Published June 10, 2013

Indoleamine 2,3-dioxygenase is a critical resistance mechanism in antitumor T cell immunotherapy targeting CTLA-4

Rikke B. Holmgaard,[1,2] Dmitriy Zamarin,[1,2,3] David H. Munn,[4] Jedd D. Wolchok,[2,3,5,6] and James P. Allison[1,7]

[1]Howard Hughes Medical Institute, Department of Immunology, Memorial Sloan-Kettering Cancer Center, New York, NY 10065
[2]Swim Across America Laboratory/Ludwig Collaborative Research Laboratory, Immunology Program, Sloan-Kettering Institute for Cancer Research, New York, NY 10065
[3]Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY 10065
[4]Cancer Center and Department of Pediatrics, Georgia Regents University, Augusta, GA 30912
[5]Weill Cornell Medical College and Graduate School of Medical Sciences of Cornell University, New York, NY 10065
[6]Ludwig Institute for Cancer Research, New York, NY 10065
[7]The University of Texas, MD Anderson Cancer Center, Department of Immunology, Houston, TX 77030

The cytotoxic T lymphocyte antigen-4 (CTLA-4)–blocking antibody ipilimumab results in durable responses in metastatic melanoma, though therapeutic benefit has been limited to a fraction of patients. This calls for identification of resistance mechanisms and development of combinatorial strategies. Here, we examine the inhibitory role of indoleamine 2,3-dioxygenase (IDO) on the antitumor efficacy of CTLA-4 blockade. In IDO knockout mice treated with anti–CTLA-4 antibody, we demonstrate a striking delay in B16 melanoma tumor growth and increased overall survival when compared with wild-type mice. This was also observed with antibodies targeting PD-1–PD-L1 and GITR. To highlight the therapeutic relevance of these findings, we show that CTLA-4 blockade strongly synergizes with IDO inhibitors to mediate rejection of both IDO-expressing and nonexpressing poorly immunogenic tumors, emphasizing the importance of the inhibitory role of both tumor- and host-derived IDO. This effect was T cell dependent, leading to enhanced infiltration of tumor-specific effector T cells and a marked increase in the effector-to-regulatory T cell ratios in the tumors. Overall, these data demonstrate the immunosuppressive role of IDO in the context of immunotherapies targeting immune checkpoints and provide a strong incentive to clinically explore combination therapies using IDO inhibitors irrespective of IDO expression by the tumor cells.

CORRESPONDENCE
Rikke B. Holmgaard:
HolmgaaR@mskcc.org
OR
James P. Allison:
jallison@mdanderson.org

Abbreviations used: 1MT, 1-methyl-tryptophan; CTLA-4, cytotoxic T lymphocyte antigen-4; GrB, Granzyme B; IDO, indoleamine 2,3-dioxygenase; MDSC, myeloid-derived suppressor cell; TDO, tryptophan 2,3-dioxygenase; TIL, tumor-infiltrating lymphocyte; T reg cell, regulatory T cells.

Cytotoxic T lymphocyte antigen-4 (CTLA-4) is a potent negative regulator of T cell responses. It is expressed on activated T cells and a subset of regulatory T cells (T reg cells; Chambers et al., 2001). CTLA-4 engagement by its ligands, B7-1 and B7-2, decreases IL-2 transcription, T cell proliferation, and T cell–APC contact times (Krummel and Allison, 1996; Schneider et al., 2006). The presumptive effect is suboptimal triggering of co-stimulatory signaling. Blocking CTLA-4 function with monoclonal antibodies can augment antitumor T cell responses and induce long-term regression of melanoma in mice (Leach et al., 1996; van Elsas et al., 1999) and humans (Phan et al., 2003; Sanderson et al., 2005; Hodi et al., 2010; Robert et al., 2011). The CTLA-4 blocking antibody ipilimumab has been approved by the U.S. Food and Drug Administration for treatment of advanced melanoma; however, CTLA-4 blockade is only effective in a subset of patients and the impact on survival remains limited, calling for identification of resistance mechanisms.

Data from clinical studies demonstrated significant infiltrates of effector T cells in tumors responding to anti–CTLA-4, but not in nonresponding tumors (Hodi et al., 2003; Ribas

The Rockefeller University Press $30.00
J. Exp. Med. 2013
www.jem.org/cgi/doi/10.1084/jem.20130066

Published June 10, 2013

et al., 2009). One proposed explanation for this finding suggested that accumulation of tumor-infiltrating T cells may be impeded by an immunosuppressive microenvironment, resulting in resistance to therapy.

The cytosolic enzyme indoleamine 2,3-dioxygenase (IDO) has been proposed as a potential contributor to melanoma-derived immunosuppression. IDO is produced mainly by the tumor cells and the host immune cells such as macrophages and DCs that reside in the draining lymph nodes or are recruited by the tumor (Uyttenhove et al., 2003; Munn et al., 2004). It catalyzes the rate-limiting step in tryptophan degradation and the combination of local reduction in tryptophan levels and production of bioactive tryptophan metabolites (kynurenine) appear to exert suppressive activity on T cells (Munn et al., 1998, 2005; Fallarino et al., 2002; Frumento et al., 2002; Terness et al., 2002). In vitro studies have shown that IDO can mediate suppressive effects directly on effector T cells and activate suppressive populations of T reg cells (Munn and Mellor, 2004, 2007).

IDO is commonly found in primary melanoma and draining lymph nodes (Munn et al., 2004; Polak et al., 2007; Brody et al., 2009), and its presence has been shown to correlate with tumor progression and invasiveness (Munn et al., 2004; Lee et al., 2005; Harlin et al., 2006; Polak et al., 2007; Weinlich et al., 2007). Pharmacological inhibition of IDO with 1-methyl-tryptophan (1MT) has been shown to result in T cell–dependent antitumor responses in murine models (Friberg et al., 2002; Muller et al., 2005a; Uyttenhove et al., 2003). However, although treatment with 1MT was observed to retard tumor outgrowth, it was unable to trigger complete tumor regression as a single intervention (Muller et al., 2005b; Hou et al., 2007; Gu et al., 2010).

It is unclear whether IDO expression by tumor cells can be used as a predictive marker for response to therapy with IDO inhibitors or whether such therapy can also benefit patients who have no detectable IDO expression in the tumor cells. In addition to being constitutively expressed by many malignant cells (Muller et al., 2005a), IDO can be induced in tumor cells and APCs by proinflammatory stimuli such as IFN-γ, which is generated by the host immune response against the tumor (Taylor and Feng, 1991; Belladonna et al., 2009). IDO induction as a result of anticancer immunotherapy may thus counteract the effectiveness of an otherwise beneficial treatment. Combining immunotherapies with IDO blockade may therefore prove advantageous.

To this end, in this study we explored the inhibitory role of IDO in the context of therapies targeting immune checkpoints and set out to determine whether inhibition of IDO expressed by either tumor cells, host cells, or both would be important for successful immunotherapy. Our data suggest that host-derived IDO suppresses infiltration and accumulation of tumor-reactive T cells in B16 tumors in the context of anti–CTLA-4 immunotherapy, and attenuates the antitumor efficacy. Along these lines, we demonstrate that inhibition/absence of IDO in combination with therapies targeting immune checkpoints such as CTLA-4, PD-1/PD-L1, and GITR synergize to control tumor outgrowth and enhance overall survival in different tumor models. We further show that the combination therapy is synergistic irrespective of detectable IDO expression in tumor cells, though therapeutic efficacy was reduced against the B16 melanoma engineered to overexpress IDO, supporting previous findings of the inhibitory role of tumor-derived IDO. To our knowledge, this is the first study to demonstrate the inhibitory role of IDO in antitumor therapies targeting immune checkpoints. We believe that these data highlight an important mechanism of immunotherapy resistance and provide important implications for development of combinatorial approaches in the clinical setting.

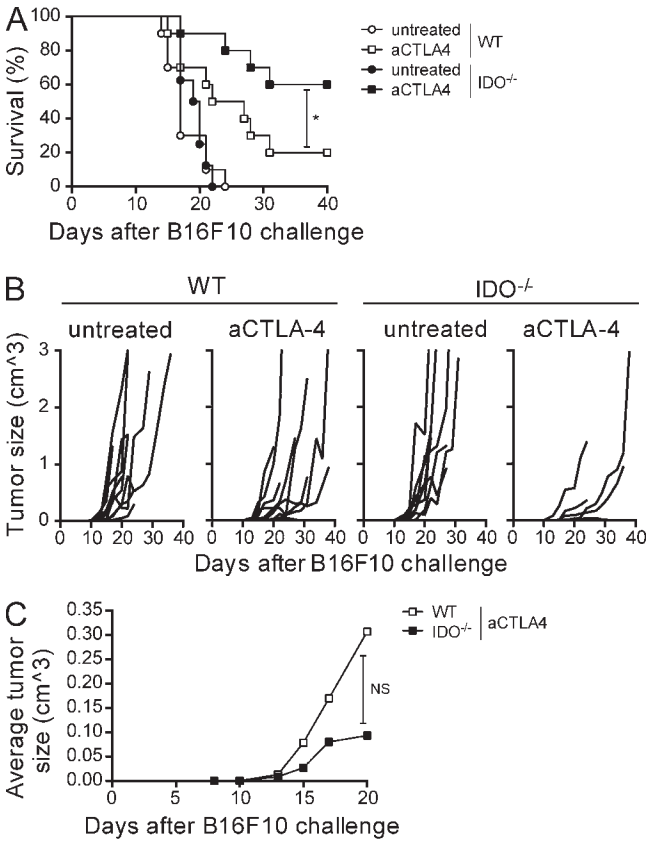

Figure 1. Delayed development of tumors and increased tumor-free survival with anti–CTLA-4 in IDO-deficient hosts. WT and $IDO^{-/-}$ mice were challenged with B16F10 tumor cells i.d. and treated with anti–CTLA-4. Tumor growth and rejection were followed over time in the different groups. (A) Cumulative survival. Statistical significance was determined by Log-Rank test (*, $P < 0.05$). (B) Individual tumor growth. The numbers of mice/group rejecting tumors were: WT/untreated (0/10 mice), WT/anti-CTLA-4 (2/10), $IDO^{-/-}$/untreated (0/8), and $IDO^{-/-}$/anti-CTLA-4 (6/10). (C) Mean tumor size. Two-way ANOVA was used to evaluate statistical significance (p-value toward significance, $P = 0.06$). Data represent cumulative results from two independent experiments with four to five mice/group.

Published June 10, 2013

RESULTS

Antitumor effect of anti–CTLA-4 is significantly increased in IDO-deficient hosts

To address the inhibitory role of the host-derived IDO in the context of anti–CTLA-4 therapy, we first tested the antitumor efficacy of CTLA-4 blockade in tumor-bearing IDO knockout ($IDO^{-/-}$) mice. For this purpose, we used the immunosuppressive B16 melanoma cell line. IDO expression is not detectable in B16 cells, but B16 is known to be a potent inducer of IDO in other cells (Sharma et al., 2007). $IDO^{-/-}$ and corresponding age-matched WT C57BL/6 mice were challenged with B16F10 cells and treated with anti–CTLA-4 as described in the Materials and methods. In the untreated groups, $IDO^{-/-}$ mice did not demonstrate any survival advantage or delay in tumor progression over WT mice (Fig. 1, A and B). Thus, $IDO^{-/-}$ mice were not inherently protected from B16 tumors. However, $IDO^{-/-}$ mice that received anti–CTLA-4 treatment displayed significantly increased survival relative to similarly anti–CTLA-4–treated WT mice (Fig. 1 A), suggesting that IDO expressed by host cells suppresses the antitumor effect of anti–CTLA-4 therapy in the B16 model. In addition, there was a trend towards a decrease in tumor growth, but this was not significant (Fig. 1, B and C).

IDO deficiency enhances the accumulation of tumor-infiltrating $CD4^+$ and $CD8^+$ effector T cells after anti–CTLA-4 therapy

To compare anti–CTLA-4–induced antitumor immune responses in $IDO^{-/-}$ and WT mice, we isolated the tumors and assayed them for T cell infiltration by flow cytometry. Tumors were harvested 15 d after implantation. Although anti–CTLA-4 antibody therapy resulted in tumor growth inhibition in both WT and $IDO^{-/-}$ mice, the tumor weights were significantly lower in the $IDO^{-/-}$ mice (Fig. 2 A), which also corresponded to an increase in absolute number of $CD45^+$ cells (Fig. 2 B). In addition, the absolute number of $CD4^+$ T cells was increased by approximately threefold in $IDO^{-/-}$ mice compared with WT mice, and, even more strikingly, the absolute number of $CD8^+$ T cells/gram tumor was increased by almost fivefold (Fig. 2 B); however, these differences did not reach statistical significance. The tumor-infiltrating lymphocyte (TIL) fraction composed of $CD4^+Foxp3^+$ T reg cells, in contrast, was significantly decreased in the $IDO^{-/-}$ mice (Fig. 2 C). As a result, intratumoral ratios of T effector cells to T reg cells were markedly enhanced in $IDO^{-/-}$ mice compared with WT mice after anti–CTLA-4 therapy (Fig. 2 D). Anti–CTLA-4 treatment also resulted in high $CD8^+$ and $CD4^+$ effector T cell proliferation and activation within the tumors of $IDO^{-/-}$ mice, as measured by expression of the cell cycle associated protein Ki67, as well as the activation markers, PD-1 and ICOS (Fig. 2 E), as previously reported in WT mice (Curran et al., 2011). In contrast to effector T cells, there was no significant increase in proliferation or up-regulation of PD-1 or ICOS by the tumor-infiltrating T reg cells with anti–CTLA-4 therapy (Fig. 2 E). Furthermore, we observed up-regulation of CD44 and down-regulation of the lymph node–homing marker CD62L in response to anti–CTLA-4 (not depicted). Collectively, these observations suggest that IDO expressed by host cells suppresses infiltration and accumulation of tumor-reactive T cells in the context of anti–CTLA-4 therapy and increases intratumoral frequencies of T reg cells.

IDO deficiency synergizes with immunotherapeutic approaches targeting PD-1–PD-L1 and GITR

To evaluate the inhibitory role of IDO on other T cell immunotherapies, we tested the antitumor activity of blocking anti–PD-1/PD-L1 and agonistic anti-GITR antibodies in IDO-deficient hosts. Anti–PD-1/PD-L1 treatment delayed tumor progression in B16F10-bearing WT mice, but resulted in only modest improvement in survival (Fig. 3 A). However, anti–PD-1/PD-L1 treatment in $IDO^{-/-}$ mice resulted in significantly reduced tumor growth and improved survival (Fig. 3 A). Survival was also increased in $IDO^{-/-}$ hosts compared with WT hosts after treatment with agonistic anti-GITR antibody (Fig. 3 B). These findings demonstrate that the inhibitory effects of IDO are not restricted to anti–CTLA-4 therapy, but are also relevant within the context of other immunotherapies targeting immune checkpoints.

Pharmacological inhibition of IDO cooperates with CTLA-4 blockade to promote rejection of B16 melanoma cells

To explore these findings in a clinically relevant model, we examined the antitumor effects of combining anti–CTLA-4 treatment with pharmacological inhibition of IDO with 1MT, which is currently in clinical trials. C57BL/6 mice were challenged with B16F10 tumor cells and treated with anti–CTLA-4 antibody and/or 1MT, as described in the Materials and methods (Fig. 4 A). Mice receiving 1MT treatment alone did not demonstrate any survival advantage over untreated control mice (Fig. 4 B). Conversely, 20% of mice that received anti–CTLA-4 monotherapy survived to 60 d (Fig. 4 B). In comparison, combination therapy resulted in a marked delay in tumor growth (Fig. 4, C and D) and, most significantly, resulted in almost 55% long-term survival, with both L- and D-1MT isoforms (Figs. 4 B). Furthermore, surviving mice from the combination therapy-treated groups were immune to a rechallenge with two times the initial dose of live tumor cells, suggesting the presence of a potent B16-specific memory response. Thus, the combination of 1MT and anti–CTLA-4 antibody appears to be efficacious in inhibiting tumor growth and prolonging survival in the B16F10 melanoma model. Importantly, combined treatments with anti–CTLA-4 and 1MT at the dose and schedule tested were well tolerated by mice, with no weight loss or other clinical signs of acute or delayed toxicity observed. Histopathology was conducted on major organs such as colon, liver, kidney, lungs, and intestines, and no evidence of toxicity or autoimmunity was observed (unpublished data). To determine if the antitumor effects of combination treatment could be extended to less immunogenic tumors, mice were challenged with poorly immunogenic B16BL6 melanoma cells and treated with anti–CTLA-4 and/or 1MT. No curative effect was observed with anti–CTLA-4/1MT alone;

Published June 10, 2013

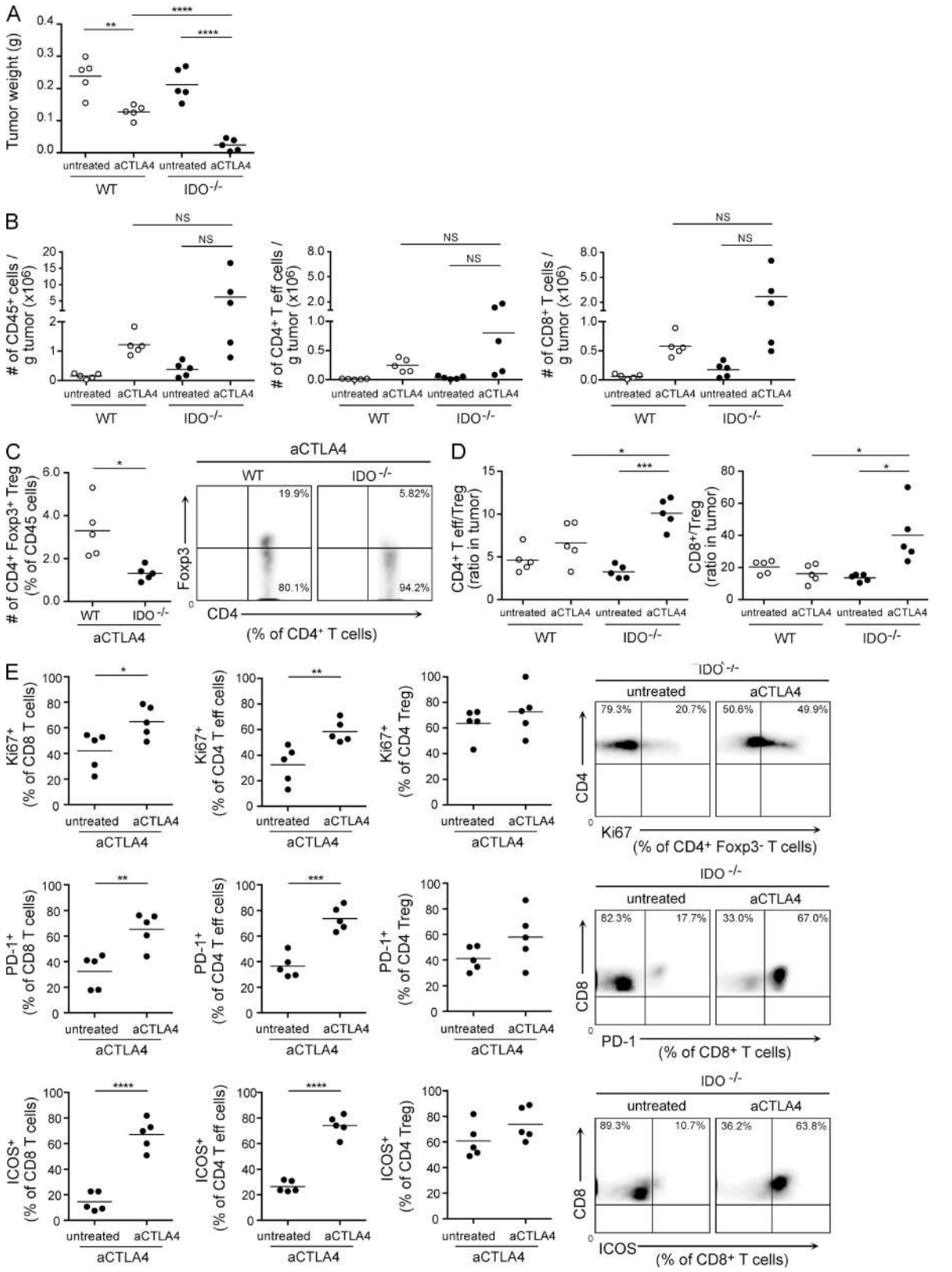

Figure 2. IDO deficiency is associated with enhanced infiltration and accumulation of T cells in tumors after anti–CTLA-4 therapy. B16F10 tumors from untreated and anti–CTLA-4–treated WT and IDO$^{-/-}$ mice were harvested 15 d after tumor challenge and analyzed by flow cytometry for their content of effector T cells and T reg cells. (A) Tumor weights. (B) Absolute numbers of CD45$^+$, CD4$^+$Foxp3$^-$, and CD8$^+$ cells per gram of tumor. (C) Percentage of CD4$^+$Foxp3$^+$ T cells of total CD45$^+$ cells, and representative dot plots, for anti–CTLA-4–treated WT and IDO$^{-/-}$ mice. (D) Ratio of

Published June 10, 2013

however, when administered with a cellular vaccine (GM-CSF–producing irradiated B16BL6 whole-cell vaccine [Gvax]), anti–CTLA-4/1MT combination therapy promoted rejection in ∼45% of B16BL6-challenged mice versus <10% and 0% for anti–CTLA-4/Gvax or 1MT/Gvax alone (Fig. 4, E and F). The antitumor effects of 1MT were independent of delivery route, as we observed comparable antitumor effects of 1MT when it was administered orally (Fig. 4 E) or as a subcutaneous time-release pellet (Fig. 4 F).

Anti–CTLA-4/1MT antitumor therapy markedly increases T eff/T reg cell ratios at the tumor site

To address the cellular mechanisms mediating tumor rejection in the anti–CTLA-4/1MT system, we studied the effector T cell and T reg cell compartments within the B16F10 tumors by flow cytometry. Tumors were isolated and assayed 15 d after implantation. As shown in Fig. 5 A, the average tumor weight on day 15 was notably reduced in anti–CTLA-4/1MT–treated mice compared with anti–CTLA-4–treated and untreated mice (Fig. 5 A). The absolute number of tumor infiltrating $CD45^+$ cells/g tumor was significantly increased in mice treated with anti–CTLA-4/1MT as compared with anti-CTLA4–treated or untreated mice (not depicted). More importantly, there was a significant increase in the percentage of $CD8^+$ T cells recruited to the tumor site, indicating that B16F10-reactive $CD8^+$ T cells were trafficking to the tumor (Fig. 5 B). Furthermore, we observed a significant decrease in the percentage of tumor infiltrating $CD4^+Foxp3^+$ T reg cells in anti–CTLA-4/1MT–treated mice (Fig. 5 B), whereas the percentage of $CD4^+Foxp3^-$ T cells (Fig. 5 B), $CD19^+$ B cells, $CD3^+NK1.1^+$ NKT cells, $CD3^-NK1.1^+$ NK cells, $CD11b^+$ cells, and $CD11c^+$ DCs remained unchanged (Fig. 5 C). Finally, we observed a slight decrease in the percentage of $CD11b^+Gr\text{-}1^+$ myeloid-derived suppressor cells (MDSCs) in the anti–CTLA-4/1MT treated mice (Fig. 5 D). The increase in $CD8^+$ T cell infiltration was also reflected in the dramatic increase in $CD8^+$ T cell to T reg cell ratio in the tumor (Fig. 5 E), which correlated with tumor rejection (Fig. 4, B–D). Ratios of $CD4^+Foxp3^-$ T cells to T reg cells in tumors were also significantly elevated with anti–CTLA-4/1MT therapy compared with anti–CTLA-4 alone (Fig. 5 E). Collectively, these observations suggest that inhibition or knockout of IDO in the context of anti–CTLA-4 therapy increases infiltration of effector T cells while attenuating accumulation of T reg cells.

$CD8^+$ T cells infiltrating tumors after anti–CTLA-4/1MT therapy express activation markers and respond to tumor antigens

The increase in the relative percentage of $CD8^+$ T cells corresponded to their absolute increase per gram of tumor (Fig. 5 F).

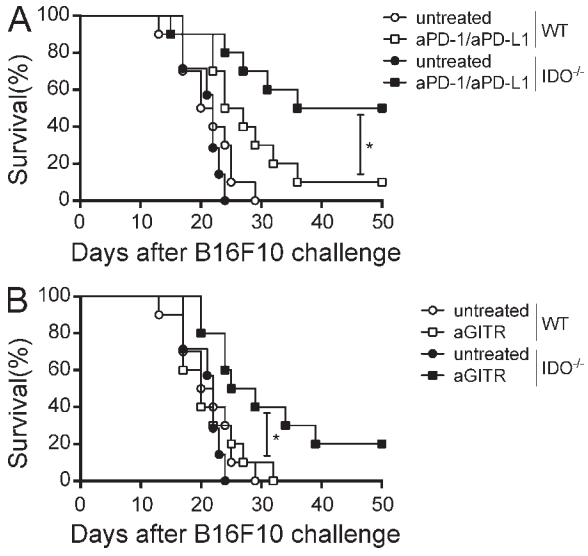

Figure 3. Antitumor effects of anti-PD-1/PD-L1 and anti-GITR are enhanced in IDO$^{-/-}$ hosts. Survival curves for WT and IDO$^{-/-}$ mice challenged with B16F10 tumor cells i.d. and treated with anti-PD-1/anti-PD-L1 (A) or anti-GITR (B). Data shown are cumulative results from two independent experiments with five mice/group and analyzed by Log-Rank Test (*, $P < 0.05$).

To determine if infiltrating T cells were functional, we analyzed their intracellular expression of Granzyme B (GrB) and IFN-γ. The cytolytic capacity of the cells as determined by GrB expression was increased (Fig. 5 G), and the cells were capable of producing higher amounts of IFN-γ in response to in vitro stimulation with PMA/ionomycin (Fig. 5 H). These data indicate that anti–CTLA-4/1MT combination therapy augments the number effector $CD8^+$ T cells at the tumor site, likely resulting in a qualitatively more effective cytotoxic T cell response. To determine whether the immune response was tumor-specific, TILs from five mice in each group were pooled and stimulated with syngeneic DCs loaded with B16F10 lysate, and specific IFN-γ production was determined by intracellular cytokine staining. As shown in Fig. 5 I, in vitro stimulation with B16F10 tumor antigen-loaded DCs resulted in up-regulation of IFN-γ expression by the tumor-infiltrating $CD8^+$ T cells. These results indicate that anti–CTLA-4/1MT therapy resulted in an increase in $CD8^+$ T cells specific for an antigen (or antigens) expressed by B16F10. Hence, successful rejection of B16F10 coincides with generation of tumor-specific T cell reactivity.

$CD8^+$ T cells and IFN-γ are required for anti–CTLA-4/1MT-induced tumor rejection

To identify specific cell types important for the significant antitumor effect observed with anti–CTLA-4/1MT treatment, mice were treated with depleting antibodies to CD8 and

$CD4^+Foxp3^-$ to $CD4^+Foxp3^+$ cells and $CD8^+$ to $CD4^+Foxp3^+$ cells. (E) Percentage and representative dot plots of $CD8^+$, $CD4^+Foxp3^-$ and $CD4^+Foxp3^+$ T cells expressing Ki67, PD-1 and ICOS, for untreated and treated IDO$^{-/-}$ mice. Two-way ANOVA (A–B and D) and Student's *t* test (C and E) were used to evaluate statistical significance (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; NS, $P < 0.1$). Data shown are representative of two independent experiments with five independently analyzed mice/group.

Published June 10, 2013

CD4 T cells, NK cells, and IFN-γ before injection of tumor cells and during the course of therapy. Adequate depletion of cell subsets was confirmed by flow cytometry of peripheral blood (Fig. 6 B). As expected, mice with no depletion of immune cells responded to treatment with anti-CTLA4/1MT (Fig. 6 A). Depletion of $CD8^+$ T cells or IFN-γ, however, completely abrogated the effect of the treatment. At day 14, the estimated average tumor size for mice depleted of $CD8^+$ T cells (0.478 CM^3) or IFN-γ (0.518 CM^3) was significantly higher than the average tumor size for mice with no depletion of immune cells (0.060 CM^3). The antitumor effect of anti–CTLA-4/1MT was decreased, but not absent in mice depleted of $CD4^+$ T cells or NK cells (Fig. 6 A). These data show that the tumor-eradicating effect of anti–CTLA-4/1MT is dependent on $CD8^+$ T cells and IFN-γ, and partially $CD4^+$ T cell- and NK cell-dependent.

B16F10 tumors overexpressing IDO are resistant to anti–CTLA-4 treatment, but not anti–CTLA-4/1MT

Whereas the aforementioned experiments demonstrate that IDO expressed by the host cells is important in inhibition of the antitumor immune responses, the contribution of the IDO expressed by the tumor cells is less clear. To this end, we examined the effectiveness of CTLA-4 blockade against B16F10 tumor cells transduced with *IDO–GFP* fusion construct. IDO expression by the transduced tumor cells was verified by flow cytometry (Fig. 7 A) and Western blot (not depicted). The in vitro growth rate of IDO/GFP-B16F10 and GFP-B16F10 cells was identical (Fig. 7 B), and both cell lines produced progressive tumors in naive mice (Fig. 7 C). As expected, ~50% of the mice injected with GFP-B16F10 completely rejected the tumor challenge when treated with anti–CTLA-4/1MT (Fig. 7 D). Anti–CTLA-4 therapy alone prolonged survival of mice with GFP-B16F10 tumors mice to some extent, but had no effect on the outgrowth of IDO-expressing B16F10 tumors (Fig. 7 D). The majority of the mice with IDO-expressing B16F10 tumors treated with anti–CTLA-4/1MT also developed progressive tumors and died; however, survival was significantly increased compared with anti–CTLA-4–treated and untreated mice (Fig. 7 D). Importantly, the progression of IDO-expressing tumors and their resistance to anti–CTLA-4 was not related to higher intrinsic tumorigenicity, because irradiated naive mice

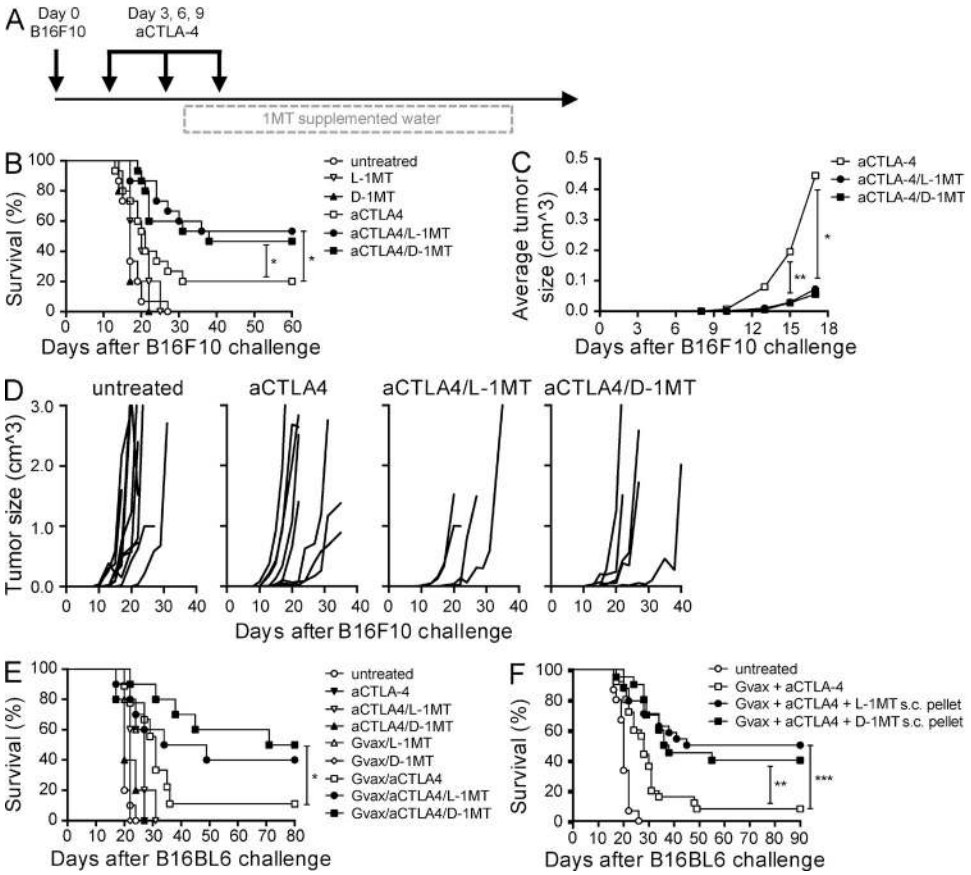

Figure 4. Anti–CTLA-4 and 1MT synergize to mediate tumor rejection. Treatment schedule (A), Kaplan-Meier survival curves (B), mean tumor size (C) and individual tumor growths (D) for C57BL/6 mice challenged with B16F10 melanoma cells i.d. and treated with anti–CTLA-4 and/or 1MT. The numbers of mice/group rejecting tumors was: untreated (0/10 mice), anti–CTLA-4 (2/10), anti–CTLA-4/L-1MT (6/10), and anti–CTLA-4/D-1MT (5/10). Kaplan-Meier survival curves for C57BL/6 mice challenged with B16BL6 and treated with anti–CTLA-4, Gvax, and/or 1MT. 1MT was administered in the drinking water (E) or as time-release subcutaneous pellets (F). Data shown are pooled from two (C and D) or three (B, E, and F) independent experiments with five mice/group. Statistical significance was evaluated by Log-Rank Test(B, E, and F; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) and two-way ANOVA (C; *, $P < 0.05$; **, $P < 0.01$).

Published June 10, 2013

injected with IDO/GFP-B16F10 or GFP-B16F10 all developed tumors quickly and with identical kinetics (Fig. 7 C). These data indicate that IDO expression by the tumor cells increases the tumor aggressiveness and decreases the tumor response to CTLA-4 blocking therapy, though this inhibition could be partially overcome with combination CTLA-4/1MT therapy.

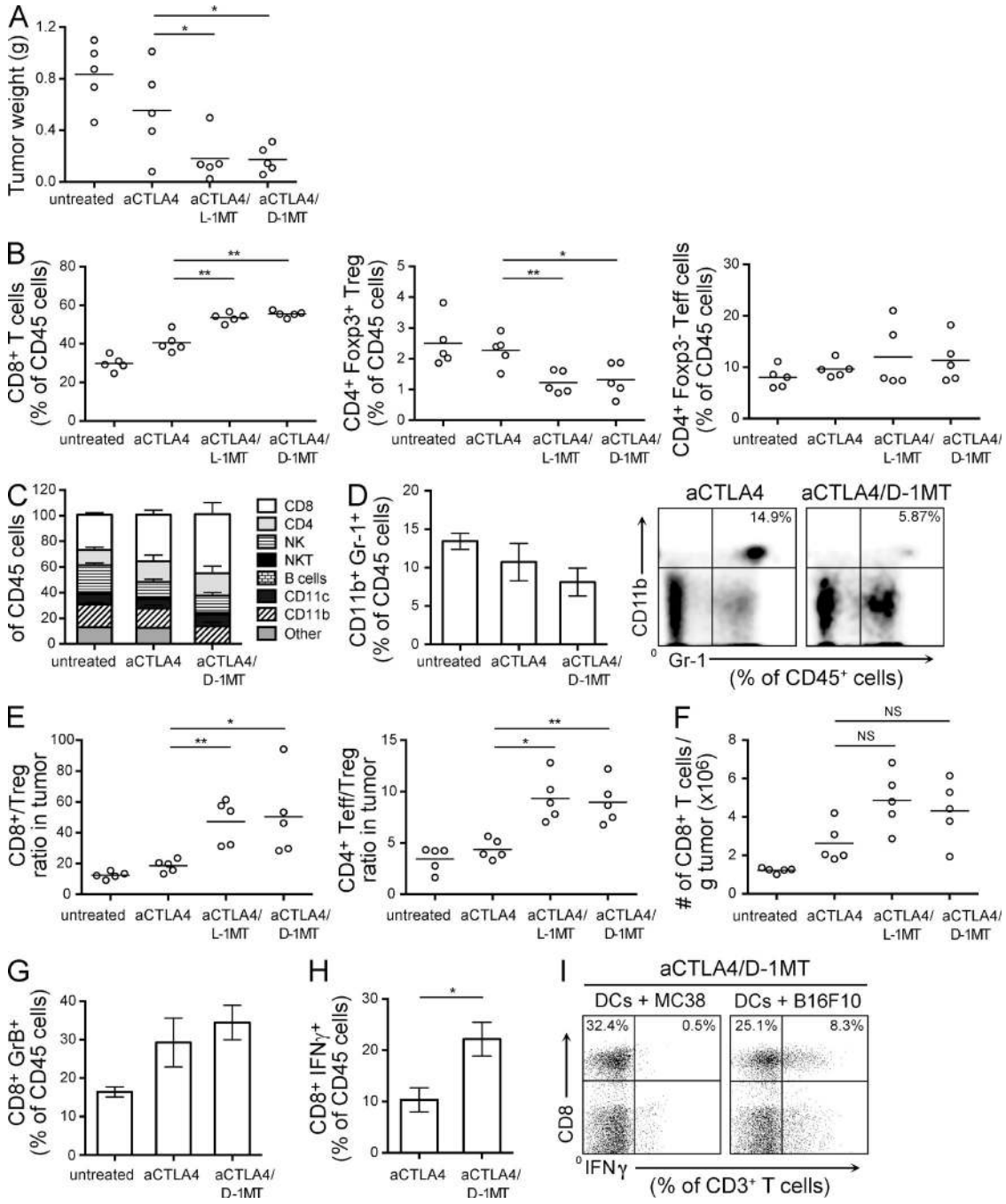

Figure 5. Anti–CTLA-4/1MT treatment increases the ratio of effector T cells to T reg cells in tumor and elicits a tumor-specific T cell response. B16F10 tumors from untreated, anti–CTLA-4–treated, and anti–CTLA-4/1MT–treated C57BL/6 mice were harvested 15 d after tumor challenge and analyzed by flow cytometry for their content of effector T cells and T reg cells. (A) Tumor weights. (B) Percentage of $CD8^+$, $CD4^+Foxp3^-$, and $CD4^+Foxp3^+$ T cells of total $CD45^+$ cells. (C) Immune infiltrate analysis expressed as a percentage of total $CD45^+$ cells. (D) Percentage of $CD11b^+Gr\text{-}1^+$ MDSCs of total $CD45^+$ cells and representative dot plots. (E) Ratio of $CD4^+Foxp3^-$ to $CD4^+Foxp3^+$ cells and $CD8^+$ to $CD4^+Foxp3^+$ cells. (F) Absolute numbers of $CD8^+$ T cells per gram of tumor. (G) Frequency of $CD8^+GrB^+$ T cells of total $CD45^+$ cells. TILs were restimulated for 4 h with PMA/Ionomycin (H) or overnight with DCs loaded with B16F10 tumor lysate or DCs with MC38 lysate as a nonmelanoma control tumor (I), and production of IFN-γ was determined by flow cytometry. Data were analyzed by two-way ANOVA (A, B, E, and F; *, $P < 0.05$; **, $P < 0.01$; NS, $P = 0.06$, $P = 0.1$) and Student's *t* test (H; *, $P < 0.05$). Data represents one of three experiments with five independently analyzed mice/group.

Published June 10, 2013

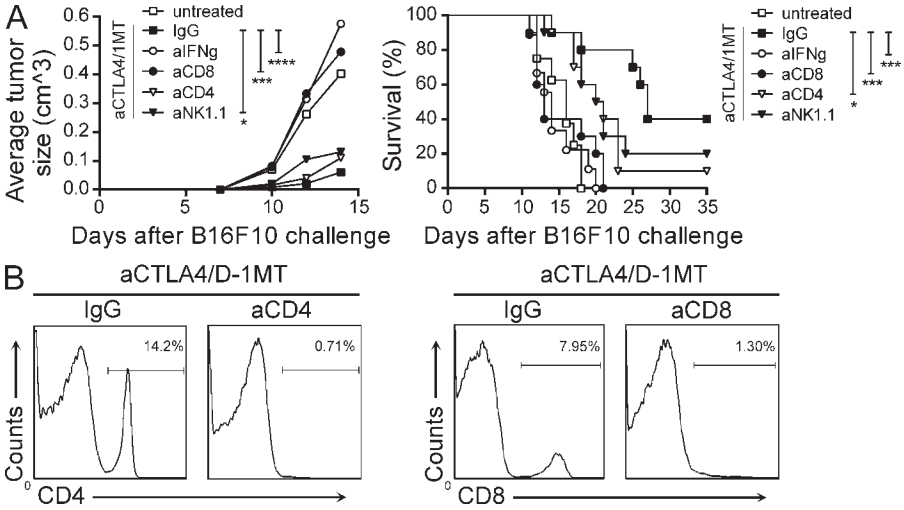

Figure 6. Anti–CTLA-4/1MT antitumor effect is T cell dependent. (A) Mean tumor growth and tumor-free survival curves for C57BL/6 mice challenged with B16F10 tumors i.d. and treated with anti–CTLA-4/1MT plus depleting antibodies for IFN-γ, CD8, CD4, or NK/NKT, or a corresponding dose of IgG isotype control (IgG). P-values are for two-way ANOVA and Log-Rank test (*, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$), respectively. (B) Percentage of $CD4^+$ and $CD8^+$ T cells in peripheral blood after depletion assessed by flow cytometry. The horizontal bars indicate positive staining. Data represent cumulative results from two independent experiments with five mice/group.

Anti-CTLA4/1MT therapy induces regression of established IDO-expressing 4T1 breast tumors

We next sought to determine whether CTLA-4 blockade combined with 1MT would be effective in nonmelanoma tumor models naturally expressing high levels of IDO. Analysis of several murine cancer cell lines revealed high levels of IDO expression in the 4T1 breast cancer cell line when cocultured with recombinant IFN-γ (unpublished data). BALB/c mice were challenged with 4T1 tumor cells and treated as described in the Materials and methods. Anti–CTLA-4 and D-1MT as single agents induced only a transient delay in tumor growth of preimplanted 4T1 breast tumors and had no significant effect on survival (Fig. 8, A and B). However, anti–CTLA-4/D-1MT combination therapy resulted in sustained growth delay and prolonged survival (Fig. 8, A and B). Similar synergistic effects were observed with anti–CTLA-4 plus the L isomer of 1MT (unpublished data). Overall, these data thus indicate that combination of IDO inhibition with

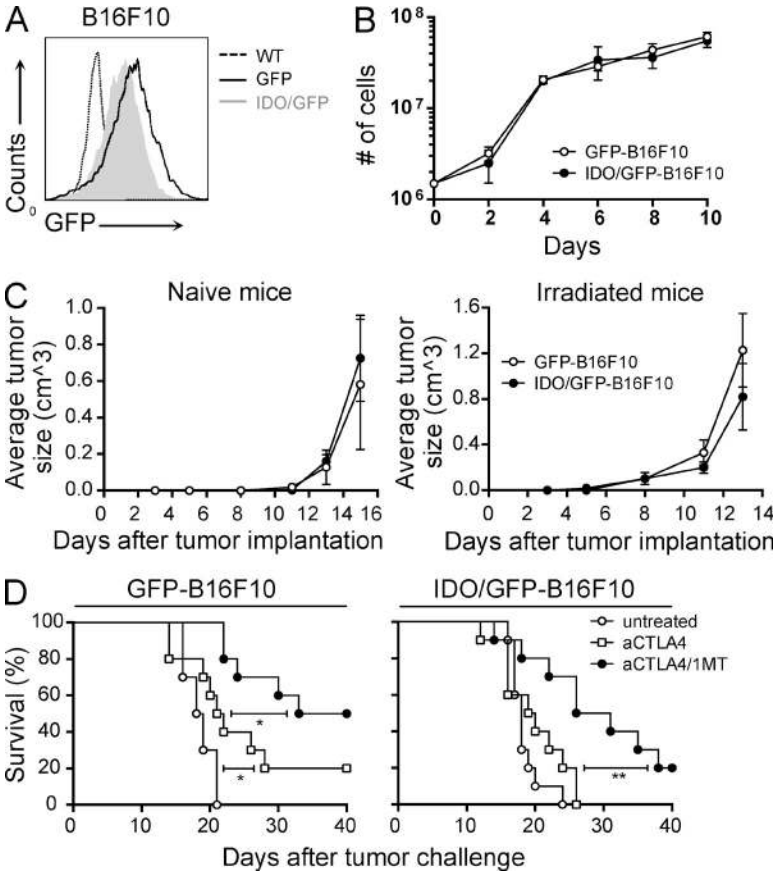

Figure 7. IDO impairs antitumor effects of anti-CTLA4 in the context of B16 tumors engineered to overexpress IDO. (A) Expression of GFP (black line) or IDO/GFP (gray) in B16F10 cells determined by flow cytometry. WT B16F10 cells were used for comparison (dotted line). (B) In vitro growth rate of GFP-B16F10 and IDO/GFP-B16F10 cells. (C) Mean growth of GFP-B16F10 and IDO/GFP-B16F10 tumors in naive and irradiated (450 cGy) mice. (D) Tumor-free survival curves for C57BL/6 mice challenged with GFP-B16F10 or IDO/GFP-B16F10 tumor cells i.d. and treated with anti–CTLA-4 or anti-CLTA-4/1MT. Data represent cumulative results from two independent experiments with five mice/group. Statistical significance was determined with Log-Rank test (*, $P < 0.05$; **, $P < 0.01$).

Published June 10, 2013

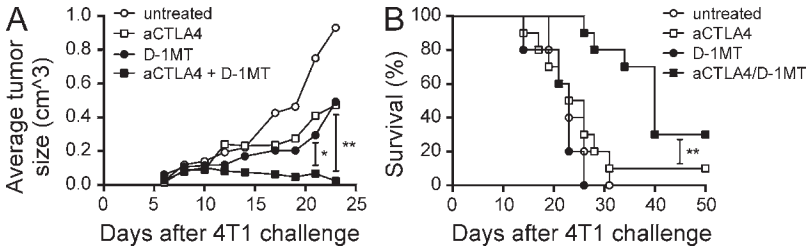

Figure 8. Anti–CTLA-4 and 1MT synergize to mediate rejection of IDO-expressing breast tumor cells. Mean tumor growth (A) and cumulative survival (B) for BALB/c mice challenged with 4T1 mammary cancer cells i.d. and treated with anti-CLTA4 and/or 1MT. Data shown are pooled from two independent experiments with five mice/group and analyzed by two-way ANOVA (A) and Log-Rank test (B; *, $P < 0.05$; **, $P < 0.01$).

CTLA-4 blockade is an effective therapeutic strategy that is active against both IDO-expressing and nonexpressing tumors. Finally, these data show that anti-CTLA4/1MT synergism is not restricted to melanoma and can be applicable to other tumor types.

DISCUSSION

Numerous studies have shown that anti-CTLA4 monotherapy fails to induce rejection of poorly immunogenic tumors, such as B16 melanoma (Hurwitz et al., 1998; van Elsas et al., 1999). Our findings show that IDO strongly contributes to this resistance and could be targeted by systemic inhibition with a clinically relevant drug (1MT) currently in Phase I clinical trials. We show that administration of anti–CTLA-4 antibody combined with the IDO inhibitor 1MT resulted in rejection of established tumors and resistance to secondary challenge in mice inoculated with B16 melanoma. Tumor rejection by the anti–CTLA-4/1MT combination therapy was associated with enhanced infiltration of functional $CD8^+$ and $CD4^+$ T cells in the tumor. We found that tumor rejection was indeed dependent on the presence of the $CD8^+$ T cells and IFN-γ, and partially dependent on $CD4^+$ T cells and NK cells.

The presence of IDO has been correlated with increased tumor-infiltrating T reg cells (Curti et al., 2007; Park et al., 2008; Witkiewicz et al., 2008; Yu et al., 2008; Mansfield et al., 2009), and it has been demonstrated that IDO-expressing APCs can recruit and activate existing T reg cells or create new T reg cells to potently suppress antitumor T cell responses (Fallarino et al., 2006; Sharma et al., 2007; Chen et al., 2008; Sharma et al., 2009). Along these lines, we found that the $CD4^+Foxp3^+$ T reg cell fraction of TILs was significantly reduced as a result of anti–CTLA-4/1MT combination therapy, which directly correlated with tumor rejection. Importantly, the intratumor ratio of effector T cells to T reg cells was also increased, a finding that was previously shown to be a marker of a favorable immunological response to immunotherapy in murine tumor models (Quezada et al., 2006; Curran et al., 2010) and has been associated with improved survival (Bui et al., 2006; Sato et al., 2005) and increased tumor necrosis (Hodi et al., 2008) in clinical studies. Our data thus suggest that IDO suppresses antitumor responses in the context of anti–CTLA-4 therapy by suppressing tumor-infiltrating effector T cells, while favoring accumulation of T reg cells. Whether suppression occurs primarily via local depletion of tryptophan or also involves direct inhibition by toxic tryptophan metabolites remains to be investigated.

IDO expression has been demonstrated in tumor cells and APCs in a range of human and murine cancers (Uyttenhove et al., 2003; Okamoto et al., 2005). IDO-expressing APCs include human monocyte-derived macrophages and DCs (Munn et al., 1999, 2002; Hwu et al., 2000; Orabona et al., 2006), and a small population of murine plasmacytoid DCs ($CD11c^+B220^+CD19^+$; Munn et al., 2004; Johnson et al., 2010). It is, however, unclear whether inhibition of IDO as a therapeutic strategy is applicable only to IDO-expressing tumors, or whether inhibition of IDO expressed by the host cells is equally important. Using IDO-deficient mice, we were able to demonstrate that the host-derived cells and not the tumor cell line were primarily responsible for the suppressive IDO activity in our B16 model. Consistent with this finding, we did not detect IDO expression in tumor cells of established B16F10 tumors by Western blotting or RT-PCR (unpublished data). We found that IDO deficiency in the host resulted in reduced tumor burden and improved survival in the context of anti–CTLA-4 treatment. Similar to the mice treated with IDO inhibitors, this effect was accompanied by enhanced accumulation of functional effector T cells at the tumor site and a reduction in frequencies of T reg cells.

In face of these findings, expression of IDO by tumor cells has been associated with faster tumor outgrowth (Uyttenhove et al., 2003; Yu et al., 2008), and it has been demonstrated that expression of IDO by immunogenic tumor cell lines protects them from immune-mediated rejection, even in the face of an otherwise protective T cell immunity (Uyttenhove et al., 2003). To determine whether the combination therapy would in addition be applicable to IDO-expressing tumors and to nonmelanoma cancers, we examined if treatment with anti–CTLA-4/1MT was sufficient to foster rejection of experimental tumors, which either naturally express high levels of IDO (4T1), or are engineered to overexpress IDO (IDO/GFP-B16F10). In both cases, the anti–CTLA-4/1MT combination therapy was significantly more effective than anti–CTLA-4 monotherapy and resulted in slower tumor growth and improved survival. Overall, these findings indicate that IDO inhibition also enhances the effectiveness of anti–CTLA-4 in the setting of IDO-expressing tumors, indicating that IDO enzymatic activity is important for the immunosuppressive effect independent of the cell types expressing the enzyme. These findings thus establish a strong rationale for the anti–CTLA-4/1MT combination therapy for all tumors, regardless of levels of IDO expression in tumor cells.

Published June 10, 2013

To examine the efficacy of IDO inhibition within the context of other immunotherapeutic approaches, we used the poorly immunogenic B16BL6 melanoma model, which can only respond to anti–CTLA-4 therapy when combined with cellular GM-CSF–expressing vaccine (Gvax; van Elsas et al., 1999). We found that in the context of 1MT, anti–CTLA-4/Gvax led to rejection of high burdens of B16BL6 melanoma, suggesting that anti–CTLA-4 and 1MT also synergize in the context of other treatments.

It has been proposed that ligation of B7 by CTLA-4 induces IDO activity in certain APCs (Grohmann et al., 2002; Baban et al., 2011; Mellor et al., 2004; Sharma et al., 2007). However, data from clinical studies using CTLA-4 blockade to treat melanoma show a positive correlation between IDO expression in pretreatment biopsies and clinical efficacy (Hamid et al., 2011; Ji et al., 2012), and demonstrate no decrease in IDO expression after treatment (Ribas et al., 2009), suggesting that IDO expression may be dependent on other factors. As IDO expression is induced by proinflammatory stimuli such as IFN-γ, it is possible that IDO activity is enhanced in patients with an ongoing, albeit suboptimal, antitumor immune response and that these patients could be more likely to respond favorably to immunotherapies such as CTLA-4 blockade. To this end, we show that IDO expression compromises the activity of anti–CTLA-4 therapy and other types of immunotherapies, such as anti–PD-1/PD-L1 and anti-GITR antibodies. These data suggest that IDO activity, in our model, may not be solely regulated by the CTLA-4 pathway, but instead is likely up-regulated by an active immune microenvironment primed by anti–CTLA-4 or other T cell immunotherapies. This is supported by a preclinical study reporting that imatinib, which potentiates antitumor T cell responses in gastrointestinal stromal tumors through the inhibition of IDO, is also synergistic with CTLA-4 blockade (Balachandran et al., 2011).

Both D-1MT and L-1MT have been described in multiple mouse and human studies for inhibition of IDO activity and they appear to possess distinct biological properties (Johnson and Munn, 2012). The literature indicates that, in vitro, the D-1MT is less effective than L-1MT against purified, cell-free recombinant IDO (Hou et al., 2007; Qian et al., 2012). However, in vivo (i.e., in mice with tumors), the D-1MT showed equivalent or even superior antitumor potency (Gu et al., 2010; Hou et al., 2007). This is perhaps due to the long half-life of the D-isomer, and/or its stronger activating effect on the mTOR pathway (Metz et al., 2012). In our system, the D and L isomers were equivalent, and both demonstrated therapeutic efficacy that approached the effect seen in mice with genetic knockdown of IDO1. Thus, our models strongly implicate an inhibitory role for IDO1. The potential immunosuppressive roles of IDO2 and Tryptophan 2,3-dioxygenase (TDO) in our system is, however, more elusive. Because 1MT is not a strong inhibitor of IDO2 (Yuasa et al., 2010; Qian et al., 2012) and does not inhibit TDO (Suzuki et al., 2001), it is possible that these enzymes exert additional immunosuppression that could compensate for IDO1 inhibition, although the evidence for such compensatory mechanism is lacking in the literature. Further studies with selective inhibitors of IDO2 and TDO are needed to demonstrate whether there is additional benefit to blocking these potential escape pathways as well.

Along similar lines, potential off-target effects are a consideration for L-1MT and D-1MT. Previous studies using RNAi-knockdown techniques have concluded that the major target of the DL-mixture of 1MT was IDO, rather than another target (Belladonna et al., 2006). Moreover, other studies have demonstrated that D-1MT was not effective in $IDO^{-/-}$ mice (Hou et al., 2007). Similarly, to rule out the contribution of off-target effects of IDO inhibition, we treated IDO-deficient mice with anti–CTLA-4 and the two different 1MT isomers and saw no additional beneficial effect from the addition of the drugs (unpublished data). Furthermore, though the possibility of both isomers affecting the same off-target pathway cannot be fully excluded, the likelihood of this is low. Thus, in our system, it appears that the potential off-target effects of D- and L-1MT do not provide significant contribution to the observed antitumor effect.

The anti–CTLA-4/1MT regimen was not associated with noticeable toxicity in our models. There were no observed clinical or histological signs of treatment-related toxicity or autoimmunity in anti–CTLA-4/1MT–treated mice as compared with control mice throughout the observation period. With these observations, there are obvious limitations of mouse models for predicting toxicities for patients, especially when evaluating immunotherapeutic agents. The development of the immune system is dependent on a complex interaction between the genetic and environmental factors, and discrepancies in such factors between mice and humans may contribute to differences in immunity and responses to immunotherapy (Haley, 2003; Mestas and Hughes, 2004). For example, although no apparent toxicity was observed in mice treated with CTLA-4 blockade, a significant percentage of patients treated with ipilimumab developed immune adverse effects such as colitis and hypophysitis (Wolchok and Saenger, 2008). It should be noted, however, that these events were more common in patients with the best tumor response to treatment (Attia et al., 2005; Downey et al., 2007). Interestingly, although D-1MT therapy was in general well tolerated by patients who received no prior immunotherapy, three patients who had received prior ipilimumab developed hypophysitis while receiving D-1MT on clinical trial (Johnson and Munn, 2012). Treatment of patients with combination therapies will thus require careful attention to potential immune toxicities, which cannot be predicted by animal models alone.

In conclusion, the data presented here suggest that combination of CTLA-4 blockade and systemic inhibition of IDO can result in more effective therapeutic antitumor immunity than when these interventions are applied separately. Moreover, we suggest that inhibition of IDO may augment the effectiveness of T cell immunotherapies in general. Our

data do not exclude that a functional overlap between these pathways exists. Nonetheless, simultaneous intervention with both regulatory mechanisms appears to be a highly promising strategy for the induction of tumor specific T cell immunity in immunotherapy of cancer. We hypothesize that an IDO inhibitor drug may trigger or exaggerate a potent T cell response that has been primed or initiated by anti–CTLA-4. Clinical trials to validate the efficacy of anti–CTLA-4 and IDO blockade therapy in humans are currently under way for the treatment of melanoma.

MATERIALS AND METHODS

Mice. BALB/c mice (6–8 wk old), congenic IDO$^{-/-}$ mice (6–8 wk old) on C57BL/6 strain background, and corresponding WT C57BL/6 mice were purchased from The Jackson Laboratory. Mice were maintained according to National Institutes of Health Animal Care Guidelines, under protocol 04–07-019 approved by the Memorial Sloan-Kettering Cancer Center Institutional Animal Care Committee.

Cell lines. The melanoma cell lines B16F10 and B16BL6 were used for tumor challenge. GM-CSF–producing B16BL6 cells (Gvax) were used for therapy (van Elsas et al., 1999), and Flt3L-secreting B16BL6 cells were used to increase the number of APCs recruited to the spleen before purification of DCs (Curran and Allison, 2009). The parental 4T1 mouse mammary carcinoma cells were used for breast cancer experiments (Pulaski and Ostrand-Rosenberg, 2001).

Lentiviral vectors and virus production. GFP-tagged murine *IDO* cDNA (OriGene Technologies) was cloned into the pMDG lentiviral vector. Recombinant virus production and infection of target cells were done as previously described (Diatta et al., 2005).

Antibodies. Therapeutic anti–CTLA-4 (clone 9H10), anti-GITR (clone DTA-1), anti–PD-1 (clone RMP1-14), anti–PD-L1 (clone 9G2), anti-CD8 (clone 2.43), anti-CD4 (clone GK1.5), anti-IFN-γ (clone XMG1.2), and anti-NK1.1 (clone PK136) monoclonal antibodies were produced by BioXCell. Antibodies used for flow cytometry were purchased from eBioscience and Invitrogen.

Tumor challenge and treatment experiments. B16F10 tumors were implanted by injection of 3×10^4 B16F10 cells in the flank i.d. at day 0. Mice were treated with anti–CTLA-4 antibody i.p., 1MT (administration of 1MT is described below), or a combination of both. Dosing of anti–CTLA-4 antibody per injection was 200 μg on day 3 plus 100 μg on days 6 and 9. For treatment experiments with GFP- and IDO/GFP-transduced B16F10 cells, 10^5 cells were implanted. For the B16BL6 model, 2×10^4 B16BL6 cells were implanted and, in addition to treatment with anti–CTLA-4 and/or 1MT, mice were vaccinated with 10^6 irradiated (150 Gy) B16BL6 tumor cells expressing GM-CSF (Gvax) on the contralateral flank. For the 4T1 model, 5×10^5 4T1 cells were injected and mice were treated with 200 μg anti-CTLA4 on day 6. For experiments with anti–PD-1/PD-L1 or anti-GITR antibodies, mice were challenged with 5×10^4 B16F10 cells. Dosing of therapeutic antibody per injection was 250 μg anti–PD-1 plus 250 μg anti-PD-L1 on day 3, 6, and 9, or 1 mg anti-GITR on day 7. Control groups received a corresponding dose of antibody isotype (herein referred to as no treatment or untreated). Tumor size and incidence were monitored over time by physical examination. To assess potential toxicities, mice were monitored daily for clinical signs such as respiratory distress, mobility, weight loss, diarrhea, hunched posture, and failure to eat, while histopathology was conducted on major organs (i.e., liver, kidney, intestines, lungs, and colon). For depletion of immune cells, mice were injected i.p. with 500 μg of monoclonal antibodies to CD8, CD4, NK1.1, or IFN-γ, 1 d before and two days after tumor challenge, followed by injection of 250 μg every 5 d throughout the experiment. The efficacy of cell depletion was verified by staining peripheral blood leukocytes for specific subsets after depletion.

Administration of 1MT. Administration of 1MT by implantable subcutaneous pellets was performed according to manufactures instructions and as described (Muller et al., 2005a; Munn et al., 1998). L-1MT (280 mg, 140 mg/pellet) and D-1MT (70 mg, 70 mg/pellet) were formulated in 14-d release pellets (Innovative Research of America). Control groups received placebo pellets without the active product (Innovative Research of America). Pellets were implanted under the dorsal skin on day 7 after tumor challenge. For administration of 1MT in drinking water, 1MT (Sigma-Aldrich) was prepared at 4 mg/ml (pH 9–10) in water as previously described (Uyttenhove et al., 2003; Hou et al., 2007). Before being delivered to the mice, the water was filter sterilized and supplemented with a small amount of aspartame to improve acceptance by the mice. Mice drank 4–5 ml/day, similar to consumption of water without drug. 1MT was administered in the drinking water for 3 wk, starting at day 7 after tumor challenge (freshly made 1MT water was given on days 7, 10, 13, 16, 19, 22, and 25).

Isolation and analysis of TILs. Mice were injected in the flank i.d. with 1.5×10^5 B16F10 melanoma cells and treated as described above. 15 d after tumor challenge, mice were sacrificed, and excised tumors were digested in Liberase (Roche) and DNase (Roche). TILs were obtained from tumors after a Ficoll gradient was performed to eliminate dead cells (Sigma-Aldrich). Cells were stained using a fixation and permeabilization kit (eBioscience) and analyzed by flow cytometry (LSRII; BD) for the expression of CD45, CD3, CD4, CD8, Foxp3, CD44, CD62L, Ki67, PD-1, ICOS, GrB, CD11c, CD19, NK1.1, CD11b, and Gr-1.

T cell assays. For cytokine production, TILs were incubated for 4 h with 50 ng/ml PMA (Invitrogen) and 500 ng/ml ionomycin (Invitrogen), or overnight with DCs loaded with tumor lysate and 20 U/ml IL-2 (PeproTech) plus Brefeldin A (BD). After in vitro stimulation, TILs were stained for intracellular cytokines with a fixation and permeabilization kit (eBioscience) using antibodies to IFN-γ.

DC purification and loading. Mice were injected subcutaneously with 2×10^6 Flt3L-secreting B16BL6 cells and sacrificed on day 14. Spleens were digested with collagenase DNase mix (Roche) before positive selection of CD11c$^+$ DCs (Miltenyi Biotec). DCs were cultured overnight with recombinant GM-CSF and tumor lysate. After loading, DCs were purified by Ficoll gradient.

Statistics. Where indicated, data were analyzed for statistical significance and reported as p-values. Data were analyzed by two-tailed Student's *t* test when comparing means of two independent groups and two-way ANOVA when comparing more than two groups. $P < 0.05$ was considered statistically significant (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$). Evaluation of survival patterns in tumor-bearing mice was performed by the Kaplan-Meier method, and results were ranked according to the Mantel-Cox Log-Rank test. $P < 0.05$ was considered statistically significant (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$). Survival was defined as mice with tumors < 0.300 cm^3.

We would like to thank Sebastian Monette (Laboratory for Comparative Pathology, Memorial Sloan-Kettering Cancer Center), Yuri Igarashi, and Herbert Francisco for technical assistance. Additionally, we thank Emily Corse for critical evaluation of the manuscript.

This work was supported by the Howard Hughes Medical Institute (James P. Allison). Rikke B. Holmgaard is the recipient of postdoctoral fellowships through The Danish Cancer Society, Denmark, and Carlsberg Foundation, Denmark.

James P. Allison is a paid consultant for Bristol-Myers Squib and is the primary inventor on the Patent "Blockade of T lymphocyte down-regulation associated with CTLA-4 signaling." David H. Munn has intellectual property interests in the therapeutic use of IDO and IDO inhibitors, and receives income and research support from NewLink Genetics, Inc. The authors have no additional financial interests.

Submitted: 9 January 2013
Accepted: 13 May 2013

REFERENCES

Attia, P., G.Q. Phan, A.V. Maker, M.R. Robinson, M.M. Quezado, J.C. Yang, R.M. Sherry, S.L. Topalian, U.S. Kammula, R.E. Royal, et al. 2005. Autoimmunity correlates with tumor regression in patients with metastatic melanoma treated with anti-cytotoxic T-lymphocyte antigen-4. *J. Clin. Oncol.* 23:6043–6053. http://dx.doi.org/10.1200/JCO.2005.06.205

Baban, B., P.R. Chandler, B.A. Johnson III, L. Huang, M. Li, M.L. Sharpe, L.M. Francisco, A.H. Sharpe, B.R. Blazar, D.H. Munn, and A.L. Mellor. 2011. Physiologic control of IDO competence in splenic dendritic cells. *J. Immunol.* 187:2329–2335. http://dx.doi.org/10.4049/jimmunol.1100276

Balachandran, V.P., M.J. Cavnar, S. Zeng, Z.M. Bamboat, L.M. Ocuin, H. Obaid, E.C. Sorenson, R. Popow, C. Ariyan, F. Rossi, et al. 2011. Imatinib potentiates antitumor T cell responses in gastrointestinal stromal tumor through the inhibition of Ido. *Nat. Med.* 17:1094–1100. http://dx.doi.org/10.1038/nm.2438

Belladonna, M.L., U. Grohmann, P. Guidetti, C. Volpi, R. Bianchi, M.C. Fioretti, R. Schwarcz, F. Fallarino, and P. Puccetti. 2006. Kynurenine pathway enzymes in dendritic cells initiate tolerogenesis in the absence of functional IDO. *J. Immunol.* 177:130–137.

Belladonna, M.L., C. Orabona, U. Grohmann, and P. Puccetti. 2009. TGF-beta and kynurenines as the key to infectious tolerance. *Trends Mol. Med.* 15:41–49. http://dx.doi.org/10.1016/j.molmed.2008.11.006

Brody, J.R., C.L. Costantino, A.C. Berger, T. Sato, M.P. Lisanti, C.J. Yeo, R.V. Emmons, and A.K. Witkiewicz. 2009. Expression of indoleamine 2,3-dioxygenase in metastatic malignant melanoma recruits regulatory T cells to avoid immune detection and affects survival. *Cell Cycle.* 8:1930–1934. http://dx.doi.org/10.4161/cc.8.12.8745

Bui, J.D., R. Uppaluri, C.S. Hsieh, and R.D. Schreiber. 2006. Comparative analysis of regulatory and effector T cells in progressively growing versus rejecting tumors of similar origins. *Cancer Res.* 66:7301–7309. http://dx.doi.org/10.1158/0008-5472.CAN-06-0556

Chambers, C.A., M.S. Kuhns, J.G. Egen, and J.P. Allison. 2001. CTLA-4-mediated inhibition in regulation of T cell responses: mechanisms and manipulation in tumor immunotherapy. *Annu. Rev. Immunol.* 19:565–594. http://dx.doi.org/10.1146/annurev.immunol.19.1.565

Chen, W., X. Liang, A.J. Peterson, D.H. Munn, and B.R. Blazar. 2008. The indoleamine 2,3-dioxygenase pathway is essential for human plasmacytoid dendritic cell-induced adaptive T regulatory cell generation. *J. Immunol.* 181:5396–5404.

Curran, M.A., and J.P. Allison. 2009. Tumor vaccines expressing flt3 ligand synergize with ctla-4 blockade to reject preimplanted tumors. *Cancer Res.* 69:7747–7755. http://dx.doi.org/10.1158/0008-5472.CAN-08-3289

Curran, M.A., W. Montalvo, H. Yagita, and J.P. Allison. 2010. PD-1 and CTLA-4 combination blockade expands infiltrating T cells and reduces regulatory T and myeloid cells within B16 melanoma tumors. *Proc. Natl. Acad. Sci. USA.* 107:4275–4280. http://dx.doi.org/10.1073/pnas.0915174107

Curran, M.A., M. Kim, W. Montalvo, A. Al-Shamkhani, and J.P. Allison. 2011. Combination CTLA-4 blockade and 4-1BB activation enhances tumor rejection by increasing T-cell infiltration, proliferation, and cytokine production. *PLoS ONE.* 6:e19499. http://dx.doi.org/10.1371/journal.pone.0019499

Curti, A., S. Pandolfi, B. Valzasina, M. Aluigi, A. Isidori, E. Ferri, V. Salvestrini, G. Bonanno, S. Rutella, I. Durelli, et al. 2007. Modulation of tryptophan catabolism by human leukemic cells results in the conversion of CD25- into CD25+ T regulatory cells. *Blood.* 109:2871–2877.

Diatta, A., E. Piver, C. Collin, P. Vaudin, and J.C. Pagès. 2005. Semliki Forest virus-derived virus-like particles: characterization of their production and transduction pathways. *J. Gen. Virol.* 86:3129–3136. http://dx.doi.org/10.1099/vir.0.81103-0

Downey, S.G., J.A. Klapper, F.O. Smith, J.C. Yang, R.M. Sherry, R.E. Royal, U.S. Kammula, M.S. Hughes, T.E. Allen, C.L. Levy, et al. 2007. Prognostic factors related to clinical response in patients with metastatic melanoma treated by CTL-associated antigen-4 blockade. *Clin. Cancer Res.* 13:6681–6688. http://dx.doi.org/10.1158/1078-0432.CCR-07-0187

Fallarino, F., U. Grohmann, C. Vacca, R. Bianchi, C. Orabona, A. Spreca, M.C. Fioretti, and P. Puccetti. 2002. T cell apoptosis by tryptophan catabolism. *Cell Death Differ.* 9:1069–1077. http://dx.doi.org/10.1038/sj.cdd.4401073

Fallarino, F., U. Grohmann, S. You, B.C. McGrath, D.R. Cavener, C. Vacca, C. Orabona, R. Bianchi, M.L. Belladonna, C. Volpi, et al. 2006. The combined effects of tryptophan starvation and tryptophan catabolites down-regulate T cell receptor zeta-chain and induce a regulatory phenotype in naive T cells. *J. Immunol.* 176:6752–6761.

Friberg, M., R. Jennings, M. Alsarraj, S. Dessureault, A. Cantor, M. Extermann, A.L. Mellor, D.H. Munn, and S.J. Antonia. 2002. Indoleamine 2,3-dioxygenase contributes to tumor cell evasion of T cell-mediated rejection. *Int. J. Cancer.* 101:151–155. http://dx.doi.org/10.1002/ijc.10645

Frumento, G., R. Rotondo, M. Tonetti, G. Damonte, U. Benatti, and G.B. Ferrara. 2002. Tryptophan-derived catabolites are responsible for inhibition of T and natural killer cell proliferation induced by indoleamine 2,3-dioxygenase. *J. Exp. Med.* 196:459–468. http://dx.doi.org/10.1084/jem.20020121

Grohmann, U., C. Orabona, F. Fallarino, C. Vacca, F. Calcinaro, A. Falorni, P. Candeloro, M.L. Belladonna, R. Bianchi, M.C. Fioretti, and P. Puccetti. 2002. CTLA-4-Ig regulates tryptophan catabolism in vivo. *Nat. Immunol.* 3:1097–1101. http://dx.doi.org/10.1038/ni846

Gu, T., R.B. Rowswell-Turner, M.O. Kilinc, and N.K. Egilmez. 2010. Central role of IFNgamma-indoleamine 2,3-dioxygenase axis in regulation of interleukin-12-mediated antitumor immunity. *Cancer Res.* 70:129–138. http://dx.doi.org/10.1158/0008-5472.CAN-09-3170

Haley, P.J. 2003. Species differences in the structure and function of the immune system. *Toxicology.* 188:49–71. http://dx.doi.org/10.1016/S0300-483X(03)00043-X

Hamid, O., H. Schmidt, A. Nissan, L. Ridolfi, S. Aamdal, J. Hansson, M. Guida, D.M. Hyams, H. Gómez, L. Bastholt, et al. 2011. A prospective phase II trial exploring the association between tumor microenvironment biomarkers and clinical activity of ipilimumab in advanced melanoma. *J. Transl. Med.* 9:204. http://dx.doi.org/10.1186/1479-5876-9-204

Harlin, H., T.V. Kuna, A.C. Peterson, Y. Meng, and T.F. Gajewski. 2006. Tumor progression despite massive influx of activated CD8(+) T cells in a patient with malignant melanoma ascites. *Cancer Immunol. Immunother.* 55:1185–1197. http://dx.doi.org/10.1007/s00262-005-0118-2

Hodi, F.S., M.C. Mihm, R.J. Soiffer, F.G. Haluska, M. Butler, M.V. Seiden, T. Davis, R. Henry-Spires, S. MacRae, A. Willman, et al. 2003. Biologic activity of cytotoxic T lymphocyte-associated antigen 4 antibody blockade in previously vaccinated metastatic melanoma and ovarian carcinoma patients. *Proc. Natl. Acad. Sci. USA.* 100:4712–4717. http://dx.doi.org/10.1073/pnas.0830997100

Hodi, F.S., M. Butler, D.A. Oble, M.V. Seiden, F.G. Haluska, A. Kruse, S. Macrae, M. Nelson, C. Canning, I. Lowy, et al. 2008. Immunologic and clinical effects of antibody blockade of cytotoxic T lymphocyte-associated antigen 4 in previously vaccinated cancer patients. *Proc. Natl. Acad. Sci. USA.* 105:3005–3010. http://dx.doi.org/10.1073/pnas.0712237105

Hodi, F.S., S.J. O'Day, D.F. McDermott, R.W. Weber, J.A. Sosman, J.B. Haanen, R. Gonzalez, C. Robert, D. Schadendorf, J.C. Hassel, et al. 2010. Improved survival with ipilimumab in patients with metastatic melanoma. *N. Engl. J. Med.* 363:711–723. http://dx.doi.org/10.1056/NEJMoa1003466

Hou, D.Y., A.J. Muller, M.D. Sharma, J. DuHadaway, T. Banerjee, M. Johnson, A.L. Mellor, G.C. Prendergast, and D.H. Munn. 2007. Inhibition of indoleamine 2,3-dioxygenase in dendritic cells by stereoisomers of 1-methyl-tryptophan correlates with antitumor responses. *Cancer Res.* 67:792–801. http://dx.doi.org/10.1158/0008-5472.CAN-06-2925

Hurwitz, A.A., T.F. Yu, D.R. Leach, and J.P. Allison. 1998. CTLA-4 blockade synergizes with tumor-derived granulocyte-macrophage colony-stimulating factor for treatment of an experimental mammary carcinoma. *Proc. Natl. Acad. Sci. USA.* 95:10067–10071. http://dx.doi.org/10.1073/pnas.95.17.10067

Hwu, P., M.X. Du, R. Lapointe, M. Do, M.W. Taylor, and H.A. Young. 2000. Indoleamine 2,3-dioxygenase production by human dendritic cells results in the inhibition of T cell proliferation. *J. Immunol.* 164:3596–3599.

Ji, R.R., S.D. Chasalow, L. Wang, O. Hamid, H. Schmidt, J. Cogswell, S. Alaparthy, D. Berman, M. Jure-Kunkel, N.O. Siemers, et al. 2012. An immune-active tumor microenvironment favors clinical response to

ipilimumab. *Cancer Immunol. Immunother.* 61:1019–1031. http://dx.doi.org/10.1007/s00262-011-1172-6

Johnson, T.S., and D.H. Munn. 2012. Host indoleamine 2,3-dioxygenase: contribution to systemic acquired tumor tolerance. *Immunol. Invest.* 41: 765–797. http://dx.doi.org/10.3109/08820139.2012.689405

Johnson, B.A. III, D.J. Kahler, B. Baban, P.R. Chandler, B. Kang, M. Shimoda, P.A. Koni, J. Pihkala, B. Vilagos, M. Busslinger, et al. 2010. B-lymphoid cells with attributes of dendritic cells regulate T cells via indoleamine 2,3-dioxygenase. *Proc. Natl. Acad. Sci. USA.* 107:10644–10648. http://dx.doi.org/10.1073/pnas.0914347107

Krummel, M.F., and J.P. Allison. 1996. CTLA-4 engagement inhibits IL-2 accumulation and cell cycle progression upon activation of resting T cells. *J. Exp. Med.* 183:2533–2540. http://dx.doi.org/10.1084/jem.183.6.2533

Leach, D.R., M.F. Krummel, and J.P. Allison. 1996. Enhancement of antitumor immunity by CTLA-4 blockade. *Science.* 271:1734–1736. http://dx.doi.org/10.1126/science.271.5256.1734

Lee, J.H., H. Torisu-Itakara, A.J. Cochran, A. Kadison, Y. Huynh, D.L. Morton, and R. Essner. 2005. Quantitative analysis of melanoma-induced cytokine-mediated immunosuppression in melanoma sentinel nodes. *Clin. Cancer Res.* 11:107–112.

Mansfield, A.S., P.S. Heikkila, A.T. Vaara, K.A. von Smitten, J.M. Vakkila, and M.H. Leidenius. 2009. Simultaneous Foxp3 and IDO expression is associated with sentinel lymph node metastases in breast cancer. *BMC Cancer.* 9:231. http://dx.doi.org/10.1186/1471-2407-9-231

Mellor, A.L., P. Chandler, B. Baban, A.M. Hansen, B. Marshall, J. Pihkala, H. Waldmann, S. Cobbold, E. Adams, and D.H. Munn. 2004. Specific subsets of murine dendritic cells acquire potent T cell regulatory functions following CTLA4-mediated induction of indoleamine 2,3 dioxygenase. *Int. Immunol.* 16:1391–1401. http://dx.doi.org/10.1093/intimm/dxh140

Mestas, J., and C.C. Hughes. 2004. Of mice and not men: differences between mouse and human immunology. *J. Immunol.* 172:2731–2738.

Metz, R., S. Rust, J.B. Duhadaway, M.R. Mautino, D.H. Munn, N.N. Vahanian, C.J. Link, and G.C. Prendergast. 2012. IDO inhibits a tryptophan sufficiency signal that stimulates mTOR: A novel IDO effector pathway targeted by D-1-methyl-tryptophan. *Oncoimmunology.* 1:1460–1468. http://dx.doi.org/10.4161/onci.21716

Muller, A.J., J.B. DuHadaway, P.S. Donover, E. Sutanto-Ward, and G.C. Prendergast. 2005a. Inhibition of indoleamine 2,3-dioxygenase, an immunoregulatory target of the cancer suppression gene Bin1, potentiates cancer chemotherapy. *Nat. Med.* 11:312–319. http://dx.doi.org/10.1038/nm1196

Muller, A.J., W.P. Malachowski, and G.C. Prendergast. 2005b. Indoleamine 2,3-dioxygenase in cancer: targeting pathological immune tolerance with small-molecule inhibitors. *Expert Opin. Ther. Targets.* 9:831–849. http://dx.doi.org/10.1517/14728222.9.4.831

Munn, D.H., and A.L. Mellor. 2004. IDO and tolerance to tumors. *Trends Mol. Med.* 10:15–18. http://dx.doi.org/10.1016/j.molmed.2003.11.003

Munn, D.H., and A.L. Mellor. 2007. Indoleamine 2,3-dioxygenase and tumor-induced tolerance. *J. Clin. Invest.* 117:1147–1154. http://dx.doi.org/10.1172/JCI31178

Munn, D.H., M. Zhou, J.T. Attwood, I. Bondarev, S.J. Conway, B. Marshall, C. Brown, and A.L. Mellor. 1998. Prevention of allogeneic fetal rejection by tryptophan catabolism. *Science.* 281:1191–1193. http://dx.doi.org/10.1126/science.281.5380.1191

Munn, D.H., E. Shafizadeh, J.T. Attwood, I. Bondarev, A. Pashine, and A.L. Mellor. 1999. Inhibition of T cell proliferation by macrophage tryptophan catabolism. *J. Exp. Med.* 189:1363–1372. http://dx.doi.org/10.1084/jem.189.9.1363

Munn, D.H., M.D. Sharma, J.R. Lee, K.G. Jhaver, T.S. Johnson, D.B. Keskin, B. Marshall, P. Chandler, S.J. Antonia, R. Burgess, et al. 2002. Potential regulatory function of human dendritic cells expressing indoleamine 2,3-dioxygenase. *Science.* 297:1867–1870. http://dx.doi.org/10.1126/science.1073514

Munn, D.H., M.D. Sharma, D. Hou, B. Baban, J.R. Lee, S.J. Antonia, J.L. Messina, P. Chandler, P.A. Koni, and A.L. Mellor. 2004. Expression of indoleamine 2,3-dioxygenase by plasmacytoid dendritic cells in tumor-draining lymph nodes. *J. Clin. Invest.* 114:280–290.

Munn, D.H., M.D. Sharma, B. Baban, H.P. Harding, Y. Zhang, D. Ron, and A.L. Mellor. 2005. GCN2 kinase in T cells mediates proliferative arrest and anergy induction in response to indoleamine 2,3-dioxygenase. *Immunity.* 22:633–642. http://dx.doi.org/10.1016/j.immuni.2005.03.013

Okamoto, A., T. Nikaido, K. Ochiai, S. Takakura, M. Saito, Y. Aoki, N. Ishii, N. Yanaihara, K. Yamada, O. Takikawa, et al. 2005. Indoleamine 2,3-dioxygenase serves as a marker of poor prognosis in gene expression profiles of serous ovarian cancer cells. *Clin. Cancer Res.* 11:6030–6039. http://dx.doi.org/10.1158/1078-0432.CCR-04-2671

Orabona, C., P. Puccetti, C. Vacca, S. Bicciato, A. Luchini, F. Fallarino, R. Bianchi, E. Velardi, K. Perruccio, A. Velardi, et al. 2006. Toward the identification of a tolerogenic signature in IDO-competent dendritic cells. *Blood.* 107: 2846–2854. http://dx.doi.org/10.1182/blood-2005-10-4077

Park, M.J., S.Y. Min, K.S. Park, Y.G. Cho, M.L. Cho, Y.O. Jung, H.S. Park, S.H. Chang, S.G. Cho, J.K. Min, et al. 2008. Indoleamine 2,3-dioxygenase-expressing dendritic cells are involved in the generation of CD4+CD25+ regulatory T cells in Peyer's patches in an orally tolerized, collagen-induced arthritis mouse model. *Arthritis Res. Ther.* 10:R11. http://dx.doi.org/10.1186/ar2361

Phan, G.Q., J.C. Yang, R.M. Sherry, P. Hwu, S.L. Topalian, D.J. Schwartzentruber, N.P. Restifo, L.R. Haworth, C.A. Seipp, L.J. Freezer, et al. 2003. Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. *Proc. Natl. Acad. Sci. USA.* 100:8372–8377. http://dx.doi.org/10.1073/pnas.1533209100

Polak, M.E., N.J. Borthwick, F.G. Gabriel, P. Johnson, B. Higgins, J. Hurren, D. McCormick, M.J. Jager, and I.A. Cree. 2007. Mechanisms of local immunosuppression in cutaneous melanoma. *Br. J. Cancer.* 96:1879–1887. http://dx.doi.org/10.1038/sj.bjc.6603763

Pulaski, B.A., and S. Ostrand-Rosenberg. 2001. Mouse 4T1 breast tumor model. *Curr Protoc Immunol* Chapter 20:Unit 20 22.

Qian, F., J. Liao, J. Villella, R. Edwards, P. Kalinski, S. Lele, P. Shrikant, and K. Odunsi. 2012. Effects of 1-methyltryptophan stereoisomers on IDO2 enzyme activity and IDO2-mediated arrest of human T cell proliferation. *Cancer Immunol. Immunother.* 61:2013–2020. http://dx.doi.org/10.1007/s00262-012-1265-x

Quezada, S.A., K.S. Peggs, M.A. Curran, and J.P. Allison. 2006. CTLA4 blockade and GM-CSF combination immunotherapy alters the intratumor balance of effector and regulatory T cells. *J. Clin. Invest.* 116:1935–1945. http://dx.doi.org/10.1172/JCI27745

Ribas, A., B. Comin-Anduix, J.S. Economou, T.R. Donahue, P. de la Rocha, L.F. Morris, J. Jalil, V.B. Dissette, I.P. Shintaku, J.A. Glaspy, et al. 2009. Intratumoral immune cell infiltrates, FoxP3, and indoleamine 2,3-dioxygenase in patients with melanoma undergoing CTLA4 blockade. *Clin. Cancer Res.* 15:390–399. http://dx.doi.org/10.1158/1078-0432.CCR-08-0783

Robert, C., L. Thomas, I. Bondarenko, S. O'Day, J.W. M D, C. Garbe, C. Lebbe, J.F. Baurain, A. Testori, J.J. Grob, et al. 2011. Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *N. Engl. J. Med.* 364:2517–2526. http://dx.doi.org/10.1056/NEJMoa1104621

Sanderson, K., R. Scotland, P. Lee, D. Liu, S. Groshen, J. Snively, S. Sian, G. Nichol, T. Davis, T. Keler, et al. 2005. Autoimmunity in a phase I trial of a fully human anti-cytotoxic T-lymphocyte antigen-4 monoclonal antibody with multiple melanoma peptides and Montanide ISA 51 for patients with resected stages III and IV melanoma. *J. Clin. Oncol.* 23:741–750. http://dx.doi.org/10.1200/JCO.2005.01.128

Sato, E., S.H. Olson, J. Ahn, B. Bundy, H. Nishikawa, F. Qian, A.A. Jungbluth, D. Frosina, S. Gnjatic, C. Ambrosone, et al. 2005. Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. *Proc. Natl. Acad. Sci. USA.* 102:18538–18543. http://dx.doi.org/10.1073/pnas.0509182102

Schneider, H., J. Downey, A. Smith, B.H. Zinselmeyer, C. Rush, J.M. Brewer, B. Wei, N. Hogg, P. Garside, and C.E. Rudd. 2006. Reversal of the TCR stop signal by CTLA-4. *Science.* 313:1972–1975. http://dx.doi.org/10.1126/science.1131078

Sharma, M.D., B. Baban, P. Chandler, D.Y. Hou, N. Singh, H. Yagita, M. Azuma, B.R. Blazar, A.L. Mellor, and D.H. Munn. 2007. Plasmacytoid dendritic cells from mouse tumor-draining lymph nodes directly activate mature Tregs via indoleamine 2,3-dioxygenase. *J. Clin. Invest.* 117:2570–2582. http://dx.doi.org/10.1172/JCI31911

Sharma, M.D., D.Y. Hou, Y. Liu, P.A. Koni, R. Metz, P. Chandler, A.L. Mellor, Y. He, and D.H. Munn. 2009. Indoleamine 2,3-dioxygenase controls conversion of Foxp3+ Tregs to TH17-like cells in tumor-draining lymph nodes. *Blood*. 113:6102–6111. http://dx.doi.org/10.1182/blood-2008-12-195354

Suzuki, S., S. Toné, O. Takikawa, T. Kubo, I. Kohno, and Y. Minatogawa. 2001. Expression of indoleamine 2,3-dioxygenase and tryptophan 2,3-dioxygenase in early concepti. *Biochem. J.* 355:425–429. http://dx.doi.org/10.1042/0264-6021:3550425

Taylor, M.W., and G.S. Feng. 1991. Relationship between interferon-gamma, indoleamine 2,3-dioxygenase, and tryptophan catabolism. *FASEB J.* 5: 2516–2522.

Terness, P., T.M. Bauer, L. Röse, C. Dufter, A. Watzlik, H. Simon, and G. Opelz. 2002. Inhibition of allogeneic T cell proliferation by indoleamine 2,3-dioxygenase-expressing dendritic cells: mediation of suppression by tryptophan metabolites. *J. Exp. Med*. 196:447–457. http://dx.doi.org/10.1084/jem.20020052

Uyttenhove, C., L. Pilotte, I. Théate, V. Stroobant, D. Colau, N. Parmentier, T. Boon, and B.J. Van den Eynde. 2003. Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase. *Nat. Med*. 9:1269–1274. http://dx.doi.org/10.1038/nm934

van Elsas, A., A.A. Hurwitz, and J.P. Allison. 1999. Combination immunotherapy of B16 melanoma using anti-cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and granulocyte/macrophage colony-stimulating factor (GM-CSF)-producing vaccines induces rejection of subcutaneous and metastatic tumors accompanied by autoimmune depigmentation. *J. Exp. Med*. 190:355–366. http://dx.doi.org/10.1084/jem.190.3.355

Weinlich, G., C. Murr, L. Richardsen, C. Winkler, and D. Fuchs. 2007. Decreased serum tryptophan concentration predicts poor prognosis in malignant melanoma patients. *Dermatology (Basel)*. 214:8–14. http://dx.doi.org/10.1159/000096906

Witkiewicz, A., T.K. Williams, J. Cozzitorto, B. Durkan, S.L. Showalter, C.J. Yeo, and J.R. Brody. 2008. Expression of indoleamine 2,3-dioxygenase in metastatic pancreatic ductal adenocarcinoma recruits regulatory T cells to avoid immune detection. *J. Am. Coll. Surg.* 206:849–854, discussion :854–856. http://dx.doi.org/10.1016/j.jamcollsurg.2007.12.014

Wolchok, J.D., and Y. Saenger. 2008. The mechanism of anti–CTLA-4 activity and the negative regulation of T-cell activation. *Oncologist*. 13(Suppl 4):2–9. http://dx.doi.org/10.1634/theoncologist.13-S4-2

Yu, G., H. Dai, J. Chen, L. Duan, M. Gong, L. Liu, P. Xiong, C.Y. Wang, M. Fang, and F. Gong. 2008. Gene delivery of indoleamine 2,3-dioxygenase prolongs cardiac allograft survival by shaping the types of T-cell responses. *J. Gene Med*. 10:754–761. http://dx.doi.org/10.1002/jgm.1201

Yuasa, H.J., H.J. Ball, C.J. Austin, and N.H. Hunt. 2010. 1-L-methyltryptophan is a more effective inhibitor of vertebrate IDO2 enzymes than 1-D-methyltryptophan. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 157: 10–15. http://dx.doi.org/10.1016/j.cbpb.2010.04.006