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Anti–PD-1 Checkpoint Therapy Can Promote the Function and Survival of Regulatory T Cells

Sarah C. Vick,†,* Oleg V. Kolupaev,‡ Charles M. Perou,†,* and Jonathan S. Serody,*†

We have previously shown in a model of claudin-low breast cancer that regulatory T cells (Treg) are increased in the tumor microenvironment (TME) and express high levels of PD-1. In mouse models and patients with triple-negative breast cancer, it is postulated that one cause for the lack of activity of anti–PD-1 therapy is the activation of PD-1–expressing Tregs in the TME. We hypothesized that the expression of PD-1 on Treg would lead to enhanced suppressive function of Tregs and worsen antitumor immunity during PD-1 blockade. To evaluate this, we isolated Tregs from claudin-low tumors and functionally evaluated them ex vivo. We hypothesized that the expression of PD-1 on Tregs would lead to enhanced suppressive function of Tregs and worsen antitumor immunity.

We found several genes associated with survival and proliferation pathways; for example, Jun, Fos, and Bcl2 were significantly upregulated in Treg exposed to anti–PD-1 treatment. Based on these data, we hypothesized that anti–PD-1 treatment on Treg results in a prosurvival phenotype. Indeed, Treg exposed to PD-1 blockade had significantly higher levels of Bel-2 expression, and this led to increased protection from glucocorticoid-induced apoptosis. In addition, we found in vitro and in vivo that Treg in the presence of anti–PD-1 proliferated more than control Treg. PD-1 blockade significantly increased the suppressive activity of Treg at biologically relevant Treg/T naïve cell ratios. Altogether, we show that this immunotherapy blockade increases proliferation, protection from apoptosis, and suppressive capabilities of Tregs, thus leading to enhanced immunosuppression in the TME.

Immunotherapy has been a promising new approach to cancer treatment in the last decade. Immunotherapy involves enhancing the patient’s immune cells to kill tumor cells. PD-1/PD-L1 signaling is an important adaptive immune response pathway to ensure the immune system is activated only at the appropriate times to minimize inflammation in the setting of persistent Ag. PD-1 expression on T cells from patients with cancer is critical to the progressive dysfunction of these cells in the tumor microenvironment (TME). Tumors can use this immune suppression mechanism by over-expressing PD-L1, the ligand to the PD-1 receptor, thus dampening antitumor immune activity in the TME. Most of the previous studies evaluating the function of PD-1 have been focused on cytotoxic CD8+ T cell function in the context of both chronic viral infections and cancer. CD8+ T cell exhaustion is characterized by the loss of proliferation, reduction in proinflammatory cytokine production, and diminished cytotoxic activity. This loss of function can be reversed by blocking the PD-1/PD-L1 signaling axis, restoring cytokine production, proliferation, and leading to an enhanced immune response.

The role of PD-1 on other types of immune cells in the TME is much less well understood.
Our group has previously shown that TNBC is typically heavily infiltrated with both adaptive and innate immune cells (12). Most recently, the IMPassion130 Study demonstrated a significant improvement in progression-free survival in patients treated with the anti-PD-L1 mAb atezolizumab plus nab-paclitaxel, a microtubule-disrupting chemotherapy agent, compared with those receiving the chemotherapy alone (13). Despite this, the clinical response for patients with TNBC treated with anti-PD-1 or PD-L1 mAb therapy alone was modest, with 6–19% of patients responding to therapy and with none of these patients responding persistently. In addition, in many cancers refractory to PD-1 blocking therapy, it has been reported that a subset of these patients can experience hyperprogression of cancer (14–16) from anti–PD-1 immunotherapy. The reason for this hyperprogression is not well understood, although it is noteworthy that regulatory T cells (Tregs) are increased after PD-1 blockade in these patients (14, 17, 18). Thus, although PD-1 expression on cytotoxic CD8 T cells may be the primary target of immune checkpoint inhibition, it is becoming evident that PD-1 expressed by other immune cell subsets could contribute significantly to the effectiveness of checkpoint blockade (19–22).

A major limitation to characterizing the function of PD-1 on non-CD8 T cells has been the lack of tumor models with substantial expression of PD-1 on immune cells other than CD8 Tumor-infiltrating lymphocytes (TILs). Although it has been demonstrated in various tumor settings that Tregs often express high levels of PD-1, until now a suitable model for studying checkpoint blockade in tumors highly infiltrated with PD-1–Tregs was not feasible. Work from our group has shown that in a mouse model of claudin-low breast cancer, the frequency of CD4 T cells expressing PD-1 was greater than the frequency of PD-1–CD4 conventional T cells and CD8 T cells subsets (23). Because Tregs provide an important mechanism of immune suppression and evasion in cancer progression (24), we used our previous model and two additional models to evaluate the hypothesis that PD-1–Tregs could be enhancing immune suppression in the TME after PD-1 blockade and potential mechanisms for this finding.

Materials and Methods

Mice and cell lines

BALB/cJ, BALB/c Foxp3-GFP, BALB/c Thy1.1, and C57BL/6 (B6) females were purchased from The Jackson Laboratory (Bar Harbor, ME). Female mice (8–14 wk) were used for all experiments. T11 and T12 (claudin-low) tumor models have been described (23, 25). T12 cells were prepared by harvesting a T12 tumor from a tumor-bearing mouse, followed by manual and chemical digestion to form a single-cell suspension. E0771 cells were obtained from the American Type Culture Collection (ATCC). All tumor cells lines were found to be free of mycoplasma as determined by PCR testing. BALB/c mice were injected with 1 × 105 T11 (claudin-low) cells in PBS or 1 × 106 T12 (claudin-low) cells in Matrigel high concentration low-growth factor. B6 mice were injected with 2.5 × 106 E0771 (luminal) cells in PBS. Tumors were orthotopically transplanted by intradermal injection into a mammary fat pad. Isolated cells were then B220 and CD25 depleted using PE-conjugated Abs (130-049-82; BioLegend). E0771 cells and T11 cells were stained with the Cell Proliferation Dye eFluor 670 (65-0840; eBioscience) and plated in media and Lympholyte-M (CL5031; Cedarlane) gradient.

Abs and flow cytometry reagents

Flow cytometry mAbs against murine CD45 (30-F11, #11-0451-82), Foxp3 (FJK-16S, #45-5773-82), PD-1 (J43, #48-9981-82), Ki67 (SolA15, #17-5698-80), Thy1.1 (HIS51, #45-0900-80), CTLA-4 (UC10-489, #12-1522-82), and GC-induced TNF receptor (GITR; DTA-1, #25-5874-82) were purchased from Invitrogen. mAbs against murine CD4 (GL1.5, #100414), CD8 (53-6.7, #100722), PD-1 (RMP-1, #109103), LAP-TGF-β (TW7-16B8, #141405), CD25 (PC6/1, #102051), and Breg (Bo2a, #339808) were purchased from BioLegend. mAbs against murine Bcl-2 (3F11, #556537) were purchased from BD Biosciences (San Jose, CA), and mAbs against murine Bim (C34C5, #948055) were purchased from Cell Signaling Technology. Cell viability was determined using Aqua Fluorescence Reactive Dye (#F34965; Life Technologies). For flow cytometry, cells were surface stained and fixed/permeabilized overnight using the Foxp3/Transcription Factor Staining Buffer Set (000-5523-00; eBioscience), and intracellular staining was performed the following day according to the manufacturer’s instructions. Apoptosis was measured using PE Annexin V Apoptosis Detection Kit (#559763; BD Pharmingen). Data were acquired using the BD FACSanto or BD LSRFortessa (BD Biosciences). Acquired data were analyzed using FlowJo Flow Cytometry Analysis Software (FlowJo, Ashland, OR).

Proliferation assays using Breg incorporation

Tumor-bearing BALB/c mice were injected with 2 mg Breg1.p in 200 μl DPBS 24 h before TIL isolation. Isolated TILs were stained using allophycocyanin Breg1 Flow Kit (51-900019AK; BD Biosciences) adapting the manufacturer’s protocol. In brief, cells were stained for surface Ags, then resuspended in BD CytoFx/Cytoperm buffer for 30 min on ice. Cells were washed with Perm/Wash and resuspended in BD Cytoperm Permeabilization Buffer Plus for 10 min on ice. Cells were then refixed/permeabilized overnight using the Foxp3/Transcription Factor Staining Buffer Set (000-5523-00; eBioscience). Cells were then treated with 30 μg DNase for 1 h at 37°C. Cells were then stained for intracellular proteins including BrdU for 30 min at room temperature. Data were acquired using the BD FACSanto (BD Biosciences). Acquired data were analyzed using FlowJo Flow Cytometry Analysis Software.

In vivo Abs

mAbs used for in vivo Ab inhibition were purchased from BioXCell (#BE0033-2). Mice undergoing immune checkpoint inhibition received i.p. injection of 200 μg anti–PD-1 (J43) or 200 μg anti–PD-1 (J43) Ag binding fragments (Fabs) created using Pierce Fab Preparation Kit (44985; Thermo Fisher) on day +7 posttumor implantation when the tumor was palpable and then every 3–4 d throughout the experiment.

RNA sequencing

Foxp3 GFP Tregs isolated from tumors were sorted using a MoFlo XDP (Beckman Coulter, Pasadena, CA) to >90% purity. RNA was isolated from sorted Tregs using RNeasy Micro Kit (Qiagen, Germantown, MD). RNA sequencing (RNA-seq) libraries were constructed with NuGen Ovation SoLo (NuGen Technologies, Redwood City, CA). Samples were sequenced using Illumina HiSeq 2500 Rapid Run (Illumina, San Diego, CA). Differential gene expression analysis was performed using DESeq2 (27). Ingenuity Pathway Analysis (IPA) was performed in a Web portal (https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis/).

Treg suppression and proliferation assays

For the Treg suppression assays, we evaluated tumor-infiltrating Tregs. Foxp3 GFP cells were sorted from tumors of T11 (claudin-low)-bearing mice using a MoFlo XDP (Beckman Coulter, Pasadena, CA) to >90% purity. APCs were isolated from wild-type BALB/c splenocytes after CD90 microbead depletion (130-049-82). Mice undergoing immune checkpoint inhibition received i.p. injection of 200 μg anti–PD-1 (J43) or 200 μg anti–PD-1 (J43) Ag binding fragments (Fabs) created using Pierce Fab Preparation Kit (44985; Thermo Fisher) on day +7 posttumor implantation when the tumor was palpable and then every 3–4 d throughout the experiment.

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Isolation of murine TILs

Murine tumors were resected and digested in Liberase TL (5401020001; Roche), DNase I (D4527; Sigma), Hyaluronidase (Sigma), and Collagenase XI (C9697; Sigma), as previously described (26). Single-cell suspensions were enriched by lysotyping by isolating cells at the interface of a 44% Percoll (P1644; Sigma) in media and Lympholyte-M (CL5031; Cedarlane) gradient.

For the assays measuring proliferation of Tregs ex vivo, we evaluated tumor-infiltrating Tregs. Foxp3 GFP cells were sorted on a cell sorter similar to described earlier to >90% purity. The sorted Tregs were then stained with the Cell Proliferation Dye eFluor670 (65-0840; eBioscience) and plated in media and Lympholyte-M (CL5031; Cedarlane) gradient.
with irradiated APCs and soluble anti-CD3 with or without anti–PD-1 Fabs in the cell culture. Fabs of PD-1 made from Ab clone J43 were used in vitro cultures to eliminate effects from Fc-mediated activity of the Abs. Cells were cultured for 3 d, stained, and FACS analyzed.

**Treg apoptosis assays**

For the assays measuring ex vivo Treg apoptosis, we evaluated tumor-infiltrating Tregs. After isolation of TILs, the isolated lymphocytes were enriched for total T cells using a T recovery column kit (CL101; Cedarlane). T cells were then cultured with 10^5 MTT assay with ABT-199

T11 cells were plated in a 96-well plate in complete media and incubated overnight. Venetoclax (ABT-199) was dissolved in DMSO, diluted in complete media, and added to the T11 cells at a starting concentration of 20 μM. T11 cells with ABT-199 were incubated at 37°C, 5% CO₂ for 48 h. Cells were then harvested, and cell death was determined using MTT Cell Growth Assay (CGD1; Sigma) following the manufacturer’s protocols. ABT-199 dose–response curve and IC₅₀ were calculated using Prism (GraphPad, San Diego, CA).

**Results**

In our model of claudin-low breast cancer, a large number of Tregs infiltrating the tumor expressed PD-1. The level of PD-1 expression on Tregs was not uniform (Fig. 1A), with the majority of PD-1⁺ Tregs expressing low levels of the protein, while some Tregs expressed higher levels of PD-1 (Fig. 1B). Although fewer in proportion, the PD-1⁺ Treg population had a significant increase in suppressive molecules, such as CTLA-4 (p = 0.05) and proteins critical to Treg function, such as the high-affinity IL-2 receptor α subunit, CD25 (p = 0.028) (Fig. 1C).

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**FIGURE 1.** Infiltrating Tregs increase in the tumor after PD-1 blockade. Mice were injected with 1 × 10⁶ T11 (claudin-low) tumor cells. Tumors were harvested at 150 mm², digested, enriched for lymphocytes, and analyzed by FACS. Cells were gated on lymphocytes/single cells/live/CD3⁺/CD4⁺ Foxp3⁺ and then analyzed for Treg markers. (A) Representative flow plots gated on CD4⁺ Foxp3⁺ Tregs showing PD-1 expression levels. (B) Percent PD-1⁻/PD-1⁺, PD-1⁻/PD-1⁺, and PD-1⁻/PD-1⁺ CD4⁺ Foxp3⁺ Treg (n = 6). (C) Percent CD4⁺ Foxp3⁺ Treg expressing CD25 or CTLA-4 in PD-1⁻ versus PD-1⁺ populations (n = 4). (D and E) Mice were untreated or treated with 200 μg anti–PD-1 Ab (J43) injected i.p. twice a week for the duration of the experiment. (D) Percent CD4⁺ Foxp3⁺ Treg from the CD45⁻ gated population (n = 9). (E) Geometric (Geo) MFI of Foxp3 in CD4⁺ Foxp3⁺ cells (n = 9). Statistical significance was determined by Mann-Whitney U test. *p < 0.05, **p < 0.01, ****p < 0.0001.
Although the functional differences between these PD-1+ Treg populations are unknown, it has been shown that only intermediate PD-1–expressing CD8+ T cells can be rescued by PD-1 blockade, while PD-1high T cells are committed to exhaustion (28). Because we observed a low percentage of PD-1high–expressing cells, we assessed the outcome of PD-1 blockade on the PD-1+ Treg infiltrating the claudin-low tumors. We compared CD4+Foxp3+ TILs from untreated mice with mice treated with anti–PD-1 Ab and saw a significant increase in the frequency of Tregs in mice treated with PD-1 blockade (p = 0.004) (Fig. 1D). We also observed a significant increase in Foxp3 levels measured by the geometric mean fluorescence intensity (MFI) of Foxp3 in Tregs treated with PD-1 blockade (p < 0.001) (Fig. 1E). Higher Foxp3 levels have been directly associated with increased suppressive capabilities in Tregs (29), thereby suggesting that Tregs treated with PD-1 blockade could lead to increased immunosuppression in the TME in claudin-low tumors.

To determine whether there were transcriptional differences between Tregs isolated from untreated claudin-low tumors versus Tregs from tumors treated with PD-1 blockade, we sorted GFP+ Tregs from Foxp3GFP reporter mice and performed RNA-seq. This demonstrated transcriptional changes in the Tregs from tumors treated with PD-1 blockade (Fig. 2A). We found 27 significantly differentially regulated genes in Tregs isolated from mice treated with PD-1 blockade when compared with untreated controls (p adjusted value [padj] < 0.05) (Table I). We used IPA to determine whether any biological pathways were affected by PD-1 blockade in our RNA-seq data. IPA predicted that the apoptosis pathway was inhibited when Tregs were treated with PD-1 blockade. In addition, Jun and Fos (p = 0.001), genes responsible for T cell proliferation (30), were significantly upregulated in Tregs from tumors treated with PD-1 blockade (Fig. 2B). Bcl-2, an antiapoptotic protein, was also significantly upregulated (p = 0.028) in Tregs isolated from tumors treated with PD-1 blockade (Fig. 2B). Based on these data, we hypothesized that PD-1 blockade in claudin-low tumors was promoting a prosurvival phenotype in Tregs.

To test this hypothesis, we first evaluated the proliferative potential of Tregs in vitro. Tregs cultured with anti–PD-1 Fabs proliferated significantly more than Tregs without anti–PD-1 in the culture (p < 0.0001) (Fig. 3A, 3B). To confirm that the significant proliferation of Tregs resulted from activation through CD3/CD28 engagement rather than an artifact of the anti–PD-1 Fabs, we cultured Tregs with anti–PD-1 Fabs alone without anti-CD3. PD-1 blockade alone did not lead to Treg proliferation (Supplemental Fig. 1), suggesting that the increase in proliferation is due to the release of the inhibitory signal from PD-1, thus allowing the Tregs to proliferate. We next investigated whether the increase in Treg proliferation was also present in vivo in the TME. To address this question, we evaluated cellular proliferation by BrdU incorporation. When immune cells were

![Volcano plot](http://www.jimmunol.org/)
isolated during early tumor growth (tumor size of 50 mm²), the Tregs proliferated significantly more than CD8⁺ or CD4⁺ Foxp3⁺ T cells (p = 0.029) (Fig. 3C). We could not detect a difference in proliferation between Tregs from mice treated with anti–PD-1 versus untreated (data not shown) on day 15 after tumor injection (50 mm²). We then evaluated proliferation at day 23 after tumor injection, by measuring expression of proliferation marker Ki-67 (31). We saw a nonsignificant increase in the frequency and total number of proliferating Tregs from mice treated with anti–PD-1 Fab compared with untreated mice (Fig. 3D, 3E). We have previously published in this model of claudin-low breast cancer that Tregs infiltrating into the tumors have significantly higher levels of PD-1 expression than CD8⁺ T cells (23). We predicted that PD-1 blockade would have a reduced impact on CD8⁺ T cells than it does on Tregs because of the reduced PD-1 expression. Indeed, PD-1 blockade did not increase the frequency of CD8⁺ Ki67⁺ T cells compared with untreated mice (Fig. 3F). Tregs not only have increased proliferation when exposed to anti–PD-1, but in our model of claudin-low breast cancer, the Tregs proliferate at a higher rate than other T cell subsets (Fig. 3C), suggesting an increased potential for Treg-mediated suppression in the TME.

From our RNA-seq data, we found that Bcl-2 was significantly upregulated in Tregs during treatment with anti–PD-1 (Fig. 2B). As an antiapoptotic protein, Bcl-2 expression can protect cells from apoptosis induced by various stimuli (32). To validate our RNA-seq data, we confirmed that Bcl-2 protein was upregulated in Tregs isolated from tumors treated with anti–PD-1. Mice were injected with claudin-low tumors, treated with anti–PD-1, or left untreated, and then tumors were harvested at 150 mm² to analyze protein expression by flow cytometry. The frequency of Bcl-2⁺ CD4⁺ Foxp3⁺ cells was approximately eight times higher in Tregs from anti–PD-1 treated mice when compared with untreated mice (Fig. 4A, 4B). Mice treated with anti–PD-1 also had a significant increase in the levels of Bcl-2 in Tregs when compared with Tregs from untreated mice (p = 0.018) (Fig. 4D). We also measured the proapoptotic protein Bim and saw no difference in Bim frequency or expression levels between the two treatment groups of Tregs (Fig. 4C, 4E). Bcl-2/Bim ratios are often used as a measure for survival potential in cells. Tregs exposed to anti–PD-1 had significantly higher Bcl-2/Bim ratios than untreated Tregs (p = 0.028) (Fig. 4F), suggesting a potential for increased protection of Tregs from apoptosis in the TME. Thus, both the increased frequency of Bcl-2⁺ Tregs and the increased expression of Bcl-2 in Tregs could enhance resistance of Tregs to apoptosis on treatment with anti–PD-1 mAb therapy.

Because we found a significant increase in levels of Bcl-2 in the Tregs from T11 (claudin-low) tumors (Fig. 4A), we sought to test whether these Tregs were protected from apoptosis ex vivo. Antia apoptotic Bcl-2 expression has been shown to inhibit glucocorticoid (GC)-induced apoptosis, so we tested whether Bcl-2 expressed in Tregs could protect them from Dex-induced apoptosis. Tumor-infiltrating T cells were isolated from control Foxp3-GFP T11 (claudin-low) tumor-bearing mice, as well as from mice treated with anti–PD-1 twice weekly, and then cultured ex vivo with or without Dex. Apoptosis in Tregs was assessed using Annexin V/7-aminomycin D (7-AAD) staining. There was greater protection from apoptosis in Tregs from mice treated with anti–PD-1 and cultured in Dex than Tregs from untreated mice (p < 0.0001) (Fig. 5A, 5B). Interestingly, we did not see this significant decrease in cell death in CD8⁺ T cells from mice treated with anti–PD-1 (Fig. 5C), suggesting that this protection from apoptosis may be specific to Tregs in the TME. We confirmed our findings in an additional model of claudin-low breast cancer (T12). There was a decrease in Tregs undergoing apoptosis when treated with PD-1 blockade, and this decrease was sustained with the addition of Dex (Supplemental Fig. 2A). To determine whether this protection from apoptosis could be attributed to Bcl-2, we added Venetoclax (ABT-199), a potent and selective Bcl-2 inhibitor. When Bcl-2 was inhibited in vitro, there was no longer a reduction in Tregs undergoing apoptosis with PD-1 blockade (Supplemental Fig. 2B). To determine whether this protection from apoptosis after PD-1 blockade was specific to claudin-low breast cancer, we employed a model of luminal breast cancer (E0771). We determined that unlike the T11 and T12 claudin-low models of breast cancer where there is a greater frequency of Tregs than CD8⁺ T cells expressing PD-1, the E0771 model of breast cancer had a higher frequency of CD8⁺ T cells that were PD-1⁺ (Supplemental Fig. 2B). When we assessed apoptosis in Tregs in mice with E0771 tumors, these cells were not protected from apoptosis induced by Dex (Supplemental Fig. 2C, 2D). Interestingly, CD8⁺ T cells from mice treated with PD-1 blockade were protected from apoptosis in the E0771 luminal breast cancer model (Supplemental Fig. 2E). Thus, protection from apoptosis was directly correlated with the difference in the expression of PD-1 by Tregs and CD8⁺ T cells.

We also treated mice with a Bcl-2 inhibitor to determine whether there would be increased apoptosis in Tregs. Mice treated with Bcl-2 inhibitor ABT-199 had delayed tumor growth and increased survival irrespective of anti–PD-1 treatment (Supplemental Fig. 3A, 3B). Although it is possible that ABT-199 had a direct effect on the T11 tumor cells themselves, the EC₅₀ against T11 cells in vitro was 2 μM (Supplemental Fig. 3D), while the IC₅₀ of ABT-199 on Bcl-2—expressing hematopoietic cells is 4 nM (33), suggesting that in our system ABT-199 does not have potent activity against T11 (claudin-low) tumor cells and is likely acting by inhibiting Treg function. However, ABT-199 therapy did not enhance the efficacy of anti–PD-1 mAb in this model. Although the total number of Tregs infiltrating into the tumor after treatment with ABT-199 was similar, the number of CD8⁺ T cells was significantly decreased (Supplemental Fig. 3C), indicating that the effect of Bcl-2 inhibition on the presence of T cells in the...
Treg function (CD25) in limiting the availability of IL-2. All of these are known to contribute to their suppressive capabilities (36).

We wanted to confirm our findings in an additional model of claudin-low breast cancer (T12) that we have previously demonstrated to be enriched in Tregs and refractory to PD-1 blockade therapy (23). After PD-1 blockade in the T12 (claudin-low) model, there was an increase in the mean frequency of Tregs expressing GITR and TGF-β (Supplemental Fig. 4A). There was no difference in CD25 expression on Tregs from mice treated with PD-1 blockade, but the MFI of CD25 was increased on Tregs after PD-1 blockade (Supplemental Fig. 4B). Based on these findings, we then sought to test whether Tregs exposed to PD-1 blockade had increased suppressive capabilities. To address this, we treated Foxp3-GFP T11 (claudin-low) tumor-bearing mice with anti-PD-1 or left them untreated, and the tumors were harvested around 150 mm² for isolation of TILs. Tregs that had been exposed in the TME to PD-1 blockade were significantly better at suppressing naive CD8⁺ T cell proliferation in an ex vivo setting than Tregs from mice that were untreated (Fig. 6C). These differences in suppression were significant for these studies (Fig. 6A). There were no significant differences in the level of expression of these molecules in Tregs as determined by the MFI (Fig. 6B).
significantly at a 2:1 ($p = 0.005$) and a 1:1 ($p = 0.02$) ratio of $T_{reg}$ to CD8$^+$ T cells (Fig. 6C). Based on our previous work, the ratio of $T_{reg}$ to CD8$^+$ T cells in the T11 (claudin-low) TME is $\sim$1:5:1 (23); thus, the suppressive effect observed in our experiment is biologically relevant to $T_{reg}$-dependent inhibition of conventional T cell activation at the ratios used in vitro.

**Discussion**

TNBC has the worst prognosis of the breast cancer subtypes despite being heavily immune infiltrated (37). The standard dogma in cancer immunotherapy is that tumors with immune infiltration have the capacity to mount a productive antitumor immune response and are therefore good candidates for immune checkpoint blockade. However, PD-1 is not only expressed on CD8$^+$ cytotoxic T lymphocytes but also on different populations of CD4$^+$ T and NK cells. Here, we show in a murine model that faithfully reproduces tumors found in patients with claudin-low breast cancer that PD-1 is most frequently expressed on Foxp3$^+$ Tregs. Blockade of PD-1 was associated with enhanced suppression, increased proliferation, and diminished apoptosis of $T_{reg}$ in vitro, which was also reproduced in the TME. These data suggest that the activity of checkpoint
inhibitors is more complicated than currently evaluated. The presence of a substantial immune infiltrate may not predict a response to immune checkpoint therapy if a significant number of the immune cells that express PD-1 are Tregs, which behave differently from conventional T cells on checkpoint inhibition.

The mechanism(s) for the enhanced function of Tregs in the presence of anti–PD-1 mAb therapy is not currently clear. Our data indicate that anti–PD-1 therapy affects at least three different pathways for Treg activity. First, we found increased proliferation of Tregs in the presence of anti–PD-1 mAb therapy. This is consistent with findings evaluating the effects of anti–PD-1 mAb on the proliferation of CD8+ T cells (11) and could be related to the increased expression of Jun and Fos in Tregs from anti–PD-1–treated animals. The second pathway is increased resistance to apoptosis. Previous work has demonstrated a critical role for the expression of Bcl family member proteins and decreased expression of Bim in the maintenance of Tregs (38). We found that anti–PD-1 therapy enhanced Bcl-2 expression and diminished GC-induced apoptosis in Tregs. Interestingly, we found that the Bcl-2 inhibitor ABT-199 could improve the median time for tumor growth in mice receiving T11 tumors, which was independent of coadministration with anti–PD-1 therapy. Given the extremely modest activity of ABT-199 in vitro against T11 tumor cells, these data suggest that inhibition of Bcl-2 in T11 tumors may also be because of diminished function of Tregs. Finally, Tregs exposed to anti–PD-1 therapy had enhanced suppressive function, which correlates with the increased expression of Foxp3 by those cells.

There are currently multiple ongoing clinical trials in TNBC where pembrolizumab (humanized anti–PD-1 Ab) is being given as a monotherapy (39). In all reported trials to date, the overall response rate to PD-1 inhibition in TNBC is reported to be between 4 and 20%, with only a small fraction of patients seeing any benefit from therapy (40). Our previous work has suggested that immune infiltration alone is not a reliable biomarker to predict overall response rate to immune checkpoint therapy, but instead the complete microenvironment including immunosuppression in the TME should be considered (23). Although the expected outcome of PD-1 therapy is that the inhibitory signal on cytotoxic T cells will be blocked, thereby allowing them to remain functional and lead to tumor killing, it is unknown whether PD-1 blockade functions similarly on other immune cell subsets that express PD-1. It has been hypothesized that therapeutic benefit from immune checkpoint blockade could be masked because of enhanced immunosuppression in the TME, leading to hyperprogression of cancer (14–16). Our study supports this hypothesis by demonstrating that PD-1 blockade promoted a prosurvival phenotype and enhanced suppression from PD-1+ Tregs in the TME.

Most of the previous studies looking at the role of PD-1 on Tregs have been in vitro studies from peripheral Tregs. These studies broadly demonstrate that Tregs cultured in vitro with PD-1 blocking Ab enhance proliferation of Tregs (14, 17, 41, 42), although these
Tregs would be exposed to saturating amounts of the Ab. Additional therapy. Furthermore, we could not assume that all PD-1 significance was determined by multiple tests. (A) Percent CD4\(^+\)Foxp3\(^+\) T\(_{\text{reg}}\) expressing suppressive molecules; CTLA-4, GITR, TGF-\(\beta\), and CD25 from mice treated with anti–PD-1 versus untreated (\(n = 9\)). (B) Geometric MFI of suppressive molecules in CD4\(^+\)Foxp3\(^+\) cells (\(n = 9\)). Statistical significance determined by Mann–Whitney U test. (C) Tumors were harvested at 150 mm\(^2\), digested, and enriched for lymphocytes, and GFP\(^+\) T\(_{\text{reg}}\) were sorted using MoFlo-XDP cell sorter. Naïve T cells were stained with proliferation dye and were incubated with sorted T\(_{\text{reg}}\), irradiated APCs, and soluble anti–CD3 in culture for 72 h. Statistical significance was determined by multiple t tests. *\(p < 0.05\), **\(p < 0.01\).

FIGURE 6. PD-1 blockade results in increased suppressive capabilities in T\(_{\text{reg}}\). Mice were injected with 1 \(\times\) 10\(^4\) T11 (claudin-low) tumor cells. Mice were untreated or treated with 200 μg anti–PD-1 Ab (J43) injected i.p. twice a week for the duration of the experiment. (A and B) Tumors were harvested at 150 mm\(^2\), digested, enriched for lymphocytes, and analyzed by FACS. Cells were gated on lymphocytes/single cells/live/CD3\(^-\)/CD4\(^+\)Foxp3\(^+\) and then analyzed for T\(_{\text{reg}}\) markers. (A) Percent CD4\(^+\)Foxp3\(^+\) T\(_{\text{reg}}\) expressing suppressive molecules; CTLA-4, GITR, TGF-\(\beta\), and CD25 from mice treated with anti–PD-1 versus untreated (\(n = 9\)). (B) Geometric MFI of suppressive molecules in CD4\(^+\)Foxp3\(^+\) cells (\(n = 9\)). Statistical significance determined by Mann–Whitney U test. (C) Tumors were harvested at 150 mm\(^2\), digested, and enriched for lymphocytes, and GFP\(^+\) T\(_{\text{reg}}\) were sorted using MoFlo-XDP cell sorter. Naïve T cells were stained with proliferation dye and were incubated with sorted T\(_{\text{reg}}\), irradiated APCs, and soluble anti–CD3 in culture for 72 h. Statistical significance was determined by multiple t tests. *\(p < 0.05\), **\(p < 0.01\).

studies are limited by the fact that T\(_{\text{reg}}\) function and proliferation were measured from peripheral T\(_{\text{reg}}\) rather than tissue-infiltrating T\(_{\text{reg}}\). Our study is novel in that we directly measure the proliferative capacity and suppressive function of tumor-infiltrating T\(_{\text{reg}}\) treated with PD-1 blockade in vivo.

It was somewhat unexpected that the number of significantly expressed genes on T\(_{\text{reg}}\) in mice treated with or without anti–PD-1 mAb was quite modest. The evaluation of persistent expression of PD-1 on CD8\(^+\) T cell exhaustion in vitro is difficult, and as a consequence, we chose to perform our screen using T\(_{\text{reg}}\) isolated from mice after in vivo treatment with anti–PD-1 mAb or control. One limitation to this approach was the performance of bulk RNA-seq on T\(_{\text{reg}}\) sorted from tumors, only 50% of which express PD-1 (Fig. 1B). Inclusion of PD-1-negative T\(_{\text{reg}}\) in our gene expression data may have minimized any changes to transcript regulation of T\(_{\text{reg}}\) from anti–PD-1 therapy. Furthermore, we could not assume that all PD-1–expressing T\(_{\text{reg}}\) would be exposed to saturating amounts of the Ab. Additional factors that might limit changes in gene expression could be a result of the timing of the administration of the Ab in relationship to the time of the RNA-seq evaluation. Nonetheless, we confirmed our findings by measuring protein expression of the relevant genes, thus allowing us to evaluate pathways that could mediate changes in T\(_{\text{reg}}\) function in the presence of anti–PD-1 mAb therapy.

In summary, we have shown in claudin-low tumors that T\(_{\text{reg}}\) express significant levels of PD-1. Blockade of PD-1 on these cells by anti–PD-1 therapy leads to enhanced T\(_{\text{reg}}\) proliferation, suppressive function, and resistance to apoptosis. The increased proliferation we observe is accompanied by increased expression of Jun and Fos, while the resistance to apoptosis is associated with increased expression of Bcl-2. These studies suggest that the activity and toxicity of checkpoint inhibitor therapy may be correlated with differences in expression of PD-1 on CD8\(^+\) versus T\(_{\text{reg}}\). We demonstrate in this study a model of breast cancer refractory to checkpoint inhibition that can be used to determine mechanistically how PD-1\(^{\text{high}}\) T\(_{\text{reg}}\) in the TME alter outcomes to immunotherapy. This hypothesis should be tested clinically and specifically evaluated in the treatment of patients with TNBC, especially those of the claudin-low/mesenchymal subtype.
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Disclosures

C.M.P. is an equity stockholder and consultant of BioClassi. The authors declare no other relevant conflicts of interest.

References


Supplementary Figure S1: PD-1 antibody alone does not result in T<sub>reg</sub> proliferation. Mice were injected with 1 x 10<sup>4</sup> T11 (claudin-low) tumor cells. (A-B) Tumors were harvested at 150mm<sup>2</sup>, digested, enriched for lymphocytes, and GFP+ T<sub>regs</sub> were sorted using MoFlo-XDP cell sorter. T<sub>regs</sub> stained with proliferation dye were incubated with or without α-PD-1 Fabs and irradiated APCs without α-CD3 in culture for 72 hours. (A) Flow cytometry gating strategy for proliferation of T<sub>regs</sub> cultured without or with α-PD-1 Fabs. (n=3) (B) Percent proliferating CD4<sup>+</sup>Foxp3<sup>+</sup> T<sub>regs</sub> from in vitro culture.
Supplementary Figure S2. Apoptosis of T\textsubscript{regs} after PD-1 blockade in T12 (claudin-low) and E0771 (luminal) breast cancer models. (A) BALB/c Foxp3-GFP mice were injected with 1 x 10\textsuperscript{5} T12 (claudin-low) tumor cells (n=3) or (B-E) B6 Foxp3-GFP mice were injected with 2.5x10\textsuperscript{5} E0771 cells (n=4). Mice were untreated or treated with 200 µg α-PD-1 antibody (J43) injected IP twice a week for the duration of the experiment. Isolated total T cells were cultured in 96 well plate in complete media or complete media + 10µM Dexamethasone + 20µM ABT-199. Apoptosis was measured using Annexin V and 7-AAD staining. (A) Percent CD4\textsuperscript{+}Foxp3\textsuperscript{+}7-AAD/Annexin V\textsuperscript{+} T\textsubscript{regs}. (B) Percent PD-1\textsuperscript{+} of T\text{regs} or CD8 T cells among E0771 TILs. (C) Representative flow plots gated on GFP\textsuperscript{+} T\text{regs}. (D) Percent CD4\textsuperscript{+}Foxp3\textsuperscript{+}7-AAD/Annexin V\textsuperscript{+} T\textsubscript{regs} from CD45\textsuperscript{+} parent population. (E) Percent CD8\textsuperscript{+}/7-AAD/Annexin V\textsuperscript{+} T cells from CD45\textsuperscript{+} parent population. Statistical significance determined by Mann-Whitney test. * denotes p < 0.05.
Supplementary Figure S3: Inhibition of Bcl-2 leads to delay of tumor growth and increase in survival. BALB/c mice were injected with $1 \times 10^4$ T11 (claudin-low) tumor cells. Mice were untreated or treated with 200µg α-PD-1 antibody (J43) injected IP twice a week for the duration of the experiment. Mice were also given ABT-199 (100mg/kg) daily, or vehicle daily by oral gavage from day +1 for the duration of the experiment. (A) Individual replicates of tumor growth curves. (B) Mice receiving Bcl-2 inhibitor ABT-199 and ABT-199 + α-PD-1 ($n = 3$) have a significant survival benefit compared to mice receiving vehicle ($n=3$) or α-PD-1 alone ($n = 3$) ($p = 0.0046$; log-rank test for Vehicle + α-PD-1 vs ABT-199 + α-PD-1) ($p = 0.0042$; log-rank test for Vehicle vs ABT-199). (C) Tumors were harvested at 100mm$^2$, digested, enriched for lymphocytes, and analyzed by FACS. (Vehicle + α-PD-1 $n=6$, ABT-199 + α-PD-1 $n=8$) (C) Total number of CD4$^+$Foxp3$^+$ Tregs. (D) T11 (claudin-low) tumor cells were plated in a 96 well plate at 1.5x10$^4$ cells/well and incubated for 24 hours at 37°C. ABT-199 was added at a starting concentration of 20µM and serially diluted. T11 cells plus ABT-199 were incubated at 37°C 5% CO2 for 48 hours. Cell death was measured using Sigma MTT Cell Growth Assay.
Supplementary Figure S4: Characterization of T<sub>regs</sub> from T12 (claudin-low) tumor model. Mice were injected with 1 x 10<sup>5</sup> T12 (claudin-low) tumor cells in Matrigel low-growth factor. Mice were untreated or treated with 200 µg α-PD-1 antibody (J43) injected IP twice a week for the duration of the experiment. (A-B) Tumors were harvested at 150 mm<sup>2</sup>, digested, enriched for lymphocytes, and analyzed by FACS. (A) Percent CD4<sup>+</sup>Foxp3<sup>+</sup> T<sub>regs</sub> expressing suppressive molecules; GITR, TGFβ, and CD25 from mice treated with α-PD-1 versus untreated (n=5). (B) Geometric Mean Fluorescence Intensity of suppressive molecules in CD4<sup>+</sup>Foxp3<sup>+</sup> cells (n=5). Statistical significance determined by Mann-Whitney test.