Dual PD-1 and CTLA-4 Checkpoint Blockade Promotes Antitumor Immune Responses through CD4⁺Foxp3⁻ Cell–Mediated Modulation of CD103⁺ Dendritic Cells

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Abstract

Immunotherapy is widely accepted as a powerful new treatment modality for the treatment of cancer. The most successful form of immunotherapy to date has been the blockade of the immune checkpoints PD-1 and CTLA-4. Combining inhibitors of both PD-1 and CTLA-4 increases the proportion of patients who respond to immunotherapy. However, most patients still do not respond to checkpoint inhibitors, and prognostic biomarkers are currently lacking. Therefore, a better understanding of the mechanism by which these checkpoint inhibitors enhance antitumor immune responses is required to more accurately predict which patients are likely to respond and further enhance this treatment modality. Our current study of two mouse tumor models revealed that CD4⁺Foxp3⁻ cells activated by dual PD-1/CTLA-4 blockade modulated the myeloid compartment, including activation of conventional CD103⁺ dendritic cells (DC) and expansion of a myeloid subset that produces TNFα and iNOS (TIP-DCs). CD4⁺Foxp3⁻ T cell–mediated activation of CD103⁺ DCs resulted in enhanced IL12 production by these cells and IL12 enhanced the therapeutic effect of dual PD-1/CTLA-4 blockade. Given the importance of these myeloid subsets in the antitumor immune response, our data point to a previously underappreciated role of CD4⁺Foxp3⁻ cells in modulating this arm of the antitumor immune response.

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Introduction

The immune system plays a vital role in the control of malignant neoplasms, and infiltration of tumors with immune cells correlates with favorable prognosis in several cancer types (1–3). Immunotherapy is now a major treatment modality in cancer (4–8), particularly the use of checkpoint inhibitors, which block the interactions used by the tumor to suppress effector T-cell function (9). The combination of anti–CTLA-4 and inhibitors of the PD-1: PDL-1/PDL-2 interaction is highly efficacious (6, 8, 10, 11), but the mechanism by which this synergy occurs remains relatively unknown, and predictive prognostic markers are lacking (12). These checkpoint inhibitors work in distinct ways (6, 13): PD-1 blockade is thought to predominantly enhance the function of CD8⁺ T cells within the tumor where the CD8⁺ T cells encounter high-expressing PDL-1⁺ tumor cells and PDL-1⁺/PDL-2⁺ stromal cells (14–16). Although CTLA-4 is also expressed on intratumoral CD8⁺ T cells and potentially limits their activity through interaction with CD80/CD86 at the tumor site (17), anti–CTLA-4 also offers therapeutic benefit through the priming of antitumor T-cell responses in the draining lymph nodes (DLN). The therapeutic efficacy of anti–CTLA-4 may also be partly attributed to the depletion of CD4⁺Foxp3⁻ cells, due to their constitutively high expression of CTLA-4 (18, 19). These distinct mechanisms of action for anti–PD-1 and anti–CTLA-4 may partially explain the increased efficacy observed following combination therapy.

Although CD4⁺ T cells can recognize neoantigens expressed by cancers (20–23) and it has been reported that anti–PD-1/anti–CTLA-4 combination therapy activates CD4⁺Foxp3⁻ cells (24, 25), little is known of the mechanism and consequence of this activation. In the current study, we investigated whether the combination of anti–PD-1/anti–CTLA-4 therapy could activate CD4⁺Foxp3⁻ cells and explored the mechanism underpinning the increase in antitumor efficacy. We observed that combination therapy significantly activated CD4⁺Foxp3⁻ effector cells resulting in the activation of tumor-infiltrating CD103⁺ DCs, a cell important for tumor-antigen presentation and efficacy of checkpoint inhibitors (26–30). Activation of CD4⁺Foxp3⁻ cells was sufficient to stimulate CD103⁺ DCs, shown by the increased IL12 production by these cells in mice depleted of both CD8⁺ T cells and CD4⁺Foxp3⁻ cells. The activation of CD4⁺ T cells was partially
dependent on IL12, indicating a previously undescribed link between tumor-infiltrating CD103+ DCs and T11-like CD4+ Foxp3+ cells in the context of checkpoint inhibition. These results indicate that dual PD-1/CTLA-4 blockade can robustly activate a CD4+ T11-like response that may influence overall treatment efficacy in patients undergoing combination immunotherapy through modulation of the tumor-infiltrating myeloid compartment, including CD103+ DCs.

Materials and Methods

Cell lines and mice

The C57BL/6 mouse breast carcinoma cell line AT-3ova/dim CD73+ and the colon carcinoma line MC38ova/dim were generated as previously described and utilized within 3 weeks of thawing from a master stock generated in 2012 (31–33). United Kingdom Coordinating Committee on Cancer Research guidelines for the use of cell lines in cancer research were followed. Cells were not authenticated in the last year. Tumor lines were also verified to be mycoplasma negative by Victorian Infectious Diseases References Lab (Melbourne, Victoria) by PCR analysis. Tumor cells were grown in DMEM supplemented with 10% FCS, glutamax, and penicillin/streptomycin. For in vivo experiments, the indicated number of cells were resuspended in PBS and injected subcutaneously in a 100 μL volume. C57BL/6 WT and OTII mice were obtained from the Walter and Eliza Hall Institute of Medical Research (Melbourne), DEREG, IFNγ−/−, and Batf3−/− mice were bred in house at the Peter MacCallum Cancer Centre, and IL12p35−/− or IL12p40−/− mice were either bred at the Peter MacCallum Cancer Centre or obtained from Prof. Hartland and Prof. van Driel (University of Melbourne).

Antibodies, cytokines, and peptides

Antibodies to PD-1 (RMP1-14), CTLA-4 (9H10), CD4 (GK1.5), or CD8α (YTS 169.4) IL12 p75 (R2-9A5) or isotype control (2A3) were purchased from Bio X Cell. IL2 used for T-cell stimulation was obtained from the National Institutes of Health (Bethesda, MD). OVA323-339 peptide was purchased from GenScript.

Treatment of tumor-bearing mice

C57BL/6 mice were injected subcutaneously with 5 × 105 AT-3ova/dim CD73+ or 1 × 106 MC38ova/dim tumor cells. Once tumors were established (20–50 mm3), mice were treated with either isotype control (2A3), anti–PD-1 (RMP1-14), or anti–CTLA-4 (9H10) with 3 to 4 doses given 4 days apart. For depletion of CD8+ or CD4+ cells, mice were dosed with the respective antibodies at 250 μg/mouse on days –1, 0, 4, 7, and 14 relative to treatment onset. Diphtheria toxin (0.5 μg/mouse) was administersed on the same days for the depletion of Foxp3+ cells. For IL12 neutralization experiments, anti–IL12 was given on days –1, 0, and 4 prior to FACS analysis on day 7.

Analysis of tumor-infiltrating immune subsets

Seven days after treatment, tumors were excised and digested postmortem using a cocktail of 1 mg/mL collagenase type IV (Sigma-Aldrich) and 0.02 mg/mL DNase (Sigma-Aldrich). After digestion at 37°C for 30 minutes, cells were passed through a 70-μm filter. Inguinal lymph nodes were also harvested and cells were filtered through a 70-μm filter. Cells were then analyzed by flow cytometry as previously described (31) and Fixable Yellow (Thermo Fisher) used as a viability dye.

Intracellular cytokine staining

For detection of IFNγ production by T cells ex vivo, TILs and DLNs cells were cultured for 3 hours with PMA (5 ng/mL) ionomycin (1 μg/mL) in the presence of GolgiPlug (BD Pharmingen) and GolgiStop (BD Pharmingen). After 3 hours, cells were analyzed by flow cytometry. For the detection of IL12p40, cells were cultured for 4 hours in GolgiPlug/GolgiStop either without further stimulation or with LPS (10 ng/mL).

Ex vivo culture of CD4+ T cells derived from DLNs of tumor-bearing mice

Seven days after therapy onset, 2 × 105 cells derived from DLNs were cultured with 300 nmol/L OVA323-339 peptide and IL2 for 5 days in a total volume of 200 μL. Supernatants were then taken for analysis of cytokine content by cytometric bead array.

Ex vivo assessment of antigen-presenting cells (APC) function of cells derived from DLNs of tumor-bearing mice

To assess the antigen-presenting function of cells derived from DLNs, 2 × 105 cells were cultured with naïve splenocytes derived from OTII mice in the presence of 300 nmol/L OVA323-339 peptide for 48 hours. Supernatants were then taken for analysis of cytokine content by cytometric bead array.

Statistical analysis

Statistical differences were analyzed by one-way or two-way ANOVA where indicated with P < 0.05 considered statistically significant.

Results

Induction of CD8+/CD4+ antitumor immune responses by dual PD-1/CTLA-4 blockade

We first evaluated the potential of monoclonal antibodies (mAb) to PD-1 and CTLA-4 to induce antitumor immune responses in a triple-negative breast cancer line AT-3ova/dim, CD73+, a variant of AT-3ova/dim that is resistant to anti–PD-1 single-agent activity (31). Mice were injected with AT-3ova/dim, CD73+ cells, and once tumors were established (20–50 mm3), mice were treated with either isotype control (2A3), anti–PD-1, anti–CTLA-4, or a combination of both checkpoint inhibitors. Mice were treated with 4 doses of antibody, 4 days apart, and tumor growth and survival was monitored. The combination of PD-1 and CTLA-4 blockade significantly reduced tumor growth (Fig. 1A) and enhanced the survival of mice (Fig. 1B), whereas single blockade of either PD-1 or CTLA-4 had only modest antitumor activity. Similar results were obtained using the colon cancer cell line MC38 ova/dim (Fig. 1C and D).

To investigate the involvement of the CD4+ and CD8+ T-cell subsets in the antitumor immune response, mice bearing AT-3ova/dim-CD73 tumors were treated with anti–PD-1/anti–CTLA-4 in mice depleted of CD4+ or CD8+ T cells (Fig. 1E). Depletion of CD8+ and CD4+ T cells was effective, resulting in depletion of more than 90% of CD8+ and CD4+ T cells, respectively, as shown by FACS analysis of tumor-infiltrating lymphocytes at day 7 after treatment (Supplementary Fig. S1A–S1C). In the absence of therapeutic intervention, CD8+ T-cell depletion had no significant effect on tumor outgrowth but significantly reduced the efficacy of anti–PD-1/anti–CTLA-4 combination therapy (Fig. 1E). In contrast, in the absence of checkpoint blockade, CD4+ T-cell depletion resulted in decreased tumor growth, likely
Dual blockade of PD-1 and CTLA-4 results in robust antitumor immune responses. C57BL/6 WT (A-E) or DEREG (F and G) mice were injected s.c. with (A, B, E-G) $5 \times 10^5$ AT-3ova<sup>tm</sup> CD73<sup>+</sup> cells or (C and D) $1 \times 10^5$ MC38 ova<sup>tm</sup> tumor cells. Fourteen (A-E) or 17 (F and G) days after tumor inoculation, mice were treated with either PBS, anti-CD4 (250 µg/mouse), or anti-CD8 (250 µg/mouse). Mice were treated on days 16, 17, and 21 with either PBS, anti-CD4 (250 µg/mouse), and/or diphtheria toxin (DT; 0.5 µg/mouse). Data shown as the mean ± SEM of 6 mice per group of a representative experiment ($n = 2$). B and D, Survival was determined as when tumor size exceeded 100 mm<sup>2</sup>. E, On days 13, 14, and 21, mice were treated with either PBS, anti-CD4 (250 µg/mouse), or anti-CD8 (250 µg/mouse). F and G, Mice were treated on days 16, 17, and 21 with either PBS, anti-CD4 (250 µg/mouse) and/or diphtheria toxin (DT; 0.5 µg/mouse). G, Mean tumor size ± SEM at day 0 after treatment. Results shown as the mean ± SEM of 6-10 mice per group. **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant (two-way ANOVA).

PD-1/CTLA-4 Blockade Stimulates Adaptive and Innate Immunity

due to the depletion of CD4<sup>+</sup> Foxp3<sup>+</sup> cells and the induction of an antitumor immune response. Treatment with anti-PD-1/anti-CTLA-4 did not further enhance the antitumor immune response in the context of CD4<sup>+</sup> T-cell depletion, suggesting that CD4<sup>+</sup> Foxp3<sup>+</sup> T cells contribute to the efficacy of treatment following combination therapy. To further investigate the potential role of CD4<sup>+</sup> Foxp3<sup>+</sup> cells in the therapeutic effect following dual PD-1/CTLA-4 blockade, we used DEREG mice, in which Foxp3<sup>+</sup> regulatory T cells (Treg) express a receptor for diphtheria toxin and so can specifically be depleted through the administration of diphtheria toxin. As expected, the depletion of Tregs led to a reduction in tumor growth and significantly enhanced the efficacy of anti-PD-1/anti-CTLA-4 therapy (Supplementary Fig. S1D and S1E; Fig. 1F). In this context, concurrent treatment with anti-CD4 (thus specifically depleting the CD4<sup>+</sup> Foxp3<sup>+</sup> cells) significantly reduced the efficacy of combination treatment (Fig. 1F and G). Indeed, in the context of total CD4<sup>+</sup> depletion, anti-PD-1/anti-CTLA-4 did not significantly reduce tumor growth compared with 2A3 isotype–treated mice (Fig. 1F and G), consistent with our observations in wild-type mice (Fig. 1E). These data are therefore consistent with our hypothesis that CD4<sup>+</sup> Foxp3<sup>+</sup> cells are required for the therapeutic efficacy of anti-PD-1/anti-CTLA-4.

Dual blockade of PD-1 and CTLA-4 activates both CD4<sup>+</sup> Foxp3<sup>+</sup> and CD8<sup>+</sup> T cells

To investigate the mechanism by which dual blockade of PD-1 and CTLA-4 induces an antitumor immune response, we analyzed the phenotype of CD4<sup>+</sup> Foxp3<sup>+</sup> and CD8<sup>+</sup> T cells isolated from the
tumors and DLNs of mice following combination therapy. Mice bearing AT-3ovadim-CD73 tumors were treated with anti–PD-1 and anti–CTLA-4 as previously and tumor-infiltrating lymphocytes were analyzed at day 7 after therapy. The combination of anti–PD-1 and anti–CTLA-4 significantly enhanced the proportion of CD8⁺ tumor-infiltrating T cells expressing PD-1, CTLA-4, and TIM-3 (Fig. 2A; Supplementary Fig. S2A). Similarly, combination therapy significantly increased the expression of PD-1 on

Figure 2.
Dual blockade of PD-1 and CTLA-4 results in the activation of both CD8⁺ and CD4⁺Foxp3⁻ subsets. C57BL/6 mice were injected s.c. with 5 x 10⁵ AT-3ova⁺⁺⁺⁺ CD73⁺ tumors. Fourteen and 18 days after tumor inoculation, mice were treated with either anti–PD-1 (200 μg/mouse), anti–CTLA-4 (150 μg/mouse), isotype control (2A3; 200 μg/mouse), or a combination of anti–PD-1 and anti–CTLA-4 (P+C). On day 21 (7 days after treatment), CD8⁺ and CD4⁺Foxp3⁻ cells from tumors (A, B, D–F) and DLNs (C and D) were analyzed by flow cytometry. Proportion of CD8⁺ or CD4⁺Foxp3⁻ cells expressing PD-1, CTLA-4, CD62L, Tbet, IFNγ, and MFI of CD40L is shown. Data, mean ± SEM of 4–12 mice per group. B and C, Bottom, Representative flow cytometry plots are shown from concatenated samples. *, P < 0.05; **, P < 0.01; ***, P < 0.001; n.s., not significant (one-way ANOVA/Tukey).
CD4+Foxp3+ cells and showed a trend for increased CTLA-4 expression on these cells (Fig. 2A). Combined PD-1 and CTLA-4 blockade also significantly reduced the proportion of both CD8+ and CD4+Foxp3+ cells expressing CD62L in tumors (Fig. 2B) and DLNs (Fig. 2C), indicating a transition to an effector cell phenotype. Similarly, combination therapy significantly enhanced the expression of Tbet and IFNγ in CD4+Foxp3+ T cells isolated from tumors or DLNs (Fig. 2D and E; Supplementary Fig. S2B), the expression of CD40L on CD4+Foxp3+ TILs (Fig. 2F) as well as the proportion of CD8+ TILs expressing granzyme B (Supplementary Fig. S2C). IFNγ is critical for the antitumor immune response evoked by PD-1 blockade (31), so we investigated the requirement for IFNγ in CD4+Foxp3+ and CD8+ T-cell activation through the use of IFNγ−/− mice. This analysis revealed that IFNγ was critical for CD4+Foxp3+ and CD8+ T-cell activation in the tumors, but not in the DLNs (Supplementary Fig. S2D). Consistent with previous studies using the 9H10 clone of anti-CTLA-4 (18), treatment with anti-CTLA-4 alone, or the combination of anti-CTLA-4/anti-PD-1, resulted in a significant reduction in the proportion of CD4+Foxp3+ cells, and significantly enhanced the CD8+Treg and CD4+Foxp3+Treg ratios (Supplementary Fig. S2E). Thus, dual blockade with anti-PD-1 and anti-CTLA-4 resulted in activation of both CD8+ and CD4+Foxp3+ TILs and/or increased infiltration of activated CD8+ and CD4+Foxp3+ cells. To confirm that these effects were broadly applicable, we also investigated the T-cell phenotype in MC38ovaLm tumors treated with anti-PD-1 and/or anti-CTLA-4 (Supplementary Fig. S3). Similarly to the AT-3ovaLmCD73 model, dual blockade of PD-1 and CTLA-4 resulted in significant activation of CD4+Foxp3+ cells. This was shown by increased expression of IFNγ within CD4+Foxp3+ cells isolated from tumors (Supplementary Fig. S3A) and CD4+Foxp3+ cells from DLNs exhibited an increased expression of IFNγ/Tbet and a transition toward an effector cell phenotype as shown by an enhanced proportion of CD62L− cells (Supplementary Fig. S3B).

Dual blockade of PD-1 and CTLA-4 results in direct activation of CD4+Foxp3+ cells

Given the significant activation of both CD8+ and CD4+Foxp3+ cells in the tumors and DLNs following combination therapy, we next investigated whether CD4+Foxp3+ cells were activated directly by therapy, or whether their activation was an indirect consequence of modulation of CD8+ and/or CD4+Foxp3+ cells. To investigate this, we first treated mice bearing established AT-3ovaLmCD73 tumors with anti-PD-1/anti-CTLA-4 and compared the phenotype of CD4+Foxp3+ cells in control mice to those that had undergone CD8 depletion. As expected, combined PD-1 and CTLA-4 blockade activated CD4+Foxp3+ T cells as shown by the significant increase in Tbet expression (Fig. 3A), reduced proportion of CD62L+ cells (Fig. 3B), and increased proportion of IFNγ+ cells (Fig. 3C) in CD4+Foxp3+ cells isolated from either tumors or DLNs. Surprisingly, in mice depleted of CD8+ T cells, CD4+Foxp3+ cells were activated to a similar extent as in control mice, indicating that CD8+ T cells were not required for the activation of CD4+Foxp3+ cells (Fig. 3A–C).

We next investigated a potential role for the modulation of CD4+Foxp3+ cells in this effect. This is particularly relevant because the 9H10 clone of anti-CTLA-4 depletes CD4+Foxp3+ Tregs (Supplementary Fig. S1D and S1E), which would consequently be expected to result in the activation of CD4+Foxp3+ cells by an indirect mechanism. To investigate this, we again utilized DEREG mice to specifically deplete CD4+Foxp3+ cells, thereby allowing investigation of the effect of anti-PD-1/anti-CTLA-4 therapy on CD4+Foxp3+ cells in the absence of CD4+Foxp3+ cells. Using this model, we concurrently depleted CD8+ and CD4+Foxp3+ cells in mice bearing AT-3ovaLmCD73 tumors. Therefore, in this setting, the only remaining CD8+ T lymphocyte population was CD4+Foxp3+ cells. Activation of CD4+Foxp3+ cells was then determined following dual PD-1/CTLA-4 blockade. Analysis of DLNs revealed that although Treg depletion in itself resulted in significant activation of CD4+Foxp3+ cells, anti-PD-1 and anti-CTLA-4 co-blockade further enhanced the activation of CD4+Foxp3+ cells, as shown by significantly increased expression of Tbet (Fig. 3D), a decreased proportion of CD62L+ cells (Fig. 3E), and significantly increased expression of IFNγ (Fig. 3F). Within the tumors, the expression of Tbet and IFNγ by CD4+Foxp3+ cells was also significantly increased by Treg depletion (Fig. 3D and F). No further increase in these parameters was observed following dual PD-1/CTLA-4 blockade, likely indicative of the highly activated state of these cells following Treg depletion. However, the proportion of CD4+Foxp3+ TILs expressing CD62L was significantly decreased (Fig. 3E), suggesting that CD4+Foxp3+ cells at the tumor site may still be directly modulated by PD-1/CTLA-4 dual blockade in the absence of CD8+ and Treg populations. Thus, dual PD-1/CTLA-4 blockade resulted in robust activation of CD4+Foxp3+ cells in the absence of CD8+ and CD4+Foxp3+ cells. Although we cannot formally exclude a role for other PD-1/CTLA-4 expressing cell types in directly activating CD4+Foxp3+ cells, these data suggest that PD-1/CTLA-4 blockade can directly modulate CD4+Foxp3+ cells.

CD4+Foxp3+ cells modulate CD103+ DCs following combination therapy

We next investigated the role of APCs in this effect and analyzed IL12 production ex vivo as a marker for APC activation. We analyzed the CD11c+ compartment (Supplementary Fig. S4A) and observed that the production of IL12 was predominantly associated with the CD103+ DC population (Supplementary Fig. S4B). The CD103+ DCs produced significantly more IL12 than the CD11b−, CD11c+, and CD11c+CD103+ populations (Supplementary Fig. S4C), consistent with previous studies in which these cells were shown to secrete high levels of IL12, be highly effective at tumor-antigen presentation (26–28), and implicated in the therapeutic efficacy of checkpoint inhibition (29, 34). The anti-PD-1/anti-CTLA-4 combination therapy resulted in the activation of these cells, as shown by their enhanced IL12 production in both AT-3ovaLmCD73 tumors (Fig. 4A) and MC38ovaLm tumors (Supplementary Fig. S3C). The frequency of CD11c−CD103+ DCs within the DLNs increased with a concurrent reduction in the tumors following combination therapy, possibly representing DC egress following antigen uptake (Fig. 4B; Supplementary Fig. S3D) as has been reported previously (36). To investigate the role of CD4+Foxp3+ cells in the modulation of CD103+ DC function and trafficking, we analyzed the effect of PD-1/CTLA-4 blockade in the context of CD8 and Treg depletion. These experiments revealed that even in the absence of CD8+ and CD4+Foxp3+ cells, treatment of AT-3ovaLmCD73 tumor–bearing mice with anti-PD-1/anti-CTLA-4 resulted in a significant increase in the proportion of IL12+CD103+CD11c+ DCs, implying a role for CD4+Foxp3+ cells in the regulation of DC function.
Figure 3. 
Dual blockade of PD-1 and CTLA-4 directly activates CD4⁺Foxp3⁻ cells in the absence of CD8⁺ or CD4⁺Foxp3⁺ cells. A–C, C57BL/6 WT or D–F, DEREG mice were injected s.c. with 5 × 10⁶ AT-3ovadim CD73⁺ tumor cells. Mice were treated with 2A3 or anti-PD-1 and anti-CTLA-4 as per Fig. 2. On days 13, 14, and 18, mice were treated where indicated with anti-CD8 (250 µg/mouse) and/or diphtheria toxin (DT; 0.5 µg/mouse). On day 21, leukocytes were isolated from tumors (TILs) or DLNs and analyzed by flow cytometry. The expression of (A, D) Tbet, (B, E) CD62L, and (C, F) IFNγ by CD4⁺Foxp3⁻ cells was determined. Data shown as the mean ± SEM of 3–13 mice per group. ***, P < 0.001; **, P < 0.01; *, P < 0.05; n.s., not significant (one-way ANOVA/Tukey).

this response (Fig. 4C). Similarly, the increased frequency of CD103⁺CD11c⁻ DCs in the DLNs following dual PD-1/CTLA-4 blockade was not significantly affected by depletion of both CD8⁺ and CD4⁺Foxp3⁻ T cells (Fig. 4D), and this was prevented by the concurrent depletion of the remaining CD4⁺Foxp3⁺ cells (Supplementary Fig. S4D), indicating the important role of CD4⁺Foxp3⁻ cells in this effect.

To confirm the requirement of CD103⁺ DCs for the therapeutic response, we investigated the efficacy of PD-1/CTLA-4 dual blockade in Batf3⁻/⁻ mice. Combination therapy was ineffective in these mice, resulting in no enhancement of mouse survival (Fig. 4E) and an attenuated upregulation of Tbet and IFNγ in CD4⁺Foxp3⁻ T cells, compared with the effect observed in WT mice (Supplementary Fig. S4E). Supernatants collected from tumor samples cultured ex vivo contained no detectable IFNγ or TNFα from tumors grown in Batf3⁻/⁻ mice, whereas these cytokines were significantly increased in tumors from WT mice treated with anti–PD-1/anti–CTLA-4 (Supplementary Fig. S4F). Thus, anti–PD-1/anti–CTLA-4 treatment enhanced IL12 production by CD103⁺ DCs and increased their frequency in DLNs, even in the absence of CD8⁺ and CD4⁺Foxp3⁻ T cells.

This implied that activation of CD4⁺Foxp3⁻ T cells by the combination therapy was sufficient to trigger activation of CD103⁺ DCs, possibly indicating direct modulation of these cells by CD4⁺Foxp3⁻ T cells.

CD4⁺Foxp3⁻ cell-mediated enrichment of a "TIP-DC" population following therapy

We further investigated the myeloid compartment of the tumor microenvironment (TME) following dual PD-1/CTLA-4 blockade, which revealed that treatment of AT-3ovadim⁺CD73 tumor-bearing mice with anti–PD-1/anti–CTLA-4 combination therapy modulated a CD11b⁺Ly6C⁺F4/80⁺ myeloid cell population within the tumors (Fig. 5A and B). Although neither anti–PD-1 nor anti–CTLA-4 modulated the proportion of these cells when given as a single therapy, dual blockade resulted in a significant increase in this subset (Fig. 5A and B). The enrichment of CD11b⁺Ly6C⁺ cells within the TME was dependent on T cells, because the frequency of these cells in RAG⁻/⁻ mice treated with anti–PD-1/anti–CTLA-4 was not changed (Fig. 5C). Further phenotypic analysis showed that these cells were MHCIIRöhCD86⁺ iNOS⁺TNFα⁺, and a subset of these cells
expression of CD11c and CXCRI (Fig. 5D). The putative MDC markers (35, 36) Ly6G, CD43, or CD115 were not detected on these cells (Fig. 5F). Thus, the phenotype of these cells was most consistent with the reported phenotype of TNF-α and IFN-γ-producing DCs (TIP-DC; refs. 36–38). Although these cells are called “TIP-DCs,” it has been suggested that these cells can be considered inflammatory macrophages (38). Further analysis of these cells after a cytospin revealed that these cells appeared macrophage-like in appearance, consistent with our hypothesis that they represent an inflammatory macrophage-like cell type (Fig. 5E). These CD11b+Ly6CintF4/80+ cells are therefore herein referred to as “TIP-DCs.”

Blockade of PD-1 and CTLA-4 significantly enhanced the proportion of TIP-DCs within tumors to a similar extent in the presence of absence of CD8+ T cells (Fig. 5F) and was also observed in the context of CD8/Treg dual depletion (Fig. 5G). However, CD4+ T cell depletion ablated the increased frequency of TIP-DCs following anti-PD-1/anti-CTLA-4 treatment of mice depleted of both CD8+ T cells and CD4+ Foxp3+ T cells, showing the importance of the CD4+ Foxp3+ cells in this effect (Fig. 5G).

To evaluate the functional consequences of PD-1/CTLA-4 blockade on CD4+ T cell and APC function in cells isolated from the DLNs of AT-3ovaCEM tumor-bearing mice. DLN cells were isolated and stimulated with 300 ng/mL of the ovalbumin MHCI restricted peptide OVA257-264 and IL2. After 5 days of culture, cytokine production was measured. In this setting, treatment of mice with PD-1/CTLA-4 significantly increased the production of IFNγ and TNFα, but not IL17 (Fig. 6A). The DLN cells isolated from mice depleted of CD8+ T cells produced anti-CD4 treatment could not fully account for this effect (Supplementary Fig. S5).

Enhanced CD4+ T-cell activation and APC function in DLNs after dual blockade

Due to the increased frequency of CD103+ DCs observed in DLNs following PD-1/CTLA-4 blockade, we hypothesized that this would lead to enhanced APC function. To investigate this, we assessed CD4+ T cell and APC function in cells isolated from the DLNs of anti-PD-1/anti-CTLA-4-treated mice or 2A3 isotype control in both control mice and those treated in the context of CD8 depletion.

To evaluate the functional consequences of PD-1/CTLA-4 blockade on CD4+ T cells, we evaluated the ex vivo cytokine production of CD4+ T cells isolated from the DLNs of AT-3ovaCEM tumor-bearing mice. DLN cells were isolated and stimulated with 300 ng/mL of the ovalbumin MHCI restricted peptide OVA257-264 and IL2. After 5 days of culture, cytokine production was measured. In this setting, treatment of mice with PD-1/CTLA-4 significantly increased the production of IFNγ and TNFα, but not IL17 (Fig. 6A). The DLN cells isolated from mice depleted of CD8+ T cells produced
equivalent amounts of these cytokines following anti-PD-1/anti-CTLA-4 treatment (Fig. 6A). Thus, increased CD4$^+$ T-cell cytokine production was achieved independently of CD8$^+$ T cells, suggestive of direct modulation of this subset by anti-PD-1/anti-CTLA-4.

Given our previous observations concerning the CD4$^+$Foxp3$^+$ T cell–dependent enrichment of CD103$^+$ DCs in the DLNs following PD-1/CTLA-4 blockade (Fig. 4), we next investigated the antigen-presentation function of DLN cells isolated from mice bearing AT-3ovadim$^{CD73}$ tumors. DLN cells were irradiated (30 Gy) and then cocultured with naïve OTII cells in the presence of 300 nmol/L of the OTII specific OVA157-163 peptide. After 48 hours, supernatants were harvested and concentrations of IFN$\gamma$, IL2, TNF$\alpha$, and IL17 were determined. DLNs isolated from mice treated with anti-PD-1/anti-CTLA-4 induced significantly higher amounts of IFN$\gamma$, IL2, TNF$\alpha$, and IL17 (Fig. 6B). CD8$^+$ T-cell depletion did not significantly affect this enhanced APC capacity of DLNs, suggesting that direct modulation of the CD4$^+$ population by PD-1/CTLA-4 blockade was sufficient to enhance APC functional activity. Thus, combined PD-1/CTLA-4 blockade results in direct activation of CD4$^+$ T cells and consequent licensing of APCs within DLNs, which further promotes CD4$^+$ T-cell responses to tumor antigens at this site.

**Activation of CD4$^+$ T cells by dual PD-1/CTLA-4 blockade is partly IL12 dependent**

Having shown that combined PD-1/CTLA-4 blockade resulted in enhanced IL12 production of tumor-infiltrating CD103$^+$ DCs, we next investigated the significance of IL12 in the therapeutic effect. The growth of AT-3ova$^{CD73}$ tumors in WT and IL12p$^{--}$ mice following treatment with anti-PD-1/anti-CTLA-4 or isotype control was compared. Although the initial
antitumor effect mediated by dual blockade was similar in WT and IL12 p35−/− mice, tumors outgrew significantly faster in IL12 p35−/− mice, resulting in a significantly longer survival of WT mice compared with IL12 p35−/− mice (Fig. 7A). As the p35 subunit of IL12 is also expressed by IL35, we confirmed this effect using IL12 p40−/− mice. IL12p40−/− mice showed a similar phenotype to IL12p35−/− mice with regard to a less potent antitumor immune response (Fig. 7B), thus confirming the role of IL12 in this response. To investigate the underlying mechanism in this effect, we analyzed the phenotype of tumor-infiltrating T cells. In wild-type mice, as expected, anti–PD-1/anti–CTLA-4 induced robust CD4+Foxp3+ T-cell activation (Fig. 7C). However, in IL12p35−/− mice, we observed that the ability of anti–PD-1/anti–CTLA-4 treatment to induce Tbet expression and IFNγ production by CD4+Foxp3+ cells was significantly reduced (Fig. 7C). Similarly, IL12 neutralization with anti-IL12p75 reduced the expression of Tbet in tumor-infiltrating CD4+Foxp3+ cells, confirming that CD4+Foxp3+ T-cell activation following dual PD-1/CTLA-4 blockade was partly IL12 dependent (Fig. 7D). Although there was no significant induction of Tbet+ cells in IL12p35−/− mice (Fig. 7C), a significant increase was observed in the context of IL12p70 neutralization (Fig. 7D), albeit to a lesser extent than in WT mice. These differences are potentially explained by incomplete neutralization of IL12 in these experiments. In contrast to effects observed with CD4+ T cells, the induction of IFNγ by CD8+ T cells was not significantly different in IL12p35−/− mice compared with wild-type mice (Fig. 7E). Taken together, our data indicate that the activation of CD4+Foxp3+ cells following checkpoint blockade results in the modulation of CD103+ DCs to secrete more IL12, which plays an important role in the antitumor response (Supplementary Fig. S6).

Discussion

Our understanding of the mechanism by which anti–PD-1 and anti–CTLA-4 evoke antitumor immune responses is incomplete, and prognostic markers that can predict responses to checkpoint inhibitors are lacking. Our current study reveals that the interplay between CD4+ Foxp3+ T cells and CD103+ DCs may be crucial in the antitumor immune response evoked by dual blockade of PD-1 and CTLA-4. The original description of the efficacy of dual PD-1/CTLA-4 blockade by Jim Allison’s group revealed that this therapeutic combination resulted in the activation of both CD8+ and CD4+ T cells (24). Further studies have shown that CTLA-4 blockade modulates both Treg and non-Treg compartments (18, 39, 40), although none of these studies could exclude the possibility that anti–CTLA-4 activates CD4+ Foxp3+ cells indirectly through modulation of CD8+ or Foxp3+ T cells. In patients, it has been observed that the CD4+ T-cell compartment becomes activated following immunotherapy with checkpoint inhibitors including anti–PD-1 and anti–CTLA-4 combination therapy (13, 41–43). Furthermore, neoantigen-specific CD4+ T cells that may be responsive to immunotherapy have been observed in patients with cancer (20–23), and thus robust activation of tumor-antigen...
specific CD4⁺ T cells is likely to be important for optimal therapeutic efficacy. However, the importance of CD4⁺ Foxp3⁻ cells in response to PD-1 and CTLA-4 is currently not fully understood.

Our study revealed that CD4⁺ Foxp3⁻ cells were robustly activated by the combination of anti–PD-1 and anti–CTLA-4. This led to the increased expression of CD40L, Tbet, and IFNγ in CD4⁺ Foxp3⁻ cells isolated from tumors and DLNs, in part due to a direct activation of these CD4⁺ Foxp3⁻ cells. This activation of CD4⁺ Foxp3⁻ cells was sufficient to induce an enhanced proportion of a myeloid cell with a phenotype consistent with previously reported TNFα, iNOS-producing DCs (TIP-DC; refs. 36–38).

Although the functional importance of these cells remains to be determined, these TIP-DCs may be capable of modulating T-cell responses at the tumor site through antigen presentation (44, 45) or conversely through the iNOS-mediated suppression (46).

Activation of CD4⁺ Foxp3⁻ cells was sufficient to induce the activation of tumor-residing CD103⁺ DCs, even in the absence of CD8⁺ and CD4⁺ Foxp3⁺ cells, highlighting an interaction...
between TH1 effector cells and CD103⁺ DCs. CD103⁺ DCs are highly efficient at tumor-antigen presentation (26, 28, 47) and have been shown to enhance the therapeutic activity of anti-PD1-1/BRAF inhibition (29) or anti-TIM3 (48). CD103⁺ DCs are thus likely to play a critical role in the therapeutic responses to checkpoint inhibitors. Our study shows that combining PD-1 and CTLA-4 blockade induces CD103⁺ DCs to produce IL12, in part through the activation of CD4⁺ Foxp3⁺ cells, which contributed to the therapeutic effect of the combination therapy. The CD103⁺ DCs were the highest per cell producers of IL12p40 in the tumor microenvironment following dual PD-1/CTLA-4 blockade, consistent with previous publications showing that CD103⁺ DCs were the major producers of IL12 in the tumor microenvironment following paclitaxel therapy (27) and that the production of IL12 by Batf3-dependent DCs enhances NK cell–mediated antitumor effects (49). IL12 is required for the antitumor effect of CpG (50) and anti-CD40 (51) and here we demonstrate the importance of IL12 in the context of checkpoint inhibition. We observed that IL12 production was particularly important for long-term antitumor immune responses. Thus, the activation of CD103⁺ DCs by CD4⁺ Foxp3⁺ cells may contribute to memory T-cell responses. CD8⁺ memory formation is most efficient when CD4⁺ and CD8⁺ cells recognize cognate antigen on the same APC (52–54), and our data imply that this mechanism may be leading to more robust antitumor CD8⁺ T-cell activity in our system.

The increased frequency of CD103⁺ DCs in the DLNs following PD-1/CTLA-4 dual blockade could represent enhanced trafficking of these cells from the tumor site and transfer of tumor antigens to lymph node–residing APCs, as observed by Krummel and colleagues (30). Taken together, our data highlight the importance of CD4⁺ Foxp3⁺ cells in the activation and migration of these cells.

In summary, our data show that CD4⁺ Foxp3⁺ cells can be activated by dual PD-1/CTLA-4 blockade in the absence of CD8⁺ and CD4⁺ Foxp3⁺ cells, inferring a direct activation of CD4⁺ Foxp3⁺ cells. This activation was sufficient to induce the activation of CD103⁺ DCs as shown by their enhanced IL12 production in turn. IL12 production following anti-PD-1/anti–CTLA-4 was required for optimal CD4⁺ Foxp3⁺ cell activation and therapeutic activity. These data are consistent with the observation that PD-1 and CTLA-4 blockade activate distinct transcriptional pathways in CD4⁺ T cells in treated mice and that the induction of a CD4⁺ PD-1⁻/Foxp3⁺ T-cell population negatively correlated with tumor growth (55). Our data revealed an additional mechanism by which CD4⁺ Foxp3⁺ cell activation contributes to the overall efficacy of checkpoint inhibition.

**Disclosure of Potential Conflicts of Interest**

S. Loi reports receiving a commercial research grant from Bristol-Myers Squibb and Merck, reports receiving other commercial research support from Novartis, Roche-Genentech, and Pfizer, and is a consultant/advisory board member for Bristol-Myers Squibb, Merck, Roche-Genentech, Puma Biotechnology, Novartis, Seattle Genetics, and Pfizer. No potential conflicts of interest were disclosed by the other authors.

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**References**


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