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 that 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 (DCs) 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 points to a previously underappreciated role of CD4⁺Foxp3⁻ cells in modulating this arm of the antitumor immune response.
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 (DLNs). The therapeutic efficacy of anti–CTLA-4 may also be partly attributed to the depletion of CD4+Foxp3+ cells, due to their constitutive 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 \(T_H^1\) 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\(^+\) \(T_H^1\) 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\textsuperscript{dim} CD73\textsuperscript{+} and the colon carcinoma line MC38ova\textsuperscript{dim} were generated as previously described and utilized within 3 weeks of thawing from a master stock generated in 2012 (31-33). United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) 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 \textit{in vivo} experiments, the indicated number of cells were resuspended in PBS and injected sub-cutaneously in a 100 \textmu l volume. C57BL/6 WT and OTII mice were obtained from the Walter and Eliza Hall Institute of Medical Research (Melbourne), DEREG, IFN\gamma\textsuperscript{-/-} and Batf3\textsuperscript{-/-} mice were bred in-house at the Peter MacCallum Cancer Centre and IL12p35\textsuperscript{-/-} or IL12p40\textsuperscript{-/-} 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 CD8\textalpha\ (YTS 169.4) IL12 p75 (R2-9A5) or isotype control (2A3) were purchased from BioXcell (West Lebanon, USA). IL2 used for T-cell stimulation was obtained from the National Institutes of Health (Bethesda, USA). OVA\textsubscript{323-339} peptide was purchased from Genscript.

Treatment of tumor-bearing mice
C57/BL6 mice were injected sub-cutaneously with $5 \times 10^5$ AT-3ova$^{\text{dim}}$ CD73$^+$ or $1 \times 10^6$ MC38 ova$^{\text{dim}}$ tumor cells. Once tumors were established (20-50 mm$^2$) mice were treated with either isotype control (2A3), anti–PD-1 (RMPI-14) or anti–CTLA-4 (9H10) with 3-4 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. Diptheria toxin (0.5 µg/mouse) was administered 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 post treatment, tumors were excised and digested post-mortem 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 twice. Inguinal lymph nodes were also harvested and cells filtered through a 70 µm filter. Cells were then analyzed by flow cytometry as previously described (31) and Fixable Yellow (ThermoFisher) used as a viability dye.

**Intracellular cytokine staining**

For detection of IFNγ production by T cells *ex vivo*, TILs and draining lymph node cells were cultured for 3 hours with PMA (5 ng /ml) ionomycin (1 µg/ml) in the presence of Golgi Plug (BD Pharmingen) and Golgi Stop (BD Pharmingen). After 3 hours, cells were analyzed by flow cytometry. For the detection of IL12p40, cells were cultured for 4 hours in Golgi Plug/Stop 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 post therapy onset, 2 x 10^5 cells derived from DLNs were cultured with 300nM OVA_{323-339} 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 APC function of cells derived from DLNs of tumor-bearing mice**

To assess the antigen presenting function of cells derived from DLNs, 2 x 10^5 cells were irradiated (30 Gy) and then cultured with naïve splenocytes derived from OTII mice in the presence of 300 nM OVA_{323-339} 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 significant.
Results

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

We first evaluated the potential of monoclonal antibodies (mAbs) 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 (34). Mice were injected with AT-3ova^dim-CD73 cells and once tumors were established (20-50 mm^2), 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-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 post treatment (Supplementary Fig. S1A-C). 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 due to the depletion of CD4+Foxp3+ 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+ T-cell depletion, suggesting that CD4+Foxp3− T
cells contribute to the efficacy of treatment following combination therapy. To further investigate the potential role of CD4+Foxp3− cells in the therapeutic effect following dual PD-1/CTLA-4 blockade, we used DEREG mice, in which Foxp3+ Treg cells 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-E, Fig. 1F). In this context, concurrent treatment with anti-CD4 (thus specifically depleting the CD4+Foxp3− cells) significantly reduced the efficacy of combination treatment (Fig. 1F-G). Indeed, in the context of total CD4+ depletion, anti–PD-1/anti–CTLA-4 did not significantly reduce tumor growth compared to 2A3 isotype–treated mice (Fig. 1F-G) consistent with our observations in wild-type mice (Fig. 1E). This data is therefore consistent with our hypothesis that CD4+Foxp3− 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+Foxp3− and CD8+ 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+Foxp3− and CD8+ T cells isolated from the tumors and DLNs of mice following combination therapy. Mice bearing AT-3ova−dim−CD73 tumors were treated with anti–PD-1 and anti–CTLA-4 as previously and tumor-infiltrating lymphocytes were analyzed at day 7 post 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 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-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 these effects were broadly applicable we also investigated the T-cell phenotype in MC38ova dim tumors treated with anti–PD-1 and/or anti–CTLA-4 (Supplementary Fig. S3). Similarly to the AT-3ova dim-CD73 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 towards 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 draining lymph nodes following combination therapy, even in the context of CD8+ cell depletion, 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-3ova\textsuperscript{dim-CD73} 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+ Treg cells (Supplementary Fig. S2C), 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 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-3ova\textsuperscript{dim-CD73+} tumors. Therefore, in this setting the only remaining αβ 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, 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 indirectly activating CD4+Foxp3− cells, this data suggests 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 antigen presenting cells (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, 35). 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-3ova<sup>dim</sup>-CD73 tumors (Fig. 4A) and MC38-ova<sup>dim</sup> 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 (30). 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-3ova\(^{dim}\)-CD73 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 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 IFN\(\gamma\) in CD4\(^+\)Foxp3\(^-\) T cells, compared to the effect observed in WT mice (Supplementary Fig. S4E). Supernatants collected from tumor samples cultured ex vivo contained no detectable IFN\(\gamma\) or TNF\(\alpha\) 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-3ova\textsuperscript{dim}-CD73 tumor-bearing mice with anti–PD-1/anti–CTLA-4 combination therapy modulated a CD11b\textsuperscript{+}Ly6C\textsuperscript{int}F4/80\textsuperscript{+} myeloid cell population within the tumors (Fig. 5A-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-B). The enrichment of CD11b\textsuperscript{+}Ly6C\textsuperscript{int} cells within the TME was dependent on T cells, because the frequency of these cells in RAG\textsuperscript{−/−} mice treated with anti–PD-1/anti–CTLA-4 was not changed (Fig. 5C). Further phenotypic analysis showed that these cells were MHCII\textsuperscript{high}CD86\textsuperscript{+}iNOS\textsuperscript{+}TNFα\textsuperscript{+}, and a subset of these cells expressed CD11c and CXCR3 (Fig. 5D). The putative MDSC markers (36, 37) Ly6G, CD43, or CD115 were not detected on these cells (Fig. 5D). Thus, the phenotype of these cells was most consistent with the reported phenotype of TNFα and iNOS producing DCs (TIP-DCs) (37-39). Although these cells are called “TIP-DCs” it has been suggested that these cells can be considered inflammatory macrophages (39). Further analysis of these cells after a cytopsin 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\textsuperscript{+}Ly6C\textsuperscript{int}F4/80\textsuperscript{+} 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 or absence of CD8\textsuperscript{+} T cells (Fig. 5F) and was also observed in the context of CD8/Treg dual depletion (Fig. 5G). However, CD4\textsuperscript{+} T-cell depletion ablated the increased frequency of TIP-DCs following anti–PD-1/anti–CTLA-4 treatment of mice depleted of both CD8\textsuperscript{+} T cells and CD4\textsuperscript{+}Foxp3\textsuperscript{+} cells, showing the importance of the CD4\textsuperscript{+}Foxp3\textsuperscript{−} cells in this effect (Fig. 5G). A minor population of TIP-DCs expressed CD4, but most were CD4 negative, indicating that direct depletion of TIP-DCs
following 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-3ova⁺dim-CD73 tumor-bearing mice. Draining lymph node cells were isolated and stimulated with 300 nM of the ovalbumin MHCII-restricted peptide OVA₃₂₃-₃₃₉ and IL2. After 5 days culture, cytokine production was analyzed. In this setting, treatment 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-3ova⁺dim-CD73 tumors. DLN cells were irradiated (30 Gy) and then cocultured with naïve OTII cells in the presence of 300 nM of the OTII specific OVA₃₂₃-₃₃₉ peptide. After 48 hours, supernatants were harvested and concentrations of IFNγ, IL2, TNFα, and IL17 were determined. DLNs
isolated from mice treated with anti–PD-1/anti–CTLA-4 induced significantly higher amounts of IFNγ, IL2, TNFα, 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⁺dim-CD73 tumors in WT and IL12p35⁻/⁻ 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 to 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 regards 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-IL12 p75 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 IL-12p35−/− mice (Fig 7C), a significant increase was observed in the context of IL-12p70 neutralization (Fig 7D), albeit to a lesser extent than in WT mice. These differences are potentially explained by incomplete neutralization of IL-12 in these experiments. However, the induction of IFNγ by CD8+ T cells was not significantly different in IL12p35−/− mice compared to 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 IL-12 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 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, 40, 41), 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, 42, 43) (44). Furthermore, neoantigen-specific CD4⁺ T cells that may be responsive to immunotherapy have been observed in cancer patients (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-DCs) (37-39). 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 (45, 46) or conversely through the iNOS-mediated suppression (47).

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 T\(_{\text{H}}\)1 effector cells and CD103\(^+\) DCs. CD103\(^+\) DCs are highly efficient at tumor antigen presentation (26, 28, 48) and have been shown to enhance the therapeutic activity of anti-PDL-1/ BRAF inhibition (29) or anti-TIM3 (49). 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 antimetastatic effects (50). IL12 is required for the antitumor effect of CpG (51) and anti-CD40 (52) 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 (53-55), and our data implies 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 \textit{et al.} (30).
together, our data highlights the importance of CD4+Foxp3– cells in the activation and migration of these cells.

In summary, our data shows 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. This data is 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-1hiTbet+ population negatively correlated with tumor growth (56). Our data revealed an additional mechanism by which CD4+Foxp3– cell activation contributes to the overall efficacy of checkpoint inhibition.
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References


Figure Legends

Figure 1. Dual blockade of PD-1 and CTLA-4 results in robust antitumor immune responses

C57BL/6 WT (A-E) or DEREG (F-G) mice were injected s.c. with (A, B, E-G) 5 x 10^5 AT-3ova^{dim} CD73^{+} cells or (C-D) 1 x 10^6 MC38 ova^{dim} tumor cells. Fourteen (A-E) or seventeen (F-G) 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). Treatment was repeated on days 18, 22 and 26. A, C, E. Data shown as the mean ± SEM of 6 mice per group of a representative experiment (n = 2). B, D. Survival was determined as when tumor size exceeded 100 mm^2 n= 9-12 per group. 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-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 11 post 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).

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 10^5 AT-3ova^{dim} CD73^{+} tumor cells. Fourteen and eighteen 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 post treatment), CD8^{+} and CD4^{+}Foxp3^{−} cells from tumors (A, B, D-F) and DLNs (C, 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 is presented as the mean ± SEM of 4-8 mice
per group. B and C Bottom panels; 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’s).

**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 $5 \times 10^5$ AT-3ova$^{\text{dim}}$ 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 draining lymph nodes (DLNs) and analyzed by flow cytometry. The expression of (A, D) Tabt, (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. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, n.s. not significant (one-way ANOVA/ Tukey’s).

**Figure 4. Dual blockade of PD-1 and CTLA-4 results in the activation of CD103$^+$ DCs and their accumulation in DLNs**

A, B, E. C57BL/6 WT. C-D. DEREG or E. Batf3$^{-/-}$ mice were injected s.c. with $5 \times 10^5$ AT-3ova$^{\text{dim}}$ CD73$^+$ tumor cells. Mice were treated with 2A3 or anti-PD-1 and anti–CTLA-4 (P+C) and where indicated with anti-CD8 and diphtheria toxin (DT) as per Fig. 3. Seven days post treatment (A-C) tumor-infiltrating leukocytes and (B, D) DLNs were analyzed by flow cytometry. A, C. The proportion of CD103$^+$CD11c$^+$ (top panel) or CD103$^+$CD11c$^+$ (bottom panel) DCs expressing IL12 following a 4 hour incubation in Golgi plug/ Golgi stop. Right panel- Pooled data. B, D. The proportion of CD45$^+$ cells in the tumor or draining lymph node (DLN) that were CD103$^+$CD11c$^+$ DCs. B-D. Data is the mean ± SEM of 8-14 per group analyzed with a one-way ANOVA/ Tukey’s test. E. Survival of mice is shown for $n = 5-8$
mice per group with survival being determined as when tumors exceeded 100 mm². Statistical significance determined by Mantel-Cox test. *P < 0.05, **P < 0.01, ***P < 0.001, n.s. not significant.

Figure 5. Dual blockade of PD-1 and CTLA-4 results in an enrichment of CD11b⁺Ly6Cint TIP-DCs in a CD4⁺ T cell dependent manner.

A, B, D-F. C57BL/6 WT, C. RAG⁻/⁻ or G. DEREG mice were injected s.c. with 5 x 10⁵ AT-3ova⁺dim CD73⁺ tumor cells. Mice were treated with either isotype control (2A3; 200 µg/mouse) or a combination of anti–PD-1 (200 µg/mouse) and/or anti–CTLA-4 (150 µg/mouse; P+C) and where indicated with anti-CD8 (250 µg/mouse), anti-CD4 (250 µg/mouse) and/or diphtheria toxin (DT; 0.5 µg/mouse) as per Fig. 3. On day 21, leukocytes were isolated from tumors and analyzed by flow cytometry or morphology assessed following cytospin. A-C, F, G. The percentage of tumor-infiltrating CD11b⁺Ly6Cint cells as a proportion of CD45⁺ live cells. A. Representative FACS plot from 5 concatenated samples. B-C, F, G. Data shown as the mean ± SEM of 3-10 mice per group. D. Expression of indicated markers on all CD45⁺ cells (dashed histograms), all CD45⁺ cells following combination therapy (grey histograms) and on CD11b⁺Ly6Cint cells following combination therapy (black histograms) E. Cytospin of CD11b⁺Ly6Cint cells following combination therapy. *p<0.05, **P < 0.01, ***P < 0.001, n.s. not significant (one-way ANOVA/Tukey’s).

Figure 6. Dual blockade of PD-1 and CTLA-4 directly activates CD4⁺ T cells resulting in enhanced APC function in DLNs and enhanced tumor-antigen specific responses.

C57BL/6 WT mice were injected s.c. with 5 x 10⁵ AT-3ova⁺dim CD73⁺ tumor cells and treated with 2A3 or anti–PD-1 and anti–CTLA-4 and in some cases anti-CD8 depletion antibody as per Fig. 3. On day 21, leukocytes were isolated from DLNs (DLNs). A. 2 x 10⁵ lymph node
cells were stimulated for 5 days with 300 nM OVA_{323-339} peptide and IL2 (100 IU/ml). B. 2 x 10^5 lymph node cells were irradiated (30 Gy) and cocultured with naïve OTII splenocytes (2 x 10^5) for 48 hours. A-B. Cytokine concentration was determined by cytometric bead array. Data is shown as the mean SEM of 4-5 mice per group from a representative experiment of n = 2. * P < 0.05, ** P < 0.01, *** P < 0.001, n.s. not significant (One-way ANOVA/ Tukey’s).

Figure 7. Activation of CD4^+Foxp3^- cells and therapeutic activity of anti–PD-1/anti–CTLA-4 combination therapy is partly IL12 dependent

A-E. C57BL/6 WT, A, C, E. IL12p35^-/- or B. IL12p40^-/- mice were injected s.c. with 5 x 10^5 AT-3ova_{dim} CD73^+ tumor cells and treated with 2A3 or anti–PD-1 and anti–CTLA-4 (P+C) as per Fig. 3. A-B. Tumor growth and survival of WT, IL12 p35^-/- and IL12p40^-/- mice. Tumor growth data is shown as the mean ± SEM of 7-8 mice per group from a representative experiment and survival, determined as when tumor size exceeded 100 mm^2, of n = 7-21 per group from pooled experiments is shown. Statistical test: two-way ANOVA/ Mantel Cox test. C-E. On day 21, leukocytes were isolated from tumors and analyzed by flow cytometry. D. Where indicated mice were treated with 500µg per mouse anti-IL12 (R2-9A5) on days 13, 14 and 18. Expression of IFNγ and Tbet in C-D. CD4^+Foxp3^- and E. CD8^+ T cells is shown. Data shown as the mean ± SEM of 8-25 mice per group. * p<0.05, ** p<0.01, ***p<0.001, n.s. not significant (one-way ANOVA/ Tukey’s).
Figure 1

A. AT-3 ova<sup>dim</sup> CD73

B. AT-3 ova<sup>dim</sup> CD73

C. MC38 ova<sup>dim</sup>

D. MC38 ova<sup>dim</sup>

E. Control

F. Control

G. Control

- 2A3
- anti-PD-1
- anti-CTLA-4
- anti-PD-1 / anti-CTLA-4
- 2A3
- anti-PD-1 / anti-CTLA-4
- 2A3
- anti-PD-1 / anti-CTLA-4
- 2A3
- P+C
- 2A3+DT
- 2A3+DT+ anti-CD4
- P+C
- P+C+DT
- P+C+DT+ anti-CD4
- Control
- Treg depleted
- Treg & CD4 depleted
- Treg depleted
- P+C
- Treg depleted
- Treg & CD4 depleted
- Treg depleted
- P+C

Legend:
- *p < 0.05
- **p < 0.01
- ***p < 0.001
- n.s. = not significant
Figure 4

A

2A3 PD-1/CTLA-4

CD103+ DCs

IL-12p40

CD103-

IL-12+ DCs

CD103-

IL-12+ DCs

B

DLN Tumor

% CD11c+CD103+

% CD11c+CD103+

% IL-12+

anti-CD8+DT

C

Tumor

% IL-12+

% CD11c+CD103+

anti-CD8+DT

D

DLN

% CD11c+CD103+

anti-CD8+DT

E

Percent Survival

Days post treatment

WT 2A3

WT P + C

BATF-/- 2A3

BATF-/- P + C

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Figure 6

A

B

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Figure 7

A

IL-12p35\(^{-/-}\)

\[
\begin{align*}
\text{WT 2A3} & \quad \text{WT PD-1/CTLA-4} \\
\text{IL-12p35\(^{-/-}\) 2A3} & \quad \text{IL-12p35\(^{-/-}\) PD-1/CTLA-4}
\end{align*}
\]

Days post treatment

B

IL-12p40\(^{-/-}\)

\[
\begin{align*}
\text{WT 2A3} & \quad \text{WT PD-1/CTLA-4} \\
\text{IL-12p40\(^{-/-}\) 2A3} & \quad \text{IL-12p40\(^{-/-}\) PD-1/CTLA-4}
\end{align*}
\]

Days post treatment

C

CD4\(^{+}\)foxp3\(^{-}\)

\[
\begin{align*}
\text{% IFN}\gamma & \quad \text{% tbet} \\
\text{2A3 P+C} & \quad \text{2A3 P+C} \\
\text{WT} & \quad \text{IL-12p35\(^{-/-}\)}
\end{align*}
\]

D

CD4\(^{+}\)foxp3\(^{-}\)

\[
\begin{align*}
\text{% IFN}\gamma & \quad \text{% tbet} \\
\text{2A3 P+C} & \quad \text{2A3 P+C} \\
\text{Control} & \quad \text{anti- IL-12}
\end{align*}
\]

E

CD8\(^{+}\)

\[
\begin{align*}
\text{% IFN}\gamma & \quad \text{n.s.} \\
\text{2A3 P+C} & \quad \text{2A3 P+C} \\
\text{WT} & \quad \text{IL-12p35\(^{-/-}\)}
\end{align*}
\]
Cancer Immunology Research

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