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Recurrent solid malignancies are often refractory to standard therapies. Although adoptive T cell transfer may benefit select individuals, the majority of patients succumb to their disease. To address this important clinical dilemma, we developed a mouse melanoma model in which initial regression of advanced disease was followed by tumor recurrence. During recurrence, Foxp3⁺ tumor-specific CD4⁺ T cells became PD-1⁺ and represented >60% of the tumor-specific CD4⁺ T cells in the host. Concomitantly, tumor-specific CD4⁺ T effector cells showed traits of chronic exhaustion, as evidenced by their high expression of the PD-1, TIM-3, 2B4, TIGIT, and LAG-3 inhibitory molecules. Although blockade of the PD-1/PD-L1 pathway with anti-PD-L1 Abs or depletion of tumor-specific regulatory T cells (Tregs) alone failed to reverse tumor recurrence, the combination of PD-L1 blockade with tumor-specific Treg depletion effectively mediated disease regression. Furthermore, blockade with a combination of anti-PD-L1 and anti-LAG-3 Abs overcame the requirement to deplete tumor-specific Tregs. In contrast, successful treatment of primary melanoma with adoptive cell therapy required only Treg depletion or Ab therapy, underscoring the differences in the characteristics of treatment between primary and relapsing cancer. These data highlight the need for preclinical development of combined immunotherapy approaches specifically targeting recurrent disease. *The Journal of Immunology*, 2013, 190: 4899–4909.

Adoptive transfer of tumor-specific cytotoxic CD4⁺ T cells into lymphopenic hosts can eradicate large, established, vascularized tumors (1–3). Despite the efficacy of such cytotoxic CD4⁺ T cell transfer in the primary setting, tumor relapse remains a significant concern. Although the mechanisms underlying tumor recurrence are not completely defined, they are postulated to include increases in regulatory T cells (Tregs), loss of tumor Ag expression, and enhanced tumor expression of inhibitory ligands (4–7).

Foxp3⁺ Tregs suppress immunity to cancer (8–11). Although removing Tregs has generally enhanced the efficacy of primary therapy (12–14), depletion of these cells in more established cancers does not confer the same therapeutic benefit (15, 16). These data

suggest that, in the setting of disease recurrence, Tregs work in combination and/or synergy with other mechanisms to suppress antitumor immunity.

One plausible mechanism for this increased tolerance observed in the setting of tumor recurrence is through the coexpression of molecules that inhibit effector T cell (T_E) function (17), including PD-1 (18, 19), LAG-3 (20), TIGIT (21), and TIM-3 (22). PD-1 is part of the B7 family of molecules and regulates T_E. PD-1 was originally shown to be highly expressed on CD8⁺ T cells from chronically infected mice (19) and was later observed on CD8⁺ T cells in humans with chronic infections and cancer (22–26). Importantly, the ligand for PD-1, PD-L1 (B7-H1), is abundant on human carcinomas of lung, ovary, and colon and melanoma (6), and it functions as a “biologic shield,” protecting tumors from T cell–mediated death. LAG-3 can regulate CD8⁺ T cells during antitumor responses (27) and is thought to play a role in Treg-mediated suppression (28). TIGIT was recently shown to down-regulate CD8⁺ T cell responses (21, 29), and blockade of TIM-3 was shown to enhance therapy of primary tumors when combined with anti-PD-1 Abs (22, 26). The role of each of these inhibitory receptors on cytotoxic CD4⁺ T_E is unknown.

From a functional perspective, blockade of PD1/PD-L1 interactions can restore antitumor immunity in mice (30). These observations have now been translated into humans, with phase I data clearly demonstrating that either PD-L1 (B7-H1) or PD-1 blockade can lead to meaningful disease regression and survival improvements in patients with large tumor burdens (18, 31, 32). Unfortunately, in the setting of widely metastatic disease, anti-PD-1 treatment, like other single-agent mAbs, is seldom curative (33).

Based on these collective data showing the potential import of CD4⁺ T cells combined with lymphopenia and PD-1/PD-L1 interactions in tumor recurrence, in this study, we investigated how these diverse mechanisms interact to dictate antitumor function in this setting. To accomplish this goal, we built upon a model system in which, through adoptive cell transfer, naive tumor-specific CD4⁺

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Abbreviations used in this article: DT, diphtheria toxin; T_E, effector T cell; Treg, regulatory T cell.

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T cells differentiate into Th1 cytotoxic T cells (1) capable of mediating the regression of primary melanoma in tumor-bearing lymphopenic mice through class II recognition and subsequent eradication through perforin and granzyme (1, 2, 34–36). Despite such initial efficacy, ~50% of mice ultimately relapse. Using this model, we now demonstrate that during recurrence, tumor-specific Tregs increase concomitantly with chronically exhausted tumor-specific CD4⁺ T_E. Although Foxp3 Tregs increased during recurrence, their removal by targeted cell-specific ablation was not sufficient to initiate tumor regression. Instead, removal of tumor-specific Tregs in combination with anti-PD-L1 (B7-H1) Abs was necessary to restore immune function of tumor-specific CD4⁺ T_E during cancer recurrence. In addition, combination immunotherapy against two inhibitory receptors with anti-PD-L1 and anti-LAG-3 Abs overcame the necessity to deplete tumor-specific Tregs and restored antitumor immunity, providing a clinically relevant alternative to Treg depletion for the treatment of recurrent disease (37). Strikingly, for the treatment of primary disease, Treg depletion or anti-PD-L1 Abs were effective singularly when combined with adoptive cell transfer. These data suggest that the immunobiology of T cell suppression differs in the setting of primary and recurrent malignancies and defines a combinatorial treatment strategy for clinical study.

Materials and Methods

Mice

Tyrr1^{B-w}RAG^{-/-} TRP-1-specific CD4⁺ TCR transgenic mice (B6.Cg-*Rag1^{tm1Mom}* *Tyrr1^{B-w}* Tg[Tcrα, Tcrβ]9Rest/J) were described previously (1). Foxp3-DTR mice were provided by A. Rudensky (Memorial Sloan-Kettering Cancer Center, New York, NY) (38). Foxp3-DTR mice were crossed with transgenic TRP-1 mice to create *tyrr1^{B-w}RAG^{-/-}* Foxp3-DTR TRP-1-specific CD4⁺ TCR transgenic mice. *RAG1^{-/-}* (*Rag1^{tm1Mom}*) mice were purchased from The Jackson Laboratory. All mice were used in accordance with guidelines from the University of Maryland Institutional Animal Care Committee.

Tumor lines and measurement

B16.F10 (H-2^b), hereafter called B16, is a TRP-1⁺ spontaneous murine melanoma that was obtained from the American Type Culture Collection and maintained in culture media, as described previously (1). Tumors were injected s.c. at 2×10^5 cells/mouse. Tumors were measured blindly with digital calipers. The perpendicular diameters were determined and multiplied to generate the area (in mm²), as described previously (1).

Sorting and adoptive cell transfer

TRP-1 Foxp3-DTR CD4⁺ T cells were sorted from spleens of donor *Tyrr1^{B-w}RAG-1^{-/-}* Foxp3-DTR TRP-1-specific transgenic male mice. NonDTR⁺ TRP-1 CD4⁺ T cells were sorted from spleens of donor *Tyrr1^{B-w}RAG-1^{-/-}* TRP-1-specific transgenic male mice. Spleens were harvested and made into single-cell suspensions. Cells were made devoid of RBCs by ACK lysis. Subsequently, cells were counted and enriched for CD4⁺ T cells by magnetic bead sorting using a CD4⁺ T cell enrichment kit (Miltenyi Biotec). Enriched CD4⁺ T cells were counted and resuspended in PBS and used in adoptive-transfer studies (2×10^5 cells/mouse). T cells were injected i.v. through the tail vein. For tumor-sensitized TRP-1 Foxp3-DTR CD4⁺ T cells, *tyrr1^{B-w}RAG^{-/-}* Foxp3-DTR TRP-1-specific CD4⁺ TCR transgenic mice were given B16.F10 tumor at 1×10^6 cells, and T cells were harvested from spleen, as described above, when tumors reached ~400 mm².

Flow cytometry

Anti-CD4 (RM4-5), anti-IFN-γ (XMG1.2), anti-TNF-α (MP6-XT22), and anti-CD244.2 (2B4) were obtained from BD Biosciences. Anti-IL-7Rα (SB/199), anti-CXCR3 (CXCR3-173), anti-Foxp3 (FJK-16s), anti-PD-1 (RMP1-30), anti-PD-L1 (MIH5), anti-TIM3 (RMT3-23), and anti-CD160 (BY55) were obtained from eBiosciences. Anti-LAG-3 (C9B7W) and anti-TIGIT (1G9) were obtained from BioLegend. All flow cytometry scales are log scales, if not otherwise specified. Intracellular staining for cytokines was done with the BD Biosciences Cytofix/Cytoperm intracel-

lular staining kit. Foxp3 staining was done with the Foxp3 staining kit. All samples were run on a BD FACSCalibur (Department of Surgery, University of Maryland School of Medicine) and analyzed by FlowJo software (TreeStar).

Ab and Treg-depletion therapy

Anti-PD-L1 (10F.9G2), anti-CTLA-4 (9H10), and anti-LAG-3 (C9B7W) were purchased from Bio X Cell (West Lebanon, NH). Diphtheria toxin (DT) was purchased from Sigma and was reconstituted according to the manufacturer's protocol. Frozen DT stocks were thawed once, and 50 μg/kg DT was injected i.p., as described previously (38).

Measurement of serum cytokines and chemokines

Serum was collected via tail vein using serum-collection tubes (BD Biosciences). Serum was analyzed by MILLIPLEX 32-Plex assay (Cytokine Core Lab, University of Maryland Baltimore).

Statistics

An unpaired Student *t* test was used to compare the differences between cytokines and chemokines. Tumor curves were compared using two-way ANOVA, nonrepeated measures. The *p* values ≤0.05 were considered significant. PRISM 5.0d software was used to analyze the data (GraphPad).

Results

Tumor-specific Foxp3⁺ Tregs expand during recurrence

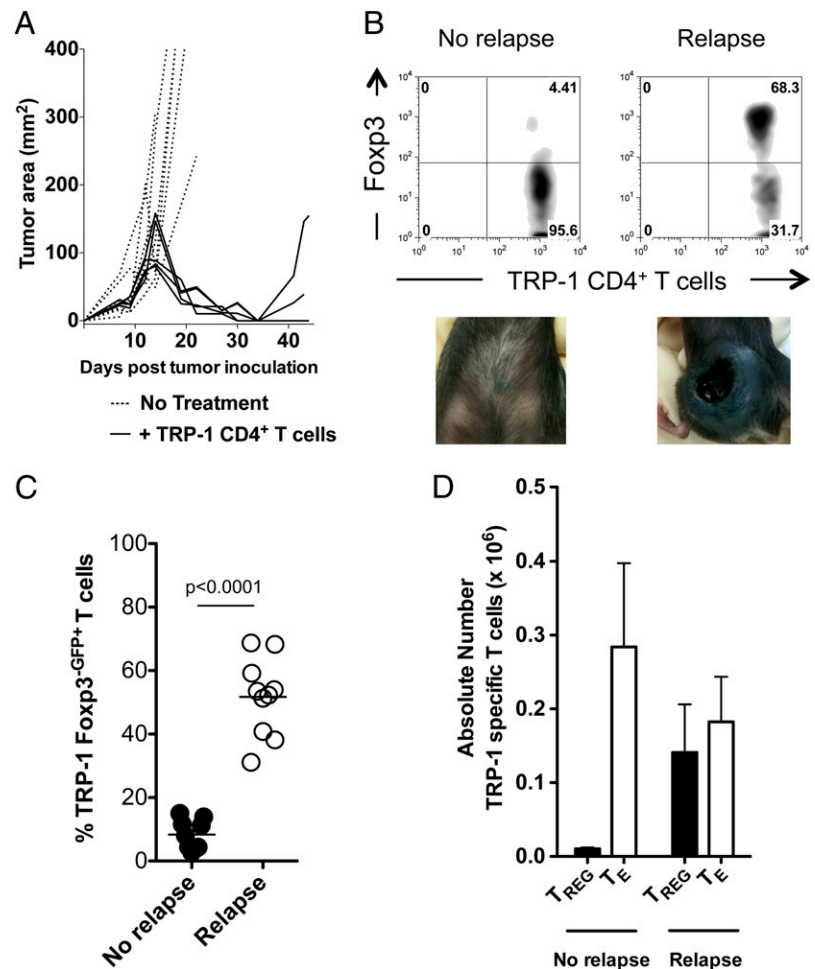
We showed that tumor Ag-specific TRP-1 CD4⁺ T cells can eradicate large established melanoma in lymphopenic RAG^{-/-} mice by direct killing of melanoma cells without the aid of CD8⁺ T cells (1). This therapy is enhanced by blockade of negative costimulation through anti-CTLA-4 (2). Preliminary evidence suggests that the enhanced immunity associated with CTLA-4 blockade is related to inhibition of tumor-specific TRP-1 Treg expansion (2). Based on these findings in primary tumors, in this study, we first investigated whether tumor-specific Tregs are expanded during tumor recurrence. Using our previously published model (1), we found that select tumors recurred, even after successful initial treatment (Fig. 1A). Recurrence was variable, with events occurring from 30 to 120 d. Analysis of TRP-1 CD4⁺ T cells from mice with recurrent disease and paired animals without relapse demonstrated that relapsing mice had consistently higher levels of tumor-specific CD4⁺Foxp3⁺TRP-1 Tregs, with levels varying from ~25 to 80% (Fig. 1B–D). Importantly, the number of T_E was approximately the same in each group, indicating that T_E were not deleted (Fig. 1D). These data demonstrate that, in this model system, tumor recurrence is associated with a dramatic increase in the tumor-specific inhibitory/effector T cell ratio.

Depletion of tumor-specific Foxp3⁺ T cells does not treat recurrence

To determine whether TRP-1 Foxp3⁺ tumor-specific CD4⁺ T cells were causing relapse, we crossed TRP-1 transgenic mice with the Foxp3-DTR transgenic reporter mouse, which allows both unequivocal cell-specific ablation of Foxp3⁺ Tregs (38) and cell-specific tracking (38). Similar to our initial studies, transfer of TRP-1^{Foxp3-DTR} CD4⁺ tumor-specific T cells resulted in analogous rates of tumor relapse, indicating no difference in antitumor T cell function associated with DTR expression (Fig. 2A).

Interestingly, in animals undergoing tumor recurrence, depletion of tumor-specific Tregs failed to prevent tumor regrowth (Fig. 2A). Importantly, tumor relapses were not secondary to loss of tumor-specific T_E, because flow cytometric analysis revealed that T_E remained present during depletion and that GFP expression faithfully marked Foxp3-DTR TRP-1 Tregs that had expanded (Fig. 2B). Furthermore, depletion of tumor-specific Tregs (i.e., TRP-1-specific

FIGURE 1. Foxp3⁺ tumor-specific Tregs increase during melanoma recurrence. **(A)** Melanoma recurs after successful tumor-specific CD4⁺ T cell-adoptive cell immunotherapy. C57BL/6 lymphopenic RAG^{-/-} mice (five mice/group) were inoculated with B16.F10 melanoma (2×10^5 cells). Tumor-bearing mice were treated with 2×10^5 naive TRP-1 CD4⁺ T cells by i.v. tail vein injection on day 10 after tumor inoculation. Tumors were followed until recurrence of melanoma. The experiments were repeated ≥ 10 times. **(B)** Foxp3⁺ tumor-specific CD4⁺ T cells increase during relapsing melanoma. TRP-1 CD4⁺ T cells were analyzed for Foxp3 expression by flow cytometry from relapsing mice (68.3%) and compared with cells from mice with no tumors (4.4%). Data represent 10 independent experiments. **(C)** Percentage of Foxp3-eGFP⁺ cells from 10 experiments. Using Foxp3-DTR TRP-1 T cells, which fluoresce with eGFP when expressing Foxp3, similar results were obtained in relapsing and non-relapsing mice (no recurrence, ~5%; recurrence ~50%). **(D)** Absolute numbers of tumor-specific CD4⁺ Tregs and Foxp3⁻ CD4⁺ T_E during recurrence compared with nonrelapsing mice. Experiments were repeated 10 times.



Tregs) at the time of primary treatment failed to prevent tumor recurrence, suggesting that Tregs present at the time of adoptive transfer cannot mediate relapse (Fig. 2C). To rule out the possibility that our findings were secondary to the induction of Tregs from T_E after initial depletion, we depleted tumor-specific Tregs continuously for ~100 d. Even under these stringent conditions, tumors still recurred in select animals (Supplemental Fig. 1).

Because depletion of tumor-specific Tregs alone failed to mediate the regression of recurrent tumors, we postulated that functional differences in T_E populations might exist in the setting of tumor relapse. To test this supposition, we tested T cell functional capabilities by IFN- γ and TNF- α release in vitro. Strikingly, although TRP-1^{Foxp3-DTR} CD4⁺ T cells from relapsing mice depleted of tumor-specific Tregs produced only low levels of IFN- γ and TNF- α , cells from nonrelapsing mice generated high levels of both cytokines (Fig. 2D, 2E). These data indicate an intrinsic dysfunction of effector CD4⁺ T cells in relapsing mice.

Tumor-specific CD4⁺ T cells become exhausted during cancer recurrence

In the setting of recurrent disease, our data suggest that tumor-specific CD4⁺ T cells are functionally crippled, even in the absence of cell-extrinsic suppression. To understand the mechanisms underlying these observations, we compared the expression of inhibitory receptors on CD4⁺ T cells during recurrence and successful primary therapy. Given the knowledge that the inhibitory receptor PD-1 is highly expressed on exhausted T cells from chronically infected mice (19), we first compared its expression on T cells from

nonrelapsing and recurring mice. Even in the absence of Tregs, PD-1 expression on CD4⁺ T cells was enhanced in animals with recurrent disease (Fig. 3A). Next, we screened a broad panel of markers, including PD-1, LAG-3, TIM3, TIGIT, 2B4, CD160, and Klr1 (17). Consistent with being chronically exhausted, CD4⁺ T cells from animals with recurrent disease exhibited higher expression of PD-1, LAG-3, TIGIT, and TIM3, slightly enhanced expression of 2B4, and similar expression of CD160 (data not shown, Fig. 3B). Interestingly, Klr1 was shown to be downregulated on exhausted cells, suggesting loss of cytotoxic function (Supplemental Fig. 2). Taken in concert, these phenotypic and functional observations suggest that CD4⁺ T_E become chronically exhausted during melanoma recurrence.

Because ligation of PD-1 mediates diverse functional properties, suppressing T_E and stimulating Tregs (39, 40), we subsequently analyzed PD-1 expression on both Foxp3⁻ TRP-1^{Foxp3-DTR} CD4⁺ T_E and Foxp3⁺ TRP-1^{Foxp3-DTR} Tregs (Fig. 3C). During recurrence, both Tregs and T_E expressed PD-1 at high levels (Fig. 3C). However, compared with each other, Tregs always expressed lower levels of PD-1 than did T_E and always had higher levels of PD-1 than did Tregs from nonrelapsing mice (Fig. 3C). Like T_E, tumor-specific Tregs from relapsing mice also expressed higher levels of TIM-3, TIGIT, and LAG-3 (data not shown). These findings suggest that, during recurrence, Tregs acquire distinctive functional properties that may interfere with antitumor immunity and that blockade of PD-L1 might restore antitumor immune function of tumor-specific CD4⁺ T cells in vivo by simultaneously blocking exhaustion and Treg-mediated suppression.

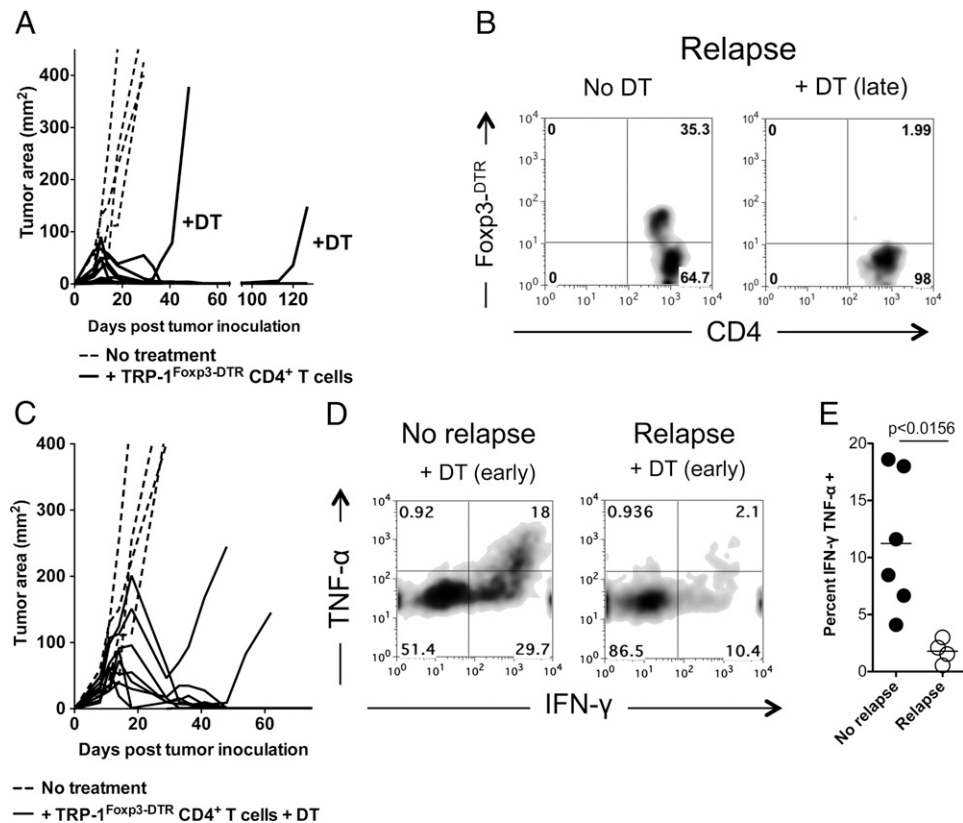


FIGURE 2. Depletion of tumor-specific T_{reg} cells does not prevent or treat relapsing melanoma. **(A)** Depletion of tumor-specific Fcγ3⁺ Tregs during recurrence (late) does not treat melanoma. C57BL/6 lymphopenic RAG^{-/-} mice (five mice/group) were inoculated with B16.F10 melanoma (2×10^5 cells). Tumor-bearing mice were treated with 2×10^5 naive TRP-1 Fcγ3-DTR CD4⁺ T cells by i.v. tail vein injection on day 10 after tumor inoculation. Tumors were followed until recurrence of melanoma. DT was injected i.p. at the prescribed concentration of 50 μ g/kg every other day for three doses total. PBS controls had no effect on Tregs (data not shown). Experiments were repeated five times. **(B)** Flow cytometry of Fcγ3 eGFP expression in TRP-1 Fcγ3-DTR CD4⁺ T cells from relapsing mice treated or not with DT at time of sacrifice. (No DT, 35.3%; +DT [late], ~2%). Experiments were repeated at least five times. **(C)** Depletion of tumor-specific Fcγ3⁺ Tregs before recurrence (early) does not prevent melanoma recurrence. C57BL/6 lymphopenic RAG^{-/-} mice (five mice/group) were inoculated with B16.F10 melanoma (2×10^5 cells). Tumor-bearing mice were treated with 2×10^5 naive TRP-1 Fcγ3-DTR CD4⁺ T cells by i.v. tail vein injection on day 10 after tumor inoculation. Tumors were followed until recurrence of melanoma. At injection of T cells (day 10), DT was injected i.p. at the prescribed concentration of 50 μ g/kg every other day for three doses total. PBS controls had no effect on Tregs (data not shown). Experiments were repeated five times. **(D)** Functional characteristics of tumor-specific CD4⁺ T cells during recurrence with Tregs depleted early. Flow cytometry of IFN- γ and TNF- α expression in TRP-1 Fcγ3-DTR CD4⁺ T cells from nonrelapsing and relapsing mice. Data represent at least three experiments. **(E)** Percentage of TRP-1 CD4⁺ T cells that are dual producers of IFN- γ and TNF- α during recurrence. Experiments were repeated four times.

PD-L1-specific blockade restores antitumor immunity and treats recurrence only in combination with tumor-specific Treg ablation

Because PD-1 increased during recurrence on both TRP-1-specific Tregs and T_E CD4⁺ T cells, we hypothesized that blocking PD-1 on Tregs would prevent their suppression and/or expansion and that blocking PD-1 on T_E would restore their function (6, 17, 19, 22–26, 41–43). To avoid any confounding effects associated with the potential stimulatory effects associated with anti-PD-1 administration on Tregs, we opted to block its ligand, PD-L1. Treatment with anti-PD-L1 Abs alone afforded no benefit (Fig. 4A). This lack of response was not due to timing, because later treatment of recurrences was met with similar results (data not shown).

Because DT-mediated Treg depletion alone also failed to treat recurrence, as shown in Fig. 2, we evaluated a combinatorial approach. Depletion of tumor-specific Tregs and concomitant PD-L1 blockade with anti-PD-L1 Abs effectively mediated regression of recurrent B16 melanoma. Importantly, these effects were observed in the absence of tumor-specific T cell retransfer, a vaccine, or induction of lymphopenia. These data indicate that tumor-specific

CD4⁺ T cells were present and capable of being reinvigorated by PD-L1 blockade and Treg depletion.

Next, we examined the changes that occurred to the T cells and the serum during a successful treatment with combination therapy. We looked for expression of markers of exhaustion, IL-7R α , and CXCR3. Re-expression of IL-7R α indicates proper memory cell transitioning from naive T cells, and CXCR3 expression indicates a functional Th1 phenotype that allows Th1 CD4⁺ T cells to become properly differentiated (44–46). Most notably, PD-1 and LAG-3 expression decreased when Tregs were depleted and anti-PD-L1 therapy was given (Fig. 4B). IL-7R α and CXCR3 expression was restored to levels seen on functional CD4⁺ T cells. However, either therapy alone did not induce any change in PD-1 or LAG-3 expression compared with nonrecurrent mice. IL-7R α and CXCR3 also stayed low. Changes in phenotype predicted changes in function because CD4⁺ T cells were reinvigorated, as evidenced by their re-expression of IFN- γ and TNF- α , similar to nonrecurrent mice (Fig. 4C), as well as their ability to treat recurring tumors (Fig. 4D). Again, Treg depletion alone or anti-PD-L1 therapy alone had little to no effect on T cell function or their treatment ability (Fig. 4C, 4D), as evidenced by low coexpression of IFN- γ and TNF- α . In-

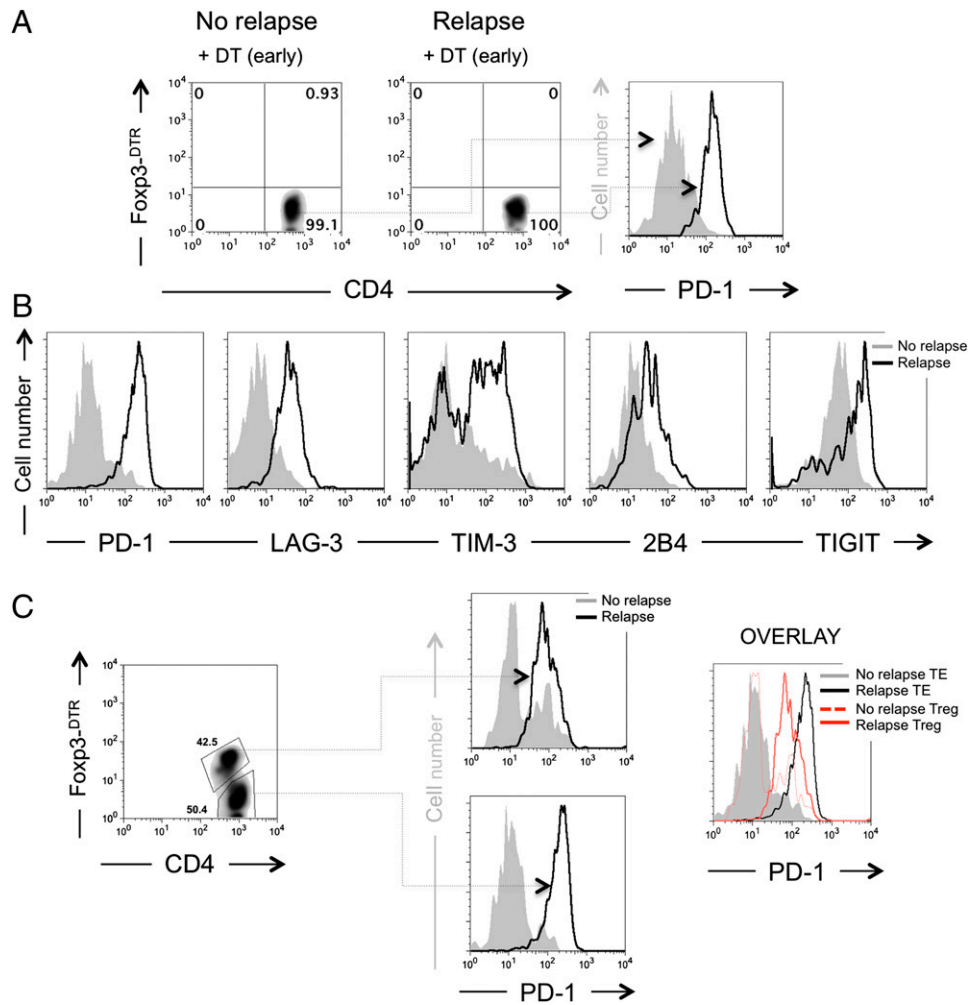


FIGURE 3. Tumor-specific CD4⁺ T cells express multiple inhibitory receptors during recurrence of melanoma. **(A)** Depletion of tumor-specific Tregs with DT before recurrence does not prevent exhaustion of TRP-1 Foxp3-DTR CD4⁺ T cells. Flow cytometry of TRP-1 Foxp3-DTR CD4⁺ T cells showing complete depletion of Tregs during recurrence compared with nonrelapsing (left two panels) and expression of PD-1 on TRP-1 Foxp3-DTR CD4⁺ T_E (right panel) from either group. The experiment was repeated five times. **(B)** Tumor-specific TRP-1 CD4⁺ T_E express multiple inhibitory receptors during relapsing melanoma. Foxp3⁻ tumor-specific CD4⁺ T_E were analyzed by flow cytometry for expression of inhibitory receptors, as indicated. Filled graphs represent nonrelapsing groups. Open graphs represent relapsing groups. Data are representative of 3–10 experiments. **(C)** Foxp3⁺ tumor-specific Tregs express PD-1 during relapsing melanoma, although at lower levels than tumor-specific TRP-1 CD4⁺ T_E. Foxp3⁺ and Foxp3⁻ tumor-specific CD4⁺ T cells were analyzed by flow cytometry for expression of the inhibitory receptor PD-1 (left panel). PD-1 is expressed on both CD4⁺ Tregs and T_E (middle panels). Overlay of flow cytometry shows that PD-1 expression is higher on CD4⁺ T_E than on Foxp3⁺ Tregs (right panel). Data are representative of three experiments.

terestingly, IFN- γ was still expressed at low levels in the chronically exhausted cells, suggesting that low levels of IFN- γ in vivo may be sustaining PD-L1 expression through an adaptive-resistance mechanism (7).

Previously, we showed that specific chemokines and cytokines changed their expression during treatment of primary melanoma when compared with no-treatment groups (1). Therefore, we wanted to determine whether similar changes took place during recurrence and how they compared with nonrelapsing mice and the original primary treatment. We found that CXCL9 and CXCL10 could predict treatment success (Fig. 4E). Interestingly, in contrast to our prior observations in the setting of primary disease, chemokines decreased during resolution of tumor and increased during recurrence (1). Chemokine levels tended toward those seen in nonrelapsing mice during treatment with anti-PD-L1 and DT and correlated with increased CXCR3 expression on tumor-specific T cells (Fig. 4B). CD4⁺ T cell numbers in the spleen in mice being treated with both therapies tended toward levels in nonrelapsing mice (Fig. 4F). These results clearly indicate that blockade of PD-L1 and loss of Tregs allow tumor-specific CD4⁺ T cells to become rein-

vigorated in vivo without retransfer of additional T cells or administration of a vaccine and no reinstatement of lymphopenia with cytotoxic drugs or radiation.

Disparate treatment requirements between primary and recurrent cancer

Surprisingly, treatment of recurring tumors was not predicted from treatment of primary tumors. CD4⁺ T cells from tumor-bearing transgenic mice (where tumors were growing unimpeded) were sorted and retransferred into tumor-bearing animals that were treated with a combination of DT or anti-PD-L1 Ab (early treatment; Fig. 5A). CD4⁺ T cells from tumor-bearing tyrl^{BW}RAG^{-/-} TRP-1 transgenic mice, termed tumor sensitized, predictably expressed PD-1 and had increased levels of Foxp3 (Supplemental Fig. 3A). Because of this, tumor recurred faster and at the same time in individual mice. Like mice, which recurred naturally, tsACT cells expressed higher levels of Foxp3 and PD-1 during recurrence (Supplemental Fig. 3B). However, early treatment of primary tumors with anti-PD-L1 or DT therapy worked alone when given with tsACT (Fig. 5A). When mice recurred in the same experi-

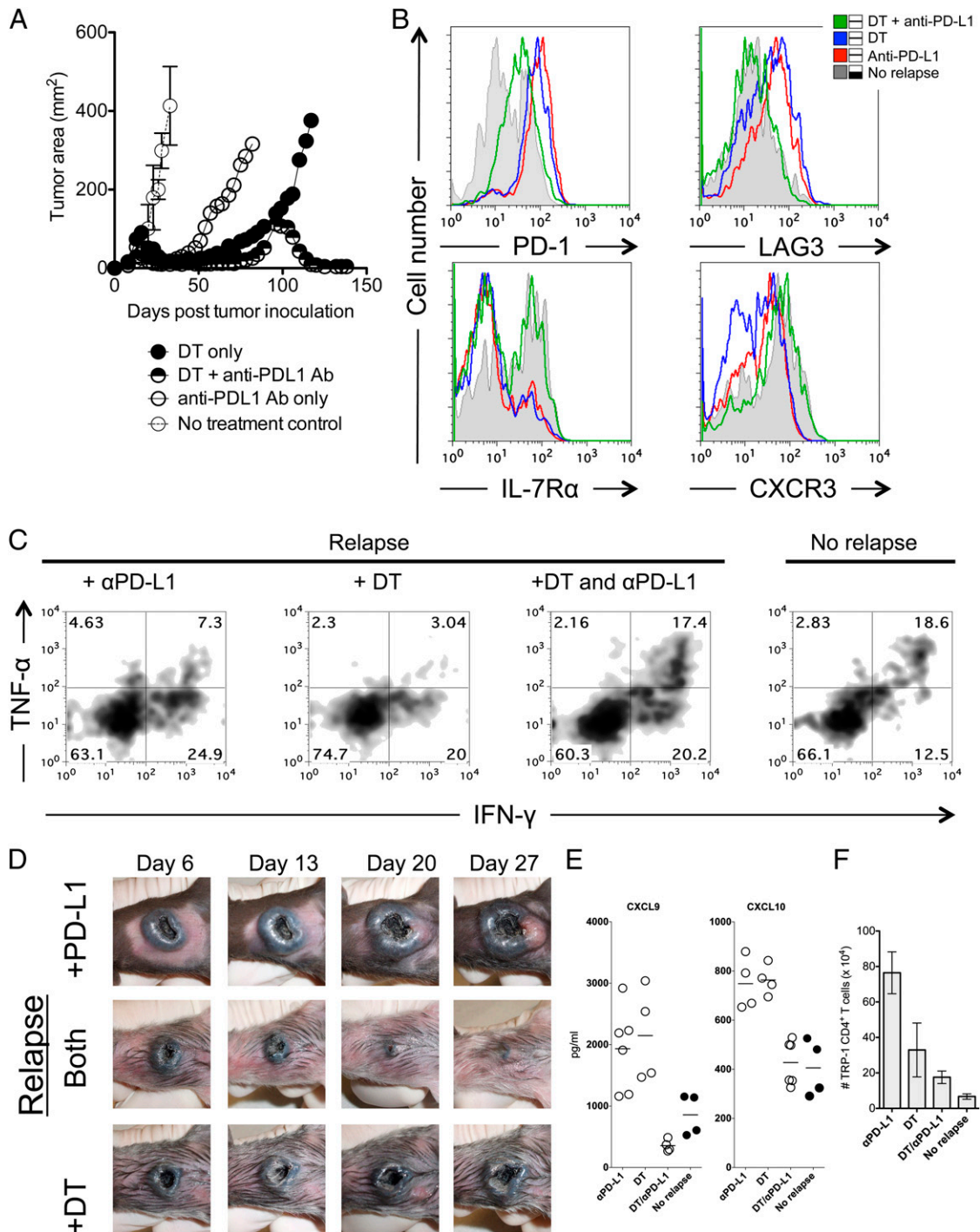


FIGURE 4. Treatment of relapsing melanoma requires both Treg depletion and blockade of PD-L1. **(A)** Treatment of relapsing melanoma requires combination Treg depletion and anti-PD-L1 therapy. C57BL/6 lymphopenic RAG^{-/-} mice (5–10/group) were inoculated with B16.F10 melanoma (2×10^5 cells). Tumor-bearing mice were treated with 2×10^5 naive TRP-1 Foxp3-DTR CD4⁺ T cells by i.v. tail vein injection on days 7–10 after tumor inoculation. Tumors were followed until recurrence of melanoma. At recurrence, DT was injected i.p. at the prescribed concentration of 50 mg/kg every other day for three doses total. At recurrence, anti-PD-L1 was given as a bolus injection i.p. at 500 μ g for the first dose and every 3 d thereafter at 200 μ g/injection for five doses. For combination therapy, both were given at the same time as described above. PBS controls had no effect (data not shown). Data shown are representative of more than five experiments. **(B)** Blockade of PD-L1 and depletion of tumor-specific Tregs reinvigorate tumor-specific CD4⁺ T cells by reducing inhibitory receptor expression of PD-1 and LAG-3 and increase IL-7R and CXCR3. Flow cytometry shows four groups (no recurrence, DT only, DT plus anti-PD-L1 Ab, and anti-PD-L1 Ab only). All therapies were given at the time of recurrence. Flow cytometry was performed on each group 3–5 d after the last dose of therapy. Data shown are representative of five experiments. **(C)** Tumor-specific CD4⁺ T cells regain effector function with dual therapy as defined by re-expression of IFN- γ and TNF- α compared with relapsing groups with single therapies (anti-PD-L1 [7.3%], DT [3.04%], +DT and anti-PD-L1 [17.4%], no recurrence [18.6%]). The experiment was repeated three times. **(D)** Gross depiction of tumor regression after combination therapy. Days indicate day after therapy was given. All tumors depicted are relapsing tumors that were previously treated as a primary tumor. **(E)** IFN- γ -inducible chemokines (CXCL9 and CXCL10) are highly increased during recurrence and return to treatment levels with combination therapy. Each symbol represents an individual mouse. **(F)** Total number of tumor-specific CD4⁺ T cells in the spleen stabilizes to nonrelapsing levels with combination therapy. Absolute number of TRP-1 CD4⁺ T cells during different treatments. Each bar in the graph represents 5–10 individual mice.

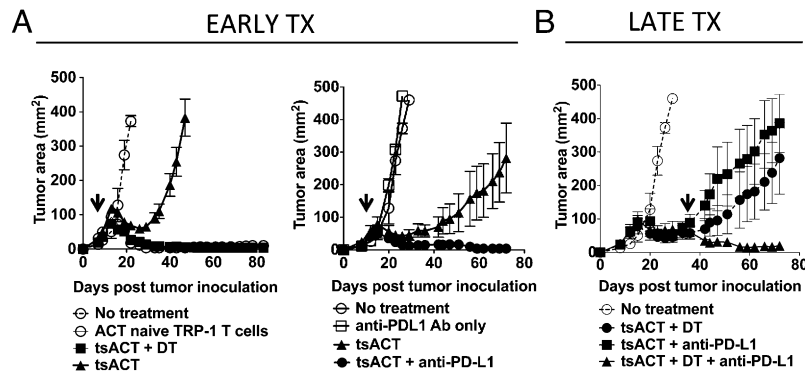


FIGURE 5. Disparate treatment requirements between primary and relapsing melanoma. **(A)** Early treatment of melanoma with tsACT from mice with progressively growing tumors fails in all mice compared with naive TRP-1 CD4⁺ T cell transfer. TRP-1 Foxp3-DTR tyrp1^{B^wRAG} transgenic mice (5–10 mice/group) were inoculated with B16.F10 melanoma (1×10^6 cells). When tumors reached ~ 400 mm², tumor-sensitized CD4⁺ T cells were harvested and sorted, and 2×10^5 cells were transferred i.v. into 7-d tumor-bearing lymphopenic mice. tsACT alone failed to control tumor, but when depleted of Tregs (+DT), they treated primary tumor (*left panel*). Anti-PD-L1 without T cell transfer fails to treat primary tumors. Anti-PD-L1 with tsACT treated primary tumor (*right panel*). **(B)** Single therapies given late during relapsing tumor fail to treat tumor, as in (A). Combination therapy with anti-PD-L1 and Treg depletion was still required. Arrows indicate beginning of therapy with DT, anti-PD-L1, or both. Experiments were repeated three times.

ment, only the combination of anti-PD-L1 Ab and Treg depletion was therapeutic (late treatment; Fig. 5B). These findings underscore the differences between primary tumor and recurring tumor microenvironments.

Simultaneous blockade of PD-L1 and LAG-3 in vivo treats recurring tumors, overcoming the necessity to deplete tumor-specific Tregs

Although combination Treg depletion with anti-PD-L1 therapy can treat relapsing poorly immunogenic B16 melanoma, this type of therapy is not practical in humans (47, 48). Depletion of Tregs is also potentially dangerous in humans because of the autoimmune diseases that could follow. Therefore, we looked for another means to overcome Treg-mediated suppression that was controllable without having to deplete Tregs. First, we blocked CTLA-4, an Ab that was recently approved for melanoma (known clinically as ipilimumab or Yervoy). We surmised that it could block the generation of peripherally derived Tregs or enhance therapy, like shown before in many models (2, 49). It was given in combination with tumor-specific Treg-depletion therapy, and there was no effect on relapsing cancer (Supplemental Fig. 4A), thus showing the dominance of the PD-1 pathway in tumor recurrence.

We noticed that CD4⁺ T cells from relapsing mice expressed both PD-1 and LAG-3 (Fig. 6A). We also noticed that, during successful therapy with anti-PD-L1 and tumor-specific Treg depletion, the expression of both PD-1 and LAG-3 decreased on tumor-specific CD4⁺ T cells, reaching levels similar to nonrelapsing mice (9–13%, Fig. 6A). We reasoned that if we could block both PD-1 and LAG-3, this would reproduce the therapeutic effects of anti-PD-L1 and Treg depletion therapy.

Anti-PD-L1 and anti-LAG-3 Abs were given in combination at time of relapse without Treg depletion. As with anti-PD-L1 and Treg depletion, tumors regressed (Fig. 6B). Analysis of mice regressing showed lower amounts of tumor-specific Tregs compared with relapsing mice (Fig. 6C), decreases in inhibitory receptors on tumor-specific CD4⁺ T cells, as well as an increase in CXCR3 similar to anti-PD-L1 therapy combined with Treg depletion (Fig. 6D). LAG-3 was blocked with unlabeled anti-LAG-3 and, therefore, could not be assessed by flow cytometry (data not shown). Anti-LAG-3 given with DT therapy also had no effect on relapsing cancer (Supplemental Fig. 4B). Thus, tumor-specific Treg-mediated suppression and chronic exhaustion could be simul-

taneously overcome with dual Ab therapy to PD-L1 and LAG-3 inhibitory receptors.

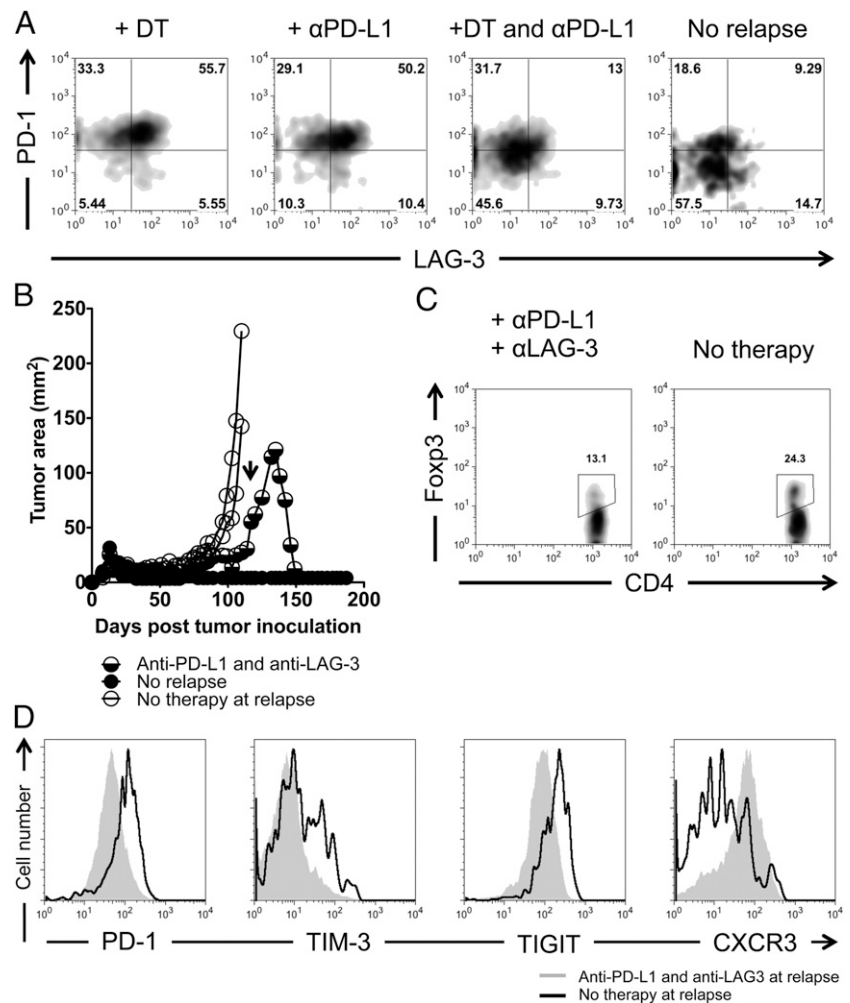
Discussion

We demonstrate that Treg-mediated suppression and chronic exhaustion of CD4⁺ T cells are intertwined during cancer recurrence. Although blockade of either pathway alone can successfully treat primary tumors, these monotherapies cannot reactivate tumor-specific CD4⁺ T cells in vivo for the treatment of recurrent disease. In contrast, combination blockade of the inhibitory molecule PD-L1 and depletion of tumor-specific Tregs or, more practically, combination blockade of the inhibitory molecules PD-L1 and LAG-3 effectively treated recurrent melanoma, and therapeutic responses were predicted by changes in well-defined serum biomarkers. Taken in concert, our data delineate a new potential approach for the treatment of recurrent solid tumors and define biomarkers that may be useful for predicting treatment response.

CD4⁺ T cells can effectively enhance immunotherapy of cancer (3, 50–53) and, in certain cases, are superior to traditional CD8⁺ T cell-based approaches for the treatment of melanoma (34, 54). For example, the combination of tumor-specific CD4⁺ Th cells, anti-PD-1, and Cytosin enhanced antitumor immunity by reactivating tumor-specific CD8⁺ T cells (55). Despite their therapeutic potential, recent observations suggest that Ag-specific CD4⁺ T cells are a “double-edged sword,” capable of both directly targeting class II-expressing tumors and suppressing antitumor immunity. Based on these data, we first asked whether depletion of Tregs could improve antitumor immunity against recurrent melanoma. Unlike the primary tumor setting, in which Treg depletion enhances the efficacy of adoptive transfer (14, 15, 56), elimination of tumor-specific Tregs was ineffective in mediating regression of recurrent disease. Importantly, this lack of therapeutic usefulness was not secondary to loss of tumor-specific T_E. Furthermore, our findings cannot be explained by incomplete Treg depletion, because we demonstrate unequivocal elimination with targeted cell-specific ablation, as shown previously (38). These data indicate that, in our model system, T_E cannot mediate regression of recurrent tumors, even in the setting of Treg depletion.

To understand why Ag-specific CD4⁺ T cells are permissive to disease recurrence, we evaluated them for markers of exhaustion. Interestingly, tumor-specific CD4⁺ T_E expressed the hallmark exhaustion markers PD-1, LAG-3, TIM-3, 2B4, and newly described TIGIT; lost expression of IL-7R α and CXCR3; and lost

FIGURE 6. Simultaneous blockade with combination therapy to PD-L1 and LAG-3 inhibitory receptors treats recurring melanoma. **(A)** PD-1 and LAG-3 are coexpressed on chronically exhausted tumor-specific CD4⁺ T cells and decrease during anti-PD-L1 and Treg-depletion therapy or during a potential cure. C57BL/6 lymphopenic RAG^{-/-} mice (5–10 mice/group) were inoculated with B16.F10 melanoma (2×10^5 cells). Tumor-bearing mice were treated with 2×10^5 naive TRP-1 Foxp3-DTR CD4⁺ T cells by i.v. tail vein injection on days 7–10 after tumor inoculation. Tumors were followed until recurrence of melanoma. At recurrence, DT was injected i.p. at the prescribed concentration of 50 mg/kg every other day for three doses total. At recurrence, anti-PD-L1 was given as a bolus injection i.p. at 500 μ g for the first dose and every 3 d thereafter at 200 μ g/injection for five doses. For combination therapy, both were given at the same time, as described above. The experiment was repeated at least five times. **(B)** Anti-PD-L1 and anti-LAG-3 Abs given during relapse treat recurring tumor. Arrow indicates the time at which the Abs were given. Treatment was similar to (A). However, a 500- μ g loading dose of each Ab (anti-PD-L1 and anti-LAG-3) was given initially, with 200- μ g doses given every 3 d for five doses total. **(C)** Treg frequency during treatment with dual Ab therapy in (B). **(D)** Inhibitory receptor expression on tumor-specific T_E during dual Ab therapy. CD4⁺ T cells were isolated from mice undergoing regression after dual Ab therapy ~3–7 d after the last dose of Ab and analyzed by flow cytometry.



coexpression of INF- γ and TNF- α . Furthermore, the quintessential chronic inhibitory receptor PD-1 was highly expressed on both tumor-specific CD4⁺ Tregs and T_E. This expression of PD-1 on Ag-specific CD4⁺ T cells was limited to mice with recurrent disease and was not observed in the primary setting.

Interestingly, despite the increased level of PD-1 expression on CD4⁺ T cells from mice with recurrent tumors, anti-PD-L1 alone could not treat relapsing cancer. Based on this surprising observation, we evaluated the possibility that anti-PD-L1 might have both positive and adverse effects on CD4⁺ T cells in the setting of disease recurrence: stimulating CD4⁺ PD-1⁺ Tregs and suppressing T_E. Our data indicate that, following CD4⁺ tumor-specific Treg depletion, anti-PD-L1 administration can effectively mediate the regression of recurrent tumor *in vivo*. Although these findings appear analogous to previous studies, using anti-PD-L1 and Treg depletion with IL-2DT (IL-2 conjugated to DT) to treat a murine model of acute myelogenous leukemia, close scrutiny of this report reveals that the treatment regimen was administered 4–10 d after tumor inoculation, indicating a therapy of primary tumors. In addition, IL-2DT therapy can target non-Tregs, especially T_E expressing CD25.

To begin to understand the mechanisms through which anti-PD-L1 restores T_E function following tumor-specific Treg depletion, we analyzed phenotypic markers of T cell function. In this context, the inhibitory markers PD-1, LAG-3, TIGIT, and TIM-3 decrease, and IFN- γ and TNF- α increase. Furthermore, IL-7R α , a marker of memory cell differentiation, and CXCR3, which allows cells to traffic to peripheral sites, were higher. KlrG1 is upregulated on

CD4⁺ T cells from nonrecurring mice but downregulated during recurrence. This phenotype is associated with terminal differentiation and cytolytic potential (57). Hence, KlrG1 loss and PD-1 expression were associated with loss of effector function. These data indicate that the combination of tumor-specific Treg depletion and anti-PD-L1 rejuvenates T_E function, enabling them to mediate the regression of recurrent disease. In addition, these observations delineate phenotypic changes on T_E that may be useful in monitoring clinical response to therapy.

These phenotypic changes pointed to a possible mechanism that could be exploited by Ab therapies. PD-1 and LAG-3 both decreased on T_E during a potential cure or treatment with anti-PD-L1 and tumor-specific Treg depletion. Therefore, we reasoned that blockade of these two negative-inhibitory pathways together might restore effector function in the absence of Treg depletion. Administration of anti-PD-L1 and LAG-3 mirrored the therapy with anti-PD-L1 and tumor-specific Treg depletion, with similar phenotypic changes occurring on T_E. This therapy overcomes a major translation hurdle in clinical medicine: the depletion of Tregs. This combination of Abs has also benefited treatment of chronic infections, such as malaria and lymphocytic choriomeningitis virus, in mice (17, 58).

So what could cause melanoma recurrence? Whether or not exhaustion is caused directly by Tregs in general is not answerable in this study, because the role of endogenous Tregs was not assessed. Endogenous Tregs may contribute to tumor recurrence given their wide Ag-specific repertoire and their ability to suppress Ag-specific transgenic pmel CD8⁺ T cells (12, 13). Our data suggest that tumor-specific Tregs may have some contribution to exhaustion, because

continuous depletion of TRP-1 Tregs appeared to attenuate relapsing cancer but did not prevent it. These same tumor-specific Tregs also became PD-1⁺ during recurrence and expanded to high levels, suggesting that they may be acting as a secondary layer of adaptive tolerance similar to the expansion of PD-1⁺ Tregs during chronic hepatitis C virus infection (59). Because tumor may be like a chronic infection, analogous mechanisms may be used whereby Tregs become simultaneously exhausted like T_E to control tissue homeostasis (60). It is possible that the opposite occurs during spontaneous remissions, similarly to severe flares in hepatitis C virus (60). Whether the expanded TRP-1 Tregs in our model are peripherally derived Tregs or an expansion of Tregs already present upon transfer is not known, but we are currently exploring these possibilities.

Relapsing melanoma may also be caused by inflammatory mediators themselves. In a recent study, TNF- α caused melanoma cells to dedifferentiate and express lower levels of class I and tumor-associated melanocytic Ags, including TRP-1 (4). Another group showed that MHC class I and another melanocytic Ag, gp100, were decreased during recurrence (5). Class II expression in the tumor microenvironment is dependent on IFN- γ (1, 2); therefore, class II might not be susceptible to loss like MHC class I. Although both studies showed loss of Ag as a cause of recurrence, recurring tumors in the current study were still amenable to treatment. CD4⁺ T cells also reacquired TNF- α during reinvigoration with combination therapy. Thus, treatment of recurrent cancer seems to require TNF- α in our studies, because tumor-specific CD4⁺ T cells from nonrecurring mice express it, and reinvigorated cells re-express it after Treg depletion and anti-PD-L1 blockade. Conversely, more in line with our results, a recent study showed that Th1 cytokines, IFN- γ and TNF- α , induced tumor cell senescence and arrested cancer progression (61). Thus, both cytokines in our model may be helping to prevent recurrence, because during cancer relapse TNF- α expression is lost by tumor-specific Th1 cytotoxic TRP-1 CD4⁺ T cells.

Another possible underappreciated mechanism of recurrence and cancer metastasis is the expression of high levels of IFN- γ -inducible chemokines CXCL9 and CXCL10. Although these chemokines are known to attract T cells to sites of inflammation, they are also known to be highly expressed during melanoma and other cancer burdens, allowing malignant cancer cells to migrate to distant nodes (45, 62). CXCR3⁺ T cells in the presence of these high chemokines also can lose CXCR3 expression, preventing the cells from migrating into the cancerous tissue (63). In fact, we see high levels of these chemokines during recurrence and loss of CXCR3 on the tumor-specific CD4⁺ T cells. With resolution of tumor during combination therapy, CXCL9 and CXCL10 decrease in the serum, and CXCR3 is re-expressed on tumor-specific CD4⁺ T cells. Because CXCL9 and CXCL10 are important for Th1 priming (46), we suspect that they may also be important in the initiation of exhaustion and suppression.

More and more, CD4⁺ T cells in many contexts are being shown to enhance immunotherapy of cancer. Recently, a group reported in vivo modulation of cytotoxic TRP-1 CD4⁺ T cells through the Ab OX40, which enhanced the cytotoxic abilities of TRP-1 CD4⁺ T cells through expression of *Eomes* (34). TRP-1 CD4⁺ T cells that differentiated into Th17 cells in vitro appeared to resist exhaustion and treat better than Th1 CD4⁺ T cells through acquiring a stem-like signature (3), CD4⁺ T cells that differentiated into Th9 cells also were beneficial in preventing melanoma (53), and lastly, CD4⁺ T cells that differentiated into Th17 cells can assist CD8⁺ T cells to clear tumor better than can CD4⁺ Th1 cells (50). Although treatment of recurrence itself was not the focus of those studies, their data in combination with our previous work (1) and the results presented in this article show how CD4⁺ Th (whether

Th1, Th9, or Th17) and cytotoxic CD4⁺ T cells themselves can be harnessed and combined with unique Ab therapies to fight cancer at different stages of disease.

In many respects, PD-L1 is an ideal system for tumor-mediated immune evasion. Specifically, its expression is enhanced by IFN- γ , one of the most potent weapons that T cells use to mediate tumor cell death. Our findings now suggest that, unlike in the setting of primary disease, in which blockade of this molecule can restore T cell function, recurrent tumors have built a second line of biologic defense. Specifically, in this setting, Tregs showed enhanced expression of PD-1, which may become activated upon PD-L1 binding (59) or, contradictorily, by anti-PD-L1 blockade (59, 60). This inhibition can be effectively overcome by a “double-pronged” therapeutic approach, in which Treg depletion and systemic anti-PD-L1 or anti-PD-L1 and anti-LAG-3 Abs can restore the ability of resident T_E to mediate tumor regression. Furthermore, the efficacy of this response can be predicted by the loss of typical markers of exhaustion on T_E. Monotherapy may be an Achilles heel of cancer immunotherapeutics. Therefore, these data offer a testable model to define the role of PD-L1 in mediating disease recurrence and suggest a new paradigm for the use of combinatorial approaches, such as PD-L1 and LAG-3 blockade, in the clinical setting.

One caveat to the current report is that our studies were performed in lymphopenic RAG^{-/-} mice, which lack endogenous Tregs, B cells, and CD8⁺ T cells. Although we were strictly interested in the role of cytotoxic CD4⁺ T cells in this study, as in our last report (1), we cannot rule out whether the roles of the other missing cells are important during recurrence. Based on evidence from the literature, we would expect endogenous Tregs to hinder tumor immunity (12, 13), B cells to produce Abs to TRP-1 (64), and CD8⁺ T cells to become cytotoxic T cells (50, 55, 65). These may or may not affect cancer recurrence. Currently, we are exploring the role of endogenous Tregs on recurrence and whether in vivo modulation in irradiated and nonirradiated settings can overcome recurrence. Our current study is the first step in determining the basic mechanisms of cancer recurrence.

In vivo modulation is an attractive form of immunotherapy long after adoptive cell therapy has been given. Our results have a long-reaching impact on not just CD4⁺ T cells, but on any adoptive cell therapy regimen, including cytotoxic CD8⁺ T cells, as well as chimeric Ag receptor T cells (66–68), which may be susceptible to the same tolerogenic mechanisms. Understanding the mechanism of recurrence and knowing the right combinations of Abs may improve clinical care of patients with cancer.

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Disclosures

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