Dual Blockade of PD-1 and CTLA-4 Combined with Tumor Vaccine Effectively Restores T Cell Rejection Function in Tumors

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Abstract

Tumor progression is facilitated by regulatory T cells (Tregs) and restricted by effector T cells. In this study, we document parallel regulation of CD8^{+} T cells and Foxp3^{+} Tregs by programmed death-1 (PD-1, PDCD1). In addition, we identify an additional role of cytotoxic T lymphocyte antigen-4 (CTLA-4) inhibitory receptor in further promoting dysfunction of CD8^{+} T effector cells in tumor models (CT26 colon carcinoma and ID8-VEGF ovarian carcinoma). Two thirds of CD8^{+} tumor-infiltrating lymphocytes (TIL) expressed PD-1, while one third to half of CD8^{+} TIL co-expressed PD-1 and CTLA-4. Double-positive (PD-1^{+}CTLA-4^{+}) CD8^{+} TIL had characteristics of more severe dysfunction than single-positive (PD-1^{+} or CTLA-4^{+}) TIL, including an inability to proliferate and secrete effector cytokines. Blockade of both PD-1 and CTLA-4 resulted in reversal of CD8^{+} TIL dysfunction and led to tumor rejection in two-thirds of mice. Double blockade was associated with increased proliferation of antigen-specific effector CD8^{+} and CD4^{+} T cells, antigen-specific cytokine release, inhibition of suppressive functions of Tregs, and upregulation of key signaling molecules critical for T cell function. When used in combination with GVAX vaccination (consisting of GM-CSF-expressing irradiated tumor cells), inhibitory pathway blockade induced rejection of CT26 tumors in 100% of mice and ID8-VEGF tumors in 75% of mice. Our study indicates that PD-1 signaling in tumors is required for both suppressing effector T cells and maintaining tumor Tregs, and that PD-1/PD-L1 pathway (CD274) blockade augments tumor inhibition by increasing effector T cell activity, while attenuating Treg cell suppression.

Introduction

An effective immune response that leads to meaningful anti-tumor effects requires not only an increase in immune activation but also reduction of suppressive or inhibitory elements of the immune system (1–3). Mechanisms regulating immune activation in cancer progression have been extensively investigated (4–16). There are multiple inhibitory mechanisms that

suppress immune responses, including inhibitory receptors, secreted soluble inhibitors (IL-10 and TGF-β), and regulatory T cells (Tregs) (3, 16–17, 19–22). However, how these factors interact with each other in the tumor environment needs further study.

It is well established that tumors employ PD-1 and CTLA-4 inhibitory pathways to silence the immune system. Both these pathways are critical for physiological homeostasis. While PD-1 is broadly expressed on activated T cells and other hematopoietic cells (23–25), CTLA-4 is expressed on activated T cells including regulatory T cells (26). PD-1 binds two ligands, PD-L1 (B7-H1) and PD-L2 (B7-DC). Upregulation of PD-L1 occurs on a wide variety of human tumors suggesting that cancer cells coopt the PD-1/PD-L1 inhibitory pathway to evade the host immune response (4–16). PD-L1 can also interact with B7.1, resulting in inhibition of T cell activation (24–25). Despite these similarities as terminators of T-cell activation, the difference in regulatory roles of the PD-1 and CTLA-4 pathways has been recognized (27–29). $CD4^+CD25^+Foxp3^+$ T cells (Tregs) play critical roles in the control of anti-tumor immune responses (21–22, 30–38). Indeed, recent data have shown that blocking of PD-1 and CTLA-4 can modulate Treg functions and enhance antitumor responses (2, 35). However, the direct role and function of PD-1 in Tregs in the cancer environment remains unknown.

Immunoregulatory pathways involving PD-1, CTLA-4, and their ligands are highly complex. Co-expression of more than one receptor by $CD8^+$ T cells has been correlated with a more severe exhausted phenotype (39–42). Antibody blockade of either PD-1 or CTLA-4 can enhance effector T-cell responses and induce T cell-mediated tumor rejection in mouse models (2, 4–15). However, the intricacies of each pathway and cross-talk between them make it unlikely that blockade of PD-1 or CTLA-4 would have identical effects. Hence, to optimally design the next generation of immunotherapeutic strategies, careful delineation of the individual contributions of PD-L1/PD-1, PD-L2/PD-1, CTLA-4/B7-1, and PD-L1/B7-1 interactions *in vivo* will be crucial.

In this study, we provide evidence that reversal of T cell dysfunction can be achieved by simultaneously targeting effector T cells and Tregs. First, we show that CTLA-4 is preferentially expressed by $PD-1^+$ $CD8^+$ T cells, and co-expression of both PD-1 and CTLA-4 is associated with marked dysfunction of antigen-specific T cells. Second, blockade of PD-1 and CTLA-4 pathways reversed T cell dysfunction. Blockade therapy with GVAX vaccination further enhanced tumor rejection in mice. Third, adoptive transfer of $CD8^+CTLA-4^+PD-1^+$ TILs that had been pre-treated *in vitro* with αPD-1 and αCTLA-4 antibodies eliminated tumors *in vivo*. Fourth, blockade of PD-1/PD-L1 pathway in Tregs attenuates their suppressive function. Taken together, our study describes an approach involving vaccination and inhibitory receptor blockade to reverse T cell exhaustion, which we envision to be a promising anticancer immunotherapy.

Methods

Mice and cell lines

Six to 8 week-old female BALB/c or C57BL/6 mice (Jackson Laboratory) were used. CT26 colon carcinoma cell line was purchased from American Type Culture Collection (43). ID8-VEGF ovarian carcinoma cell line was developed previously from a mouse ovarian epithelial papillary serous adenocarcinoma cell line (ID8) obtained from Dr. Paul F. Terranova, University of Kansas, and used as described (44–46).

pMG-Lyt2 vector expressing murine GM-CSF was kindly provided by Dr. J.P. Allison, M.D. Anderson Cancer Center, Houston, TX (2). Development of CT26 or ID8-VEGF cells expressing murine GM-CSF (CT26/GVAX or ID8-VEGF/GVAX) was based on the

methods described previously (2, 47). CT26/GVAX and ID8-VEGF/GVAX cells showed secretion of murine GM-CSF 365 ng/10^6 cells/48 h (95% confidence interval, 231–499) and 397 ng/10^6 cells/48 h (95% confidence interval, 275–519), respectively, by ELISA (R&D Systems, Minneapolis).

Tumor experiments and *in vivo* blockade

BALB/c or C57BL/6 mice were implanted subcutaneously (s.c.) on the right flank with either 5×10^5 CT26 or 5×10^6 ID8-VEGF tumor cells, respectively. Two hundred μg of rat α-mouse PD-1 (29F.1A12), PD-L1 (10F.9G2), PD-L2 (3.2) (24), or 100 μg of α-CTLA-4 (clone 9D9) were administered intraperitoneally (i.p.), either 3, 6, and 9 days following CT26 inoculation, or 10, 13, and 16 days following ID8-VEGF inoculation. In vaccination experiments, 10^6 irradiated (150Gy) CT26-GVAX or ID8-VEGF-GVAX were given on the contralateral flank intradermally (i.d.) once either day 3, or day 10 after CT26 or ID8-VEGF inoculations, respectively. CD8$^+$ T cells and CD4$^+$CD25$^+$ T cells from tumor leucocytes were purified either on a MACS column or FACSAria.

Adoptive transfer experiments

CD8$^+$CTLA-4$^+$PD-1$^+$ TILs from CT26 tumor (25 gms pooled from 80 mice) were sorted to more than 95% purity by FACS. Sorted TILs were treated *in vitro* with 10 μg/ml of α-PD-1 and α-CTLA-4 blocking antibodies and cultured in the presence of AH-1 peptide-loaded APCs for 7 days. The cells expanded an average of 10-fold *in vitro* in the week of culture. Following expansion, 5×10^6 purified CD8$^+$ T cells per mouse were injected intratumorally (i.t) 5 times on alternate days starting from day 10 following s.c. CT26 tumor inoculation.

in vitro proliferation assay

PD-1$^+$CTLA-4$^+$ and PD1$^+$CTLA-4$^-$ CD8$^+$ TIL were sorted to more than 95% purity by FACS and co-cultured with splenocytes from naive CD45.1$^+$ C57BL/6 mice in the presence of AH-1 (CT-26) or FR-α (ID8-VEGF) peptides for 3 days (48–49). Thymidine incorporation was measured after pulsing with 0.5 μCi/well [^{3}H]-thymidine during the last 8 h.

in vitro Treg suppression and cytokine analysis

CFSE-labeled CD8$^+$ T cells (5×10^4) were stimulated with 1.5×10^5 irradiated splenic CD45.1$^-$ APCs in the presence of αCD3. CD4$^+$CD25$^+$ Tregs (10^5) from CT26 TILs, along with αPD-L1, αCTLA-4, or control rat IgG were added to the culture as indicated. Four days later, *in vitro* Treg suppression was determined based on CFSE dilution by flowcytometry.

The whole tumor TILs were isolated and adjusted to 2×10^6/ml in 24-well plates and cultured with AH-1 peptide ± 10 μg/ml αPD-1, αPD-L1, αCTLA-4, or control IgG. After 3 days, supernatants were analyzed for secretion of TGF-β, IL-10 and IFN-γ by ELISA and cytokine bead array.

Results

PD-1, CTLA-4, and their ligands are highly expressed in tumor models

To examine the interplay between the PD-1 and CTLA-4 in cancer, we used the murine CT26 colon carcinoma and ID8-VEGF ovarian carcinoma models. In the CD8$^+$ TIL population, two thirds expressed PD-1, and the majority of these (over two thirds) were PD-1^{hi} (Fig. 1A and B). We also found expression of CTLA-4 on TIL, however CTLA-4 was expressed primarily in a subset of PD-1$^+$CD8$^+$ TIL, while PD-1$^-$CD8$^+$ cells were

mostly CTLA-4$^-$. About half of PD-1$^+$CD8$^+$ CT26 TIL and one third of PD-1$^+$CD8$^+$ ID8-VEGF TIL were CTLA-4$^+$, while the remainder of PD-1$^+$CD8$^+$ TIL were CTLA-4$^-$. CTLA-4 was expressed on PD-1^{hi} as well as PD-1^{int} cells in CT26 TILs, whereas CTLA-4 was expressed on PD-1$^+$ cells in ID8-VEGF TILs. Taken together, among CD8$^+$ TIL, one third co-expressed PD-1 and CTLA-4, one third expressed PD-1 only, and the remainder were negative for either receptor (Fig. 1A and B). Among the CD4$^+$ TIL, only a third of the population expressed either of the receptors and approximately 10% were double-positive (Fig. 1A and B), while a quarter of splenic CD4$^+$ T cells expressed CTLA-4, yet there were very few PD-1$^+$ cells (Supplementary Fig. 1). We also found that CT26 and ID8-VEGF tumors upregulate PD-1 and CTLA-4 ligands on tumor and myeloid cells (Supplementary Fig. 2). Thus, CT26 and ID8-VEGF models are suitable for studying PD-1 and CTLA-4.

To begin defining the functional status of TIL, we examined their memory phenotype. Among CD8$^+$ T cells, effector memory (T$_{EM}$) cells were most prevalent in the double positive (PD-1^{hi}CTLA-4$^+$) population (approximately 90% of CT26 and 75% of ID8-VEGF cells), but also accounted for approximately 60% (CT26) and 40% (ID8-VEGF) of the PD-1^{hi}CTLA-4$^-$ population and half of the rare PD-1$^-$CTLA-4^{Int} population (CT26). One third of the PD-1^{Int} CT26 CD8$^+$ T cells were T$_{EM}$ independently of their CTLA-4 status. Both single- and double-positive CT26 CD4$^+$ T cells had a T$_{EM}$ phenotype, whereas only PD-1$^+$CTLA-4$^+$ ID8-VEGF CD4$^+$ T cells had a T$_{EM}$ phenotype (Fig. 1A and C). Thus, a significant fraction of TILs upregulated PD-1, and many of these cells were T$_{EM}$. A proportion of PD-1^{hi} TILs also upregulated CTLA-4, and these double-positive cells were mostly T$_{EM}$.

Co-expression of PD-1 and CTLA-4 correlates with more severe dysfunction of tumor-specific CD8$^+$ T cells

Given the strong association of PD-1 expression and T$_{EM}$ phenotype, we hypothesized that most tumor-reactive TIL are present in the PD-1$^+$ population. To address this, we used AH-1 (gp70$_{423-431}$) and H2-K^b folate receptor (FR$_{161-169}$), CD8$^+$ T cell specific epitopes expressed by CT26 and ID8-VEGF tumor cells, respectively (45–46). We hypothesized that the PD-1$^+$CTLA-4$^+$ cells exhibit greater dysfunction in response to cognate antigens than the single-positive (PD-1$^+$) counterparts. To address this, we sorted PD-1$^+$CTLA-4$^+$ and PD-1$^+$CTLA-4$^-$ CD8$^+$ TILs, labeled them with CFSE, and co-cultured for 3 days with peptide-pulsed APCs. CFSE dilution was observed only in the PD-1$^+$CTLA-4$^-$ population but not in the PD-1$^+$CTLA-4$^+$ population (11.39% vs. 2.45% and 32.3% vs. 4.4% CT26 or ID8-VEGF CFSE^{lo} cells, respectively), indicating a profound suppressed state of the double-positive cells (Fig. 2A).

Next, we directly compared the functional properties of sorted PD-1$^+$CTLA-4$^-$ or PD1$^+$CTLA-4$^+$ CD8 TILs by measuring cytokine secretion in response to peptide stimulation. The frequency of IFN-γ^+, TNF-α^+, IL-2$^+$ or CD107a/b$^+$ cells was approximately 2-fold higher in the PD-1$^+$CTLA-4$^-$ subset compared with the PD1$^+$CTLA-4$^+$ subset (Fig. 2B; $p<0.05$). The PD1$^+$CTLA-4$^+$ CD8 T cells displayed higher levels of other inhibitory receptors such as 2B4, LAG-3, and TIM-3, along with lower levels of CD62L, and CD127 (Fig. 2B; $p<0.01$). These results therefore indicate that dysfunction of CD8$^+$ T cells in tumor was associated with coexpression of PD-1 and CTLA-4.

Next, we tested whether checkpoint blockade could rescue the proliferation of double-positive cells and/or enhance the proliferation of single-positive cells. We isolated PD-1$^+$CTLA-4$^+$ and PD-1$^+$CTLA-4$^-$ CD8$^+$ CT26 and ID8-VEGF TIL subsets and co-cultured them with AH-1 or FR peptide pulsed splenocytes (APCs) $\pm$ αPD-1 and/or αCTLA-4 antibodies. Again, in the absence of any neutralizing antibodies we observed modest proliferation of the PD-1$^+$CTLA-4$^-$ CD8$^+$ TIL and no proliferation of the

PD-1$^+$CTLA-4$^+$ CD8$^+$ TIL (Fig. 2C; p=0.019 and p=0.015 respectively). PD-1 blockade alone was able to enhance the proliferation of both PD-1$^+$CTLA-4$^-$ and PD-1$^+$CTLA-4$^+$ CD8$^+$ TIL, although it was unable to completely restore the proliferation of double-positive cells. CTLA-4 blockade alone was able to enhance the proliferation only of PD-1$^+$CTLA-4$^+$ CD8$^+$ TIL, and had no effect on CTLA-4$^-$ cells. However, dual blockade fully restored the proliferation of PD-1$^+$CTLA-4$^+$ CD8$^+$ TIL (Fig. 2C; p=0.038 and p=0.004, respectively). Thus, the double-positive population represents cells with severely dysfunctional status, but their function can be rescued by double blockade.

Combination therapy promotes CT26 and ID8-VEGF tumor rejection

Given that a significant proportion of tumor-reactive CD8$^+$ TILs were found to be PD-1$^+$CTLA-4$^+$, and given that their proliferation in response to cognate tumor antigen was restored by double blockade *in vitro*, we tested whether simultaneous blockade of PD-1 and CTLA-4 could promote tumor rejection in mice. We found that treatment with αPD-1 or αPD-L1 showed CT26 tumor regression in 25% and 33% of the mice, respectively (Fig. 3A; Table 1). CTLA-4 blockade alone led to tumor regression in 50% of the mice. Further, we noticed that combination of either αPD-1 or αPD-L1 with αCTLA-4 induced tumor regression in 75% of mice (p<0.01). Similarly, we found that treatment with αPD-1, αPD-L1 or αCTLA-4 showed ID8-VEGF tumor regression in 25%, 37.5%, and 25% of the mice, respectively (Fig. 3B; Table 1). Combined blockade induced tumor regression in 50% of mice. In contrast, αPD-L2 blockade did not induce tumor regression in both models. Further, *in vivo* CD4$^+$ and CD8$^+$ depletion completely abolished tumor regression (Supplementary Fig. 3).

Hypothesizing that the effects of inhibitory receptor blockade could be enhanced by increasing the frequency of tumor-reactive T cells, we combined our treatment with GM-CSF transduced whole tumor cells (GVAX) (50). Remarkably, 100% of CT26 bearing mice and 75% of ID8-VEGF bearing mice in the triple combination groups (GVAX/αPD-1/ αCTLA-4) rejected their tumors (Fig. 3A & B; Table. 1). Thus, double checkpoint blockade has the potential to induce significant tumor rejection, which could be maximized by boosting antitumor immune response with vaccine.

Adoptive transfer of *in vitro* pre-treated CD8$^+$CTLA-4$^+$PD-1$^+$ TILs with αPD-1 and αCTLA-4 antibodies cause regression of CT26 tumor in mice

Next we tested whether CD8$^+$CTLA-4$^+$PD-1$^+$ TILs treated *in vitro* with αPD-1 and αCTLA-4 would kill the tumor cells *in vivo*. We sorted CD8$^+$CTLA-4$^+$PD-1$^+$ TILs from CT26 tumor and cultured in the presence of α-PD-1 and α-CTLA-4 blocking antibodies and AH-1 peptide for 7 days. We found that *in vitro* treated cells showed profound increase in IFN-γ cytokine production and Ki-67 expression (Fig. 4B; left panel). These *in vitro* treated cells were adoptively transferred into CT26 tumor bearing mice as described in the Methods. We found that adoptive therapy resulted in tumor regression in 75% of mice (Fig. 4A). In addition, we found that the adoptively transferred TILs were still functional (IFN-γ$^+$Ki67$^+$) in the regressing tumors a week after the treatment (Fig. 4B; right panel).

Combination blockade increases TIL activation and antigen-specific inflammatory cytokine production

Next, we tested whether tumor regression following *in vivo* blockade was associated with activation of TIL. We resected and analyzed tumors (n=8) that showed evidence of rejection from 13–15 day onwards. As expected, Ki-67 expression on TIL was markedly increased following PD-1, PD-L1, or CTLA-4 monotherapy (p=0.001). Confirming our observation *in vitro* (Fig. 2) combination of αPD-1 with αCTLA-4 doubled the frequency of Ki-67$^+$CD8$^+$ T cells (Fig. 5A; p=0.002), while αPD-L1 and αCTLA-4 combination increased the

frequency of Ki-67$^+$CD8$^+$ T cells by 50% (p=0.012) compared with single antibody blockade (Fig. 5A). Combined blockade also increased granzyme B expression, suggesting an enhancement in cytolytic potential of effector T cells (Fig. 5B; p=0.01).

We corroborated the above findings by testing the phosphorylation status of key transcription factors by flowcytometry (51–52). The ribosomal S6 kinase (S6K) is implicated in IL-2 induced T cell proliferation (53). Validating the increased expression of Ki-67 by TIL, we observed an increase in the expression of phosphorylated (p)-S6K in CD8$^+$ T cells following single antibody (αPD-1) blockade, and combined PD-1 and CTLA-4 blockade further enhanced pS6K levels (p=0.01; Fig. 5C). T-bet and Eomes are two T-box transcription factors regulating Th1 and cytolytic function of CD8$^+$ cells (54–56). αPD-L1 antibody alone induced high levels of pT-bet, whereas αPD-1 or αCTLA-4 alone or combined showed moderate increases in pT-bet levels (Fig. 5C). Validating the enhanced granzyme B expression, blocking antibodies increased pEomes expression levels (Fig. 5C). Thus, checkpoint blockade induced TIL activation, and combined αPD-1 and αCTLA-4 therapy significantly increased proliferation and cytolytic function of TIL relative to monotherapy.

We next tested whether the functional capacity of tumor-reactive CD8$^+$ TIL increased following single versus double blockade. CT26 tumors from the various treatment groups were dissociated, and mixed cells from these tumors were seeded in primary co-cultures with AH-1 peptide. Cultures derived from single antibody (PD-1, PD-L1, or CTLA-4) treated mice showed modestly increased IFN-γ$^+$TNF-α$^+$ or IFN-γ$^+$IL-2$^+$ CD8$^+$ TILs (Fig. 5D; p=0.05). Cultures from combined αPD-1 and αCTLA-4 treatment, exhibited increased numbers of IFN-γ$^+$TNF-α$^+$ (Fig. 5D; p=0.05), but not IFN-γ$^+$IL-2$^+$ CD8$^+$ TILs (Fig. 5D;p=0.12). These data suggest that combination blockade induces polyfunctional activation of tumor antigen-specific CD8$^+$ T cells. As expected, combining GVAX with αPD-1, αPD-L1, or αCTLA-4 inhibitory blockade further increased the frequency of CT26-specific IFN-γ$^+$TNF-α$^+$ CD8$^+$ TIL (Fig. 5E; p=0.009).

in vivo PD-1 and CTLA-4 blockade enhances infiltration of T cells and reduces Tregs in tumors

As expected based on the above experiments, combined blockade significantly increased total inflammatory infiltration and CD8$^+$ cells in tumors, but also CD4$^+$ cell infiltration (Fig. 6A; p=0.002, p=0.025, and p=0.001 respectively). Then we tested whether the effects of inhibitory receptor blockade could be further enhanced by depleting Tregs. Interestingly, we could not find any additional benefit by combining αCD25 antibody with blocking antibodies (Supplementary Fig. 4). This suggested that blocking antibodies could independently affect Treg accumulation in tumors as well as tumor draining lymph nodes (Supplementary Fig. 5).

To test this hypothesis, we analyzed the frequency of Tregs in tumors of mice following single or double blockade. PD-1 or PD-L1 blockade alone produced a moderate reduction in Treg levels in the tumor (Fig. 6B and 6C; p=0.024), while CTLA-4 blockade did not affect Treg numbers. However, combined blockade profoundly reduced the frequency of Tregs (from 9.06% to 3.24% of CD4$^+$; Fig. 6B and 6C; p=0.004). As a result, the CD8/Treg and CD4/Treg ratios increased markedly, especially after combined αPD-L1 and αCTLA-4 treatment.

We asked whether the reduction in Treg frequency was due to apoptosis. None of the blocking antibodies affected the apoptosis rate in Tregs (based on Annexin-V and 7-AAD staining - data not shown). Importantly, combined αCTLA-4 and αPD-1 reduced GITR (a functional marker of activated Tregs) expression from 66.7% to 45.8% of Tregs (Fig. 6B;

p<0.001), indicating that double blockade could affect not only the number but also the function of tumor-infiltrating Tregs.

Co-expression of PD-1 and CTLA-4 by Tregs is associated with heightened T cell dysfunction in tumor

Since we found approximately one-fourth of the CD4^{+} TIL expressing PD-1 and/or CTLA-4 (Fig. 1), we first examined whether these CD4^{+} TIL are effector cells or Tregs. We found that Tregs were primarily comprised in the CTLA-4^{+} CD4^{+} population. Importantly, among the CTLA-4^{+} cells, most were Foxp3^{+}CD25^{+} cells, representing activated effector Tregs (30, 35, 37), and were PD-1^{+}CTLA-4^{+} double-positive cells (Fig. 7A). Notably, splenic Tregs expressed much lower levels of PD-1 and CTLA-4 (Fig. 7A). We also observed not only increased expression of CTLA-4, but also of inducible T cell co-stimulator (ICOS) and folate receptor 4 (FR-4) expression on PD-1^{hi} Treg compared to PD-1^{lo} Treg (Fig. 7B; p=0.001, p=0.001 and p=0.034, respectively), indicating an activated functional status. Thus, tumor Treg were mostly CTLA-4^{+} and among them, the PD-1^{+} Tregs appeared to be activated Tregs.

To understand the specific contribution of PD-1^{+} Treg to the suppressive program, CD4^{+}CD25^{+}CTLA-4^{+} Tregs were preincubated with PD-1 blocking antibody or control IgG. Control Tregs or PD-1-neutralized Tregs were then incubated with CD8^{+} T cells stimulated by immobilized αCD3 antibody (responder cells) in the presence of splenic APCs (CD11c^{+} cells) or PD-L1 neutralized tumor-derived APCs. Responder T cell proliferation was assessed by CFSE dilution. Whereas control Tregs were able to suppress responder cell proliferation, PD-1 neutralized Tregs as well as PD-L1-neutralized tumor-derived APCs were unable to suppress CD8^{+} T cell proliferation (Fig. 7C, p=0.0014). Thus, the suppressive function of tumor-derived Tregs is critically dependent on PD-1 signaling, compatible with the notion that the PD-1^{+}CTLA-4^{+} Tregs are responsible for the suppressor function.

Next, we tested whether PD-1 blockade could attenuate the suppressive phenotype of Tregs and their regulatory cytokine programming (Fig. 7E–G). The whole tumor digests from various *in vivo* treatment groups were co-cultured with AH-1 peptide for 5 hours, and we measured TGF-β, IL-10 and IFN-γ in the supernatants. We detected significant amounts of TGF-β and IL-10 and minimal IFN-γ in leukocyte co-cultures form untreated mice, indicating ongoing activity of Tregs and no reactivity of antitumor effector cells. TGF-β secretion was suppressed *ex vivo* following αPD-1 treatment, and more effectively after αCTLA-4 treatment *in vivo* (Fig. 7E; p=0.017). In contrast, IL-10 secretion was decreased *ex vivo* following αPD-1 treatment, but not after αCTLA-4 treatment *in vivo* (Fig. 7F; p=0.024). Furthermore, dual αPD-1 and αCTLA-4 blockade elevated AH-1-specific IFN-γ induction (Fig. 7G; p=0.03). Thus, checkpoint blockade reduces the ability of Tregs to secrete immunoregulatory cytokines.

Discussion

In this study, we demonstrated a non-redundant regulation of CD8^{+} T cell exhaustion by PD-1 and CTLA-4 in CT26 colon carcinoma and ID8-VEGF ovarian carcinoma models. About one fourth of CD8^{+} TIL expressed both PD-1 and CTLA-4, and this was associated with more severe CD8^{+} T cell exhaustion. Blockade of either PD-1 or CTLA-4 pathways enhanced effector T cell infiltration into the tumor. However, simultaneous blockade of both pathways had synergistic effects in activation of tumor antigen specific CD8^{+} and CD4^{+} effector T cells as well as affecting Tregs, ultimately improving long-term survival rates. Adoptive transfer of CD8^{+}CTLA-4^{+}PD-1^{+} TILs that had been pre-treated *in vitro* with

αPD-1 and αCTLA-4 antibodies eliminated CT26 tumors *in vivo*, further demonstrating the role of the PD-1 and CTLA-4 pathways in the tumor.

These effects were further augmented through combination with a GVAX vaccine. Combining blockade therapy with a single dose of GVAX vaccination resulted in enhanced intratumoral CD45$^+$ and CD8$^+$ T cells and as a result, the CD8/Treg ratios increased markedly. Moreover, mice treated with αPD-1/CTLA-4 combined with GVAX further increased IFN-γ$^+$ TNF-α$^+$ CD8$^+$ TILs (Fig. 5E) resulting in enhanced tumor rejection. Hence our results suggest that the mechanisms underlying the synergistic effects of vaccine could possibly be due to the capacity of vaccine to increase the frequency of tumor-reactive CD8$^+$ TILs, which could be activated by CTLA-4 and PD-1 blockade. The levels of GM-CSF produced by CT26/GVAX and ID8-VEGF/GVAX vaccines are comparable to the previously published preclinical studies using B16/GVAX and Glioma/GVAX (2, 57). The lack of *in vivo* efficacy of PD-1/PD-L1 blockade with GVAX in B16 melanoma seen by Curran et al. (2) may be due to the fact that the B16 tumor line does not express PD-L1, whereas CT26 and ID8-VEGF cells express high levels. Our results are also in line with previous observations that higher dose of GVAX recruited MDSCs into the tumor (2, 57).

The PD-1/PD-L1 pathway mediates T cell exhaustion by antagonizing activation signaling pathways (28–29, 58–62). We investigated our therapy's ability to counteract these effects by comparing the levels of key signaling molecules in different treatment groups. First we looked at T-bet, which is expressed preferentially by type 1 effector T cells, and Eomes, which is expressed in memory T cells (61–62). We also monitored the S6K which, when phosphorylated, acts downstream of mTOR to activate cell proliferation, protein translation, and survival (53). In untreated mice, we found that tumor infiltrating T cells showed no or very low expression of pT-bet, pEomes, or pS6K. However, upon single αPD-L1 antibody treatment we observed a marked increase in pT-bet expression. αPD-L1 combined with αCTLA-4 antibody further increased pEomes, and αPD-1 combined with αCTLA-4 blockade further increased pS6K. That our treatments led to increased levels of these phosphorylated signaling molecules supports the contention that we were activating classical anti-tumor effector T cells. However, the differential effects of combining αCTLA-4 with αPD-1 versus αPD-L1 could be because αPD-1 blocks the inhibitory contributions of PD-1 binding to PD-L1 and PD-L2 but leaves the PD-L1/B7-1 inhibitory interaction unaffected (24–25). In contrast, αPD-L1 blocks both the PD-L1/PD-1 and the PD-L1/B7-1 inhibitory pathways but leaves the PD-1/PD-L2 inhibitory interaction unaffected (24–25).

Our experiments with sorted PD-1$^+$CTLA-4$^-$ and PD-1$^+$CTLA-4$^+$ cells showed that CT26 or ID8-VEGF antigen-specific CD8$^+$ T cells were exclusively located in the double-positive population and were highly dysfunctional, highlighting the relevance of these receptors in escape from the anti-tumor T cell response. Studies report that PD-1 mediates inhibition by blocking PI3K activation, whereas CTLA-4 functions through binding to the phosphatase PP2A, leading to inhibition of Akt phosphorylation (29, 59, 63–64). Our study proves that double blockade targets both pathways resulting in an additive response by targeting both PD-1 and CTLA-4 signaling molecules allowing more effective T-cell activation, proliferation and function of TILs, and further augmentation of therapeutic effect. Taken together, our results indicate that PD-1 and CTLA-4 pathways act non-redundantly to inhibit TIL function. This is an important lesson to bring to the clinic, highlighting why blockade of both pathways may be essential to completely regain T cell function.

Our therapy's ability to limit the Treg population *in vivo* is highly desirable. Here we found simultaneous expression of PD-1 and CTLA-4 on Tregs and distinct effects of blocking each pathway. The importance of the PD-1 pathway for Tregs, however, is not well characterized, although one recent study described PD-L1 expression by Tregs (36). While PD-1 enhanced

in vivo Treg function, CTLA-4 blockade did not impact the suppressive function of Tregs in CT26 *in vivo* (Figs. 6). However, our findings indicate that complete restoration of CD8$^+$ T cell proliferation required combined αPD-1 and αCTLA-4 blockade both *in vivo* as well as *in vitro* (Figs. 6 and 7). In our models, PD-L1 on Tregs might bind directly to PD-1 on CD8$^+$ T cells (cell-autonomous), or it may indirectly activate nearby DCs by reversing suppression through PD-1, allowing the induction of a stronger adaptive immune response (non-autonomous). We investigated which of these mechanisms was occurring. We found that blocking either PD-1 or PD-L1 on Tregs led to an increase in CD8$^+$ T cell proliferation (Fig. 7C). Also, in the absence of PD-L1 depleted APCs, the Tregs were not able to suppress CD8$^+$ T cell proliferation (Fig. 7C). This demonstrates that PD-1 expression by effector T cells and Tregs, as well as PD-L1 expression by APCs, are all involved in the suppression of CD8$^+$ TIL responses. We also found that dual blockade reduces production of other immunoinhibitory cytokines including TGF-β and IL-10. This provides further novel insights into the mechanisms of dual blockade.

αCTLA-4 has already been approved by FDA for treatment of melanoma. The antitumor activity of αPD-1 and αPD-L1 are evident in animal models, and αPD-1 and αPD-L1 antibodies are now under intense clinical evaluation (65–67). It will be critical to understand the direct effects of blockade on effector T cells as well as Tregs. Our work suggests that administration of αPD-1 antagonists may harness the therapeutic potential of effector T cells *in vivo* while concomitantly suppressing the functions of activated Tregs. Our study provides the scientific basis for a clinical trial that would involve combination of tumor-cell vaccination and simultaneous PD-1 and CTLA-4 blockade for sustained tumor control in cancer patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

|	
PD-1	programmed death-1
PD-L	PD-1 ligand
CTLA-4	cytotoxic T-lymphocyte antigen-4
CT26	colon carcinoma
ID8	ovarian carcinoma
Tregs	regulatory CD4$^+$ T cells
GVAX	Tumor cells transduced to express GM-CSF

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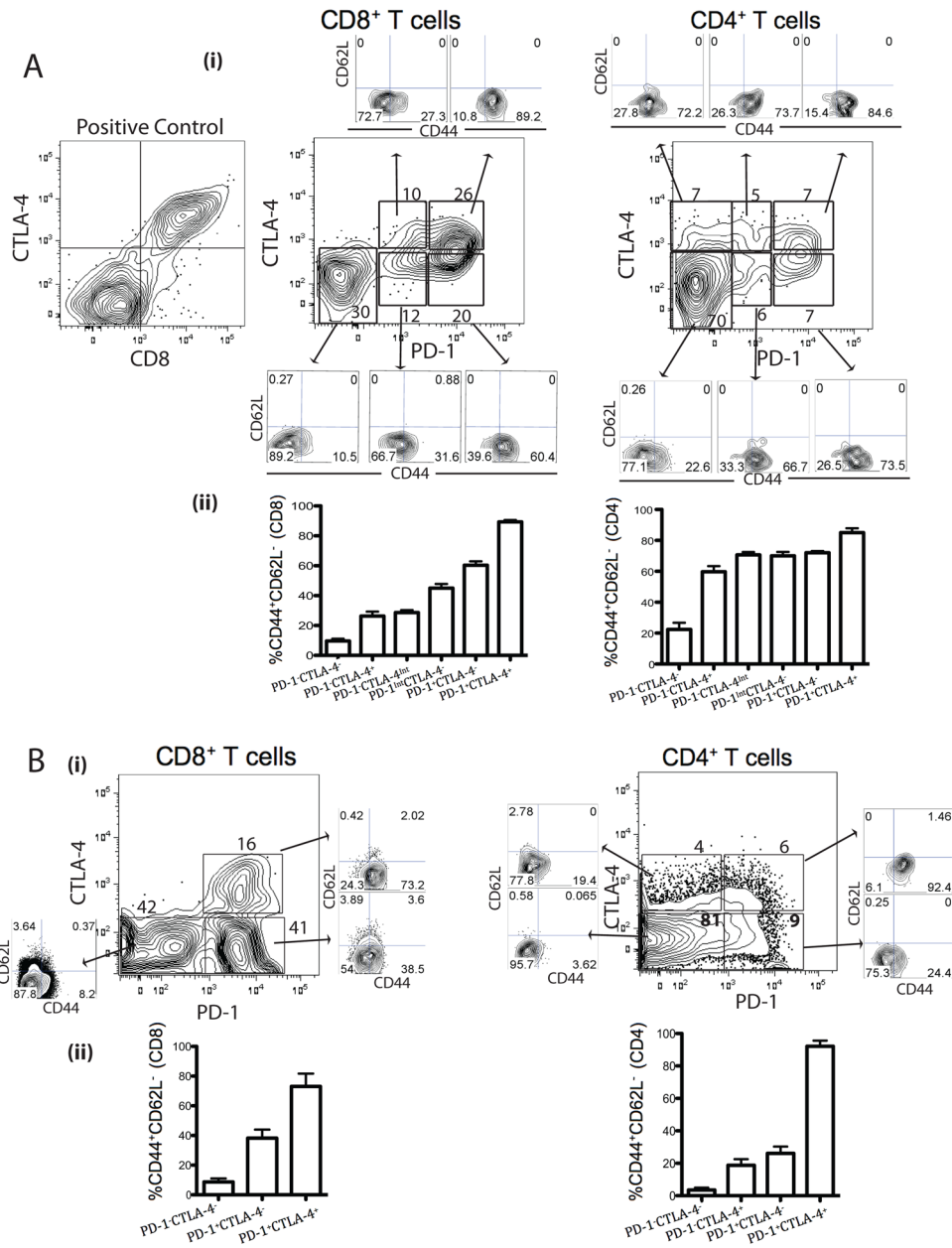
Figure 1. Phenotypic analysis of CT26 and ID8-VEGF TIL

TIL isolated from CT26 and ID8-VEGF tumors were stained with antibodies to CD45, CD8, CD4, PD-1, CTLA-4, CD44, and CD62L as well as live/dead stain. Representative CT26 (A) and ID8-VEGF (B) tumor samples showing expression of PD-1 and CTLA-4 on CD8^{+}CD45^{+} and CD4^{+}CD45^{+} TIL with distinct cell populations, which are further analyzed for CD44 and CD62L. Summary data of CTLA-4 and PD-1 expression on T_{EM} (CD44^{hi}CD62L^{lo}) CD8^{+} and CD4^{+} TIL. Data represent mean ± SD of n=6 mice per group and are representative of 4 independent analyses.
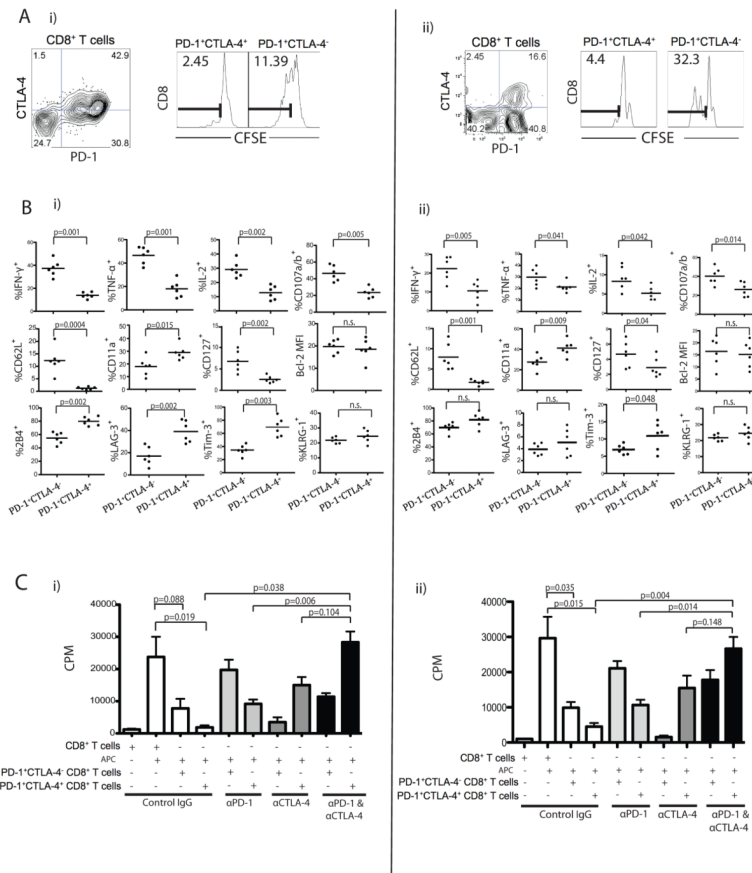
Figure 2. Co-expression of PD-1 and CTLA-4 correlates with more severe dysfunction of CT26 and ID8-VEGF antigen-specific CD8+ T cells

A) PD-1+CTLA-4+ and PD-1+CTLA-4− CD8+ TIL populations from either CT26 (i) or ID8-VEGF (ii) tumors were sorted, labeled with CFSE, and stimulated with AH-1 or FR peptide, respectively for 3 days in the presence of irradiated splenic CD45.1− APCs. CD8+ T cell proliferation was determined by dilution of CFSE; numbers indicate the percentage of CFSE[lo] (AH-1 or FR reactive cells). B) Frequency of CT26 (i) or ID8-VEGF (ii)-specific PD-1+CTLA-4− and PD1+CTLA-4+ CD8+ T cells showing cytokine and degranulation after peptide stimulation (n=6). PD-1+CTLA-4− and PD1+CTLA-4+ CD8+ TILs cells were further analyzed for differential phenotype and expression of other inhibitory receptors. C) PD-1+CTLA-4+ or PD-1+CTLA-4− CD8+ TILs from CT26 (i) or ID8-VEGF (ii) were stimulated with AH-1 or FR peptide, respectively and cultured with blocking antibodies for 3 days. Proliferation was determined by ^{3}H-thymidine incorporation. Statistical significance was determined by Student's t-test.
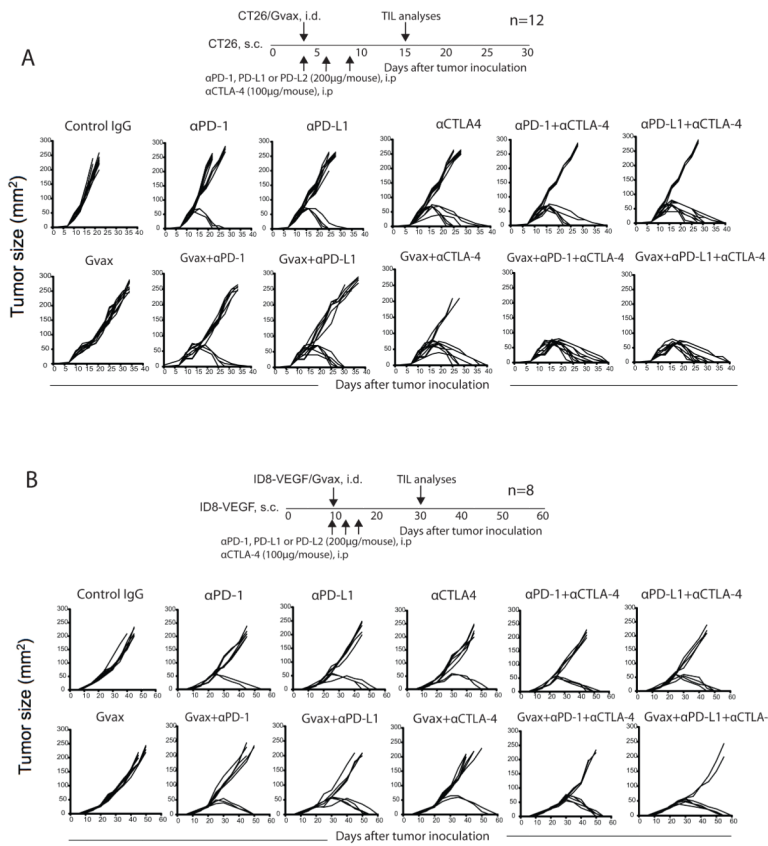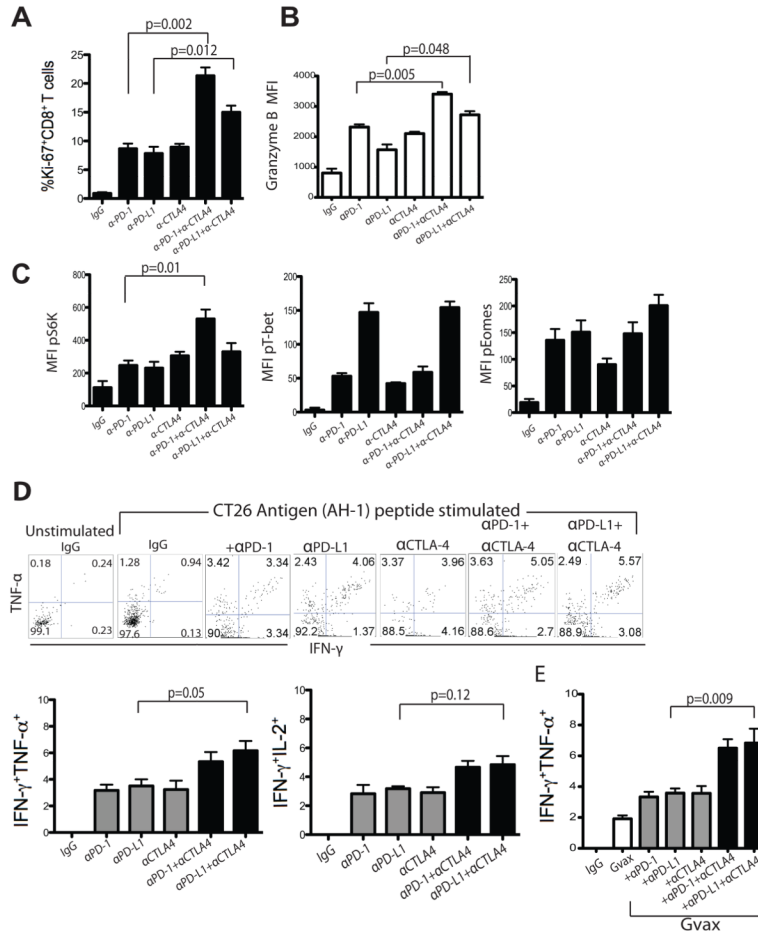
Figure 3. *In vivo* **PD-1 and CTLA-4 blockade promotes CT26 and ID8-VEGF tumor rejection in mice**

Regression and survival (<200 mm^2) of CT26 (A) and ID8-VEGF (B) inoculated mice following treatment as described in the Methods section. Figures represent one of the 5 (CT26) and 3 (ID8-VEGF) independent experiments.
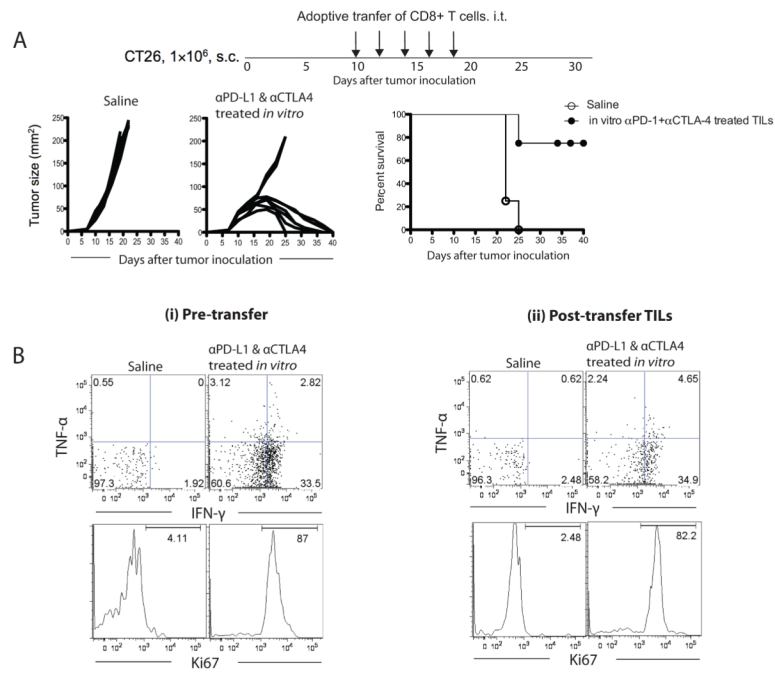
Figure 4. Therapeutic adoptive transfer of *in vitro* α-PD-1 and α-CTLA-4 pre-treated TILs cause regression of CT26 tumors in mice

A) Tumor regression in mice transferred with *in vitro* expanded CT26 antigen-specific CD8+CTLA-4+PD-1+ CT26 TILs. (B) The percent of IFN-γ+ and Ki67+ of *in vitro* pre-treated CD8+ T cells just before adoptive transfer (left panel) and the TILs recovered from tumor one week after the final transfer (right panel) are shown.

Figure 5. *In vivo* PD-1 and CTLA-4 blockade increases TIL activation and CT26 antigen-specific inflammatory cytokine production
Summary data showing percentage of Ki-67$^+$ CD8$^+$ TILs (A) and mean fluorescence intensity (MFI) of granzyme B$^+$ CD8$^+$ TILs (B) from mice treated with various antibodies, as indicated. C) Flow cytometry analysis showing MFI of pT-bet, pEomes and pS6K expression by CD8$^+$ TIL from treated mice. Representative and summary data showing IFN-γ and TNF-α production by CD8$^+$CD45$^+$ TIL from antibody (D) as well as GVAX ± antibody (E) treated mice 13–15 days following tumor inoculation. Statistical significance was determined by Student's *t*-test.
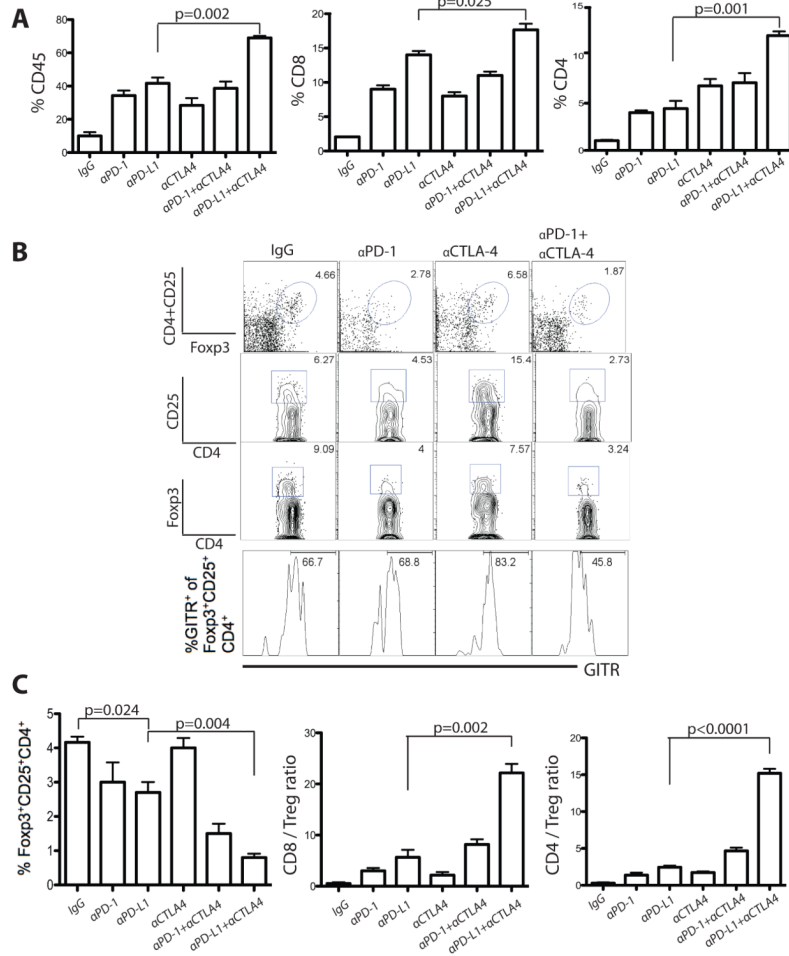
Figure 6. PD-1 and CTLA-4 blockade enhance lymphocyte infiltration and reduce the frequency of Tregs in CT26 tumors

A) CD45$^+$ leukocyte infiltration, and CD8$^+$ and CD4$^+$ TIL as percent of total CD45$^+$ cells 13–15 days after tumor inoculation. B) Representative data showing the percentage of Tregs and expression of GITR among Tregs. C) Percentage of CD45$^+$ TILs of Tregs, and the ratios of CD8$^+$ or CD4$^+$ effector T cells to Tregs in the tumors of treated mice with indicated blocking antibodies. Values are shown for mice analyzed independently and the sum of 3 independent experiments with 6–12 mice per group. Statistical significance was determined by Student's *t*-test.
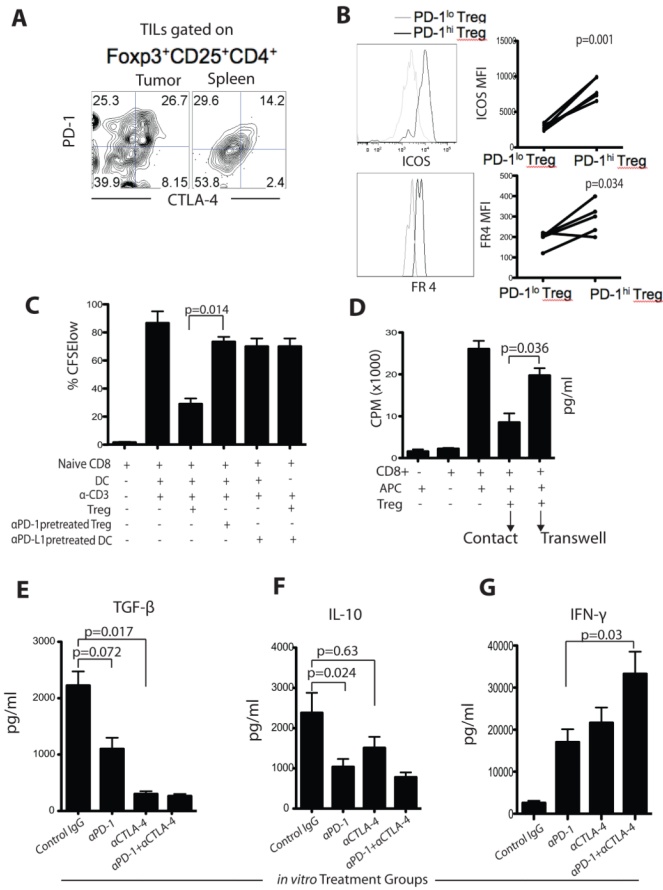
Figure 7. PD-1 and CTLA-4 pathway blockade reduces Treg-mediated suppression of CD8+ T cells *in vitro* and secretion of regulatory cytokines TGF-β and IL-10
A) Representative expression of PD-1 and CTLA-4 by intra-tumoral and splenic Tregs in the same mouse. B) ICOS and folate receptor 4 (FR4) expression and MFI in PD-1hi and PD-1lo Tregs. C) TIL Tregs (± αPD-1) were co-cultured with CFSE-labeled CD8+ T cells and DCs (pretreated) in the presence of αCD3. Dividing cells were quantified after 4-days of culture by gating on the CFSElo population. D) [3H]-thymidine incorporation into effector T cells in the presence or absence of TIL Tregs. Tregs were either in direct contact with the effector cells or were separated by a transwell membrane. The whole tumor leucocytes were isolated and cultured *ex vivo* for 72 hours in the presence of AH-1 peptide and PD-1 and CTLA-4 blocking antibodies as indicated. After culture, supernatants were analyzed for secretion of TGF-β (E), IL-10 (F), and IFN-γ (G). Results are from 3 representative experiments. Bar graphs show mean ± SD.

Table 1

Cumulative results of all mice inoculated with either CT26 and ID8-VEGF tumors.

Treatment Groups	CT26	ID8-VEGF
Control IgG	0	0
αPD-1	25	25
αPD-L1	33	37.5
αCTLA-4	50	25
αPD-1+αCTLA-4	75 [*]	50
αPD-L1+αCTLA-4	75 [*]	50
GVAX	0	0
GVAX + αPD-1	42	37.5
GVAX + αPD-L1	50	50
GVAX + αCTLA-4	75 [*]	37.5
GVAX + αPD-1+ αCTLA-4	100 [*]	75 [**]
GVAX + αPD-L1+ αCTLA-4	100 [*]	75 [**]

p values among different treatments were calculated using the log-rank test

[*] P $\leq$ 0.05;

[**] P $\leq$ 0.01.