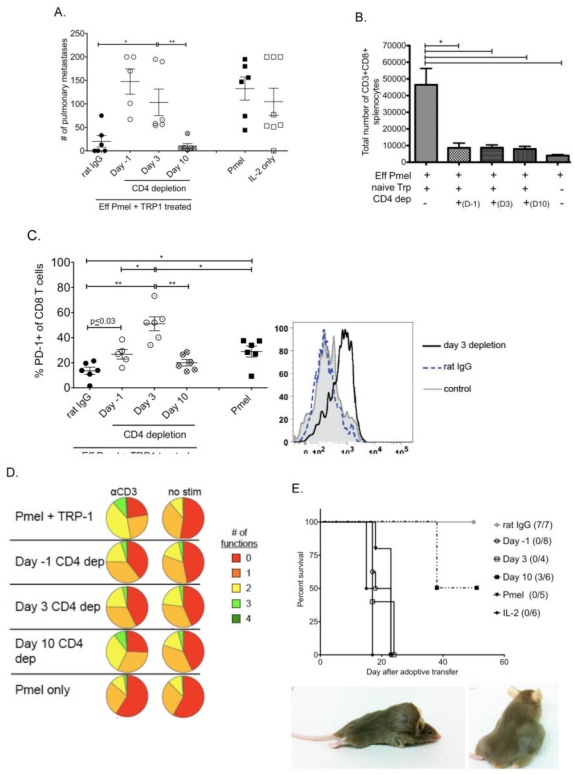

Figure 4. TRP-1 T cells are important early to reduce tumor burden

Mice treated with pmel and TRP-1 were depleted of CD4 cells one-day prior, 3 or 10 days following adoptive transfer and compared to pmel alone or no treatment. All analysis was done 17/18 days following immunotherapy. While findings were consistent in 3 of 3 experiments data is only shown for 2 of 3 experiments where all groups were included (n=5–7 mice/group). A) Total number of pulmonary metastases 17/18 days after adoptive transfer and survival (representative experiment). B) Total number of $CD3^+CD8^+$ T cells in the spleen. C) Frequency of PD-1 expressing $CD3^+CD8^+$ T cells 17/18 days after transfer. Histogram shows representative PD-1 expression on $CD3^+CD8^+$ cells comparing treatment with rat IgG treatment or CD4-depletion 3 days after transfer. D) Summary of the number of cytokines expressed by $CD8^+$ cells (IL-2, TNF-α, IFN-γ and Granzyme B) with 24 hours αCD3 or no stimulation.

E) Survival curve and representative of distant skin metastases that occurred 38 days after transfer in mice depleted of $CD4^+$ T cells 10 days after transfer. Bars are mean+SEM. D+E are one representative experiment. *p<0.05, **p<0.01

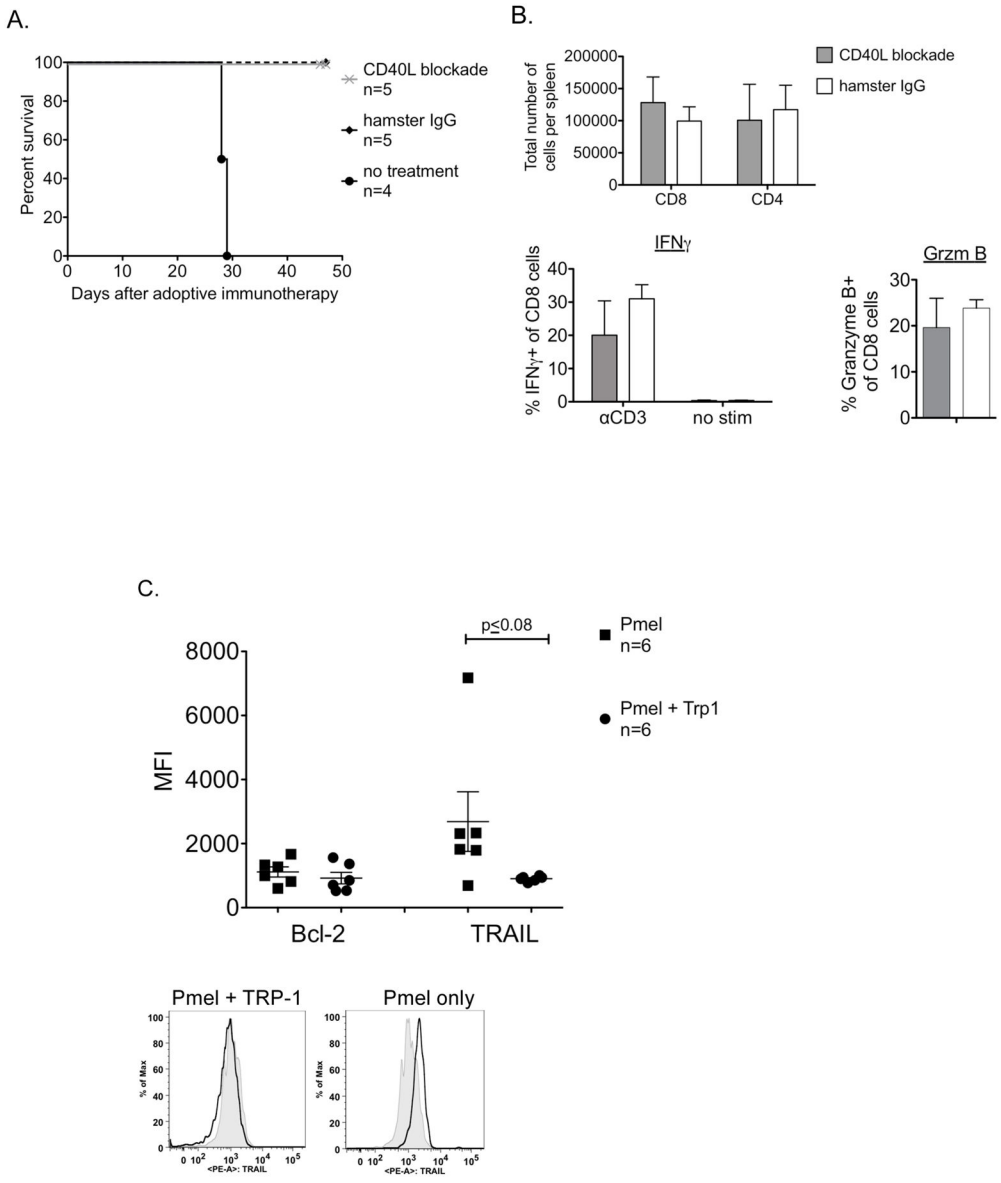

Figure 5. PD-1-blockade augments the therapeutic efficacy of pmel-alone

A) Number of pulmonary metastases from mice treated with pmel plus PD-1-blocking antibody (RMP1-14) or rat IgG. All mice received IL-2. Data are a combination of 3 experiments n=3–6/experiment. B) Mice treated with pmel and TRP-1 cells were compared to mice treated with pmel alone. Flow cytometry is shown for Live-$CD3^{+}CD8^{+}$ cells 18 days after adoptive transfer. MFI minus control (FMO) of Bcl-2 and TRAIL. Histograms representative TRAIL expression for pmel and TRP-1 or pmel alone treated mice. Black line is treatment, grey fill is FMO. Data are a combination of 2 experiments n=3. Bars are mean ±SEM.