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CD8^{+} T Cell Immunity Against a Tumor/Self-Antigen Is Augmented by CD4^{+} T Helper Cells and Hindered by Naturally Occurring T Regulatory Cells

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CD4^{+} T cells control the effector function, memory, and maintenance of CD8^{+} T cells. Paradoxically, we found that absence of CD4^{+} T cells enhanced adoptive immunotherapy of cancer when using CD8^{+} T cells directed against a persisting tumor/self-Ag. However, adoptive transfer of CD4^{+}CD25^{-} Th cells (Th cells) with tumor/self-reactive CD8^{+} T cells and vaccination into CD4^{+} T cell-deficient hosts induced autoimmunity and regression of established melanoma. Transfer of CD4^{+} T cells that contained a mixture of Th and CD4^{+}CD25^{+} T regulatory cells (T$_{reg}$ cells) or T$_{reg}$ cells alone prevented effective adoptive immunotherapy. Maintenance of CD8^{+} T cell numbers and function was dependent on Th cells that were capable of IL-2 production because therapy failed when Th cells were derived from IL-2$^{-/-}$ mice. These findings reveal that Th cells can help break tolerance to a persisting self-Ag and treat established tumors through an IL-2-dependent mechanism, but requires simultaneous absence of naturally occurring T$_{reg}$ cells to be effective. *The Journal of Immunology,* **2005, 174: 2591–2601.**

Although CD8^{+} T cells have been shown to be potent mediators of antitumor immunity, the role of CD4^{+} T cells is largely undefined (1, 2). In the absence of CD4^{+} T cell help, CD8^{+} T cells against viral or foreign Ags can become lethargic (3), deleted (4), or lose the capacity to become and remain memory CD8^{+} T cells upon rechallenge (5–7). Therefore, the use of self-reactive CD8^{+} T cells in the adoptive immunotherapy of cancer may face similar fates, because T cells must remove tumor Ag in the context of persisting self-Ag. One theoretical means of improving immunotherapy to self may involve the provision of CD4^{+} T cell help, because helper cells facilitate CD8^{+} T cell activation, function, and survival (1, 6, 8). Nonetheless, naturally occurring CD4^{+} T cells represent a double-edged immunological sword: in addition to their helper functions, one T cell subset, naturally occurring CD4^{+}CD25^{+} T regulatory cells (hereafter referred to as T$_{reg}$ cells),[2] suppresses T cells and controls immunological tolerance to self-Ags (9–12).

In recent years, naturally occurring T$_{reg}$ cells have emerged as the dominant T cell population governing peripheral self-tolerance (13–15). CD4^{+}CD25^{+} T cells develop in the thymus and represent 5–10% of the peripheral CD4^{+} T cell compartment. They constitutively express the high-affinity IL-2R or CD25 (IL-2Rα), glucocorticoid-induced TNFR, CTLA-4, and the transcription factor forkhead box P3 (Foxp3) (13). The mechanism of suppression is through cell-cell contact, but how T$_{reg}$ cells induce and maintain self-tolerance in vivo is still unknown.

In mice, autoimmune destruction of a variety of tissues can be triggered by the removal of T$_{reg}$ cells (12, 16). Organ-specific destruction of tissue expressing a self-Ag can be further enhanced by self-Ag vaccination or through the provision of inflammatory signals when functional T$_{reg}$ cells are absent (17). When lymphopenic mice are reconstituted with a normal repertoire of CD4^{+}CD25^{-} Th cells (hereafter referred to as Th cells), autoimmunity is observed in multiple tissues and cotransfer of T$_{reg}$ cells abrogates these effects (18). Furthermore, depletion of T$_{reg}$ cells can enhance tumor protection to tumor-associated Ags that are expressed as self-Ags (19, 20).

Spontaneous autoimmunity is also seen in IL-2$^{-/-}$, IL-2R$\alpha^{-/-}$, IL-2R$\beta^{-/-}$, JAK-3$^{-/-}$, STAT-5$^{-/-}$, and Foxp3-deficient mice (15, 21, 22). The CD4^{+} T cell subset that remains in each of these knockouts is devoid of functional T$_{reg}$ cells. Together, these observations suggest a role for IL-2 and T$_{reg}$ cells in controlling effector T lymphocytes specific for self-Ags in vivo.

Because T$_{reg}$ cells are able to control autoimmunity to naturally expressed self-Ags, they may play a role in T cell tolerance to self-Ags naturally or overexpressed by tumors. We hypothesized that, by removing the T$_{reg}$ cell subset, we could use the autoimmune potential present within the normal repertoire of CD4^{+} T cells to help self-reactive CD8^{+} T cells mediate antitumor immunity against tumors expressing self-Ags.

Previously, we have shown that adoptive transfer of CD8^{+} T

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[2] Abbreviations used in this paper: T$_{reg}$ cell, CD4^{+}CD25^{+} T regulatory cell; Th cell, CD4^{+}CD25^{-} Th cell; Foxp3, forkhead box P3; CM, culture medium; gp100, melanocyte differentiation Ag gp100; h, human; rFPVhgp100, recombinant fowlpox virus encoding human gp100; WT, wild type.

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cells from pmel-1 transgenic mice, which recognize the melanocyte differentiation Ag gp100 (gp100), could cause autoimmunity and the regression of established tumors in tumor-bearing syngeneic animals. This therapy was successful after vaccination with a virus encoding a tumor Ag, and exogenous administration of a γ_c-signaling cytokine, either IL-2 or IL-15 (23, 24). In the present report, we transferred different $CD4^+$ T cell subsets along with pmel-1 $CD8^+$ T cells, and vaccination into $CD4^+$ T cell-deficient tumor-bearing animals. Although help against foreign and viral Ags has been described numerous times (reviewed in Ref. 2), we sought to understand the effect of different $CD4^+$ T cell subpopulations on adoptive immunotherapy of established tumors as well as the mechanisms that cause and break tolerance to tumors in an environment of persisting self-Ag. We conclude that naturally occurring Th cells can help self-reactive $CD8^+$ T cells break tolerance to self through an IL-2-dependent mechanism, but require the absence of naturally occurring T_{reg} cells to be effective.

Materials and Methods

Mice and tumor cells

pmel-1 TCR transgenic mice have been described previously (23). pmel-1 TCR transgenic and pmel-1 Thy1.1$^+$ mice were bred and kept at the National Institutes of Health animal facilities. C57BL/6, C57BL/6 CD45.1, CD4$^{-/-}$, CD8$^{-/-}$, RAG-1$^{-/-}$, IL-2$^{+/-}$, and Thy1.1$^+$ mice were obtained from The Jackson Laboratory and bred at the National Institutes of Health animal facility. IL-2$^{-/-}$ mice were obtained by crossing IL-2$^{+/-}$ mice together and verified by PCR (The Jackson Laboratory). B16.F10 (H-2^b), hereafter called B16, is a gp100$^+$ spontaneous murine melanoma obtained from the National Cancer Institute tumor repository and was maintained in culture medium (CM) as previously described (23, 25).

Peptides and recombinant fowlpox and vaccinia viruses

All synthetic peptides were synthesized using regular F-MOC chemistry. The synthetic H-2D^b-restricted peptide, human (h)gp100$_{25-33}$, KVPRNQDWL, was synthesized by Peptide Technologies to a purity >99% by HPLC and amino acid analysis. All recombinant viruses encoding hgp100 have been described before and were kindly provided by Therion Biologics (23).

In vitro activation of pmel-1 T cells and cytokine release assay

Splenocytes from mice were depleted of erythrocytes by hypotonic lysis, cultured in CM with 30 IU/ml rhIL-2 in the presence of 1 μM hgp100$_{25-33}$ peptide and used on day 6 after start of the culture. For cytokine release assays, sorted Thy1.1$^+$ pmel-1 T cells (5.0×10^4) were cocultured in CM with 10^5 irradiated (3000 rad) splenocytes pulsed with 1 μM hgp100$_{25-33}$ peptide. Supernatants were collected after 24 h and tested using a murine IFN-γ ELISA kit (Endogen) according to manufacturer's protocol.

Flow cytometry and CFSE staining

From fresh splenocytes, erythrocytes were removed by hypotonic lysis and cells were stained with the indicated mAbs: CD8α-allophycocyanin (53-6.7), CD4-allophycocyanin (H129.19), Vβ 13-FITC (MR12-3), CD25-PE (PC61), Thy1.1-PE (OX-7). For IFN-γ intracellular staining, splenocytes were activated with lymphocyte activating mixture (BD Biosciences) for 6 h, and then fixed and stained using Cytofix and Cytoperm intracellular staining protocol (BD Biosciences). All Abs were purchased from BD Biosciences. Propidium iodide-staining cells were excluded from analysis. Samples were analyzed using a FACSCalibur flow cytometer and CellQuest software. For CFSE staining, pmel-1 T cells were activated and cultured for 1 wk before being sorted on $CD8^+$ enrichment columns (R&D Systems) and labeled with 2 μM CFSE dye (CFDA SE Cell Tracer kit; Molecular Probes). pmel-1 T cells were prepared by resuspending in prewarmed PBS (37°C) containing the appropriate concentration of CFSE dye for 15 min. Cells were then washed and incubated with fresh PBS for an additional 30 min to allow complete modification of the probe before adoptive cell transfer. Four days later, pmel-1 T cells were isolated from mouse splenocytes and analyzed by flow cytometry.

Purification of $CD4^+$ T cell subsets and pmel-1 T cells

Unfractionated $CD4^+$ T cells were purified from single-cell suspensions of IL-2$^{-/-}$, C57BL/6, or C57BL/6 CD45.1 spleens, using a $CD4^+$ enrichment column (R&D Systems). T_{reg} cells were subsequently purified using MACS $CD4^+CD25^+$ Isolation kits (Miltenyi Biotec) to a purity >95%. Th cells were purified on a LS$^+$ selection column twice (Miltenyi Biotec) to obtain >98% depletion of $CD4^+CD25^+$ T cells. Cells were either cultured overnight in CM or transferred immediately. Cells were also used for suppression assays to confirm their function. pmel-1 Thy1.1$^+$ T cells were purified from splenocytes of RAG-1$^{-/-}$ mice by labeling with Thy1.1-PE (15 μg/1.0×10^8 cells/ml) for 10 min. Cells were subsequently washed and sorted from whole splenocytes with anti-PE microbeads using LS$^+$ selection columns (Miltenyi Biotec).

In vitro suppression assays

$CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were isolated from peripheral lymph nodes of C57BL/6 mice by FACS sorting as previously described (26). Subsequently, they were activated with irradiated TΔS (T-depleted splenocytes; 1:1 ratio), soluble anti-CD3 (0.5 μg/ml), and human IL-2 (5 ng/ml, 100 U/ml) for 72 h and then were split and maintained in IL-2 medium for 7–14 days (26). pmel-1 transgenics were activated with 1 μM hgp100$_{25-33}$ peptide, which was pulsed onto gamma-irradiated (3000 rad) TΔS for 30 min and washed twice before coculture. In vitro suppression assays were performed by stimulating pmel-1 $CD8^+$ cells (5.0×10^4) alone or in the presence of titrated numbers of either freshly isolated or activated $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells. Cultures were stimulated with either soluble anti-CD3 (0.5 μg/ml) or in the presence of peptide-pulsed TΔS for 72 h, as previously described (26). Supernatants were taken on day 3 of coculture for IFN-γ release and 1 μCi of [^{3}H]TdR was added for the last 8 h. All data represent the average counts per minute of triplicate determinations. IFN-γ was measured using an ELISA kit (R&D Systems).

Adoptive cell transfer

Mice were injected s.c. with $1.0–5.0 \times 10^5$ B16 melanoma cells as depicted. The standard treatment regimen consisted of the i.v. administration of 1.0×10^6 pmel-1 T cells activated for 1 wk in vitro with 1 μM hgp100 peptide and subsequently purified using $CD8^+$ enrichment kits (R&D Systems) to a purity >98%. $CD4^+CD25^+$ (1.0×10^5), $CD4^+CD25^-$ (1.0×10^6), unfractionated $CD4^+$ T cells (1.0×10^6), IL-2$^{-/-}$ $CD4^+CD25^-$ T cells (1.0×10^6), or a mixture of T_{reg} to Th (1:10) were coinjected with pmel-1 T cells as indicated. One day before adoptive cell transfer of T cells, C57BL/6 mice underwent sublethal whole-body irradiation (500 cGy) (24). Mice were vaccinated by i.v. injection with 2.0×10^7 PFU of a recombinant fowlpox virus encoding human gp100 (rFPVhgp100) on the same day of transfer. IL-2 (Chiron) was administered for 4 days directly following vaccination by daily i.p. injections of 600,000 IU of rhIL-2 in PBS. Tumors were measured in a blinded fashion using calipers, and the products of perpendicular diameters were recorded.

Masked uveitis score

Eyes were enucleated from mice and placed in 4% gluteraldehyde for 30 min. Subsequently, eyes were transferred in 10% formalin for 48 h, and then embedded in methylacrylate. Four- to 5-μm sections were taken along pupillary-optical axis. Sections were evaluated by a masked ophthalmic pathologist using the score as follows: minimal = 0.5, mild = 1, moderate = 2, and severe = 3. Scores were given for iridiocyclitis, choroiditis, vitritis, and retinal involvement. The grading was then combined for a final masked uveitis score.

Statistics

Tumor graphs were compared using Wilcoxon rank sum test. Factorial ANOVA was used to compare autoimmunity in the eye. The *t* test for means was used to analyze IFN-γ ELISA results.

Results

Naturally occurring $CD4^+$ T cells prevent immunotherapy to established tumors

We have previously demonstrated that adoptive cell therapy using either 10^7 naive or activated pmel-1 T cells, rFPVhgp100 vaccination, and exogenous IL-2 could effectively cure established B16 melanoma in wild-type (WT) syngeneic mice (23). This therapy was not dependent on host T or B lymphocytes for its effectiveness. However, because the transfer of a large precursor frequency of $CD8^+$ transgenic T cells can be independent of the effects of $CD4^+$ T cells (27), we evaluated whether host lymphocytes could have a positive or negative effect by transferring a smaller dose of pmel-1 T cells (1.0×10^6/mouse).

To evaluate the impact of host lymphocytes on a $CD8^+$ T cell-mediated adoptive cell therapy, we tested the relative efficacy of treatment in mice with either a selected loss of lymphocyte subsets through genetic knockouts or through whole-body irradiation. RAG-1-deficient (RAG-$1^{-/-}$), $CD4^{-/-}$, $CD8^{-/-}$, or C57BL/6 mice were inoculated with the highly aggressive, poorly immunogenic B16 melanoma and treated i.v. 14 d later with the tripartite treatment regimen comprised of activated pmel-1 T cells ($CD25^+$, $CD44^{high}$, $CD62L^{low}$, $CD69^{high}$), rFPVhgp100, and IL-2 (23). Treatment of B16 melanoma in mice either sublethally irradiated (500 cGy) or on a RAG-$1^{-/-}$ background was markedly enhanced when compared with nonirradiated WT C57BL/6 mice (Fig. 1*a*). Importantly, B16 melanoma grew at the same rate in the no-treatment controls, indicating that the absence of lymphocytes did not alter the growth kinetics of the B16 tumor.

Next, we continued with a genetic dissection of the adaptive immune system by using selective knockout mice for different T cell subsets. In the same experiment, tumor regression was augmented in $CD4^{-/-}$ mice, similar to that seen in RAG-$1^{-/-}$ mice (Fig. 1*b*). Because both RAG-$1^{-/-}$ and $CD4^{-/-}$ do not develop $CD4^+$ T cells, we used our adoptive cell transfer regimen in $CD8^{-/-}$ mice, whose immune system contains $CD4^+$ T cells. As shown in Fig. 1*c*, there was no augmentation of tumor treatment in $CD8^{-/-}$ mice when compared with WT C57BL/6 controls. Tumor regression in MHC class $II^{-/-}$, athymic nude, and SCID mice was also similar to RAG-$1^{-/-}$ mice and $CD4^{-/-}$ mice in the same experiment (data not shown). Therefore, the endogenous $CD4^+$ T cell repertoire is capable of suppressing antitumor immunity to established tumors as demonstrated 20 years earlier (28).

Regression of self-Ag-expressing tumors is independent of homeostatic proliferation

Because it has been reported that homeostatic proliferation of adoptively transferred $CD8^+$ T lymphocytes can protect mice from tumor challenge (29), we evaluated whether or not regression of established B16.F10 tumors (expressing gp100) in RAG-$1^{-/-}$ mice, which also express the gp100 Ag in their skin and eyes, was due to nonspecific activation of $CD8^+$ T cells by adoptive transfer.

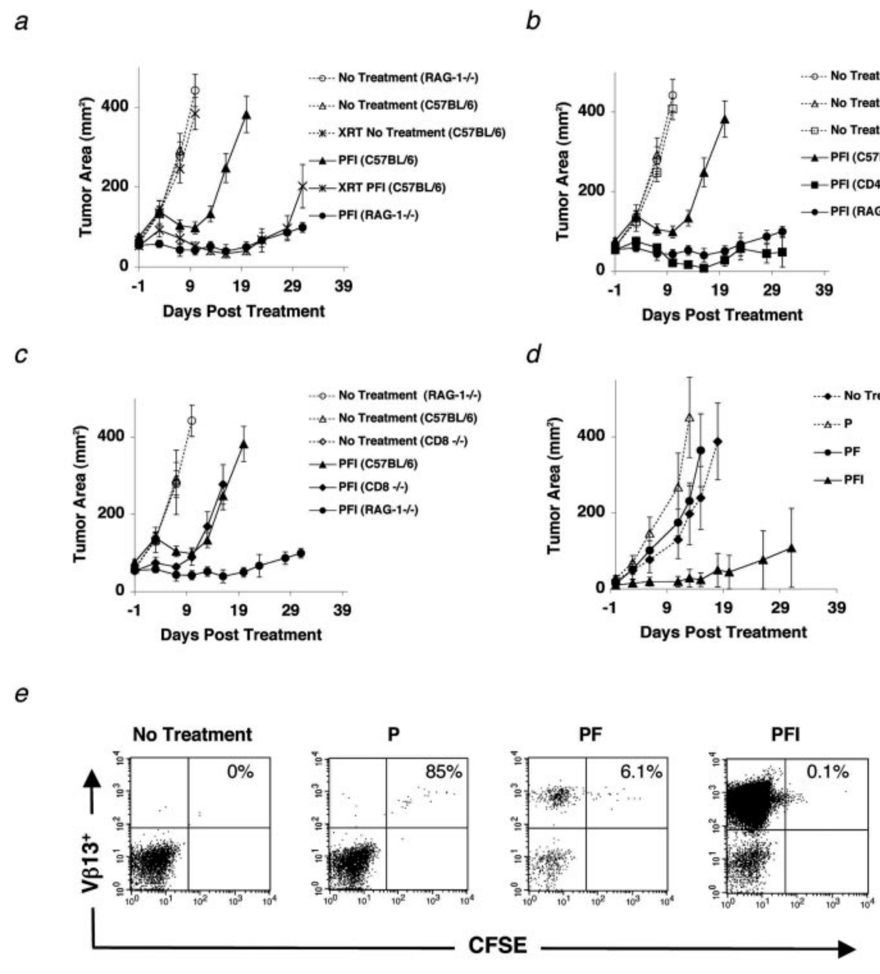

FIGURE 1. Naturally occurring $CD4^+$ T cells inhibit effective immunotherapy to established tumors. *a–d*, Mice were inoculated with 1.0×10^5 cells of B16 melanoma on day −14 before adoptive cell transfer with 1.0×10^6 pmel-1 T cells (P), 2.0×10^7 PFU of rFPVhgp100 (F), and 600,000 IU of exogenous IL-2 (I), which was given daily for 3–4 days. *a*, Tumor regression in C57BL/6 mice (▲) is compared with RAG-$1^{-/-}$ mice (●) and C57BL/6 mice receiving 500 (cGy) whole-body irradiation on day −1 of treatment (*). *b*, Tumor regression in C57BL/6 mice (▲) is compared with RAG-$1^{-/-}$ mice (●) and $CD4^{-/-}$ mice (■). *c*, Tumor regression in C57BL/6 mice (▲) is compared with RAG-$1^{-/-}$ mice (●) and $CD8^{-/-}$ mice (◆) in the same experiment. Data are represented as mean tumor size ± SEM. Experiments were independently repeated twice. *d* and *e*, Tumor regression is IL-2 dependent. *d*, Transfer of pmel-1 T cells alone (△) or pmel-1 T cells and rFPVhgp100 vaccine (●) into tumor-bearing RAG-$1^{-/-}$ hosts is similar to no treatment (◆). Addition of exogenous IL-2 with cells and vaccine is required for tumor regression (▲). *e*, CFSE profile of adoptively transferred pmel-1 T cells into RAG-$1^{-/-}$ hosts. pmel-1 $CD8^+$ T cells were labeled with CFSE and adoptively transferred into tumor-bearing RAG-$1^{-/-}$ hosts alone (P), with vaccination (PF), or with vaccination and exogenous IL-2 (PFI). Four days later, splenocytes from treated mice were analyzed by flow cytometry. Gated on $CD8^+$ T cells and displayed as Vβ13-PE vs CFSE.

In Fig. 1*d*, RAG-$1^{-/-}$ mice bearing established tumors were treated with CFSE-labeled pmel-1 T cells and given rFPVhgp100 vaccine and/or exogenous IL-2. Four days later, splenocytes were isolated from recipient mice and analyzed by flow cytometry. CFSE staining revealed that pmel-1 T cells, designated here as Vβ 13^+, transferred alone (P), divided minimally when compared with mice receiving cells and vaccination (PF), or cells, vaccination, and IL-2 (PFI) (Fig. 1*e*). Surprisingly, even though pmel-1 T cells from mice that received cells and vaccine (PF) had more T cell divisions (Fig. 1*e*) and T cell numbers (Table I) than mice receiving cells alone (P), tumor regression was similar (*d*). Durable tumor regression was only seen in mice receiving pmel-1 T cells, vaccine, and exogenous IL-2 (PFI; Fig. 1*d*). In this group, CFSE staining demonstrated that pmel-1 T cells proliferated extensively (PFI; Fig. 1*e*). The frequency of tumor-reactive pmel-1 T cells as indicated by $CD8^+V\beta\ 13^+$ staining was substantial, when compared with transfer of cells alone (>3000-fold increase; Table I). These results showed that IL-2 not only enhances T cell function in vivo (23) but also increases their T cell numbers. Thus, in this model, enhanced tumor regression (i.e., autoimmunity) seen in RAG-$1^{-/-}$ mice was dependent on exogenous IL-2 administration, not homeostatic proliferation.

T_{reg} cells suppress self-reactive $CD8^+$ T cells in vitro

Because T_{reg} cells can suppress $CD4^+$ and $CD8^+$ T cells (26), we evaluated whether T_{reg} cells could suppress transgenic pmel-1 $CD8^+$ T cells. Therefore, naturally occurring $CD4^+$ T cells were purified from lymph nodes of C57BL/6 mice and fractionated into T_{reg} and Th cells. Sorted T_{reg} and Th cell subsets were separately activated with anti-CD3 and IL-2 as previously described (26) and then cocultured with either anti-CD3-activated (0.5 μg/ml) or peptide-stimulated (1 μM hgp100) pmel-1 T cells. From this assay, we observed a dose-dependent suppression of proliferation (Fig. 2*a*) and IFN-γ production (*b*) when pmel-1 T cells were mixed with T_{reg} cells. By contrast, coculture with Th cells did not suppress proliferation or IFN-γ production. As reported in the literature, similar results were found when T_{reg} cells were cocultured with Th cells (data not shown and Ref. 26). T_{reg} cells cultured alone proliferated minimally (data not shown). These results indicated that T_{reg} cells could profoundly suppress tumor/self-Ag-specific pmel-1 T cells and naturally occurring Th cells in vitro.

Th cells are required to maintain effector self-reactive $CD8^+$ T cells in vivo

We observed that transfer of pmel-1 T cells with or without rFPVhgp100 vaccination into tumor-bearing RAG-$1^{-/-}$ hosts could not induce regression of B16 melanoma, despite the fact that RAG-$1^{-/-}$ hosts lack T_{reg} cells (Fig. 1*d*). As seen in Fig. 1*d*, the addition of exogenous IL-2 was necessary for full therapeutic effectiveness. Therefore, we hypothesized that exogenous IL-2, in this setting, was substituting for a Th cell. Because absence of T cell help can hinder the in vivo maintenance of $CD8^+$ T cells and development of memory T cells (4, 6, 7, 30), we surmised that effector $CD8^+$ T cells also needed help to induce the regression of established tumors, in addition to removal of T_{reg} cells.

Table I. *Absolute number of pmel-1 $CD8^+$ T cells after transfer into RAG-1-deficient hosts*

Treatment Group	Absolute No. ($\times 10^3$)	±SEM ($\times 10^3$)	Fold Increase
No treatment	0	0	0
Pmel-1 T cells only (P)	7	0.3	1
Pmel-1 T cells and vaccine (PF)	393	19	55
Pmel-1 T cells, vaccine, and IL-2 (PFI)	26,913	780	3,753

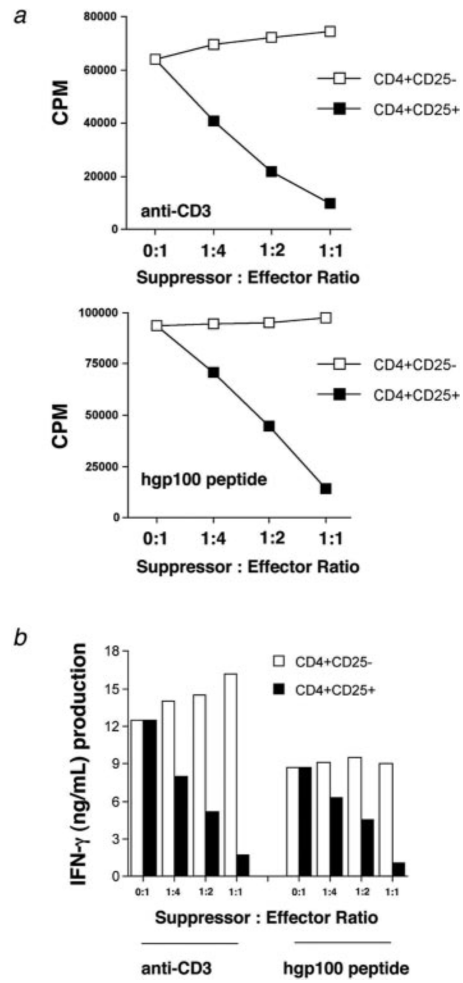

FIGURE 2. T_{reg} cells suppress pmel-1 $CD8^+$ T cell proliferation and function in vitro. *a* and *b*, Highly purified T_{reg} cells from C57BL/6 lymph nodes suppress proliferation (*a*) and IFN-γ production (*b*) of pmel-1 T cells in vitro. T_{reg} or Th cells were activated with irradiated APC (1:1 ratio) and soluble anti-CD3 Ab (0.5 μg/ml) for 3 days in the presence of 100 IU of human IL-2. Cells were then split and maintained for an additional 7–14 days in 100 IU of human IL-2. *a*, pmel-1 T cells were stimulated with soluble anti-CD3 or 1 μM $hgp100_{25-33}$ peptide-pulsed T-depleted spleen cells in the presence of activated T_{reg} (■) or Th (□) cells for 3 days in the absence of IL-2 at the indicated ratios. Maximum suppression of pmel-1 T cell proliferation is seen at a 1:1 suppressor-to-effector ratio. *b*, pmel-1 T cells were stimulated with anti-CD3 or 1 μM $hgp100_{25-33}$ peptide-pulsed TΔS cells in the presence of activated T_{reg} (■) or Th (□) cells for 3 days in the absence of IL-2 at the indicated ratios. Maximal suppression of IFN-γ production by pmel-1 T cells for both modes of stimulation is seen at a 1:1 suppressor-to-effector ratio. Experiments were independently repeated twice.

To test whether the transfer of $CD4^+$ T cells might replace the requirement for exogenous IL-2 and help pmel-1 T cells eradicate tumors, we transferred unfractionated $CD4^+$ T cells and sorted $CD4^+CD25^-$ T cells with pmel-1 T cells and rFPVhgp100 vaccine into tumor-bearing RAG-$1^{-/-}$ hosts. The combination of pmel-1 T cells, vaccine, and $CD4^+CD25^-$ T cells induced tumor regression and long-term survival without exogenous IL-2 (Fig. 3, *a* and *b*), whereas no or minimal therapeutic effect was seen with unfractionated $CD4^+$ T cells (*a*). Furthermore, adoptive transfer of $CD4^+CD25^-$ T cells alone or in combination with pmel-1 cells

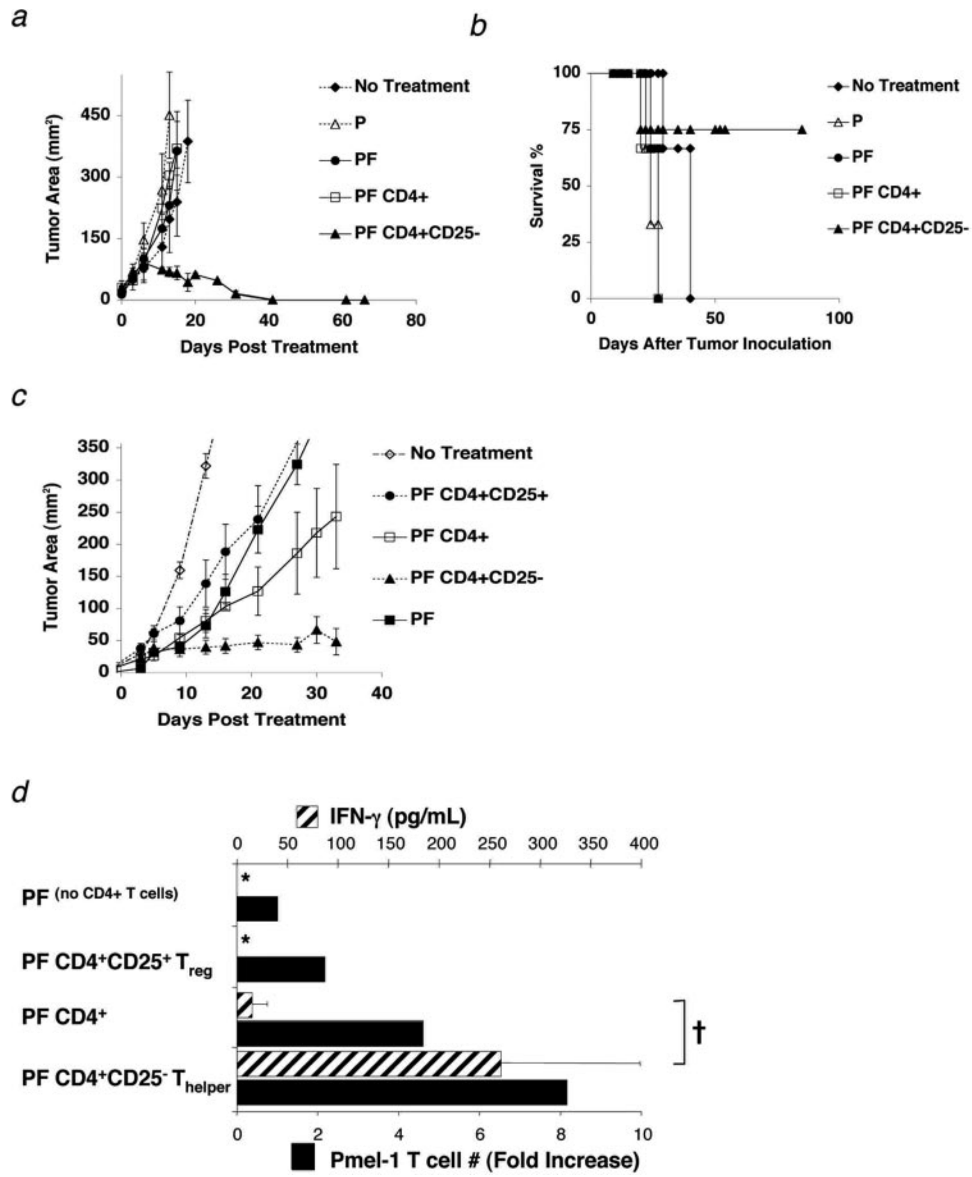

FIGURE 3. Th cells maintain self-reactive effector $CD8^+$ T cells in vivo. *a–d*, RAG-$1^{-/-}$ mice were inoculated with 3.0×10^5 cells of B16 melanoma between day −7 and −14 before adoptive cell transfer. *a*, Th cells help $CD8^+$ T cell-mediated antitumor immunity to established B16 melanoma. Mice receiving 1.0×10^6 pmel-1 T cells (P), 2.0×10^7 PFU of fowlpox virus encoding human gp100 (F), and 1.0×10^6 Th cells maintained long-term, durable regression of established B16 melanoma (▲). Data are represented as mean tumor size ± SEM. Data represent six independent experiments. *b*, Survival of mice in *a* treated with pmel-1 T cells (P), rFPVhgp100 vaccine (F), and Th cells was maintained up to 80 days posttreatment (▲). *c*, Adoptive cell transfer of sorted 1.0×10^5 T_{reg} cells (●) or a mixture of 1.0×10^5 T_{reg} and 1.0×10^6 Th cells (1:10 ratio; $CD4^+$ T cells; □) with pmel-1 T cells and vaccination does not maintain tumor regression of B16 melanoma. Removal of the T_{reg} subset from the unfractionated $CD4^+$ T cell pool allows the helper function of the remaining Th cells to become apparent (▲). Data represent five independent experiments. Experiment in *c* was stopped at 35 days posttreatment to allow for analysis of adoptively transferred pmel-1 T cells. *d*, Th cells maintain maximal pmel-1 T cell numbers (■) and function (▨) in the absence of IL-2. †, $p < 0.037$. Proliferation bar graphs represent fold increase in the absolute number of pmel-1 T cells (% $CD8^+Thy1.1^+V\beta\,13^+$ T cells × splenocyte count) taken from pooled spleens 5 wk after transfer ($n = 2$). Fold increase is defined as follows: absolute no. of pmel-T cells in a group divided by absolute no. of pmel-1 T cells from the group that received no $CD4^+$ T cells (PF). For all functional assays, pmel-1 $Thy1.1^+CD8^+$ T cells were purified from spleens of treated mice 5 wk after transfer and stimulated with 1 μM $hgp100_{25–33}$ peptide-pulsed gamma-irradiated spleen cells for 24 h ($n = 2$). All groups were also tested against non-peptide-pulsed targets, which resulted in no production of IFN-γ (data not shown). Data are represented as IFN-γ (picograms per milliliter) ± SEM. Experiments were repeated twice. *, Undetectable value.

without vaccine did not induce durable and stable tumor regression, showing the requirement for vaccination in this treatment model (data not shown).

Although in Fig. 3*a* there was no tumor regression observed when unfractionated $CD4^+$ T cells were used, we found in repeated experiments that the transfer of unfractionated $CD4^+$ T cells had a variable effect on tumor regression; ranging from negligible (Fig. 3*a*) to a modest suppression of tumor growth (*c*). We found in sorted $CD4^+$ T cell preparations that the $CD4^+CD25^+$ T cell population varied from 2 to 13% (data not shown). Therefore, we hypothesized that the variability of the antitumor responses was due to the relative percentages of regulatory and helper T cell subsets. To solve the variability between these subsets, we prepared T_{reg} and Th cells from a common pool of $CD4^+$ T cells or from different congenic strains ($CD4^+CD25^+CD45.1^+$ or $CD4^+CD25^-CD45.2^+$ T cells) and fixed the ratio at 1:10 (T_{reg}:

Th) for subsequent experiments, which is the accepted physiological ratio in vivo (13). This ratio was verified in vivo by flow cytometry (data not shown).

Adoptive transfer of a mixture of T_{reg} and Th cells at a 1:10 ratio resulted in tumor growth that was similar to transfer of unfractionated $CD4^+$ T cells that had a comparable ratio (Fig. 3*c* and data not shown). As seen in Fig. 3*a*, the transfer of sorted Th cells (1.0×10^6) with pmel-1 T cells and rFPVhgp100 vaccine resulted in durable and stable tumor regression (*c*). However, transfer of sorted T_{reg} cells (1.0×10^5) with pmel-1 T cells and vaccine resulted in minimal tumor regression that was similar to pmel-1 T cells and vaccination alone (Fig. 3*c*).

To understand the differences between the treatment groups, we analyzed the impact of these $CD4^+$ T cell subpopulations on the persistence and function of adoptively transferred pmel-1 T cells. By using a congenic marker system ($Thy1.1^+Thy1.2^+$), we were able to measure the persistence of $Thy1.1^+$ pmel-1 T cells 5 wk after vaccination. We found that the absolute numbers of pmel-1 T cells was enhanced by cotransfer with Th cells, but not with a mixture of T_{reg}:Th (1:10 ratio) (Fig. 3*d*). Function of sorted $Thy1.1^+$ pmel-1 T cells as measured by IFN-γ ELISA after peptide stimulation (1 μM hgp100) ex vivo was significantly enhanced with the addition of Th cells (Fig. 3*d*; †, $p = 0.037$).

Next, pmel-1 T cells were taken from mice treated with T_{reg} cells and analyzed ex vivo for T cell numbers and IFN-γ secretion. We found that pmel-1 T cell numbers were decreased ~4-fold when compared with Th cells and function was similar to the transfer of no $CD4^+$ T cells as measured by IFN-γ ELISA (Fig. 3*d*). Suppression could only be observed when T_{reg} cells were cotransferred with the Th population, which contained ~90% $CD4^+CD25^-$ T cells (Fig. 3*d*; †, $p = 0.037$). Thus, T_{reg} cells suppressed adoptive immunotherapy of an established tumor, but Th cells improved tumor immunotherapy by maintaining the functionality of tumor/self-reactive $CD8^+$ T cells.

Th cells enhance exogenous IL-2 therapy

In the current report, we noticed that lower dosages of pmel-1 T cells (10^6/mouse) could induce stable tumor regression when given in combination with vaccination and Th cells but not with exogenous IL-2 (Fig. 4*a*). IL-2 treatment induced transient tumor regression that lasted for ~20 days after adoptive transfer. However, in the presence of Th cells, IL-2 therapy was enhanced and induced stable tumor regression beyond 20 days (Fig. 4*a*).

Next, to understand how IL-2 and Th cells affected the adoptive immunotherapy differently, we analyzed the function of pmel-1 $CD8^+$ T cells cotransferred with vaccination, exogenous IL-2, and/or Th cells ex vivo beyond 20 days after transfer (wk 5). We found that the cotransfer of Th cells resulted in a significant increase in pmel-1 T cell function that was maintained in vivo, as measured by IFN-γ ELISA (PFI $CD25^-$; ‡, $p < 0.001$; Fig. 4*b*).

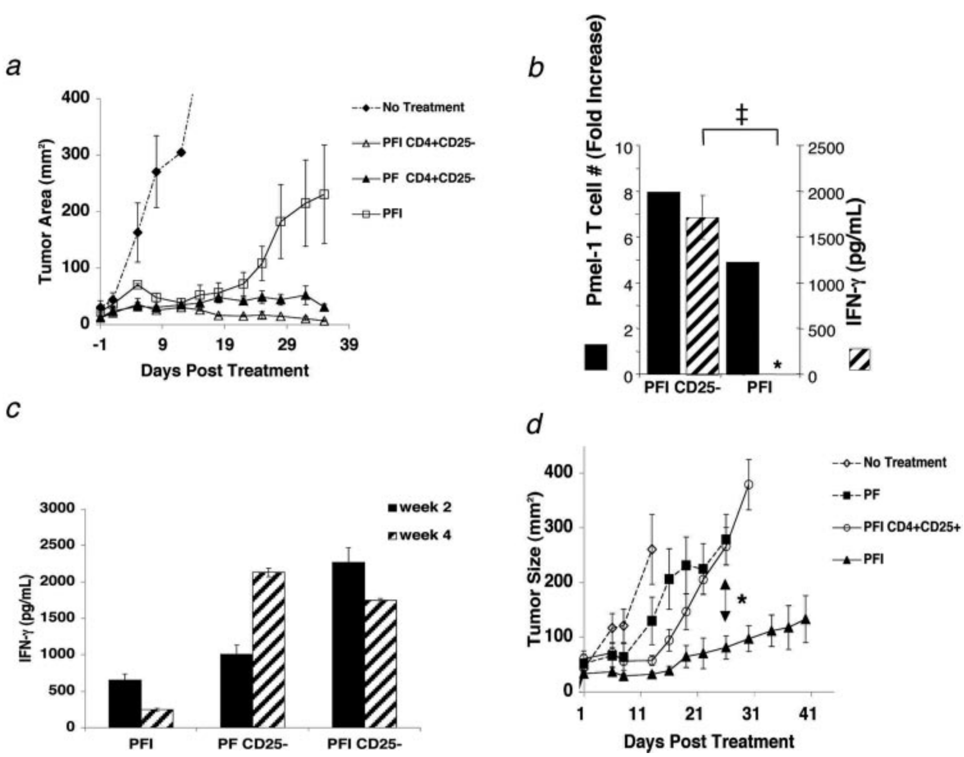

FIGURE 4. Th cells can replace exogenous IL-2 and maintain function of tumor-reactive $CD8^+$ T cells, but exogenous IL-2 therapy fails in the presence of T_{reg} cells. *a*, The combination of 1.0×10^6 pmel-1 T cells, 2.0×10^7 PFU of rFPVhgp100 vaccination, and exogenous IL-2 (600,000 IU) given daily for 3 days in RAG-$1^{-/-}$ hosts enhances but does not maintain tumor regression (□). Only cotransfer of 1.0×10^6 Th cells with pmel-1 T cells and vaccination into RAG-$1^{-/-}$ hosts helped maintain tumor regression with (△) or without (▲) exogenous IL-2. Data represent three independent experiments. *b*, Exogenous IL-2 does not maintain function (▨) of pmel-1 T cells unless given in combination with Th cells (PFI $CD4^+CD25^-$ vs PFI; ‡, $p = 0.001$). pmel-1 T cell absolute numbers were increased ~2-fold in the presence of Th cells (■). Fold increase is defined as follows: absolute no. of pmel-T cells in a group divided by absolute no. of pmel-1 T cells from the group that received no $CD4^+$ T cells (PF). *, Undetectable value. *c*, Function of adoptively transferred pmel-1 T cells 2 wk (■) and 4 wk (▨) after transfer. Function (IFN-γ (picograms per milliliter) declines with time unless Th cells are also transferred: PFI vs PF $CD25^-$ or PFI $CD25^-$. *d*, Activated pmel-1 $CD8^+$ T cells ($CD25^+$, $CD44^{high}$, $CD62L^{low}$, $CD69^{high}$; 1.0×10^6) were transferred with 1.0×10^5 sorted T_{reg} cells, rFPVhgp100 vaccination (2.0×10^7 PFU), and exogenous IL-2 on day 7 after tumor inoculation. $CD8^+$ T cells required vaccine and IL-2 for tumor treatment (▲). T_{reg} cells inhibited tumor treatment by effector $CD8^+$ T cells in the presence of exogenous IL-2 (○), and treatment was similar to groups receiving no exogenous IL-2 (■). Experiments repeated independently three times.

Surprisingly, the provision of pmel-1 T cells, vaccine, and exogenous IL-2 in the absence of Th cells was insufficient to maintain long-term $CD8^+$ T cell function (5 wk after transfer) (PFI; Fig. 4*b*). A more detailed analysis revealed that T cell function goes down with time in the absence of Th cells (Fig. 4*c*). Thus, adoptive cell transfer of Th cells could replace and/or enhance exogenous IL-2 therapy of established tumors by maintaining the effector function and numbers of adoptively transferred $CD8^+$ T cells.

Exogenous IL-2 therapy fails in the presence of T_{reg} cells

Next, we investigated the effects of exogenous IL-2 on both $CD8^+$ T cells and T_{reg} cells together in vivo. We transferred effector ($CD25^+$, $CD44^{high}$, $CD62L^{low}$, $CD69^{high}$) pmel-1 T cells along with T_{reg} cells into tumor-bearing RAG-$1^{-/-}$ mice. Vaccination with rFPVhgp100 and exogenous IL-2 were also administered and tumor size was monitored for 41 days. Again, as shown earlier, pmel-1 T cells, vaccination with rFPVhgp100, and exogenous IL-2 were required for treatment of established tumors (Fig. 4*d*). However, IL-2 therapy failed when activated pmel-1 T cells were adoptively transferred with T_{reg} cells. These results were similar to groups that received cells and vaccine alone (*, $p < 0.007$; Fig. 4*d*) and were similar to groups that had endogenous T_{reg} cells (C57BL/6 and $CD8^{-/-}$ mice; Fig. 1*c*). Thus, exogenous IL-2 therapy was effective only when T_{reg} cells were absent.

T cell help is IL-2 dependent, not programmed, and lost in the presence of T_{reg} cells

We showed earlier that treatment was either IL-2 dependent or required Th cells to be effective. Therefore, we hypothesized that tumor regression observed following adoptive transfer of Th cells was the result of IL-2 production by the Th cell population. To be able to test this hypothesis, we derived Th cells from IL-$2^{-/-}$ mice. However, because precursor T_{reg} cells may be resident in IL-$2^{-/-}$ mice (31) (Fig. 5*a*), we transferred sorted Th cells from IL-$2^{-/-}$ mice together with pmel-1 T cells and vaccine into tumor-bearing RAG-$1^{-/-}$ hosts. In more than five independent experiments, we did not observe stable tumor regression using Th cells derived from IL-$2^{-/-}$ mice, whereas sorted Th cells from IL-$2^{+/+}$ mice effectively enhanced tumor regression (Fig. 5*b*).

In an attempt to understand the kinetics of IL-2 dependency in this system, we gave exogenous IL-2 for 4 days after treatment together with Th cells derived from IL-$2^{-/-}$ mice. Initially, we observed tumor regression, but this regression was not maintained (Fig. 5*c*; *, $p = 0.021$).

To assess whether Th cells could program $CD8^+$ T cells to treat established tumors, we transferred Th cells for 4 days and then depleted with injection of 500 μg of $CD4^+$ T cell-depleting mAb (GK1.5; Fig. 5*d*), which was confirmed by flow cytometry (data not shown). Tumor treatment in mice receiving depleting mAb was similar to mice that had received no Th cells or exogenous IL-2 (Fig. 5*d*) or Th cells derived from IL-$2^{-/-}$ mice (*b*). Isotype control Ab had no effect on adoptively transferred Th cells in vivo (data not shown).

Next, the expression of CD25 on transferred $CD4^+$ T cells was determined. IL-2 up-regulates its own receptor expression (32). Therefore, to determine whether the major source of IL-2 was from the transferred Th cells or from the host, $CD4^+$ T cells were analyzed by flow cytometry 35 days after transfer into tumor-bearing RAG-$1^{-/-}$ hosts. Before transfer, CD25 expression was between 5 and 10% as expected for whole $CD4^+$ T cells (Fig. 5*e*). Sorted Th cells had 0.23% CD25 expression and Th cells from IL-$2^{-/-}$ mice had 0.8% CD25 expression (Fig. 5*e*). After 35 days in vivo, whole $CD4^+$ T cells and sorted Th cells up-regulated their receptor (Fig. 5*e*). Th cells from IL-$2^{-/-}$ mice also up-regulated their receptor, but at a much lower level (4-fold less) (Fig. 5*e*), indicating that IL-2 mainly comes from transferred activated Th cells.

Next, we looked at the persistence of tumor-reactive $CD8^+$ T cells after adoptive transfer with different $CD4^+$ T cell subsets by flow cytometry 3 wk after treatment. As shown in Fig. 5*f*, $Thy1.1^+$ pmel-1 T cells required the presence of Th cells to persist. A 10-fold reduction in $Thy1.1^+$ pmel-1 T cell frequency was seen in groups that received T_{reg} cells and Th cells at a 1:10 ratio when compared with groups receiving pmel-1 T cells and Th cells alone. The same reduction in $Thy1.1^+$ pmel-1 T cell frequency was seen in groups that received Th cells from IL-$2^{-/-}$ mice or no Th cells. Persistence of $Thy1.1^+$ pmel-1 T cells was even more depressed in groups receiving only T_{reg} cells, a ~30-fold reduction when compared with the Th cell group. As a comparison, absolute number of pmel-1 $CD8^+$ T cells was also calculated for the same experiment (Fig. 5*g*). Function, as measured by intracellular IFN-γ, of adoptively transferred pmel-1 T cells with Th cells was also assessed and shown to be suppressed when T_{reg} cells were cotransferred at 1:10 ratio, or when Th cells were derived from IL-$2^{-/-}$ mice (Fig. 5*h*). Thus, these results highlight that IL-2 from Th cells was essential for the induction of antitumor immunity to a self-Ag, and this effect was lost in the presence of T_{reg} cells.

Breakdown of tolerance to the gp100 self-Ag is IL-2 dependent

We noticed mice treated with pmel-1 T cells, vaccine, and IL-2 or Th cells developed profound autoimmune vitiligo following 5 wk after adoptive cell transfer. This vitiligo usually started periorbitally and spread in a random fashion as shown in Fig. 6*a* ($n = 26$). Conversely, limited or no autoimmune vitiligo was seen in mice that did not receive exogenous IL-2 ($n = 25$) or received Th cells derived from IL-$2^{-/-}$ mice ($n = 25$).

Because the eyes of C57BL/6 mice also express the gp100 tumor/self-Ag (33), we evaluated the requirement for IL-2 production in the destruction of normal eye tissue. We looked for the induction of autoimmunity in the eye, as evidenced by uveitis. We found in repeated experiments that exogenous IL-2 caused significant uveitis (10-fold increase) when compared with no exogenous IL-2 treatment (PFI vs PF; *, $p < 0.05$; Fig. 6*b*; and data not shown). We also found that the addition of Th cells induced uveitis that was similar to groups receiving IL-2 (PF $CD25^-$ IL-$2^{+/+}$ vs PFI; **, $p > 0.05$; Fig. 6*b*). Importantly, as seen with autoimmune vitiligo, no uveitis was observed when Th cells were derived from IL-$2^{-/-}$ mice (PF $CD25^-$ IL-$2^{-/-}$; Fig. 6*b*; §, $p < 0.05$). Together, these results indicated that naturally occurring Th cells facilitated the induction of tumor regression and autoimmunity against a tumor/self-Ag through an IL-2-dependent mechanism.

Discussion

A fundamental question unanswered in immunology is how to raise T cell help against a persisting self-Ag, which subsequently results in the breakdown of self-tolerance (2). We describe here the requirements for the initiation of autoimmunity and thus the induction of antitumor immunity to established tumors expressing the gp100 melanocyte differentiation Ag, an Ag also expressed in the skin and eyes of C57BL/6 mice (33).

Recently, depletion of T_{reg} cells has been shown to augment reactivity to tumor/self-Ags in tumor prevention models (19, 20, 34, 35), but we show for the first time that T_{reg} cells can inhibit help of self-reactive $CD4^+$ T cells and prevent effector $CD8^+$ T cells from initiating autoimmunity. T_{reg} cells control peripheral self-tolerance through yet-unknown mechanisms, but we believe that progressively growing tumors shed or secrete self-Ags that subsequently activate naturally occurring T_{reg} cells (9–11, 36). Although depletion of T_{reg} cells enhances tumor protection in

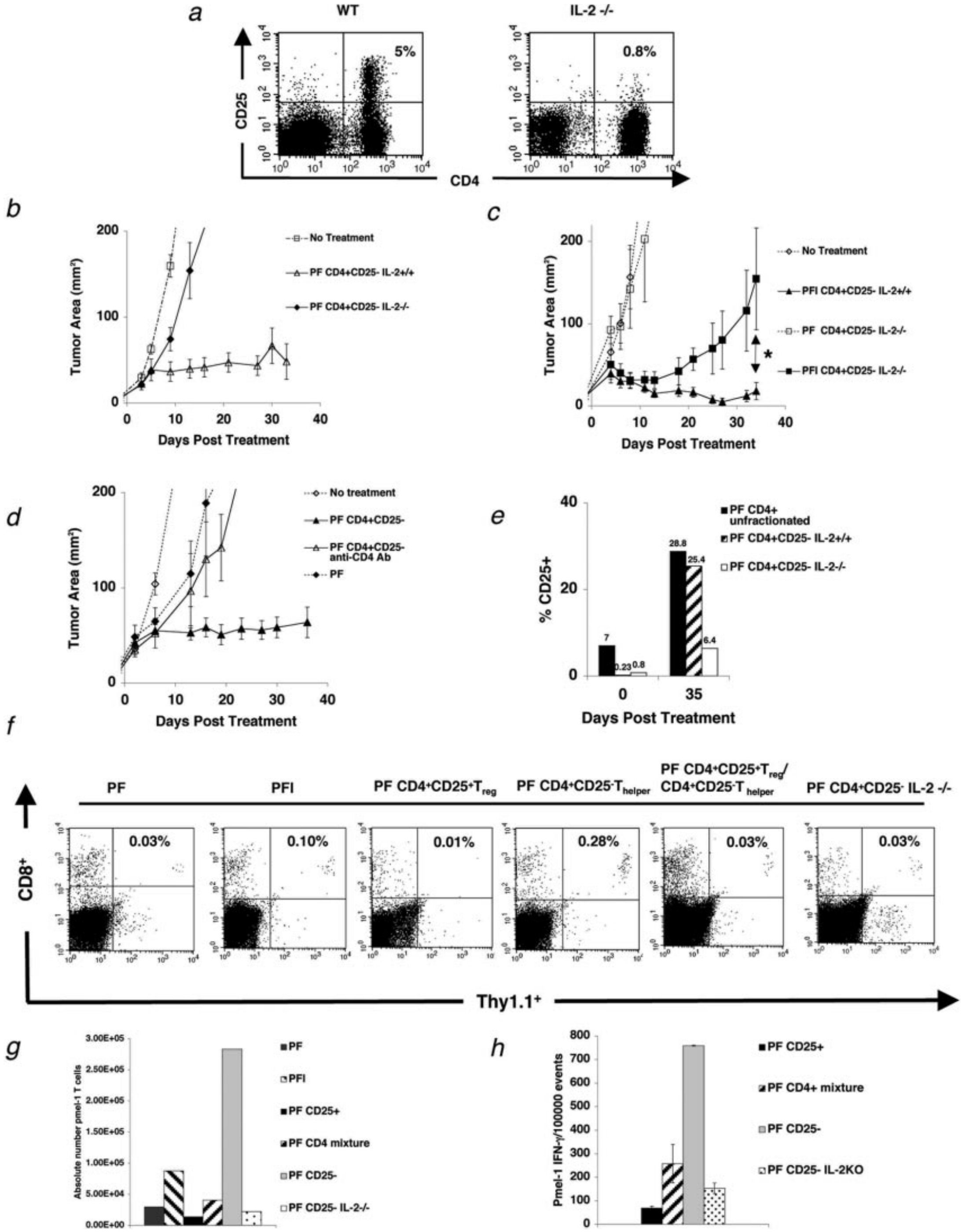

FIGURE 5. T cell help is IL-2 dependent and lost in the presence of T_{reg} cells. *a*, Flow cytometry analysis of mouse splenocytes shows that IL-$2^{-/-}$ mice do not develop T_{reg} cells ($n = 3$). *b* and *c*, RAG-$1^{-/-}$ mice were inoculated with 3.0×10^5 cells of B16 melanoma between day -7 before adoptive cell transfer with 1.0×10^6 pmel-1 T cells (P), 2.0×10^7 PFU rFPVhgp100 (F), plus Th cells from IL-$2^{-/-}$ mice or naturally occurring Th cells plus/minus exogenous IL-2 (I). *b*, Transfer of Th cells from IL-$2^{-/-}$ mice with pmel-1 T cells and vaccination into tumor-bearing RAG-$1^{-/-}$ hosts does not help treatment of established B16 melanoma (◆). *c*, Addition of exogenous IL-2 does not restore the helper function of Th cells from IL-$2^{-/-}$ mice (■). *, $p = 0.021$. Data are derived from a single experiment that was independently repeated three times. *d*, Th cells do not program tumor-reactive $CD8^+$ T cells. Depletion of Th cells 4 days after transfer with 500 μg of GK1.5 CD4-depleting mAb (△). Data represent three independent experiments with similar results. Isotype control Ab had no effect on $CD4^+$ T cells and depletion of $CD4^+$ T cells was confirmed by flow cytometry. *e*, Th cells use IL-2 in vivo. CD25 expression on adoptively transferred Th cells alone, Th cells with T_{reg} ($CD4^+$ unfractionated), and Th cells derived from IL-$2^{-/-}$ mice, 35 days after treatment. *f*, Spleens were taken from tumor-bearing RAG-$1^{-/-}$ mice and analyzed by flow cytometry for the congenic marker Thy1.1 and CD8, which represents the transferred pmel-1 T cells 3 wk after treatment with the indicated regimen. Two mice were used per group. Data are indicative of three independent experiments. *g*, Absolute number of pmel-1 T cells 3 wk after transfer from the same experiment in *f*. *h*, Intracellular IFN-γ 3 wk after adoptive cell therapy. Cells were activated with lymphocyte-activating kit and analyzed by flow cytometry 6 h later. Two mice were analyzed per group. Data are indicative of three independent experiments.

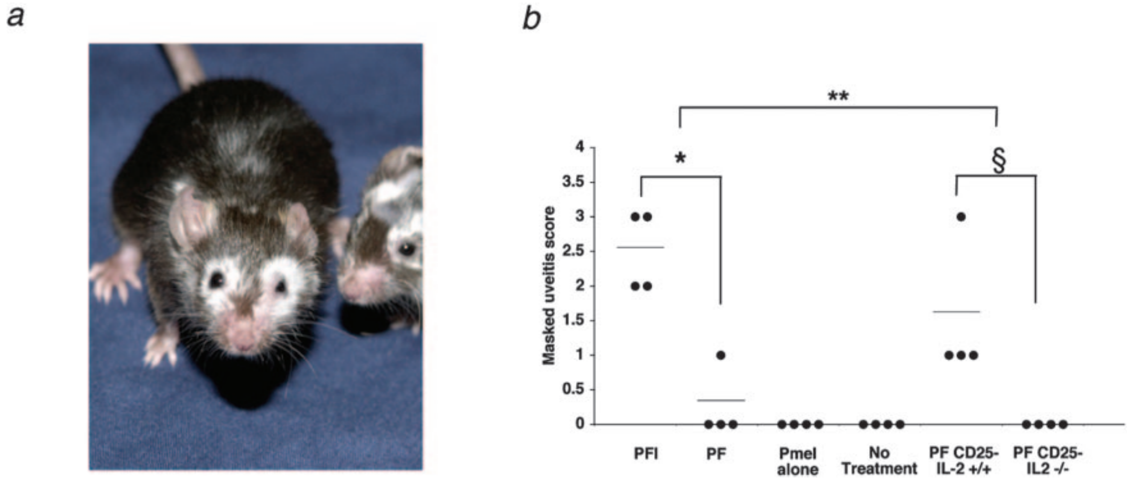

FIGURE 6. Breakdown of self-tolerance to the gp100 Ag requires IL-2. *a*, Mice receiving pmel-1 T cells, rFPVhgp100 vaccination, and Th cells develop autoimmune vitiligo that spreads in an unpredictable fashion 5 wk after adoptive cell transfer. Mice receiving pmel-1 T cells, rFPVhgp100 vaccination, and exogenous IL-2 also develop vitiligo (as shown in Ref. 23). Two representative mice receiving pmel-1 T cells, rFPVhgp100, and Th cells are shown (n = 25). *b*, Uveitis during immunotherapy of B16 melanoma is only present with the administration of pmel-1 T cells, rFPVhgp100 vaccine, and exogenous IL-2, ∗, $p < 0.05$, or with cotransfer of Th cells without exogenous IL-2; §, $p < 0.05$. Uveitis is not observed in mice receiving pmel-1 T cells, rFPVhgp100 vaccine, and Th cells derived from IL-2$^{-/-}$ mice. Uveitis in the eyes of treated mice was scored as follows: 0 = none; 1 = mild; 2 = moderate; 3 = severe. Data represent two independent experiments.

many models using artificial self-Ags, we show that complete absence of T$_{reg}$ cells is not enough for treatment of established tumors against a self-Ag when using adoptive immunotherapy (37). Even in the absence of T$_{reg}$ cells, treatment still required CD8$^+$ T cells, vaccination, and some type of help either provided through exogenous cytokines or through Th cells. Therefore, we suspect as with acute infections (6) that ongoing tumor regression will need continuous T cell help to eradicate established tumors, in addition to removing T$_{reg}$ cells, because Th cells were unable to program self-reactive CD8$^+$ T cells.

Because self-Ags can activate T regulatory cells (38), their role may have been overlooked in artificial systems modeling self-Ags (36). The modeling of self-reactivity is likely to be important in the development of new immunotherapies that target tumor/self-Ags. Many of the currently available tumor models show the complete destruction of established tumors targeting "foreign" Ags and represent tumors given for only a short period of time (8, 39–41). These models have shed valuable light on basic immunologic principles, but it is unclear to what extent the results obtained from these models reflect immune responses against true self/tumor Ags (42). The present study was designed to elucidate the requirements for raising help to a tumor Ag that was also expressed in normal tissues, a situation that models the clinical scenario in patients with cancer.

We found that CD8$^+$ T cell-mediated immunotherapy and vaccination was ineffective in CD4$^+$ T cell-deficient hosts unless given in combination with IL-2, and this effect was dramatically diminished in the presence of T$_{reg}$ cells. Additionally, we showed that Th cells were superior to exogenous IL-2 therapy, but that cotransfer of T$_{reg}$ cells also inhibited this effect. Importantly, Th cells derived from IL-2$^{-/-}$ mice contributed to neither antitumor immunity nor autoimmunity, even in mice lacking T$_{reg}$ cells. Altogether, these findings show the importance of Th cell-derived IL-2 in the help of CD8$^+$ T cells in vivo.

Next, we showed that adoptive cell therapy with Th cells or exogenous IL-2 failed in the presence of T$_{reg}$ cells. Because T$_{reg}$ cells constitutively express the high affinity IL-2R, it is plausible that T$_{reg}$ cells may be preferentially using IL-2 as shown in vitro (43), because they require IL-2 for their function and maintenance in vivo (44). It is also feasible that T$_{reg}$ cells suppress CD8$^+$ T cells or CD4$^+$ T cells by decreasing their access to IL-2 either by suppressing the production of IL-2 by Th cells (45, 46), or decreasing the surface expression of the IL-2R (28). Alternatively, T$_{reg}$ cells may condition the APC toward tolerance (47). Thus, the durable induction of an antitumor (anti-self) response by Th cells may be dependent on their continuous production of IL-2, which is lost in the presence of T$_{reg}$ cells constitutively expressing high-affinity IL-2R. Whether Th cells that become CD25$^+$ T cells are bona fide Foxp3-expressing T$_{reg}$ cells is unknown in this model, but a recent paper suggests that this may be the case during expansion in a lymphopenic environment (48). Regardless, we still see maintenance of tumor regression, and therefore, a converted Th to an induced T$_{reg}$ cell may not play a role during treatment of established tumors.

It has been argued that Th cells from IL-2$^{-/-}$ mice have a secondary deficiency that diminishes their helper effect. However, it has been reported in the literature that T cell ontogeny and function in IL-2$^{-/-}$ mice is not affected (49, 50). Furthermore, mixed bone marrow chimeras of IL-2$^{-/-}$ and CD25$^{-/-}$ cells used to reconstitute lethally irradiated hosts resulted in normal T cell homeostasis and engraftment of a stable T$_{reg}$ population (51). In addition, transfer of T$_{reg}$ cells into CD25$^{-/-}$ mice, which have the same phenotype as IL-2$^{-/-}$ mice, led to recoverable levels of T$_{reg}$ cells and suppression of autoimmune disease, because CD25$^{-/-}$ mice still have a cellular source of IL-2 (21). However, transfer of T$_{reg}$ cells into IL-2$^{-/-}$ mice did not prevent autoimmune disease (44). Taken together, these findings show that the main deficiency in IL-2$^{-/-}$ mice is the complete absence of functional T$_{reg}$ cells and not an intrinsic functional T cell defect (44). Lack of T$_{reg}$ cells, due to absence of IL-2 signaling, leads to uncontrolled CD4$^+$ T cell proliferation and activation, which paradoxically is not dependent on IL-2 (15, 21, 31, 51)

However, even though IL-2$^{-/-}$ mice get autoimmune disease, adoptive transfer of IL-2-deficient Th cells was unable to help CD8$^+$ T cells treat an established tumor or cause autoimmunity in IL-2$^{+/+}$ mice. Autoimmunity in our model is dependent on IL-2 production, whereas in IL-2$^{-/-}$ mice, it is independent of IL-2 (15). This is an important finding because it shows a disparity between how these two types of autoimmunity can manifest. Most importantly, it shows the risk of using self-Ags to immunize against self-Ag-expressing tumors (52, 53).

These findings also point to the deficiencies in the use of high-dose exogenous IL-2 in cancer clinical trials (54). Already known for its toxicity, another danger inherent in the administration of exogenous IL-2 may be the induction of T_{reg} cell function (21, 44, 46, 55). Thus, depletion of T_{reg} cells with either ONTAK (56) or another method before adoptive cell transfer may enable unencumbered delivery of IL-2 by Th cells to tumor-reactive T cells or to Th cells themselves.

A key feature of this immunotherapy regimen is that Th cells are derived from WT mice, obviating the need for the development of Th cells with specificities for tumor Ags a priori. The identities of the Ags recognized by Th cells in this setting remains of considerable interest because isolation of tumor-reactive Th cells can lead to more effective class II-restricted vaccines (57). However, exactly what the requirements of T cell help are in vivo are still being debated (2), but we report here that IL-2 plays an important role in the breakdown of self-tolerance to a persisting Ag. Whether IL-2 is acting on $CD8^+$ T cells or Th cells or both is unknown (2). It is possible that IL-2 secreted by Th cells leads to downstream events that participate in T cell help, such as release of other cytokines or activation of costimulatory molecules, which license the APC to initiate help of $CD8^+$ T cells. Thus, exogenous IL-2 therapy may lose these contributions by Th cells when used alone. However, whatever the mechanism of help in vivo, transfer of naturally occurring Th cells in combination with tumor-reactive $CD8^+$ T cells plus vaccination represents a clinically feasible approach to the immunotherapy of established, progressing tumors in humans, because isolation of tumor-reactive $CD4^+$ T cells has been difficult.

One currently used and approved immunotherapeutic approach in humans involves lymphodepletion before adoptive transfer (58). The immune-enhancing effects of lymphodepletion can be accomplished through irradiation, chemoablation (27, 58), or through genetic means as demonstrated here. The mechanisms of how lymphodepletion enhances adoptive immunotherapy remain incompletely understood (59), but our data suggest that the removal of T_{reg} cells is a major contributing factor. However, as shown here, removing T_{reg} cells is not enough to treat established tumors, T cell help must be provided. The mechanisms of lymphodepletion are multifactorial because antitumor immunotherapies in $CD4^+$ T cell-deficient mice can be further enhanced with total body gamma irradiation (data not shown). As has been shown in other systems (60), the increased availability of homeostatic γ_c-signaling cytokines such as IL-7, IL-15, or IL-21 could be enhancing T cell function in this model (24, 61, 62).

Nevertheless, we show here that naturally occurring Th cells can initiate autoimmunity and tumor regression in an environment of persisting self-Ag through self-reactive $CD8^+$ T cells, and that naturally occurring T_{reg} cells represent a formidable barrier to the breakdown of self-tolerance. Therefore, the future of immunotherapy against self-Ags will rely on ways of removing this population and augmenting T cell help of tumor-reactive T cells or tumor-infiltrating lymphocytes (54) isolated from patients. Together, these findings form a new approach for studying T cell help and suppression in vivo against self-Ags and form the basis of a new treatment for many types of cancers expressing self-Ags and chronic persisting infections.

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Disclosures

The authors have no financial conflict of interest.

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