Tumor-associated Tregs obstruct antitumor immunity by promoting T cell dysfunction and restricting clonal diversity in tumor-infiltrating CD8+ T cells

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ABSTRACT

Background Accumulation of regulatory T cells (Treg) has been described to often correlate with poor prognosis in many solid tumors. How Treg presence impinges on limited functionality and clonal composition of tumor-associated CD8+ T cells has important implications for their therapeutic targeting in the tumor microenvironment. In the present study, we investigated how accumulation of Tregs contributes to T cell dysfunction and clonal constriction of tumor-infiltrating CD8+ T cells.

Methods Resected melanoma and lung adenocarcinoma tissues from tumor-bearing mice or patients were analyzed. The proportions and phenotype as well as clonal diversity of tumor-associated CD8+ T cells were evaluated by flow cytometry and single-cell T-cell receptor (TCR) sequencing, respectively, at early or advanced tumor stages or under Treg depletion conditions. Furthermore, antigen-specific T cells were evaluated on adoptive transfer into tumor-bearing mice in the presence or absence of anti-CTLA-4 antibody or CTLA-4 Ig. Lastly, tumor-bearing mice were treated with anti-CTLA4 antibody and/or bromodomain inhibitor JQ1 with interleukin (IL)-2 immune complexes to determine therapeutic efficacy.

Results We demonstrate that the emergence of exhaustion-like phenotype and impaired effector functionality in tumor-associated CD8+ T cells is positively correlated with Treg accumulation in the tumor bed and this dysfunctional phenotype becomes reversed on Treg reduction in murine melanoma and lung cancer models. Heightened tumor-associated Treg-expressed CTLA-4 is key to emergence and sustenance of this phenotype. Furthermore, TCR sequencing revealed a clonal shrinkage of tumor-infiltrating CD8+ T cells as tumor progressed, which was associated with reduced survival profile concomitant to increasing Treg proportions. Limited IL-2 availability was a key mechanism contributing to this peripheral repertoire reshaping as Treg depletion improved IL-2 levels, rescued CD8+ T cell viability, and improved their clonal diversity. Finally, targeted reduction of tumor but not peripheral Tregs through JQ1 and/or anti-CTLA4 antibody significantly improved antitumor response in melanoma-bearing mice when supplemented with IL-2 immune complexes.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ It is well established that regulatory T cells (Tregs) contribute to T cell dysfunction and attenuation of antitumor response in many cancers. However, how they orchestrate this immune-suppressive program in the tumor milieu is not well understood.

WHAT THIS STUDY ADDS

⇒ We demonstrate that the emergence and sustenance of T cell dysfunction marked by upregulation of inhibitory receptors on tumor-infiltrating CD8+ T cells is mediated by intratumoral Tregs through limitation of costimulatory signal availability to these effector cells. This dysfunctional state is further compounded by Treg-mediated deprivation of IL-2 which facilitates reduced survival and limits clonal diversity within the effector CD8+ T cells, effects that could be rescued by Treg-depleting agents and IL-2 supplementation in tumor-bearing mice. These findings illuminate the mechanistic underpinnings of Treg-mediated contribution to T cell dysfunction in the tumor microenvironment and highlights the therapeutic potential of targeted Treg-centric combinatorial treatment strategies to enhance antitumor immunity.

Conclusion Collectively, our study reveals a bimodal program enacted by Tregs to support T cell dysfunction in the tumor bed and highlights a promising therapeutic regimen for localized reprogramming of the tumor microenvironment to curb T-cell impairment of antitumor CD8+ T cell response in favor of improved antitumor immunity.

INTRODUCTION

CD4+CD25+Foxp3+regulatory T (Treg) cells represent an active cellular mechanism that ensures peripheral self-tolerance.1 They regulate diverse immune responses against self, allogeneic, and foreign antigens.2 3 Specifically in the context of autoimmunity, Tregs suppress potentially self-reactive T cells that have escaped thymic negative selection from initiating autoimmune diseases.
This inhibitory function is well established as deficiency in FOXP3, the lineage-defining transcription factor for Tregs that is important for their generation, leads to systemic and organ-specific autoimmune disorders. In other inflammatory or pathological conditions such as allergies, infections, and tissue transplant rejection, Tregs also participate in curtailing excessive immune activation to ensure measured responses and avoid collateral damage to healthy tissues.

While their function is critical for immune homeostasis, Treg activity poses a hindrance to protective antitumor immunity in cancer. In most solid cancers, Tregs are present in the tumor microenvironment and their accumulation is associated with poor outcomes. Consistent with this notion, decreased ratios of CD8 T cells to Treg cells in the tumor microenvironment have been reported to correlate with poor prognosis in several cancers. Given their specificity for self-antigens, which most tumor antigens are although often aberrantly expressed, Treg accumulation in the tumor bed is construed to inhibit potential tumor-reactive effector T cells which results in tumor immune evasion. Treg depletion as a way to prevent this immune suppression has been explored in various investigational models, although the risk of potential autoimmune side effects stemming from systemic depletion has hampered this strategy as most Treg-targeting agents do not discriminate tumor-associated Tregs from their peripheral counterparts.

It is well established that tumor-infiltrating CD8 +T cells exhibit impaired effector function concomitant to sustained expression of one or more inhibitory receptors, a phenotype that is reminiscent of T cell exhaustion that was originally described in chronic viral infections. Existing reports also suggest that clonal reshaping occurs in tumor-infiltrating T cells which may instruct clinical outcomes. Indeed, tumor-infiltrating CD8 T cells undergo T-cell receptor (TCR) repertoire changes under therapeutic agents that may impact Treg activity within the tumor bed, although it is not known whether the degree of tumor-CD8 +T cell clonality is a function of Treg presence in the tumor. Furthermore, the contribution of Treg accumulation in the tumor microenvironment to emergence of a dysfunctional phenotype within potential tumor-reactive CD8 T cells remains to be substantiated.

The present study investigated the association of Treg presence with phenotypic changes accompanying tumor-infiltrating CD8 T cells with respect to expression of inhibitory molecular features as well as alterations to their TCR repertoire as tumor progresses, while exploring localized Treg-targeting strategies to improve antitumor immunity in a murine melanoma model.

**MATERIALS AND METHODS**

**Mice, tissues, and tumor inoculation studies**

C57BL/6J, CD45.1, CD86 (B7-2) knock-out (KO), Nu/J (athymic nude) and Foxp3DTR mice, as well as OT-I, OT-II and Pmel-1 TCR transgenic mice were purchased from the Jackson Laboratory. B7-2 KO mice were bred with CD45.1 congenic mice in our facility. KP mice, which develop spontaneous lung adenocarcinomas, have been previously described. All mice were used at 6–10 weeks of age. Mice were maintained under specific-pathogen-free conditions in an AAALAC-accredited facility and in accordance with institutional guidelines for animal welfare. B16-F10 melanoma (CRL-6475) and LLC1 (CRL-1642) cell lines were purchased from American Type Culture Collection (ATCC). B16.OVA cells were derived from B16 cells by transduction with a complementary DNA (cDNA) encoding the ovalbumin gene. Cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS, Gemini), 1% penicillin/streptomycin, and glutamine (Gibco). Cell cultures were maintained at 37°C in a humid atmosphere containing 5% CO2 and 95% air. Cell lines were routinely tested for Mycoplasma every 3 months and were authenticated by short tandem repeat authentication every 6 months. For tumor inoculation studies, C57BL/6J (B6) or CD45.1 mice were inoculated subcutaneously in the right hind flank with 1×106 B16-F10 or B16.OVA cells in 200 µl of phosphate-buffered saline (PBS). Tumor size was determined by caliper measurement and recorded as the product of perpendicular diameters of tumor mass. For orthotopic tumor inoculations, 1×106 Lewis lung carcinoma (LLC1) cells were injected intracarinally. Tumor formation was evaluated by MRI with a BioSpec USR70/30 horizontal bore system (Bruker). Tumor burden was quantified from the MRI images with 3D Slicer software. We used the following criteria to classify tumor progression into three stages: 30–50 mm2 (early), 75–100 mm2 (intermediate), and 150–200 mm2 (advanced) for melanoma models; 150–300 mm3 (early), 600–900 mm3 (intermediate), and 1200–1400 mm3 (advanced) for lung cancer models.

**In vivo treatment and cell transfer studies**

For Treg depletion studies, 0.5 µg diphtheria toxin (DT; Merck) was injected intraperitoneally (i.p.) at days 8, 14, and 20 post B16-F10 tumor cell inoculation. Alternatively, 0.25 mg of anti-CD25 (PC61) antibody (BioXCell) was administered either by i.p. or intratumoral injections 2×/week beginning from day 7 post tumor inoculation and up to day 21. For short-term treatment studies with the bromodomain inhibitor JQ1 or anti-KLRG1 antibody, tumor-bearing mice were treated i.p. with JQ1 (25 mg/kg body weight, 3×/week) or anti-KLRG1 antibody (100 µg/mouse, 2×/week) for 1 week. Control mice received vehicle (5% dimethyl sulfoxide in 10% 2-hydroxypropyl β-cyclodextrin). For long-term drug efficacy studies, similar dosage was used for both drugs. Interleukin (IL)–2/α-IL-2 antibody immune complexes (IL2C) previously described were prepared by mixing 50 µg of anti-IL-2 monoclonal antibody clone S4B6-1 or JES6-1 (BioXCell) with 1.5 µg of mouse IL-2 (PeproTech, Rocky Hill, New Jersey, USA) for 15 min on ice prior to i.p. injections in mice. Control mice received purified rat IgG2a (BioLegend) and vehicle. In all treatment studies, mice were randomly assigned into vehicle or drug treatment...
groups when tumor size was approximately 25–35 mm³. For adoptive cell transfers, CD8+ T cells or Tregs were purified from the spleens of OT-I or OT-II TCR transgenic mice, respectively. Transfer of 2.0×10⁶ OT-I cells were made intravenously with or without OT-II cells (1–5×10⁶) into B16. OVA tumor-bearing nude mice, or wild-type (WT) versus CD86 KO mice 5 days after tumor cell inoculation. Purified anti-CTLA-4 mAb (9D9; 50 µg/mouse), purified rat IgG2b (50 µg/mouse), or CTLA-4 Ig (50 µg/mouse) was administered i.p. every 3 days for 2 weeks. For OT-I and OT-II T cell transfer studies in CD45.1 tumor-bearing mice, the programmed cell death protein-1 (PD-1) negative fraction of OT-I cells and OT-II Tregs were sorted from tumor cell suspensions based on CD45.2 positivity. In some experiments, 5×10⁶ CD3+CD25拇-5×10⁶ CD4+CD25 hi cells sorted from the spleens of WT B6 mice were transferred into nude mice intravenously. For Pmel-1 TCR transgenic T cell transfer studies, 1×10⁶ cells were injected into B16-F10 melanoma-bearing B6 mice.

**Cell purifications and ex vivo T cell activation assay**

Purification of T cells or T cell subsets from spleen or resected tumors was performed using CD90.2 magnetic beads (Miltenyi) according to the manufacturer’s protocol or via cell sorting using FACSAria (BD Biosciences). For ex vivo T cell activation, 1×10⁶ T cells were stimulated at 37°C with eBioscience Cell Stimulation Cocktail plus protein transport inhibitor (Thermo Fisher Scientific) for 6 hours. Cells were washed and stained for intracellular cytokines using BD Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer’s instructions. Briefly, cells were first stained for surface markers, including CD4, CD8, and CD3, followed by intracellular staining with fluorophore-conjugated antibodies against interferon-γ (IFN-γ), granzyme B, and tumor necrosis factor (TNF-α) after fixation and permeabilization. In all stained samples, dead cells were excluded using LIVE/DEAD Fixable Aqua Dead Cell Staining Kit (Invitrogen, Carlsbad, California, USA).

**Antibodies**

Anti-KLRG1 (2F1), anti-CTLA-4 (9D9), Syrian hamster IgG, and CTLA-4-Ig were purchased from BioXCell (Lebanon, New Hampshire, USA). All antibodies used for flow cytometry analysis were purchased from BD Biosciences (San Jose, California, USA), BioLegend (San Diego, California, USA), or eBioscience (San Diego, California, USA) and are listed in online supplemental table 1.

**Cell preparation and flow cytometry**

Processing of tumor nodules into single cell suspensions has been previously described.²⁴ For immune profiling by flow cytometry, 0.5–1×10⁶ single cells were stained for surface markers in 1X PBS/2% FBS for 15 min at 4°C. Intracellular staining was performed for Foxp3 using the Foxp3 staining kit (eBioscience, Santa Clara, California, USA) according to manufacturer’s instructions. For patient tissue processing, de-identified resected tumors were gently dissociated mechanically in RPMI 1640 media containing 10 U/mL Collagenase D (Sigma Aldrich) and 25 µg/mL DNase I grade II (Sigma Aldrich) followed by incubation at 37°C for 30 min. Dissociated tissue was further digested using GentleMACS (Miltenyi) after which single cells were passed over a 100 µm filter and staining was performed as described above. Cells were acquired on the BD FACSymphony (BD Biosciences) and analyzed with FlowJo software (Treestar).

**Cell culture and in vitro studies**

PD-1 negative OT-I CD8+ T cells (1.0×10⁶) sorted from B16.OVA CD45.1 tumor-bearing mice were cultured with antigen-presenting cells (APCs, T-depleted spleen cells) pulsed with OVA25–267 in the presence or absence of OT-II Tregs (0.2×10⁶) also isolated from the same tumors. Co-cultures were maintained for 7 days with anti-CTLA-4 antibody (clone 9D9; 10 µg/mL) or CTLA-4-Ig (10 µg/mL) added to some wells. Half of the media was replaced with fresh media on day 4. For pme1-1 T cell/tumor spheroid co-cultures, tumor spheroids were generated from resected B16-F10 melanoma tumors by mechanical dissociation with a scalpel followed by transfer of the suspension over a 300 µm filter. The fraction that passed through was then added over a 100 µm filter to retain spheroids that are between 100 and 300 µm in size. The 100 µm filter was then flipped over a 6-well plate and gently rinsed with media to collect the spheroids. Pmel-1 T cells sorted from tumors were first incubated with APCs pulsed with hgp10025–33 peptide for 2 hours after which they were co-cultured with the tumor spheroids for 48 hours. Viability was assessed by staining cells with Annexin V and 7AAD (Invitrogen, Carlsbad, California, USA).

**Cytokine analysis**

Bronchoalveolar lavage (BAL) fluid was collected from the tumor-bearing lungs of orthotopically-implanted mice as previously described.³² BAL fluids were centrifuged for 5 min at 1200 rpm at 4°C and subsequently stored at −80°C and thawed on ice prior to analysis. For melanoma tissues, tumor was first manually dissociated in 1 mL of 1X PBS followed by mechanical dissociation using GentleMACS (Miltenyi) after which samples were centrifuged at 4°C 1500 rpm for 10 min and supernatant was filtered through 0.45 µm filters. Samples were stored at −80°C and thawed on ice prior to analysis. IL-2 was measured in the BAL fluids and melanoma homogenates using the IL-2 Mouse ProcartaPlex Simplex Kit, High Sensitivity (Invitrogen) according to the manufacturer’s instructions.

**TCR sequencing of tumor-infiltrating CD8+ T cells**

Tumor-infiltrating CD8+ T cells were isolated from tumor tissues by FACS sorting. Single-cell TCR sequencing was performed using the 10X Genomics Chromium system. A single-cell suspension derived from dissociated tissue was analyzed for viability using the Nexcelom Cellometer.
Auto 2K. The cell suspension was loaded onto the 10X Genomics Chromium Single Cell Controller at a concentration of 1000 cells per microliter in order to encapsulate 10,000 cells per sample. Briefly, the single cells, reagents, and 10X Genomics gel beads were encapsulated into individual nanoliter-sized Gelbeads in Emulsion (GEMs) and then reverse transcription of poly-adenylated messenger RNA was performed inside each droplet. The cDNA and VDJ-enriched libraries were completed in a single bulk reaction using the 10X Genomics Chromium NextGEM Single Cell 5’ v1.1 and V(D)J Reagent Kits. Generation of 5000 sequencing reads per cell for VDJ libraries were made on the Illumina NextSeq 500 instrument. 10X Genomics Cell Ranger (V.3.0.2) vdj pipeline was used for demultiplexing, barcode processing, sequence assembly, and paired clonotype calling. R package Immunarch (V.0.6.5) was used for clonotype analysis. Simpson Diversity Index for each sample was calculated using R package vegan (V.2.5–7). Simpson Clonality was calculated by taking square root of 1-Simpson Diversity Index.

Statistical analysis
Statistical significance was evaluated by Student’s t-test for comparisons between two groups or one-way analysis of variance for multigroup comparisons using Prism V.9 software (GraphPad). P value<0.05 values were considered statistically significant (*), p value<0.01 values were considered very significant (**), and p value<0.001 values were considered highly significant (***) For regression analysis, R² >0.5 is deemed as strong correlation.

RESULTS
Accumulation of Tregs in the tumor microenvironment correlates with emergence and establishment of dysfunctional phenotype in tumor-infiltrating CD8+ T cells
To determine whether there is a correlation between Treg presence and CD8+ T cell dysfunction as tumor progresses, we assessed the frequencies of tumor-infiltrating Tregs in B16-F10 melanoma and lung adenocarcinoma-bearing mice at defined time points. Treg proportions generally followed an increasing trend in the tumor microenvironment in both models from early to advanced disease stage (figure 1A–D, online supplemental figure S1A–D). This increase was strongly associated with higher expression of T cell immunoreceptor with Ig and ITIM domains (TIGIT), Band T lymphocyte attenuator (BTLA), and PD-1 on tumor-infiltrating CD8 +T cells as tumor progressed (figure 1E). When stimulated ex vivo, tumor-CD8 +T cells in early stage B16-F10 melanoma were comparable in their capacity to secrete IFN-γ and TNF-α compared with their splenic counterparts. However, this functional capacity was significantly impaired at the advanced stage of disease (figure 1F) Thus, a dysfunctional phenotype characterized by upregulation and sustained expression of inhibitory receptors and poor effector activity is coincident with increasing proportions of Tregs in the tumor microenvironment as tumor progressed.

Similar to the mouse models, analysis of Tregs in the tumors of patients with melanoma and non-small cell lung cancer showed that Tregs were more prevalent in advanced tumor stage compared with tissues analyzed from early stage patients (figure 1G, online supplemental figure S1E). Furthermore, higher Treg proportions strongly correlated with higher expression of PD-1, TIGIT, and CTLA-4 on tumor-associated CD8 +T cells (figure 1H, online supplemental figure S1F). These findings indicate a strong association between Treg proportions and expression of a dysfunctional phenotype in the tumor-associated CD8 +T cells of these cancer patients.

Reduced Tregs in the tumor microenvironment ameliorates the exhaustion-like phenotype of tumor-associated CD8+ T cells
Based on the findings above, we postulated that Treg presence in the tumor bed promotes the dysfunctional profile that partially resembled exhaustion phenotype17 in tumor-associated CD8 +T cells. To test this, we treated Foxp3DTR knock-in mice in which diptheria toxin receptor is expressed under the Foxp3 promoter25 with DT in order to deplete Tregs (figure 2A). Efficacy of DT-mediated depletion of Tregs was confirmed in tumor and peripheral tissues (online supplemental figure S2). Coincident with substantial reduction in the proportion of Tregs in the tumors of DT-treated mice, tumor-associated CD8 +T cells expressed much lower levels of TIGIT, BTLA, and PD-1 compared with control mice injected with PBS (figure 2B,C). Furthermore, tumor-infiltrating CD8 +T cells under DT treatment were more capable of secreting IFN-γ and TNF-α on ex vivo stimulation relative to equivalent cells from the tumors of PBS-injected control mice indicating their enhanced effector function capability in the absence of high Treg numbers (figure 2D).

In a complementary study, we transferred graded numbers of Tregs mixed with CD25-depleted conventional T cells into nude mice that were orthotopically implanted with LLC1 cell line and evaluated the phenotype of the tumor-infiltrating CD8 +T cells. Similar to the melanoma model, higher Treg transfers were associated with higher proportions of TIGIT+, BTLA + and PD-1 +CD8+T cells (online supplemental figure S3A,B). Furthermore, the capacity of the tumor-associated CD8 +T cells isolated from the lung tumors of these LLC1 tumor-bearing mice to secrete IFN-γ and TNF-α was significantly diminished under the high Treg transfer setting (online supplemental figure S3C). In genetically engineered Kras mutant, P53-deficient (KP) mice with spontaneous lung cancer development,22 treatment with the bromodomain inhibitor JQ1, which we previously reported reduced the numbers of tumor-infiltrating Tregs24 (online supplemental figure S3D), was also accompanied by reduced populations of tumor-CD8 +T cells that expressed these immune checkpoint proteins (online supplemental figure S3E,F). Together, these results indicate an active contribution of Tregs to emergence and sustenance of...
Figure 1  Accumulation of Tregs correlates with progressive CD8+ T cell dysfunction in the tumor. B16-F10 melanoma-bearing B6 mice or lung tumor-bearing Kras/p53-mutant (KP) genetically engineered mice were monitored for tumor growth and tumors were resected at indicated time points for assessment of Treg proportions and phenotype of CD8+ T cells by flow cytometry. (A, C) Tumor growth kinetics in (A) melanoma and (C) lung tumor-bearing mice. (B, D) Proportion of CD4+CD25hiFoxp3+ Tregs as a fraction of CD4+ cells in (B) melanoma and (D) lung tumors. (E) Representative histograms (top) and summary (bottom) for the expression of indicated inhibitory receptors on CD8+ T cells in melanoma tissues. CD8+ T cells were isolated from spleen or tumor of B16-F10 melanoma-bearing mice at day 8 or day 24 post tumor inoculation and equivalent numbers were stimulated with cell stimulation cocktail plus protein transport inhibitor. (F) Per cent of splenic or tumor-CD8+ T cells that secreted IFN-γ (left) or TNF-α (right) after 6 hours of culture as assessed by intracellular staining. (G) Proportion of CD4+CD25hiCD127loFoxp3+ Tregs in melanoma patient tumors that were analyzed at indicated disease stages as a fraction of total CD4+ cells. (H) Correlation between Treg proportions and expression levels of PD-1 (top) or TIGIT (bottom) on tumor-associated CD8+ T cells in patients with melanoma based on median fluorescent intensity (MFI). Data are representative of 6–7 mice per group (E) or are mean±SEM of 12 patient samples (G). *indicates p value<0.05, **p value<0.01, ***p value<0.001. Ad, advanced disease stage; Ea, early disease stage; IFN, interferon; In, intermediate disease stage; TIGIT, T cell immunoreceptor with Ig and ITIM domains; BTLA, B and T lymphocyte attenuator; PD-1, programmed cell death protein-1; TNF, tumor necrosis factor; Treg, regulatory T cells.
CD8+ T-cell dysfunction in the tumor bed as defined by aforementioned molecular and functional changes.

**Limited co-stimulation availability dictated by Treg presence precipitates the establishment of dysfunctional phenotype in tumor-infiltrating CD8+ T cells**

Beyond quantitative changes in the Tregs as tumor progresses, we also evaluated their phenotype. Although GITR and CD25 were expressed at comparable levels, CTLA-4 which is constitutively expressed in Tregs was further upregulated in advanced stage tumors compared with early stage disease (online supplemental figure S4A). Given that CTLA-4 competes with CD28 to bind the costimulatory molecules CD80/CD86,26 one possibility is that in the tumor microenvironment, augmented expression on Tregs supports its ability to outcompete CD28 and impede the CD28-CD80/86 co-stimulation axis which is important for optimal T cell activation and priming. In support of this possibility, adoptively-transferred OT-1 TCR transgenic CD8+ T cells whose TCRs are specific for

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**Figure 2**  Reversal of dysfunctional phenotype and improved functionality of tumor-infiltrating CD8+ T cells on Treg depletion. Foxp3DTR mice with established B16-F10 melanoma were injected with diphtheria toxin (DT) or with PBS as controls at indicated time points. Tumors were resected for phenotypic analysis of tumor-associated CD8+ T cells by flow cytometry. (A) Schematics of DT or PBS administration. (B) Representative histograms and (C) summary for the expression of TIGIT (left), BTLA (middle), and PD-1 (right) on CD8+ T cells in melanoma tissues. CD8+ T cells were isolated from melanoma tissue of mice treated as indicated and equivalent numbers were stimulated with cell stimulation cocktail plus protein transport inhibitor. (D) Per cent of tumor-CD8+ T cells that secreted IFN-γ (left) or TNF-α (right) after 6 hours of culture as assessed by intracellular staining. Data are representative (B) or are mean±SEM (C, D) of six to seven mice per group. *indicates p value<0.05, **p value<0.01, ***p value<0.001. IFN, interferon; PBS, phosphate-buffered saline; TIGIT, T cell immunoreceptor with Ig and ITIM domains; BTLA, B and T lymphocyte attenuator; PD-1, programmed cell death protein-1; TNF, tumor necrosis factor.
OVA\textsubscript{257-264} upregulated TIGIT, BTLA, and PD-1 in B16-OVA tumors in implanted nude mice when treated with soluble CTLA-4-Ig, which similar to endogenous Treg-expressed CTLA-4, binds available CD80/CD86 on APCs (figure 3A,B). This effect was phenocopied in mice in which CTLA-4-Ig was replaced with co-adoptive transfer of OT-II TCR transgenic Tregs, molecular changes that were absent when there was no CTLA-4-Ig treatment or Treg co-transfer. Importantly, CTLA-4 blockade prevented the OT-II Tregs-induced upregulation of these inhibitory receptors (figure 3A,B). Similar dysfunctional profile was upregulated in OT-I CD8\textsuperscript{+}T cells present in the tumors of B16-OVA melanoma-bearing WT mice when co-transferred with OT-II Tregs or when transferred alone into B16-OVA melanoma-bearing CD86 KO mice (online supplemental figure S4B), suggesting that the emergence of this phenotype is mediated by diminished co-stimulation to effector T cells which can be reproduced by sequestration of costimulatory molecules such as CD86 through antigen-specific Treg-expressed CTLA-4.

We also examined in vitro whether Tregs potentiate the emergence of this dysfunctional phenotype particularly in the setting of antigen-specific CD8\textsuperscript{+}T cell response. To this end, we co-injected OT-I CD8\textsuperscript{+} and OT-II CD4\textsuperscript{+}T cells into B16-OVA melanoma-bearing mice and sorted the PD-1 negative OT-I CD8\textsuperscript{+}T cells which are CD45.2\textsuperscript{+} were then sorted from single-cell suspensions of resected tumors and cultured with or without similarly sorted CD4\textsuperscript{+}CD25\textsuperscript{hi} OT-II Tregs in the presence of OVA\textsubscript{257-264}-pulsed T-depleted spleen cells. α-CTLA-4 antibody was added to some cultures. (C) Representative histograms and (D) summary for the expression of PD-1 on the OT-I CD8\textsuperscript{+}T cells after 7 days under indicated culture conditions. Data are representative of five mice per group (A) or three independent experiments (C). ***p value<0.001, TCR, T-cell receptor; Treg, regulatory T cells; TIGIT, T cell immunoreceptor with Ig and ITIM domains; BTLA, B and T lymphocyte attenuator; PD-1, programmed cell death protein-1.
Restriction of clonal diversity of tumor-infiltrating CD8+ T cells is facilitated by Treg accumulation in the tumor bed

Assessments of the proportions of tumor-infiltrating T cells revealed that while Tregs increased as tumor progressed, the CD8+T cells initially increased but their numbers declined in advanced stage disease resulting in a diminishing CD8:Treg ratio as tumor progressed (online supplemental figure S5A–C). These results raised the possibility that changes in clonal composition of tumor-infiltrating CD8+T cells may be directly or indirectly subject to Treg activity in the tumor. On single-cell TCR sequencing, we found that compared with early-stage tumors, there was an over-representation of some T cell clones and absence of others resulting in a skewed repertoire that reflects reduced clonal diversity of tumor-associated CD8+T cells in advanced tumors (figure 4A).

This is further supported by the higher Simpson clonality score in these cells, which is indicative of a constriction in their TCR repertoire compared with the lower scores in equivalent cells in early tumors or control splenic CD8+T cells (figure 4B). Thus, there is a shift towards TCR repertoire restriction of tumor-associated CD8+T cells with disease progression. To determine whether Tregs play a contributory role in this repertoire reshaping, we treated melanoma-bearing mice with anti-CD25 antibody to deplete Tregs (online supplemental figure S5D) and evaluated the TCR clonality of tumor-associated CD8+T cells. We found that the much reduced clonal composition of
these cells in tumors of rat IgG-injected control mice was partially reversed on Treg reduction although not to the degree of broadness that exists in the polyclonal repertoire existing in control splenic CD8+ T cells (figure 4C). Furthermore, treatment with IL-2:anti-IL-2 immune complex (IL-2C) that increases the bioavailability of IL-2 preferentially to effector T cells (α-IL-2 antibody clone S4B6-1 [25]) phenocopied the Treg reduction effect as TCR diversity under this increased IL-2 condition also led to increased clonal diversity of the tumor-associated CD8+ T cells. Consistent with these observations, clonality score was reduced from the higher value associated with Treg undepleted group (ie, rat IgG control), demonstrating some diversification in the TCR repertoire on Treg reduction in the tumor (figure 4D). In support of this outcome, the proportions of CD8+ T cells increased in the tumors of anti-CD25-treated mice (online supplemental figure S5E) in alignment with the reduction of Tregs within the tumor. Interestingly, the use of IL-2C (α-IL-2 antibody clone JES6-1 [25]) that preferentially targets CD25, the IL-2Rα chain, hence expands Tregs led to even more constriction of the TCR repertoire of the tumor-CD8+ T cells than the rat IgG-injected control mice (online supplemental figure S5F).

**Reduced effector CD8+ T cell survival is imposed by Treg-mediated IL-2 deprivation in the tumor microenvironment**

The declining trend noted for CD8+ T cells in advanced tumors prompted us to investigate whether IL-2, which is important for effector T cell survival and expansion, [27] is differentially altered in the tumor environment as tumor progressed. We found that IL-2 levels were significantly lower in tumor homogenates analyzed from B16-F10 melanoma-bearing mice at advanced tumor stage relative to early stage disease, coincident with high proportions of Tregs in the former (figure 5A, online supplemental figure S6). Furthermore, CD8+ T cells in advanced stage tumors displayed a phenotype suggestive of reduced survival evidenced by higher expression of the pro-apoptotic protein Bax and lower expression of the anti-apoptotic protein Bcl-2 (figure 5B and C). Furthermore, there was a strong positive and negative correlation between the proportion of Tregs in melanoma tissue and the expression of Bax and Bcl-2, respectively in tumor-infiltrating CD8+ T cells (figure 5D,E). Treg depletion by anti-CD25 treatment partially restored intratumoral IL-2 levels after 2 weeks of treatment concomitant to an increase in Bcl-2:Bax ratio on the CD8+ T cells, suggesting an improvement in survival profile (figure 5F,G). We conducted a similar experiment in the orthotopic LLC1 model and similar results were obtained (online supplemental figure S7).

To further substantiate the contribution of Tregs and IL-2 deprivation to detrimental survival of effector T cells, we isolated pmel-1 TCR transgenic T cells previously injected into B16-F10 melanoma-bearing mice and cultured them with tumor spheroids generated from early or advanced stage resected B16-F10 melanoma. Pmel-1 T cells bear TCRs specific for pmel-17, the mouse homolog

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**Figure 5** Improved IL-2 availability and survival profile of tumor-associated CD8+ T cells accompany Treg-depletion in the tumor microenvironment. B16-F10 cell line was subcutaneously implanted into B6 mice. Tumor homogenates from melanoma tissues resected from early (Ea) or advanced (Ad) stage tumors were processed and analyzed by ELISA. (A) IL-2 concentration in the tumor homogenates at indicated tumor stages. The phenotype of CD8+ T cells in these tumors was evaluated by flow cytometry. (B) Representative histograms and (C) summary for the expression of Bax (left) and Bcl-2 (right). Treg proportions and CD8+ T cell phenotype were evaluated in B16-F10 melanoma tissues. (D,E) Correlation between % Tregs in tumors and expression levels of Bax (D) and Bcl-2 (E) as determined by median fluorescent intensity (MFI). Tumor homogenates from melanoma-bearing mice that were treated with α-CD25 or Rat IgG isotype as controls were subjected to cytokine analysis by ELISA. (F) Intratumoral IL-2 concentration. (G) Bcl2/Bax ratio in tumor-associated CD8+ T cells under each treatment condition as determined from MFI expression values. Data are representative (B) or are mean±SEM (A, F, G) of five to six mice per group. *indicates p value<0.05, **p value<0.01, ***p value<0.001. IL, interleukin; MFI, median fluorescent intensity; Treg, regulatory T cells.
of melanocyte-associated antigen gp-100.28 We found that cell viability as assessed by Annexin V and 7AAD staining was significantly low (<20%) in pmel-1 T cells co-cultured with spheroids from advanced tumors in which higher Tregs were confirmed, and this paralleled reduced IL-2 levels in the supernatant. In contrast, pmel-1 T cells exposed to early tumor spheroid cultures were more viable (>40%) and higher IL-2 levels detected in culture supernatants mirrored lower fraction of confirmed Tregs in the starting tumor (online supplemental figure S8A-C). Given that Tregs constitutively express CD25, the alpha chain of the trimeric high-affinity IL2 receptor, our findings support the notion that the accumulation of Tregs in the tumor impedes the overall clonal diversity of tumor-infiltrating CD8 +T cells by limiting IL-2 availability that could support the survival and persistence of various clones of CD8 +T cells trafficking to the tumor bed.

Enhanced IL-2 availability via administration of IL-2 immune complexes plus local Treg depletion evokes antitumor immunity in tumor-bearing mice

Based on the observations above, we reasoned that Treg depletion plus IL-2 supplementation should favor reduced dysfunction and support improved maintenance of CD8 +T cells infiltrating the tumor to facilitate enhanced antitumor response. In a previous report, we found a discrete subset of lung tumor-confined KLRG1-expressing Tregs which exhibited enhanced suppressive function, and their proportions correlated with tumor burden.24 This Treg subset was also dominantly present in melanoma-bearing mice analyzed 6 days after the last treatment (online supplemental figure S9B). Anti-KLRG1 antibody alone led to moderate depletion of tumorous but not splenic Tregs and this was mostly evident in the KLRG1 +Treg fraction. The bromodomain inhibitor JQ1, which can also partially deplete intratumoral Tregs,26 had a more profound depleting effect that included some of the KLRG1 +Tregs (online supplemental figure S9C,D). Consistent with the reduction in Tregs, CD8 to Treg ratios were increased under the combined supplemental strategy (figure 6A). Based on these effects, we reasoned that robust targeting of tumor-associated Tregs could be achieved by combining JQ1 with anti-KLRG1 antibody as a strategy to reverse some of the T cell dysfunctional phenotype associated with the accumulation of Tregs as tumor progressed. In in vivo drug efficacy studies, we then treated another cohort of mice with established melanoma with anti-KLRG1 and/or JQ1 followed by introduction of IL-2C once every 4 days (figure 6B) and mice were monitored long-term for treatment efficacy. While moderate delay in tumor growth was observed in mice treated with either JQ1 or anti-KLRG1 supplemented with IL-2C, it was more significant when both were combined with IL-2C administration, leading to the longest survival of treated mice (figure 6C). Importantly, analysis of the tumor-infiltrating T cells revealed a sustained increase in the ratio of CD8 +T cells to Tregs (figure 6D), and these CD8 +T cells showed a gradual reversal in the increased frequencies of BTLA+, TIGIT+, and PD-1 +tumor-associated CD8 +T cells that were initially observed in untreated controls, revealing a somewhat additive effect of the combinatorial strategy (figure 6E). Consistent with the therapeutic outcomes associated with the drug treatments, tumor-infiltrating CD8 +T cells expressed significantly higher granzyme B and IFN-γ under the combined drug treatment on ex vivo stimulation compared with either agent alone (figure 6F,G). Thus, targeted depletion of Tregs plus IL-2 supplementation restored effector T cell functionality and promoted therapeutic benefits in tumor-bearing mice.

DISCUSSION

The contribution of Tregs to impairment of effective T cell response against tumors is well established.5 30 While we have come a long way in understanding their mode of suppression, our knowledge of how Tregs blunt antitumor responses against various tumor-associated antigens continues to evolve. In the present study, we demonstrate that Treg presence not only shapes the emergence of T cell dysfunction but also facilitates clonal restriction of tumor-infiltrating T cells in murine models of melanoma and lung cancer. This bimodal program could be explored for therapeutic targeting in Treg-centric approaches especially one that relies on localized functional or quantitative impairment of Tregs in the tumor milieu.

Tumor-infiltrating Tregs express or upregulate a number of proteins that may signal distinct clonal populations in the tumor microenvironment.12 31 32 Our study is in alignment with that of others in which clonal populations of Tregs are implicated in promoting T cell impairment.31 33 While our studies focused on the composite intratumoral Treg population, we speculate that various clonal Treg subsets likely function as a collective pool to enact their inhibitory program limiting effector T cell diversity and function as demonstrated in our study in which the bulk of the tumor-associated Tregs was targeted. This led to a reversal in exhaustion-like phenotype and improved the clonal representation of tumor-associated CD8 +T cells.

Persistent antigen stimulation as described in the context of chronic viral infections leads to a state of T cell exhaustion.34-36 This same phenomenon is now appreciated in cancer settings.10 15 37 Based on our findings, we surmise that chronic encounter of tumor-associated antigens by these potential tumor-reactive T cells in the absence of sufficient co-stimulation is key to their phenotypic shift towards dysfunction, orchestrated through Treg-expressed CTLA-4 that restricts effector T cell access to requisite costimulatory signals such as CD86.
This notion is supported not only by the increase in Tregs with tumor progression but by their higher expression of CTLA-4 compared with their peripheral counterparts. Thus, a ‘below-threshold’ activation program fueled by Treg-orchestrated suboptimal co-stimulation creates a recipe for priming conditions that result in T cell dysfunction. Most tumor-associated antigens are derived from self-proteins and their presentation can occur under tolerogenic conditions. Additionally, potential tumor-reactive T cells may be actively blunted through Treg activity. Under optimal TCR activation/co-stimulation, and in the absence of inhibitory mechanisms, these effector T cells in principle should be able to mount measurable effector antitumor T cell responses.

In this regard, eliminating the barriers imposed by tumor-infiltrating Tregs such as limiting co-stimulation, promoting effector T cell functional impairment, and reducing effector T cell diversity as demonstrated in our study should bode well for improved antitumor immunity. We infer from our studies that limited IL-2 availability is contributory to the shrinking of the clonal pool of tumor-associated CD8+ T cells which exhibited reduced viability and functionality as tumor progressed. Indeed, acting as an ‘IL-2 sink’ is one of the modules for Treg inhibitory programs. In support of this inference, drugs that reduce intratumoral Tregs with intermittent IL-2 supplementation as was explored in this study would be predicted to not only rescue effector T cells from IL-2 starvation that could hamper their survival and maintenance in the tumor microenvironment, it would make these cells less prone to becoming dysfunctional. While IL-2 has long been explored for cancer therapy, the toxic effects and vascular leakage syndrome that accompanies prolonged systemic use limits its clinical utility. Thus, approaches such as intratumoral administration which appears to be effective for accessible tumors would be ideal to mitigate these side effects. To extend such approach to less accessible tumors, customized delivery

Figure 6  Localized tumor-Treg targeting with IL-2 supplementation promotes enhanced antitumor immunity in melanoma. B16-F10 melanoma-bearing mice were administered α-KLRG1 antibody and/or JQ1 intraperitoneally for 2 weeks after which single-cell suspensions from resected tumors were stained for Treg markers. Control mice received vehicle and isotype antibody. (A) Per cent Tregs within tumor-associated CD4+ T cells in control and treated mice as indicated. (B) Schematics of in vivo drug treatment in B16-F10 inoculated mice in which mice were monitored long-term after treatment with α-KLRG1 antibody and/or JQ1 supplemented with IL-2:anti-IL-2 immune complex (IL-2C). (C) Kinetics of survival for each group of control and treated tumor-bearing mice. At clinical endpoints, tumors were resected and the proportions of tumor-associated CD8+ T cells and Tregs or phenotype of the CD8+ T cells was examined by flow cytometry. (D) CD8/Treg ratios in the tumors. (E) Summary for the per cent of tumor-CD8+ T cells that expressed BTLA, TIGIT, and PD-1 in indicated control and treated mice. Tumor-infiltrating CD45+CD3+T cells were isolated from tumor cell suspensions and equivalent numbers stimulated for 6 hours with Cell Stimulation Cocktail plus protein transport inhibitor after which intracellular staining was conducted. Representative circle plots (F) and summary (G) for the proportion of gated CD8+ T cells that expressed IFN-γ or Granzyme B (GZB) under each treatment condition. Data are mean±SEM of six to seven mice per group. *indicates p value<0.05, **p value<0.01, ***p value<0.001. IFN, interferon; IL, interleukin; TIGIT, T cell immunoreceptor with Ig and ITIM domains; BTLA, B and T lymphocyte attenuator; PD-1, programmed cell death protein-1; Treg, regulatory T cells.
platforms will need to be developed while navigating the delicate balance in activating effector T cells without fueling Tregs in the local tumor environment.

Although the combinatorial regimen explored in our study did not include CTLA-4 blockade, existing reports suggest that combining IL-2 with other immunotherapy agents may promote clinical benefit. Whether ipilimumab depletes or impairs Treg function in patients with cancer remains a subject of investigations, but one thing is clear: the use of agents that appear to have deleterious effects on tumor but not peripheral Tregs similar to the bromodomain inhibitor and anti-KLRG1 antibody employed in our studies would be rational partners with IL-2 supplementation. JQ1 is a purely experimental drug and is not used in humans but the novel bivalent bromodomain 4 inhibitor AZD5150 which is currently being evaluated in clinical trials (NCT03205176) may phenocopy JQ1’s deleterious effects on Tregs, a possibility that we are presently investigating.

In conclusion, our study supports the rationale that immuno-modulatory agents that could wholly or partly disrupt Treg suppressive machinery within the tumor microenvironment will reduce T cell dysfunction and promote T cell repertoire diversification to favor improved immune responses against tumors. In this regard, the drawing board for Treg-centric approaches for cancer therapy could use some newly emerging agents ranging from epigenetics-modifier drugs to antibodies targeting unique tumorous Treg clones as well as engineered cytokines that might favor enhancement of effector T cell functionality over Tregs.

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Ethics approval All breeding and treatment studies were performed with the approval of Moffitt Cancer Center Animal Care and Use Committee. All animal work was conducted in accordance with ARRIVE guidelines and in accordance with institutional guidelines for animal welfare. Analysis of patient samples was conducted with de-identified tumor specimens obtained from consenting patients under IRB-approved protocol #00000971 at the Moffitt Cancer Center.

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Data availability statement All data relevant to the study are included in the article or uploaded as supplementary information. The TCR sequencing data generated and/or reported in this study have been deposited in the NCBI GEO public data repository under GEO accession number GSE200515.

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