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Increased Frequency of Suppressive Regulatory T Cells and T Cell-Mediated Antigen Loss Results in Murine Melanoma Recurrence

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Therapeutic treatment of large established tumors using immunotherapy has yielded few promising results. We investigated whether adoptive transfer of tumor-specific CD8+ T cells, together with tumor-specific CD4+ T cells, would mediate regression of large established B16BL6-D5 melanomas in lymphopenic Rag1−/− recipients devoid of regulatory T cells. The combined adoptive transfer of subtherapeutic doses of both TRP1-specific TCR transgenic Rag1−/− CD4+ T cells and gp100-specific TCR transgenic Rag1−/− CD8+ T cells into lymphopenic recipients, who received vaccination, led to regression of large (100–400 mm2) melanomas. The same treatment strategy was ineffective in lymphoreplete wild-type mice. Twenty-five percent of mice (15/59) had tumors recur (15–180 d postregression). Recurrent tumors were depigmented and had decreased expression of gp100, the epitope targeted by the CD8+ T cells. Mice with recurrent melanoma had increased CD4+Foxp3+ TRP1-specific T cells compared with mice that did not show evidence of disease. Importantly, splenocytes from mice with recurrent tumor were able to suppress the in vivo therapeutic efficacy of splenocytes from tumor-free mice. These data demonstrate that large established tumors can be treated by a combination of tumor-specific CD8+ and CD4+ T cells. Additionally, recurrent tumors exhibited decreased Ag expression, which was accompanied by conversion of the therapeutic tumor-specific CD4+ T cell population to a Foxp3+CD4+ regulatory T cell population.


Cancer immunosurveillance suggests that malignant cells are targeted and destroyed by the immune system (1). Preclinical mouse models demonstrated that the presence of a functional immune system is critical to avoid the development of spontaneous tumors (2–4). Further, increased infiltration of human solid tumors with cytotoxic CD8+ T cells correlated with prolonged survival (5–7). These studies show the protective role of the immune system against tumors; however, the immune system occasionally fails to completely eliminate potential malignant cells, allowing tumors to escape and form larger, established tumors. As the tumor becomes established, it is often difficult to use the immune system to eradicate the tumor, because the process of tumor escape selects for reduced immunogenicity of the tumor and/or the presence of immunosuppressive mechanisms to attenuate the antitumor immune response (8, 9). It is imperative to understand these interactions to develop more effective therapeutic approaches to treat cancer patients for whom cancer immunosurveillance has failed.

The presence of T cells specific for tumor Ags among a cancer patient’s T cell repertoire verifies the existence of tumor-specific T cell clones, and the presence of T cells at the tumor site correlated with improved outcomes (10–12). However, the inability of these tumor-specific T cells to maintain immune surveillance implies that extrinsic factors limit the efficacy of these T cells and/or that these T cells intrinsically lack the properties necessary to eliminate tumor cells. To prime a therapeutic antitumor immune response, tumor-specific T cells must have TCRs with sufficient affinity for tumor Ags to enable their activation (13–15). Strategies to enhance priming of these low-affinity, tumor-specific T cells would be beneficial. Seminal work by Mackall et al. (16) provided evidence that T cells are more sensitive to T cell activation during immune reconstitution, primarily as a result of increased access to homeostatic cytokines (17, 18). Various groups have extended this observation, demonstrating that antitumor immune responses are enhanced during immune reconstitution (19–21).

Although initial interactions of tumor-specific T cells with their Ag dictate whether they will become activated, the tumor environment also influences the antitumor immune response (22). It has become increasingly clear that tumors can establish an immunosuppressive environment that blocks both the priming and the effector phase of the immune response. Factors such as TGF-β (23, 24), IL-10 (25), prostaglandins (26, 27), and IDO (28), secreted either by the tumor or by suppressive cell populations, were shown to mediate this effect. Regulatory T cells (Tregs) are present in a variety of tumors (29–31) and were shown to attenuate graft-versus-host disease (32, 33), demonstrating their potent
suppressive role in controlling the immune response. The Treg population can be subdivided into two groups: natural Tregs and peripherally induced Tregs, both of which can contribute to immune suppression during the tumor-bearing state (34, 35). Importantly, various groups showed that depletion of Tregs results in enhanced antitumor immune responses that protect against tumor challenge or treat minimal tumor burden (36–40).

These data led us to investigate whether the adoptive transfer of tumor-specific T cells into a lymphopenic environment devoid of Tregs would lead to regression of large established tumors. Previous work examined the contribution of CD4+ T cells to the development, expression, and maintenance of CD8+ T cells (41–44). Our studies demonstrated that the adoptive transfer of activated CD8+ effector T cells could mediate therapeutic effects against 3-d experimental pulmonary metastases, but complete elimination and maintenance of long-term protection required CD4+ T cell help (45). These data provided the rationale to use both a tumor-specific CD4+ TCR transgenic T cell and tumor-specific CD8+ TCR transgenic T cell to examine whether their adoptive transfer into lymphopenic hosts could regress large established tumors. To further ensure that the observed effects were due to the transgenic TCR and not an endogenous TCR, both the CD4 and CD8 TCR transgenic mice were backcrossed to the Rag1−/− background.

Materials and Methods

Mice

Female Rag1−/− mice on a C57BL/6 background (B6.129S7-Rag1−/−;J) were purchased from The Jackson Laboratory (Bar Harbor, ME), pmel-1 TCR transgenic mice were bred with Rag1−/−;B6.129S7-Ick−/−;J) and were provided from the manufacturer (Invitrogen). Twenty days after tumor challenge, 3 × 106 CFSE-labeled TRP1 TCR transgenic mice were labeled with 5 μM CFSE, according to the manufacturer’s protocol (Invitrogen). Twenty days after tumor challenge, 3 × 106 CFSE-labeled pmel splenocytes and 3 × 106 CFSE-labeled TRP1 splenocytes were adoptively transferred into the tumor-bearing mice. Some mice were vaccinated s.c. with 107 irradiated (10,000 rad) D5-G6 cells. In some experiments, TRP1 × Rag1−/− splenocytes and pmel × Rag1−/− splenocytes were plated at 2 × 107 cells/ml in CM and cultured in 24-well plates with 5 μg/ml anti-CD3 Ab (2C11). After 48 h of activation, the cells were harvested and expanded in CM containing 60 IU/ml recombinant human IL-2 (Chiron, Emeryville, CA) for 20 min at 4˚C. After washing and resuspension inPerm/Wash solution (BD Pharmingen), intracellular staining with PE-labeled anti-Typr1 Ab (M-19; Santa Cruz Biotechnology) was performed for 20 min at 4˚C. Cells were washed twice with Perm/Wash solution, resuspended in FACS buffer, and analyzed on a BD Biosciences FACSCalibur.

Adoptive transfer of transgenic T cells

C57BL/6 mice or Rag1−/− mice were injected s.c. in the hind flank with 106 tumor cells. This dose is 100 times greater than the dose (104) required to establish tumor in 100% of challenged mice. Tumors were allowed to establish and become sizeable (50–400 mm3). In our experience, all mice with tumors in this size range remained mobile and did not experience clinical signs of distress or cachexia. Animals were euthanized at signs of distress and/or cachexia. Nine to sixteen days after tumor challenge, the indicated numbers of naive TRP1 × Rag1−/− splenocytes and/or pmel × Rag1−/− splenocytes were injected i.v. into tumor-bearing mice. Recipient mice also received a s.c. injection in the contralateral flank with 107 irradiated (10,000 rad) D5-G6 cells. In some experiments, TRP1 × Rag1−/− splenocytes and pmel × Rag1−/− splenocytes were plated at 2 × 107 cells/ml in CM and cultured in 24-well plates with 5 μg/ml anti-CD3 Ab (2C11). After 48 h of activation, the cells were harvested and expanded in CM containing 60 IU/ml recombinant human IL-2 (Chiron, Emeryville, CA) at a starting cell density of 1.50 × 107 cells/ml in a six-well plate. After 72 h, "effector" T cells were harvested and adoptively transferred into tumor-bearing Rag1−/− recipients.

CFSE staining

Rag1−/− mice were injected s.c. with 5 × 105 D5 tumor cells. Splenocytes from pmel × Rag1−/− TCR transgenic mice and TRP1 × Rag1−/− TCR transgenic mice were labeled with 5 μM CFSE, according to the manufacturer’s protocol (Invitrogen). Twenty days after tumor challenge, 3 × 106 CFSE-labeled pmel splenocytes and 3 × 106 CFSE-labeled TRP1 splenocytes were adoptively transferred into the tumor-bearing mice. Some mice were vaccinated s.c. with 107 irradiated D5-G6. Four days after adoptive transfer, splenocytes were collected and stained with anti-CD4 PE, anti-CD3 PE-Cy7, and anti-CD8 allophycocyanin. Cells were analyzed on a FACScalibur (BD Biosciences).

Intracellular Foxp3 staining

Intracellular staining for Foxp3 was performed using the manufacturer’s protocol (eBioscience). Briefly, cells were stained for surface molecules with anti-CD3 PE-Cy7, anti-CD4 FITC. Cells were permeabilized using freshly prepared Fix/Perm solution and incubated for 1–2 h at 4˚C. Cells were washed once with permeabilization buffer, blocked with purified anti-mouse FCε for 15 min, stained intracellularly with PE-labeled anti-Foxp3 at 0.5 μg/106 cells, and incubated at 4˚C for 30 min. Cells were washed and resuspended in 1% paraformaldehyde and analyzed on a FACScalibur (BD Biosciences).

ELISA

Spleens from either pmel TCR transgenic or TRP1 TCR transgenic mice were collected and processed to yield single-cell suspensions. A total of 106 splenocytes was cultured with 105 D5, MCA-310, D5-CIITA, MCA310-CIITA, or D5-White tumor cells. Splenocytes stimulated or not with plate-bound anti-CD3 Ab (10 μg/ml) were used as positive and negative controls, respectively. After culture for 20 h, supernatants were harvested, and IFN-γ concentration was determined by ELISA using commercially available reagents (anti-IFN-γ; BD Biosciences). The concentration of IFN-γ was determined by regression analysis.

Statistical analysis

Statistical analysis of the data was performed using Prism (GraphPad Software). A two-tailed p value < 0.05 was considered significant for ELISA data and frequency of Foxp3+ TRP1 T cells. Statistical significance for survival studies was determined using the Gehan–Breslow–Wilcoxon Test; a p value < 0.05 was considered significant.

Results

gp100-specific TCR transgenic CD8+ T cells and TRP1-specific TCR transgenic CD4+ T cells can respond to gp100 and tyrp1 Ags expressed by the B16BL6-D5 melanoma cell line

The availability of both CD4+ and CD8+ TCR transgenic mice that are specific to endogenous proteins expressed by melanoma provided an opportunity to examine whether adoptive transfer of tumor-specific CD8+ T cells together with tumor-specific CD4+
T cells could mediate regression of established tumors. The T cell compartment of the TRP1 TCR transgenic × Rag1−/− mouse contains only CD4+ TCR transgenic T cells that recognizes the tyrp1(109–130) peptide (46, 47). Likewise, we bred the pmel TCR transgenic mouse with the Rag1−/− mouse to obtain the pmel TCR transgenic × Rag1−/− mouse that contains only CD8+ TCR transgenic T cells that recognizes gp100 25–33 of the endogenous murine gp100 protein (48). Both of these strains of mice are devoid of endogenous TCR rearrangement because they lack the Rag1 protein. We verified that D5, a poorly immunogenic subclone of B16BL6, expresses both gp100 and tyrp1 (Fig. 1A). Additionally, splenocytes from pmel mice secreted significantly increased amounts of IFN-γ when cultured with D5, but not MCA-310, verifying that processing and presentation of gp100 25–33 on MHC class I molecules was occurring in D5 (Fig. 1B). To assay whether D5 expresses sufficient tyrp1 protein to stimulate TRP1 TCR CD4+ transgenic T cells, we transduced D5 with CIITA to upregulate MHC class II on the surface of this cell line. Splenocytes from TRP1 TCR transgenic mice secreted significantly increased amounts of IFN-γ when stimulated with D5-CIITA compared with the irrelevant sarcoma cell line, MCA310-CIITA (Fig. 1C), which does not express tyrp1 protein (Fig. 1A).

Adoptive transfer of CD4+ TCR transgenic T cells together with CD8+ TCR transgenic T cells into a lymphopenic mouse with D5 tumor causes the regression of D5

D5 is classified as poorly immunogenic because vaccination with irradiated D5 does not protect mice against subsequent D5 tumor challenge. Previous work in our laboratory demonstrated the ability of a polyclonal antitumor T cell response to reproducibly treat 3-d experimental pulmonary metastases. However, adoptive transfer of these tumor-specific T cells has not been effective against established s.c. tumors. Because we had never observed effective treatment of these tumors with adoptive transfer of polyclonal tumor-specific effector T cells, in the first experiment we combined multiple strategies (Fig. 2A). Specifically, wild-type (wt) C57BL/6 mice were injected s.c. with 105 D5 tumor cells, followed 9 d later with an adoptive transfer of 106 pmel “naive” splenocytes and 5 × 107 TRP1 “naive” splenocytes. These mice were then vaccinated with irradiated GM-CSF–secreting D5 (D5-G6). On day 12 after tumor challenge, the mice received 2 × 106 in vitro-activated “effector” pmel splenocytes together with 2 × 105 in vitro-activated “effector” TRP1 splenocytes. IL-2 (90,000 IU/d) was given for 5 d, separated by 2 d of rest, for up to 5 wk. Amazingly, this combination vaccine/adoptive immunotherapy-strategy had absolutely no impact on tumor growth in lymphopenic wt C57BL/6 mice (Fig. 2B, 2D). In the same experiment, we also tested the same combination immunotherapy strategy in lymphopenic Rag1−/− recipients. D5 tumors in lymphopenic conditions grew similarly to the tumors in lymphoreplete mice (Fig. 2B, 2C). In striking contrast to wt mice, tumor-bearing Rag1−/− mice that received the same combination immunotherapy specified above began to exhibit tumor regression on day 16 (Fig. 2E), when the tumors had reached an average size of 76 mm². By day 60, all of the lymphopenic mice that had received pmel and TRP1 T cells did not have palpable tumors.

The striking result that adoptive transfer of tumor-specific “naive” and “effector” CD4 and CD8 T cells, IL-2 administration, and vaccination in a lymphopenic environment mediated regression of established tumors led us to evaluate whether regression required all elements of the therapeutic strategy. It was clear that the lymphopenic environment was required, so all further in vivo experiments were performed in lymphopenic hosts (Rag1−/− mice). We wanted to determine whether the adoptive transfer of naive tumor-specific TCR transgenic T cells together with vaccination would be sufficient to mediate tumor regression (Fig. 2F). As shown previously, Rag1−/− recipient mice challenged with D5 tumor, followed by naive TRP1 transgenic CD4 and CD8 T cells on day 12 and in vitro-activated “effector” TRC transgenic CD4 and CD8 T cells on day 16, exhibited regression of large established tumors averaging 273 mm² (Fig. 2G). However, the tumor regression was not dependent on the ex vivo activation of T cells, because the adoptive transfer of naive pmel and naive TRP1 cells, together with the D5-G6 vaccination, was sufficient to cause tumor regression (Fig. 2H).

Previous work demonstrated that the adoptive transfer of TRP1 CD4+ TCR transgenic cells can mediate regression of B16BL6 tumors (46, 47, 49). Because we were interested in the cooperative effect of the CD4+ TCR transgenic T cells combined with the CD8+ TCR transgenic T cells, we titrated the dose of naive TRP1 splenocytes to find a dose that did not cause complete regression of tumor in all vaccinated tumor-bearing Rag1−/− mice. The adoptive transfer of 5 × 105 TRP1 splenocytes led to the regression of D5 tumor in a majority of Rag1−/− mice (Fig. 3A, 3B) and 85% survival 100 d after tumor challenge (Fig. 3E). A majority of tumor-bearing mice receiving 1.5 × 105 TRP1 splenocytes also exhibited tumor regression (Fig. 3C); however, only 33% of mice survived until day 100 (Fig. 3E). The adoptive transfer of 5 × 105 TRP1 splenocytes caused tumor regression in <40% of the treated mice (Fig. 3D), and none of the mice that received this dose of TRP1 splenocytes survived to day 100 (Fig. 3E). This lower dose of TRP1 splenocytes did not provide a statistically significant increase in survival versus no T cell transfer. Based on these data, we adoptively transferred into tumor-bearing

**FIGURE 1.** B16BL6/D5 (D5) expresses gp100 and tyrp1 protein and is recognized by pmel TCR transgenic T cells and TRP1 TCR transgenic T cells. (A) Western blot analysis of gp100, tyrp1, and β-actin expression from D5 or MCA310 cell lysates. A total of 10⁶ splenocytes from pmel TCR transgenic mice (B) or TRP1 TCR transgenic mice (C) was stimulated in vitro with 10⁷ D5, MCA310, D5CIITA or MCA310CIITA or no tumor. Supernatants were harvested, and IFN-γ concentration was determined by ELISA. Data are mean of two independent experiments (± SE).
TUMORS RECUR DUE TO IMMUNOSUPPRESSIVE MECHANISMS

GM-CSF–secreting tumor vaccination induces pmel T cell proliferation and tumor regression

Because homeostatic proliferation can lead to the expansion of T cells in the lymphopenic environment, it is possible that the lymphopenic environment might activate both pmel and TRP1 T cells independent of the D5-G6 vaccine. Approximately 20% of both pmel CD8+ transgenic T cells and TRP1 CD4+ transgenic T cells undergo homeostatic proliferation when they are transferred into lymphopenic mice that are tumor free and not vaccinated (Fig. 4A). The presence of s.c. D5 tumor caused the TRP1 T cells to undergo strong proliferation, whereas there was minimal enhancement of proliferation of the pmel T cells (Fig. 4A). Vaccination with the GM-CSF–secreting tumor vaccine induced 2-fold more pmel T cells to proliferate (Fig. 4A), and, importantly, sustained tumor regression was observed only with this D5-G6 vaccination (Fig. 4B). Taken together, these data indicate that, at the doses used in the adoptive transfer, three components are needed for tumor regression: TRP1 CD4+ T cells, pmel CD8+ T cells, and D5-G6 vaccination.

Tumor recurrence is associated with tumor depigmentation and loss of Ag expression

The adoptive transfer of pmel and TRP1 T cells caused tumor regression in Rag1−/− recipient mice; however, 25% of mice had a recurrent tumor that grew out after the initial tumor regression (15 mice had tumor recurrence/59 treated animals, n = 10 experiments). Interestingly, all recurrent tumors displayed some degree of depigmentation (Fig. 5A). Because both tyrp1 and gp100 are proteins involved in the pigmentation of this melanoma, we were interested to determine whether the adoptive transfer of pmel and TRP1 TCR transgenic cells selected for an Ag-loss variant. Two cell lines were derived from a tumor that, upon resection, had both pigmented (D5-Black) and depigmented (D5-White) portions. As expected, the parental D5 and the D5-Black cell lines expressed tyrp1 protein, whereas the methylcholanthrene-induced sarcoma cell line, MCA-310, did not (Fig. 5B). The depigmented D5-White cell line continued to express tyrp1, demonstrating that it was not the loss of tyrp1 that resulted in depigmentation. D5-Black retained expression of gp100, whereas the depigmented tumor cell line, D5-White, did not express detectable amounts of gp100 protein by Western blot analysis (Fig. 5C). We also observed that D5-White had increased expression of MHC class I compared with the parental D5 tumor cell line (Fig. 5D). Interestingly, pmel T cells cultured with D5-White tumor cells секретed higher amounts of IFN-γ than did pmel T cells cultured alone (without stimulation), as well as statistically greater amounts compared with pmel T cells cultured with the irrelevant MCA-310 sarcoma cell line (p = 0.05, Fig. 5E). Although gp100 expression was undetectable by Western blot analysis, it appeared that low levels of gp100 were present to stimulate pmel T cells. However, the reduction in gp100 expression in D5-White tumors resulted in less IFN-γ secretion compared with that secreted by pmel T cells cultured with the parental D5 cell line. Culturing TRP1 T cells with the D5-White cell line resulted in significant (p < 0.05) IFN-γ secretion compared with TRP1 T cells stimulated with the MCA-310 cell line. Because the D5-White cell line had undetectable levels of MHC class II at the start of the cytokine-release experiment, we analyzed MHC class II expression again at the end of the assay. Although D5 or D5-White cells cultured alone had no detectable levels of MHC class II, D5-White cultured with TRP1 T cells for 20 h had >50% of the tumor cells expressing MHC.

FIGURE 2. The adoptive transfer of TRP1 and pmel T cells combined with systemic IL-2 and vaccination causes the regression of established D5 tumor in lymphopenic mice. (A) A total of 10⁶ D5 tumor cells was injected s.c. into wt C57BL/6 mice (B, D) or Rag1−/− mice (C, E). Nine days later, these mice received 10⁶ pmel splenocytes and 5 × 10⁵ TRP1 splenocytes (D, E) and a s.c. vaccination with lethally irradiated D5-G6 (B–E). On day 12 after tumor challenge, the mice also received 2 × 10⁶ in vitro-activated pmel splenocytes together with 2 × 10⁵ in vitro-activated TRP1 splenocytes (D, E). All mice received IL-2 (90,000 IU/d) for 5 d, separated by 2 d, for up to five cycles. Data are representative of four independent experiments. (F) A total of 10⁶ D5 tumor cells was injected s.c. into Rag1−/− mice. (G and H) Twelve days later, these mice received 7.5 × 10⁵ pmel splenocytes, 5 × 10⁵ TRP1 splenocytes, and a s.c. vaccination with 10⁴ lethally irradiated D5-G6. (H) On day 17 after tumor challenge, the mice also received 10⁶ in vitro-activated pmel splenocytes together with 2 × 10⁵ in vitro-activated TRP1 splenocytes. Data are representative of two independent experiments.
class II (data not shown). In contrast, D5 had <25% of the tumor cells expressing MHC class II (data not shown), which may explain the reduced amount of IFN-γ secreted following stimulation with D5 (Fig. 3E).

The reduced expression of gp100 in D5-White raised the possibility that tumor recurrence was due to the inability of pmel T cells to sufficiently recognize the tumor in vivo. We investigated this possibility by determining whether naive pmel and TRP1 cells could impact the growth of D5-White tumor in vivo. Rag1−/− mice were challenged with D5-White tumor, followed 15 d later with an adoptive transfer of naive pmel and TRP1 cells together with an irradiated D5-G6 vaccine. The D5-White cell line grew slightly more slowly than did the parental D5 tumor in Rag1−/− recipient mice; however, Rag1−/− mice that received the adoptive transfer of pmel and TRP1 T cells, combined with vaccination, showed regression of their D5-White tumors (Fig. 5F). This shows that the reduced expression of gp100 alone did not protect tumors from rejection and suggests that gp100 loss alone was not sufficient for
tumor recurrence. These data are consistent with tumor recurrence being the result of multiple mechanisms dampening the immune response and facilitating immune escape of the depigmented tumor.

**Immune responses from mice with recurrent tumor exhibit suppressive function**

Because pmel and TRP1 T cells could regress depigmented D5-White tumors, we examined whether mice with recurrent tumors had dysfunctional immune responses. The adoptive transfer of splenocytes from mice whose tumor regressed and remained tumor free maintained the ability to cause parenta D5 tumor regression (Fig. 6B). In contrast, splenocytes from mice whose tumor recurred were not therapeutic against D5, even though splenocytes from those mice contained similar frequencies of pmel and TRP1 T cells as did splenocytes from tumor-free mice (data not shown). The inability of TCR transgenic CD4+ and CD8+ T cells from mice whose tumor recurred to provide therapeutic impact against parenta D5 tumor (gp100+) demonstrates that mechanisms other than Ag loss are responsible for the tumor recurrence.

We previously reported in a separate model that D5 tumors can sensitize Tregs that inhibit the generation of a productive antitumor immune response (23, 36). Interestingly, in the studies reported in this article, mice with recurrent tumor had a significant increase in the frequency of Foxp3+ TRP1 T cells, with a majority of animals having >40% of their TRP1 T cells expressing Foxp3 (Fig. 6B). In contrast, mice whose tumor regressed and remained tumor free maintained frequencies of Foxp3+ TRP1 T cells similar to those that exist in naive TRP1 transgenic mice. The increase in frequency of Foxp3+ TRP1 T cells raised the question of whether these cells could exert suppressive effects. The cotransfer of splenocytes from mice whose tumor recurred mixed with splenocytes from mice whose tumor regressed and remained tumor free showed a statistically (p < 0.05) impaired therapeutic function compared with the transfer of splenocytes from tumor-free mice alone (Fig. 6C). These data demonstrate that the splenocytes from mice whose tumor recurred exert immunosuppressive function and have a high frequency of TRP1 T cells expressing a Treg phenotype.

**Discussion**

Our previous work using the D5 melanoma cell line demonstrated that complete and sustained elimination of experimental pulmonary metastases required both CD4+ and CD8+ T cell subsets (45). These studies guided our rationale to use both CD4+ and CD8+ TCR transgenic T cells to investigate the ability of the immune system to cause large established tumors to regress. Both the pmel CD8+ TCR transgenic T cell and the TRP1 CD4+ TCR transgenic T cell have been used separately to treat tumor-bearing mice (46–49). The triple combination of 103 freshly isolated pmel splenocytes with a recombinant fowlpox vaccine encoding human gp10025–33 and IL-2 administration caused the regression of B16 tumors (<100 mm2) (48). Reducing the dose to 102 transferred pmel cells was deemed suboptimal and led to resurgent tumor growth. In our studies, we transferred 0.5–1 × 106 freshly isolated pmel splenocytes (containing ∼1–2 × 106 CD8+ pmel T cells), which minimally impacted tumor growth on their own (data not shown). The TRP1 TCR transgenic T cell was reported to cause tumor regression through CD4+-dependent cytotoxicity (47, 49). However, in a nontransgenic system, we have never observed significant therapeutic effects mediated by adoptive transfer of bulk populations of wt CD4+ T cells from vaccinated recipients, which contained tumor-specific IFN-γ-secreting CD4+ T cells (B. Fox, unpublished observations). The failure to mediate significant therapeutic effect may reflect the low frequency of tumor-specific CD4+ T cells present in bulk populations. Although the frequency of tumor-specific CD4+ T cells may be too low to mediate significant regression of tumor when only CD4+ T cells are transferred, when combined with tumor-specific CD8+ T cells they can mediate the long-term cure of mice bearing 3-d established pulmonary metastases (45). In contrast, tumor-specific CD8+ T cells can mediate initial tumor regression in the absence of CD4+ T cells, but all mice exhibit tumor recurrence at later time points (45). On this basis, we designed a model that used subtherapeutic doses of both TRP1 CD4+ TCR transgenic T cells and pmel CD8+ TCR transgenic T cells. The suboptimal dose of 2.5 × 104 TRP1 splenocytes used in these studies equates to 3.75 × 105 TRP1 TCR transgenic T cells, because they account for ∼15% of freshly isolated TRP1 TCR transgenic mouse splenocytes. This dose was 13-fold less than the dose used by Quezada et al. (49), who...
transferred $5 \times 10^4$ enriched CD4$^+$ TRP1 T cells. The combined adoptive transfer of both of these suboptimal doses of TRP1 T cells and pmel T cells led to tumor regression and a statistically significant increase in the survival of mice. It is not clear from these studies whether this was due to the additive antitumor activities from each of these two cell subsets or whether it results from the increased efficacy of pmel T cells helped by TRP1 T cells. Although it is most likely a combination of both, recent data from our laboratory support the latter explanation (S. Church, manuscript in preparation).

Prophylactic vaccination prior to tumor challenge provides an advantage to the immune system. Activated T cells are primed and ready to eradicate the minimal number of tumor cells that are injected into the environment. In contrast, the number of tumor cells proliferating in a large established tumor mass raises the question whether immune-mediated tumor destruction can eliminate tumor cells faster than they are being produced. Regression of tumors as large as 400 mm$^2$ after adoptive transfer of pmel and TRP1 T cells clearly demonstrates that the immune system can outpace tumor growth. Although naive TRP1 T cells proliferated when they were transferred into tumor-bearing hosts (Fig. 4A), they were unable, on their own, to mediate sustained tumor regression. In addition to the TRP1 T cells, tumor regression required vaccination with the GM-CSF–secreting tumor vaccine that promoted proliferation and differentiation of the pmel T cells (Fig. 4). These data support the important contribution of CD8$^+$ pmel T cells to tumor regression in this model. It also supports the concept that mechanisms that reduce the function of either CD4$^+$ or CD8$^+$ T cells could impact the efficacy of tumor elimination. These studies further demonstrate that properly activated tumor-specific T cells can mount a potent antitumor immune response that can eradicate a large tumor mass. A caveat of these studies is that the frequency of tumor-specific T cells achieved using transgenic TCR T cells might be difficult to meet with polyclonal T cell populations. However, redirecting T cell specificity using tumor-specific chimeric AgRs or TCR gene transfer provides strategies that could be used to increase the frequency and avidity of tumor-specific T cells (15, 50). Our data indicate that these strategies would benefit from adoptive transfer of both CD4$^+$ and CD8$^+$ redirected T cell subsets in the lymphopenic setting.

FIGURE 5. Decreased expression of gp100 in depigmented recurrent tumor. (A) Twenty-five percent (15/59 mice, n = 10 experiments) of Rag1$^{-/-}$ mice that had D5 tumors regressed tumors recur at later time points. Recurred tumors were depigmented or exhibited a mixture of depigmented and pigmented portions. Tumor cell lines were created from depigmented (D5-White) and pigmented (D5-Black) portions of the recurrent tumor. (B) The parental D5, D5-Black, D5-White, and MCA310 (sarcoma) were analyzed for expression of gp100 protein by Western blot. (C) D5-Black, D5-White, and MCA310 were analyzed for expression of gp100 protein by Western blot. (D) D5 (left panel) and D5-White (right panel) were stained intracellularly with anti-tyrp1 protein mAb. (E) A total of $10^6$ pmel splenocytes (left panel) or $10^5$ TRP1 splenocytes (right panel) was cultured with $10^5$ tumor targets for 20 h. Supernatants were assayed for IFN-γ by ELISA. (F) Rag1$^{-/-}$ mice were injected s.c. with either $10^5$ D5 tumor cells or $2 \times 10^5$ D5-White tumor cells. On day 15 after tumor challenge, $5 \times 10^5$ pmel splenocytes and $2.5 \times 10^4$ TRP1 splenocytes were adoptively transferred into the tumor-bearing Rag1$^{-/-}$ mice, and they were s.c. vaccinated with $10^7$ irradiated D5-G6. Data are representative of two independent experiments.

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A sizeable amount of data demonstrate that immunosuppressive environments established by tumors can subvert the ability of the immune system to mount a therapeutic antitumor immune response. Mechanisms of immunosuppression are varied and include tumor-mediated and immune-mediated options. Our studies demonstrated that a delineator of therapeutic efficacy was the difference between lymphopenic and lymphoreplete environments. The lymphopenic environment may release adoptively transferred T cells from the constraints of immunoregulation. Tregs were shown to prevent homeostasis-driven proliferation of T cells displaying either low or high affinity for self-Ags (51). The absence of a Treg population in lymphopenic Rag1^{---} mice may be sufficient to allow the adoptively transferred T cells to become activated and functionally therapeutic. Previous work in our laboratory demonstrated that D5 tumor-bearing lymphoreplete mice generate a population of Tregs that blocks the generation of a therapeutic immune response. Removal of this tumor-sensitized Treg population released the immune system from immunosuppression that blocked the activation of a therapeutic immune response (23, 36). Splenocytes from mice that had recurrent tumors exhibited immunosuppressive properties, as demonstrated by their ability to suppress therapeutic efficacy in adoptive-transfer studies. The ability to adoptively transfer the immune suppression is reminiscent of previous work demonstrating T cell-mediated suppression from tumor-bearing mice (52). All of the mice in this study that had recurrent tumors had an increase in the frequency of FoxP3-expressing TRP1 T cells in the spleen. Efforts were made to analyze the frequency of Foxp3+ Trp1 T cells in the tumor; however, few T cells were recoverable (data not shown). Although these data are supportive of Foxp3+CD4+ Tregs playing a critical role in mediating the observed suppression of therapeutic efficacy, additional studies are necessary to clarify their role in tumor recurrence and in the observed suppression of therapeutic efficacy. Quezada et al. (49) showed that administration of CTLA-4 with the transfer of TRP1 T cells increased the number of effector TRP1 T cells and reduced the number of CD4+Foxp3+ TRP1 T cells. This treatment strategy blocked tumor recurrence, but it is unclear whether this was a result of complete tumor elimination as a result of the increased number of effector TRP1 T cells or the lack of immunosuppression as a result of the reduced number of TRP1 Foxp3+ Tregs.

In our studies, all recurrent tumors exhibited depigmentation, most likely as a result of the decreased expression of gp100, a protein important for melanosome biogenesis. The appearance of these depigmented tumors clearly demonstrated the immunoselective force mounted by the pmel T cells. The presence of tyrp1 protein in these depigmented tumors suggests that direct cytotoxic effects from TRP1 T cells were less important, at least under the conditions used in this model. The gp100 protein was not detected in the D5-White tumor by Western blot; however, D5-White tumor cells could stimulate pmel T cells to secrete low levels of IFN-γ. This response was lower than the response of pmel T cells to the...
parental D5 tumor, supporting reduced, but not complete, loss of gp100 expression. TRP1 T cells also secreted IFN-γ when stimulated with D5-White tumor. Although D5-White tumor did not constitutively express levels of MHC class II that were detectable by flow cytometry, coculturing TRP1 T cells with D5-White or D5 induced upregulation of MHC class II on these tumors. It is unclear what mechanism led to the upregulation of MHC class II. We know that IFN-γ can upregulate the expression of both MHC class I and class II on these tumor cell lines (data not shown), raising the possibility that low-level secretion of IFN-γ, either as background secretion by TRP1 T cells or as a TRP1 T cell response to very low levels of MHC class II (undetectable by flow cytometry), resulted in increased expression of MHC class I and MHC class II.

The observation that combination immunotherapy with naive pmel T cells, naive TRP1 T cells, and vaccination could mediate regression of D5-White in vivo demonstrated that, although pmel T cells respond less well against D5-White in vitro, the diminution of gp100 did not prevent complete regression of the established tumor. Thus, we consider it unlikely that the reduction in gp100 alone was solely responsible for tumor recurrence (Fig. 5E). We consider these data to fit the proposed cancer immunoeediting model (8, 9). During the initial elimination phase, pmel T cells and TRP1 T cells cooperate to cause the regression of large established tumors. This is followed by an equilibrium period during which the continued presence of pmel T cells select for tumor cells with decreased expression of gp100. Decreased expression of gp100 reduces the therapeutic effectiveness of pmel T cells, but it is not solely responsible for tumor escape. In all cases, tumors recur in mice that had depigmented tumors coupled with increased frequencies of Foxp3-expressing TRP1 T cells. These data reveal that crippling both CD4 and CD8 T cells through separate mechanisms correlated with progression to the tumor-escape phase of the immunoeediting model.

We consider that this preclinical system could be a relevant model for what occurs in patients with cancer, specifically, the observation that patients with a T cell infiltrate at the invading margin of their primary colon cancer do significantly better than patients without this infiltrate (12, 53). Even colon cancer patients with metastatic disease that have T cell infiltrate have a better response to chemotherapeutic intervention than do those lacking the T cell infiltrate (53). Presumably, patients who lose the T cell infiltrate (which may be secondary to Ag loss and/or development of a suppressive environment) mimic what happens in our D5-White tumor model, in which there appears to be a correlation between tumor recurrence and relative Ag loss coupled with an immunosuppressive environment. Although additional studies are underway to validate these findings in the clinic, a preponderance of evidence supports the concept that intra- and peritumoral T cell infiltrates have both a prognostic and predictive impact (54, 55).

Although it may not be possible to prevent the immune selection that leads to Ag-loss tumor variants, current research efforts are examining the immunosuppressive function of Tregs and their ability to decrease the efficiency of immunotherapy. This has led to a number of clinical trials that aim to downmodulate or remove the Treg population prior to and/or during immunotherapy, with the intent of maximizing the anticancer immune response (56). However, the resurgence of the Treg population after immunotherapy and the resulting suppression of the anticancer immune response at later time points require us to study what drives this increase in Tregs. Although our current focus is to understand the kinetics and cause of Treg expansion during the equilibrium phase, the results of the studies reported above underscore the complexity of cancer. This complexity was recently identified as a major hurdle for cancer immunotherapy, and it is becoming increasingly clear that the successful development of effective immunotherapy for cancer necessitates a better understanding of that complexity (57). In our opinion, that understanding will enable the design and preclinical testing of combination immunotherapy strategies that enhance tumor elimination, block increases in Tregs, and reduce immune escape. The translation of these strategies to patients with cancer will not be easy. The complexity present in the spectrum of known tumor-escape mechanisms will likely require the development of immune assessments that allow pairing of a specific strategy (or strategies) aimed at incapacitating a specific tumor-escape mechanism in patients that have a tumor that uses that same escape mechanism. This type of evidence-based approach to determine combination immunotherapy strategies for patients with advanced cancer represents a “next generation” of personalized cancer immunotherapy.

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Disclosures
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References


