Central Role of IFNγ–Indoleamine 2,3-Dioxygenase Axis in Regulation of Interleukin-12–Mediated Antitumor Immunity

Tao Gu, Rachael B. Rowswell-Turner, Mehmet O. Kilinc, and Nejat K. Egilmez

Abstract
Sustained intratumoral delivery of interleukin-12 (IL-12) and granulocyte macrophage colony-stimulating factor induces tumor regression via restoration of tumor-resident CD8+ T-effector/memory cell cytotoxicity and subsequent repriming of a secondary CD8+ T-effector cell response in tumor-draining lymph nodes (TDLN). However, treatment-induced T-effector activity is transient and is accompanied with a CD4+ CD25+ Foxp3+ T-suppressor cell rebound. Molecular and cellular changes in posttherapy tumor microenvironment and TDLN were monitored to elucidate the mechanism of counterregulation. Real-time PCR analysis revealed a 5-fold enhancement of indoleamine 2,3-dioxygenase (IDO) expression in the tumor and the TDLN after treatment. IDO induction required IFNγ and persisted for up to 7 days. Administration of the IDO inhibitor D-1-methyl tryptophan concurrent with treatment resulted in a dramatic enhancement of tumor regression. Enhanced efficacy was associated with a diminished T-suppressor cell rebound, revealing a link between IDO activity and posttherapy regulation. Further analysis established that abrogation of the regulatory counterresponse resulted in a 10-fold increase in the intratumoral CD8+ T-cell to CD4+ Foxp3+ T-cell ratio. The ratio of proliferating CD8+ T-effector to CD4+ Foxp3+ T-suppressor cells was prognostic for efficacy of tumor suppression in individual mice. IFNγ-dependent IDO induction and T-suppressor cell expansion were primarily driven by IL-12. These findings show a critical role for IDO in the regulation of IL-12–mediated antitumor immune responses. Cancer Res; 70(1); 129–38. ©2010 AACR.

Introduction
Cancer vaccines can break tolerance to tumor antigens and induce potent antitumor T-cell activity (1, 2). However, in the great majority of cases, antitumor T cells fail to induce effective tumor regression, independent of the intensity of the response (1–3). The immunosuppressive nature of the tumor microenvironment has been identified as a major factor contributing to the inability of T cells to mediate effective tumor regression (4, 5). Blocking of suppressive mechanisms during therapy results in improved tumor eradication; however, durable regressions are rarely achieved (6, 7). Whether the lack of long-term efficacy is due to the inherent transient nature of effector T-cell responses (8) and the associated T-cell intrinsic regulatory pathways (9), resurgence of tumor-mediated immune dysfunction (4), or a combination of these factors is not established.

Previous studies showed that sustained intratumoral delivery of interleukin-12 (IL-12) and granulocyte macrophage colony-stimulating factor (GM-CSF) restored tumor-resident CD8+ T-effector/memory cell cytotoxicity and induced de novo priming of antitumor CD8+ T-effector cells in the tumor-draining lymph nodes (TDLN; refs. 10, 11). Treatment-induced immune activation, however, subsided within a week and was followed by a T-suppressor cell rebound (12). Because IL-12 directly restored full effector function to preexisting quiescent CD8+ T-effector/memory cells in the presence of T-suppressor cells (10), we hypothesized that repeated stimulation could revive and extend the window of CD8+ T-effector/memory cell cytotoxicity and enhance long-term tumor suppression. Chronic treatment, however, resulted in progressive loss of therapeutic efficacy culminating in tumor resurgence (12). Long-term monitoring of intratumoral T-cell populations showed that multiple treatments led to the intensification of the T-suppressor cell rebound after each cycle of therapy and ultimately resulted in the loss of antitumor T-effector/memory cells (12). These findings suggested that feedback inhibition may represent an important tumor-independent mechanism that can limit long-term therapeutic efficacy.

Whereas the majority of tumor immune therapy protocols focus on the induction phase of the antitumor immune response, few studies have addressed the mechanism of postactivation regulatory rebound. Consistent with our findings, these studies showed that repeated vaccination or immune stimulation can promote the expansion of the CD4+ CD25+ Foxp3+ T-suppressor cell subset in murine models of autoimmunity and cancer (13–15). On the other hand, the molecular basis of therapy-induced T-suppressor cell expansion has not
been delineated. To this end, quantitative and qualitative monitoring of the tumor microenvironment and the TDNL was undertaken to investigate the mechanism of post-IL-12/GM-CSF T-suppressor cell amplification. The results show a central role for the IL-12/GM-CSF T-suppressor cell amplification. The results show a central role for the IL-12/GM-CSF T-suppressor cell amplification. The results show a central role for the IL-12/GM-CSF T-suppressor cell amplification.

Materials and Methods

Mice and tumors. BALB/c mice were purchased from Taconic Laboratories or bred in our facility. IFNγ-knockout (GKO) mice in BALB/c background were purchased from The Jackson Laboratory. The BALB/c syngeneic mammary carcinoma cell line 4T1 (16) was used in all experiments.

Microspheres and treatments. Recombinant murine IL-12 was a gift from Wyeth Pharmaceuticals. Recombinant murine GM-CSF was purchased from PeproTech, Inc. Cyto-
kines were encapsulated into polyacrylic microspheres via phase inversion nanoencapsulation (10). Mice were treated with a single intratumoral injection of 2 mg of each preparation (1 μg of cytokine) as described previously (10).

Surgical metastasis model. Mice were injected s.c. with 3 × 10⁵ tumor cells in 0.1 ml PBS in the mammary tissue, and tumors were allowed to grow to 6 to 7 mm in diameter (14 d) to establish metastases. Primary tumors were treated with a single intratumoral injection of microspheres, and tumors were surgically resected 1 wk after treatment (17). Mice were euthanized 3 wk after surgery, and lung tumor burden was quantified using a clonogenic metastasis assay (17).

Quantitative real-time PCR analysis. Quantitative real-
time PCR (qRT-PCR) was performed essentially as de-
scribed (17). Transcript levels were calculated by the 

\[ C_{t} \text{target} = \frac{C_{t} \text{endogenous control}}{C_{t} \text{endogenous control}} \]

\[ \Delta C_{t} = C_{t} \text{target} - C_{t} \text{endogenous control} \]

\[ \Delta \Delta C_{t} = \Delta C_{t} \text{target} - \Delta C_{t} \text{control} \]

\[ \frac{C_{t} \text{target}}{C_{t} \text{endogenous control}} \]

\[ \text{fold} = 2^{-\Delta \Delta C_{t}} \]

Results

Posttreatment CD8⁺ T-cell cytotoxicity is transient. Previous studies showed that intratumoral IL-12 and GM-
CSF promoted rapid activation of tumor-resident CD8⁺ T-effector/memory cells followed by priming of an antitumor CD8⁺ T-effector cell response in the TDNL (10, 11). Although the secondary CD8⁺ T-effector cells effectively homed to and infiltrated the tumors, whether they mediated effective long-
term tumor killing was not determined (11). To this end, we 

Intratumoral IL-12 + GM-CSF induces concurrent expansion of effector and suppressor T cells. The above findings suggested that treatment-induced effector and/or...
memory CD8+ T cells were not effective against tumors that persisted beyond 10 days. Previous studies had shown that treatment was followed by a rebound in intratumoral CD4+ CD25+ Foxp3+ T-suppressor cells (12). We therefore hypothesized that CD8+ T-cell priming could be paralleled by CD4+ Foxp3+ T-cell expansion, potentially curbing long-term T-effector cell activity in the tumor. To this end, CD8 and Foxp3 mRNA kinetics were monitored in the TDLN and tumors of treated mice via qRT-PCR. The results are shown in Fig. 2A. Consistent with previous findings (11), treatment was followed by a 4-fold enhancement of CD8 mRNA in the TDLN on day 3 and an 8-fold increase in the tumor on day 7. Importantly, a similar increase (3-fold) in Foxp3 mRNA in the TDLN on day 3 was followed by a 2-fold increase in intratumoral Foxp3 on day 7. The increase in Foxp3 expression in the TDLN was slightly delayed when compared with CD8 during the first 24 hours, suggesting that CD8+ T-cell priming preceded T-suppressor cell expansion. Collectively, these data show that treatment-induced CD8+ T-cell priming and proliferation was accompanied by a similar expansion of T-suppressor cells in the TDLN and that both populations eventually homed to tumors.

**Treatment promotes rapid IFNγ-dependent IDO expression.** The overlapping nature of posttherapy CD8 and Foxp3 mRNA expression patterns in the TDLN and tumors raised the possibility that treatment itself was responsible for the T-suppressor cell rebound. IFNγ is the primary downstream cytokine induced by IL-12 and is central to its antitumor effects (21). Among >200 immunologically relevant genes induced by IFNγ is IDO (22–24), a tryptophan-catabolizing enzyme with immunosuppressive function (25). Importantly, recent studies showed a role for IDO in CD4+ Foxp3+ T-suppressor cell activation and generation (26, 27). These findings provided the rationale for the next series of experiments in...
which the putative link between the IL-12–IFNγ–IDO axis and posttherapy immune suppression was investigated. Initially, expression of IFNγ and IDO was monitored in the TDLN and the tumors of treated mice by qRT-PCR. Treatment induced a rapid increase in IFNγ mRNA both in the TDLN and the tumor (Fig. 2B). This enhancement was more dramatic in the TDLN (5- and 34-fold increases at 6 and 24 hours over background, respectively) compared with the tumor (2- and 3-fold enhancement at 6 and 24 hours, respectively). Upregulation of IFNγ expression in tumors was
transient and declined rapidly after day 1, returning to background levels on day 7. IFNγ mRNA levels also declined in the TDLN but remained above pretherapy levels for at least 7 days. Importantly, treatment promoted a similar increase in the expression of IDO (referred to as IDO-1 from this point on) as well as the more recently described IDO-2 (28) in the TDLN, except that induction of these enzymes was delayed by 24 hours in comparison with IFNγ. In contrast, treatment resulted in the upregulation of IDO-1 but not IDO-2 in tumors (Fig. 2B). Whereas IDO-1 mRNA returned to background levels in the tumor on day 3, both IDO-1 and IDO-2 remained high in the TDLN for at least 7 days.

The sequential nature of IFNγ and IDO-1/IDO-2 expression kinetics in the TDLN between days 0 and 2 suggested that their transcription was linked. To test this hypothesis, tumors were induced in either wild-type or GKO mice, and IDO-1 mRNA was quantified in pretherapy and posttherapy tumors and TDLN. The results are shown in Fig. 2C. In wild-type mice, treatment promoted 5- and 2-fold increases in IDO-1 mRNA in the tumor and the TDLN on day 1, respectively (Fig. 2C). In contrast, treatment failed to induce detectable IDO-1 mRNA in the GKO mice (Fig. 2C). Analysis of day 3 TDLN revealed a similar loss of IDO-2 induction in GKO mice, showing that IDO-2 upregulation was also IFNγ dependent (data not shown).

**Blocking IDO activity during treatment results in complete tumor eradication.** The above findings were consistent with the notion that the IL-12–IFNγ–IDO axis represents a critical feedback inhibitory mechanism in our model. D-1MT, an inhibitor of IDO-2 activity, enhances tumor suppression in immune-competent but not in immune-deficient mice (18). To this end, we tested whether coadministration of D-1MT with IL-12 and GM-CSF to mice could enhance tumor suppression by blocking posttherapy IDO activity. Tumor-bearing mice were treated either with IL-12/GM-CSF microspheres, D-1MT alone, or a combination of IL-12/GM-CSF + D-1MT, and tumor growth was monitored. The results are shown in Fig. 3. In the control group (blank microspheres), all tumors grew rapidly and mice had to be sacrificed by day 21 or earlier. Administration of D-1MT alone resulted in delayed tumor growth during the first week but tumors grew rapidly thereafter. Administration of IL-12/GM-CSF microspheres suppressed tumor growth during the first 2 weeks but all tumors eventually resumed growth. In contrast, complete tumor regression was achieved in 5 of 11 mice in the combination group, resulting in long-term cure (see Supplementary Fig. S1 for survival analysis). Because both IDO-1 and IDO-2 were upregulated in the TDLN in response to treatment, we also tested the antitumor efficacy of L-1MT, which specifically targets IDO-1. L-1MT also delayed tumor growth when administered in combination with IL-12/GM-CSF therapy; however, complete regressions were achieved only with D-1MT, suggesting that IDO-2 was the primary mediator of posttherapy feedback inhibition in this model (Supplementary Fig. S2).

**D-1MT administration delays CD8+ Foxp3+ T-suppressor cell expansion and extends CD8+ T-effector activity window.** To determine whether IDO-2 activity and CD4+ Foxp3+ T-cell expansion were linked, posttherapy T-suppressor cell numbers were monitored in the presence or absence of D-1MT. The results shown in Fig. 4A reveal that IL-12/GM-CSF treatment resulted in a dramatic 6-fold expansion of T-suppressor cells in tumors and a more modest 1.4-fold increase in the TDLN between days 3 and 10. Importantly, coadministration of D-1MT resulted in a significant reduction (1.8- to 6-fold) in the numbers of CD4+ Foxp3+ T cells in the TDLN and the tumors on days 3 and 7. However, this inhibition was transient and the CD4+ Foxp3+ T-cell numbers in the experimental mice rebounded to levels observed in control mice on day 10. These findings establish that D-1MT transiently inhibited posttherapy T-suppressor cell expansion.

Because inhibition of T-suppressor cell expansion is expected to result in enhanced CD8+ T-cell activity, the effect of IDO-2 inhibition on CD8+ T-cell proliferation was monitored. To this end, CD8+ T-cell proliferation was quantified in both the TDLN and tumors of control and D-1MT–treated mice. The results are shown in Fig. 4B. These data show that initially TDLN CD8+ T cells proliferated equally well in both control and D-1MT–treated mice independent of Foxp3+ CD8+ T-cell levels. On day 7, however, CD8+ T-cell proliferation declined in the TDLN of control mice but remained high in D-1MT–treated animals, consistent with extended priming activity. On day 10, proliferation returned to background levels in both groups. In tumors, CD8+ T-cell proliferation on day 3 was minimal in both groups. On both days 7 and 10, however, CD8+ T cells incorporated BrdUrd more vigorously (>3-fold higher) in the D-1MT group, showing that CD8+ T-effector cells continued to proliferate in the tumors for at least until day 10. These data establish that the diminished T-suppressor cell expansion correlated with extended CD8+ T-cell activity.

**Ratio of actively dividing CD8+ T cells to T-suppressor cells predicts therapeutic efficacy.** Several studies showed a correlation between intratumoral CD8+ T-cell to CD4+ Foxp3+ T-cell ratio and tumor progression (29, 30). Because D-1MT administration resulted in reduced T-suppressor cell numbers and enhanced CD8+ T-cell proliferation, the BrdUrd+ CD8+ T-cell to CD4+ Foxp3+ T-cell ratio was evaluated as a potential prognostic marker for tumor regression. To this end, the ratios were determined between days 0 and 10 in the TDLN and the tumors of control (IL-12/GM-CSF alone) and D-1MT (IL-12/GM-CSF + D-1MT) groups. These data are presented in Fig. 5A. The results show that treatment did not significantly change the ratio in control mice between days 0 and 7 in the TDLN or tumors. On day 10, however, the ratio declined by 2-fold, suggestive of the development of a more immunosuppressive microenvironment. In contrast, the ratio of actively dividing CD8+ T cells to T-suppressor cells increased dramatically in both the TDLN and the tumors on days 3 and 7 (2- to 3-fold in the TDLN and 6- to 15-fold in the tumor compared with day 0) in the D-1MT–treated group. Although the ratio declined in both microenvironments on day 10, it still remained above pretherapy levels.

The absolute values for total or proliferating CD8+ T-cell to CD4+ Foxp3+ T-suppressor cell ratios in tumors were plotted against tumor weight to determine whether the ratio...
correlated with tumor size. More specifically, the correlation coefficients (r values) were determined in control and D-1MT groups on days 3, 7, and 10 (Supplementary Fig. S3). Of these, the r values for proliferating CD8+ T-cell to CD4+ Foxp3+ T-cell ratio on days 3 and 7 were most predictive. Data for day 7 tumors with or without D-1MT are shown in Fig. 5B. The correlation between BrdUrd+ CD8+ T-cell to CD4+ Foxp3+ ratio and tumor suppression was significant in both groups, with the D-1MT group displaying stronger linkage. In contrast, total CD8+ T-cell to CD4+ Foxp3+ T-cell ratio was found to be less predictive (Table 1).

**T-suppressor cell rebound is driven primarily by IL-12.** Because IL-12 and GM-CSF were found to be synergistic in mediating tumor regression in previous studies (19) we wanted to determine their respective roles in the observed feedback inhibition. Combined administration of IL-12 + GM-CSF promoted 5.1 ± 0.45-fold and 5.2 ± 1.01-fold increases in intratumoral IFNγ and IDO-1 mRNA on day 1, respectively. IL-12 alone resulted in similar increases in both IFNγ (4.5 ± 1.27-fold) and IDO-1 (3.1 ± 0.2-fold) expression. In contrast, GM-CSF alone did not promote any significant increases in IFNγ (1.3 ± 0.04-fold) or IDO-1 (0.7 ± 0.17-fold) expression (n = 3–4 mice per group). With regard to the T-suppressor cell rebound between days 3 and 7, combination, IL-12-alone, GM-CSF-alone, and control (blank microsphere) groups showed 13.9 ± 4.7-fold, 6.0 ± 2.0-fold, 2.5 ± 0.6-fold, and 2.3 ± 0.4-fold increases in T-suppressor cell numbers, respectively. These data show that T-suppressor cell expansion was driven by IL-12. GM-CSF seemed to contribute to the T-suppressor cell rebound when administered with IL-12 but had no effect when injected alone.

**Discussion**

The above studies show that the proinflammatory activity of IL-12 is regulated via a feedback inhibitory mechanism involving the IFNγ–IDO–T-suppressor cell axis. Our results indicate that the combination of IL-12 and GM-CSF is more effective than either agent alone in promoting T-suppressor cell rebound and tumor suppression.
also establish that blocking of IDO activity with D-1MT can be effectively used to overcome feedback inhibition and enhance IL-12–mediated antitumor responses. Finally, the above data identify the proliferating CD8+ T-cell to CD4+ Foxp3+ T-suppressor cell ratio in tumors as a highly accurate prognostic marker of therapeutic efficacy.

Numerous clinical studies have shown the tachyphylactic nature of IL-12 therapy (31–34). However, the mechanism underlying the progressive loss of therapeutic efficacy that accompanies repeated IL-12 administration has not been defined. Our findings, for the first time, show that IDO activation is, at least in part, responsible for this effect. Whether suppression occurred primarily via T-suppressor cell activity (26, 27) or also involved direct inhibition by tryptophan catabolites (35, 36) was not investigated. In our model, induction of IDO-1 in the tumor microenvironment was rapid and transient with no change in intratumoral IDO-2. These data, combined with our previous finding that tumor-resident CD8+ T-effector/memory cells display full-effector function during this interval (10), are inconsistent with a direct role for tryptophan catabolites in T-effector inhibition. To this end, it was recently reported that IDO-1 overexpression

Figure 4. Effect of D-1MT coadministration on posttreatment T-cell kinetics in mice receiving IL-12/GM-CSF therapy. A, CD4+ Foxp3+ T-suppressor cell expansion in posttherapy mice. Mice bearing tumors were treated with either IL-12/GM-CSF alone (Control) or IL-12/GM-CSF + D-1MT (D-1MT). Tumors and TDLN were harvested on days 3, 7, and 10, and T-suppressor cells were quantified. The differences between control and D-1MT groups were significant on days 3 and 7 in the TDLN (P ≤ 0.031) and on day 3 in the tumor (P = 0.036). The dot plots represent typical results (CD45+ CD3+ cells were gated on and analyzed for CD4 and Foxp3 expression) on day 7. B, analysis of proliferating CD8+ T cells. The above samples were also analyzed for proliferating CD8+ T-cell populations using the BrdUrd pulse-labeling strategy. CD45+ CD3+ cells were gated on and analyzed for CD8 and BrdUrd staining. The differences between the control and D-1MT samples for day 7 TDLN (P = 0.006) and for day 10 tumors (P = 0.026) were significant. Columns, mean (n = 4–5 mice per group); bars, SE. This experiment was repeated twice with similar results.
does not inhibit the effector function of preexisting T-effector/memory cells (36). In contrast, the kinetics of intratumoral T-suppressor cell expansion between days 3 and 10 closely paralleled the loss of posttherapy CD8\(^+\) T-effector cytotoxicity in tumors (10). This finding, combined with the well-known ability of T-suppressor cells to inhibit CD8\(^+\) T-cell cytotoxicity in vivo (37), suggest that the inhibitory activity of IDO was mediated indirectly via T-suppressor cells.

The sources of posttreatment IDO-1 and IDO-2 were not investigated in this study. Numerous reports have established that plasmacytoid dendritic cells (DC), conventional tolerogenic DCs, and macrophages are the primary producers of IDO-1 (38–40). IDO-2, on the other hand, is expressed in the kidney, testis, and liver but has also been detected in DCs (28). Our preliminary findings are consistent with the hypothesis that DCs are the primary source of IDO-1 and IDO-2 in the TDLN of post–IL-12 mice.\(^1\) The mechanistic basis of the differential IDO-1 and IDO-2 expression patterns in posttreatment tumors and TDLN, on the other hand, remain undefined. In the case of IDO-1, treatment-induced migration of DCs from tumors to TDLN (41) can potentially account for the initial intratumoral spike and the subsequent upregulation and persistence of IDO-1 in the TDLN. The restriction of IDO-2 expression to the TDLN, on the other hand, is more difficult to explain. One potential mechanism would involve selective infiltration of tumors and the TDLN by DC subsets.

\(1\) Harden and Egilmez, unpublished data.
with differential IFN-γ responsiveness and/or IDO-1 or IDO-2 expression profiles (42).

The finding that D-1MT was more efficient than L-1MT in achieving complete tumor eradication in IL-12/GM-CSF–treated mice is consistent with the findings of others in different tumor models (18). The mechanistic basis of the in vivo superiority of D-1MT to L-1MT in promoting tumor suppression is controversial (18, 43, 44). In our study, treatment promoted both IDO-1 and IDO-2 expression in the TDLN, showing that the differential antitumor efficacy of the 1MT isomers was not due to selective expression of the target enzymes in posttreatment TDLN. On the other hand, whether IDO-1 and IDO-2 differ in their abilities to induce T-suppressor cell activation/expansion and whether they are expressed by different tolerogenic DC subsets are not known. Analysis of posttherapy TDLN DC subsets with regard to IDO-1 and IDO-2 expression and defining their relative abilities to activate/expand T-suppressor cells may shed further light on the respective roles of each enzyme in the development of posttherapy regulatory rebound.

The above results suggest that selective blocking of IFN-γ-mediated regulatory pathways can overcome IL-12 tachyphylaxis. Whereas repeated activation of tumor-resident T-effector/memory cells via this strategy represents a potentially effective therapeutic approach, its utility may still be limited by T-cell intrinsic regulatory mechanisms (9) and/or the finite clonal proliferative potential of cytotoxic CD8+ T cells (45). We are currently investigating the limits of this strategy in a model that allows long-term quantitative monitoring of tumor-specific CD8+ T-effector cell activity.

Disclosure of Potential Conflicts of Interest

N.K. Egilmez: ownership interest, TherapyX, Inc. The other authors disclosed no potential conflicts of interest.

Acknowledgments

We thank Dr. Stanley Wolf (Wyeth, Inc.) for providing the murine IL-12 and his continued support of our studies.

Grant Support

NIH grant R01-CA100656-01A1 and New York State Office of Science, Technology, and Academic Research faculty recruitment award C040070 (N.K. Egilmez).

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Received 8/25/09; revised 10/14/09; accepted 10/23/09; published OnlineFirst 12/22/09.

Table 1. The ratio of proliferating CD8+ T cells to CD4+ Foxp3+ T cells is prognostic for therapeutic efficacy

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