iNOS Expression in CD4+ T Cells Limits Treg Induction by Repressing TGFβ1: Combined iNOS Inhibition and Treg Depletion Unmask Endogenous Antitumor Immunity

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Abstract

- **Purpose:** Expression of inducible nitric oxide synthase (iNOS) in different cellular compartments may have divergent effects on immune function. We used a syngeneic tumor model to functionally characterize the role of iNOS in regulation of CD4+ FOXP3+ regulatory T cells (Treg), and optimize the beneficial effects of iNOS inhibition on antitumor immunity.

- **Experimental Design:** Wild-type (WT) or iNOS knockout mice bearing established MT-RET-1 melanoma were treated with the small-molecule iNOS inhibitor L-NIL and/or cyclophosphamide alone or in combination. The effect of iNOS inhibition or knockout on induction of Treg from mouse and human CD4+ T cells in ex vivo culture was determined in parallel in the presence or absence of TGFβ1-depleting antibodies, and TGFβ1 levels were assessed by ELISA.

- **Results:** Whereas intratumoral myeloid-derived suppressor cells (MDSC) were suppressed by iNOS inhibition or knockout, systemic and intratumoral FOXP3+ Treg levels increased in tumor-bearing mice. iNOS inhibition or knockout similarly enhanced induction of Treg from activated cultured mouse splenocytes or purified human or mouse CD4+ T cells in a TGFβ1-dependent manner. Although either iNOS inhibition or Treg depletion with low-dose cyclophosphamide alone had little effect on growth of established MT-RET1 melanoma, combination treatment potently inhibited MDSC and Treg, boosted tumor-infiltrating CD8+ T-cell levels, and arrested tumor growth in an immune-dependent fashion.

- **Conclusions:** iNOS expression in CD4+ T cells suppresses Treg induction by inhibiting TGFβ1 production. Our data suggest that iNOS expression has divergent effects on induction of myeloid and lymphoid-derived regulatory populations, and strongly support development of combinatorial treatment approaches that target these populations simultaneously.

Introduction

In health, suppressive/regulatory immunocyte populations, most prominently regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSC), play critical roles in immune homeostasis. However, their aberrant induction by cancer is an important mechanism of tumor-mediated immunosuppression, leading to failure of host immuno-surveillance and impaired efficacy of cancer immunotherapy. Thus, a detailed understanding of the mechanisms regulating the induction of suppressive immunocytes is crucial for understanding host–tumor interactions and developing effective cancer immunotherapies capable of overcoming tumor-induced barriers to immune activation.

Much is already known about the regulation of Tregs in cancer. Treg can be induced from naïve CD4+ T cells by molecules expressed by tumor or tumor-infiltrating cells (1–4), and by TGFβ1, which is often expressed at high levels in the tumor microenvironment. These so-called inducible Tregs (iTreg) are supplemented by pre-existing natural Tregs (nTreg) generated in the thymus and subsequently directed to the tumor by soluble molecules such as TGFβ1 and PGE-2 (5). Tregs have also been shown to be regulated in cancer and other disease states by inflammatory molecules such as PGE-2 and COX2 (6, 7). The inflammatory molecule inducible nitric oxide synthase (iNOS) is overexpressed by many solid tumors and a known mediator of tumor-mediated immunosuppression, in part, through its well-described...
role as an effector mechanism of MDSC-mediated T-cell inactivation. We have previously shown (8) that iNOS also plays a role in the regulation of MDSC inhibition by demonstrating that iNOS drives cancer-mediated MDSC accumulation and upregulation of STAT3 and reactive oxygen species (ROS) required for inhibition of T-cell activation.

However, little is known about the effect of iNOS expression on Treg induction beyond a handful of studies showing that nitric oxide (NO) supplied by chemical donors or iNOS-expressing myeloid cells can suppress Treg induction (9, 10), and nothing about the potential role of iNOS in regulating Treg in the context of cancer. In support of a regulatory role for CD4+ T-cell–expressed iNOS, we have previously shown that T cell–expressed iNOS plays a negative regulatory role in the induction of Th17 cells by nitration of the Th17-specific transcription factor RORγT (11). Thus, there is some evidence that NO and T cell–expressed iNOS regulate uncommitted CD4 T-cell fate and could play a role in regulation of Treg induction by cancer cells.

We hypothesized that host-expressed iNOS may play a negative regulatory role in Treg induction by cancer cells, and that the beneficial effect of iNOS inhibition on MDSC is antagonized by simultaneous Treg induction, thus limiting the ability of iNOS inhibition to restore host immune function. We tested this hypothesis in the syngeneic MT-RET1 mouse melanoma model, and determined that iNOS expressed by CD4+ T cells inhibits their differentiation to Treg by antagonizing release of TGFβ1. Concurrent treatment of MT-RET1 melanoma-bearing mice with the iNOS inhibitor L-NIL and Treg depletion with low-dose cyclophosphamide simultaneously suppressed intratumoral accumulation of MDSC and Treg, while significantly enhancing CD8+ T-cell infiltration into the tumor. These beneficial immune effects were associated with marked immune-mediated growth inhibition of established tumor, demonstrating the therapeutic potential of combination treatment with iNOS inhibitors and Treg-depleting agents.

Materials and Methods

Mice and tumor models

C57BL/6, iNOS−/− (B6.129P2-Nos2tm1Lau/J), C57BL/6 Foxp3tm1(CAG)J, and Rag−/− (B6.129S7-Rag1tm1Mom/J) mice were obtained from The Jackson Laboratory and housed in the Icahn School of Medicine at Mount Sinai (ISMMS; New York, NY) animal facility under specific pathogen-free conditions. All animal experiments were performed in accordance with the regulations of the local Institutional Animal Care and Use Committee. The MT-RET-1 mouse melanoma tumor cell line (C57BL/6 background) is a transplantable tumor developed from a spontaneous melanoma growing in the MT-RET transgenic mouse (provided by Willem Overwijk, University of Texas MD Anderson Cancer Center, Houston, TX). Because this line is not available from a commercial cell bank and has not yet been genetically characterized, authentication by genetic analysis was not performed. However, MT-RET-1 cells in our laboratory are routinely examined for stability of cell morphology (bright field microscopy) and pigmentation (visual analysis of pelleted cells).

Ex vivo generation of Tregs

Splenocytes (106) or purified CD4+ T cells (purified with the Miltenyi MACS positive selection kit according to the manufacturer’s directions) were derived from spleens of C57BL/6 mice and activated with soluble anti-CD3 (0.5 µg/mL) and anti-CD28 (0.5 µg/mL) antibodies in the presence or absence of exogenous L-NIL (1 mmol/L) or TGFβ1 (10 ng/mL) for a period of 5 days after which cells were harvested and stained for CD4+FoxP3+ cells and analyzed by flow cytometry.

Intracellular staining

Cells were surface-stained with CD4 PERCP and CD8 Pacific Blue antibodies for 30 minutes in the dark at 4°C. Cells were then washed, spun, and resuspended in Fix/Perm Buffer (ebiosciences) as per the manufacturer’s instructions overnight in the dark at 4°C. Cells were then spun and resuspended in permeabilization buffer for 30 minutes in the dark, then washed with perm wash, and stained with FOXP3-PECY7, RORγt-APC, or IFN-γ FITC or monoclonal antibody against iNOS (all antibodies were purchased from ebiosciences) for 1 hour at 4°C after they were washed with perm wash and resuspended in 300 µL of staining buffer for FACS analysis using LSR Fortessa (BD Biosciences). Data were analyzed using FlowJo 7.6 software.

NO measurement

Unfractionated splenocytes derived from C57BL/6 mice were activated with soluble anti-CD3 and anti-CD28
antibodies in the presence or absence of L-NIL (1 mmol/L). Cells were harvested at 48 hours after activation and surface stained for CD4 T cells for 30 minutes in the dark at 4°C, after which they were washed twice with staining buffer and resuspended in DAF-DA FM (Millipore; Cat no: 251520 at 30 μmol/L) containing PBS buffer containing calcium and magnesium for 15 minutes and incubated at 37°C, covered in foil. Cells were then washed once and resuspended in FACS buffer and analyzed by flow cytometry.

Animal protocols
Mice were injected subcutaneously (s.c.) with 3 × 105 MT-RET tumor cells in PBS suspension. Tumors were measured every 2 to 4 days with electronic calipers. Tumor sizes were determined according to the bidimensional product of the longest measurement × its perpendicular. Once tumors became established (>30 mm², roughly 2 weeks), half the mice received L-NIL (2%) in drinking water for the remainder of the experiment (generally 7 days), and the other half received plain drinking water. Some groups of tumorbearing mice also received a one-time intraperitoneal (i.p.) administration of low-dose cyclophosphamide (100 mg/kg of bodyweight) with or without L-NIL administered in drinking water and replenished every day as mentioned above. After completing the course of L-NIL, all mice were sacrificed and spleens harvested. Blood was collected by cardiac puncture and allowed to clot at room temperature to obtain serum.

Cell harvesting/purification
Spleens were mashed on a 40-m mesh cup (Fisherbrand) placed on top of a 50-mL tube, using a syringe plunger. RPMI (10 mL) containing 1% FBS was added to the mesh cup to elute the splenocytes, and cells were centrifuged at 1,400 rpm for 10 minutes at 4°C. The pellet was resuspended in 2 mL of ACK red blood cell lysis buffer (GIBCO) and incubated at room temperature for 3 minutes to remove red cells. Cells were washed with RPMI containing 1% FBS and the pellet was resuspended in 2 mL of ACK red blood cell lysis buffer and incubated at room temperature for 30 minutes to remove red cells. Then, the cell suspension was centrifuged and the supernatant was removed. The pellet was resuspended in 2 mL of RPMI-CM. Tumor-infiltrating lymphocytes (TIL) were isolated by ficoll gradient centrifugation. FOXP3+ cells were intracellularly stained for in splenocytes and TIL described previously (8).

Treg suppression assay
Splenocytes from RFP-foxp3 reporter mice were stimulated with soluble anti-CD3 and anti-CD28 antibodies in the presence or absence of exogenous TGFβ1 (10 ng/mL) cells or L-NIL (1 mmol/L) for 5 days after which RFP+ T cells were sorted using a MoFlo sorter (Becton-Coulter). Sorted RFP+ Treg were added to CFSE (carboxyfluorescein diacetate succinimidyl ester)-labeled wild-type (WT) splenocytes at a different ratios in 96-well plates and activated with soluble anti-CD3 (0.5 μg/mL) + anti-CD28 (0.5 μg/mL) antibodies for 72 hours at 37°C. Cells were harvested in a 96-well plate and washed with staining buffer before staining with anti-CD4 and -CD8 monoclonal antibodies. CFSE dilution was measured by flow cytometry using LSR Fortessa (BD Biosciences).

TGFβ1 ELISA
Both total and bioactive (free) TGFβ1 in serum from MT-RET tumor-bearing mice, culture supernatants from activated splenocytes or CD4 T cells was determined by ELISA (PromegaTGFβ1, Emax ImmunoAssay Systems) as per the manufacturer’s instructions.

RNA isolation and quantitative real-time RT-PCR
Total RNA was extracted from WT or iNOS knockout C57/B6. Splenocytes or CD4+ T cells activated with soluble anti-CD3 and anti-CD28 antibodies in the presence or absence of L-NIL with TRIzol (Life technologies) according to the manufacturer’s instructions. cDNA was generated with an oligo (dT) primer and the SuperScript II system (Invitrogen), and quantitative PCR performed with the iCycler system with SYBR Green PCR master mix (Applied Biosystems). During analysis, results were normalized to expression of ubiquitin. The following primer sets were used:

TGFβ1 forward: CCC GAA GCG GAC TAC TAT GC
TGFβ1 reverse: CGA ATG TCT GAT GTA TIG AAG A A
ubiquitin forward: TGG CTA TTA ATT ATT CGG TCT GCA
ubiquitin reverse: GCA AGT GGC TAG AGT GCA GAG TAA

Human PBMC isolation and Treg generation
Blood was collected from healthy control individuals under an approved ISMMS Institutional Review Board (IRB) protocol (GC3-10-1219) and peripheral blood mononuclear cell (PBMC) isolated by the ficoll gradient method. Naïve CD4+ T cells (CD45 RO- cells) were sorted to 100% purity. The sorted cells were added at 1 × 10^6 cells per well to a 48-well plate coated with OKT3 antibody (1 μg/mL) for 18 hours overnight before cell culture. The CD4+ T cells were activated for 5 days in the presence or absence of L-NIL (1 mmol/L). Cells were harvested and surface-stained for CD4 and intracellularly stained for FOXP3 as per the manufacturer’s instructions and analyzed with a BD Fortessa flow cytometer.

TGFβ1 promoter luciferase reporter assay
HEK293 cells were transiently transfected with a TGFβ1 promoter luciferase reporter plasmid in Lipofectamine 2000 before incubation in the presence of the NO donor SNAP at different concentrations. The cells were incubated for 30 hours and harvested in reporter lysis buffer (Promega) for determination of luciferase activity. Cells were cotransfected with α-galactosidase reporter plasmid to normalize experiments for transfection efficiency.

Results
INOS inhibition has divergent effects on levels of MDSC and Treg in tumor-bearing mice
We have previously shown that pharmacologic inhibition of iNOS leads to decreased number and suppressive function of intratumoral GR-1+ CD11b+ MDSC (8). However, the effect of iNOS inhibition on Treg levels in
tumor-bearing hosts is unknown. We harvested splenocytes and TIL from control and L-NIL-treated (7 days) WT and iNOS knockout MT-RET-1 tumor-bearing mice 21 days after initial tumor inoculum and analyzed numbers of MDSC and Treg. We found that L-NIL treatment increased the percentages of CD4^+FOXP3^+ Treg in the spleen and tumor of WT mice (Fig. 1A and C). We also found the basal level of Tregs to be higher in spleen and tumor of untreated iNOS knockout mice, and treatment with L-NIL did not further increase Treg numbers (Fig. 1A and C). These data clearly
demonstrate that inhibition of host-derived iNOS is sufficient to drive upregulation of Treg in tumor-bearing mice. The increase in Treg is in contrast to the effect of iNOS inhibition or knockdown on GR-1\(^+\) CD11b\(^-\) MDSC, where we saw the anticipated decrease in intratumoral MDSC number (Fig. 1B and C). Treatment of tumor-bearing mice with L-NIL did not significantly alter tumor volume (Fig. 1D), a potential confounder of Treg and MDSC accumulation in tumor. These data suggest distinct and opposing effects of host iNOS expression on Treg and MDSC numbers in tumor-bearing mice. A similar increase in splenic Treg numbers was observed in L-NIL-treated or iNOS knockout non-tumor-bearing mice (Supplementary Fig. S1), confirming that inhibition of host-expressed iNOS is sufficient to drive Treg accumulation.

Because TGF\(_{\beta}\) is a well-described regulator of Treg differentiation and function, we determined the effect of iNOS inhibition/knockout on serum levels of free (bioactive) and total TGF\(_{\beta}\)1 by ELISA. We found that absolute levels of free (bioactive) and total TGF\(_{\beta}\)1 were significantly increased in L-NIL-treated or iNOS knockout mice (Fig. 1E), without a significant change in the ratio of free to total TGF\(_{\beta}\)1 (Fig. 1F). This suggests that negative regulation of TGF\(_{\beta}\)1 production by iNOS may be a potential mechanism limiting Treg accumulation in iNOS-expressing mice.

**iNOS expression is upregulated in CD4\(^+\) T cells in tumor-bearing mice, and activated CD4\(^+\) T cells**

Because the data presented above clearly implicate host iNOS as a regulator of FOXP3 expression in Treg, we sought to determine whether CD4\(^+\) T cells in our melanoma model expressed iNOS, as we have previously demonstrated in the context of regulation of Th17 responses (11). Although intracellular staining for iNOS revealed that it was expressed at low levels by CD4\(^+\) splenocytes and CD4\(^+\) FOXP3\(^+\) cells in tumor-free mice, both the percentage of iNOS-expressing CD4\(^+\) T cells and the average level of iNOS expression were significantly upregulated in tumor-bearing mice (Fig. 2A, i). When we examined iNOS expression in purified CD4\(^+\) T cells FACS sorted from spleens of WT C57/BL6 mice ex vivo (Fig. 2B), we observed relatively low levels of iNOS expression in unactivated CD4\(^+\) cells, which were markedly upregulated by activation for 5 days with anti-CD3 + anti-CD28 mAbs. iNOS was expressed by both FOXP3\(^+\) and FOXP3\(^-\) CD4\(^+\) T cells after activation. Furthermore, we found that NO levels were increased in CD4\(^+\) T cells (measured by DAF-DA staining) after anti-CD3 + anti-CD28 activation (Fig. 2C, i), and that this increase was abrogated by treatment with L-NIL. In parallel, we confirmed that nitrotyrosine immunostaining (a biomarker of cumulative NO exposure) in CD4\(^+\) T cells mirrored the NO levels measured by DAF-DA in all treatment groups (Fig. 2C, ii). Together, these data demonstrate that CD4\(^+\) T cells express iNOS, that iNOS expression is accompanied by elevated levels of intracellular NO, and that iNOS and NO expression levels are regulated dynamically across T-cell activation states, consistent with a potential role in directing the fate of activated CD4\(^+\) T cells. We also confirmed that treatment with the iNOS-selective inhibitor L-NIL downregulates intracellular NO in activated CD4\(^+\) T cells, as expected.

**iNOS expression by CD4\(^+\) T cells inhibits Treg accumulation without altering their suppressive function**

To understand the potential role of T-cell–expressed iNOS in Treg induction and function, we analyzed the effect of iNOS inhibition/knockout on Treg generated from unfractonated splenocytes and purified CD4\(^+\) T cells in ex vivo culture. Unfractionated splenocytes or purified CD4\(^+\) T cells from WT or iNOS knockout naïve (tumor-free) mice were activated with soluble anti-CD3 and anti-CD28 antibodies in the presence or absence of L-NIL or under Treg-polarizing conditions in the presence of exogenous TGF\(_{\beta}\)1 as described in the Materials and Methods. Activation of either purified CD4\(^+\) T cells (Fig. 3A and C, i) or unfractionated splenocytes (Fig. 3B and E) in the presence of TGF\(_{\beta}\)1 induced 3- to 4-fold increase in FOXP3\(^+\) Treg over baseline levels. Surprisingly, for both purified CD4\(^+\) cells (Fig. 3A and C, i) and unfractionated splenocytes (3B), WT cells cultured in the presence of L-NIL and cells isolated from iNOS knockout mice generated FOXP3\(^+\) Treg at levels comparable with culture in the presence of TGF\(_{\beta}\)1. Although iNOS inhibition during culture of CD4\(^+\) T cells under Th1 (9) or Th17 (11) polarizing conditions has been shown to inhibit (Th1) or enhance (Th17) polarization, respectively, we did not observe an effect of either L-NIL treatment or iNOS knockout on intracellular IFN-\(\gamma\) levels consistent with altered Th1 polarization (Fig. 3C, iii). We did observe significantly increased expression of ROR\(_\gamma\)T with both L-NIL treatment and iNOS knockout (Fig. 3C, ii), consistent with our previously published observations; however, increased ROR\(_\gamma\)T expression was not accompanied by enhanced Th17 function, as measured by IL17 ELISA (Fig. 3C, ii).

We next sought to determine whether iNOS inhibition also affects the suppressive function of CD4\(^+\) FOXP3\(^+\) cells. For this, we obtained splenocytes from RFP\(^+\) foxp3 reporter mice and activated them with anti-CD3 + anti-CD28 in the presence or absence of L-NIL or TGF\(_{\beta}\)1 as described above. RFP\(^+\) cells were then sorted and cocultured with CFSE-labeled, anti-CD3/anti-CD28-activated WT splenocytes at various ratios for 3 days to determine their effect on proliferation. T-cell proliferation was measured by CFSE dilution in both CD4 and CD8 T-cell populations by flow cytometry. Suppression of CD4\(^+\) and CD8\(^+\) T-cell proliferation by RFP\(^+\) foxp3 Treg on a per-cell basis was unaffected by treatment with L-NIL (Fig. 3D).

Because molecular mechanisms induced by activation of CD4\(^+\) T cells with anti-CD3/CD28 antibodies may differ from those occurring during stimulation with specific antigen, we activated unfractionated OT-2 splenocytes with Ova323–347 in the presence or absence of L-NIL or exogenously added TGF\(_{\beta}\)1 for 5 days and measured CD4\(^+\) FOXP3\(^+\) generation (Fig. 3E). We found that L-NIL treatment was similarly effective in increasing the number of CD4\(^+\) FOXP3\(^+\) Treg cultured from OT-2 splenocytes, and Treg numbers were comparable with those observed when
cells were activated in the presence of TGFβ1. The above data demonstrate that iNOS expression by CD4+ T cells themselves acts to repress differentiation of these cells to FOXP3+ Treg, without altering the suppressive function of FOXP3+ cells.

**iNOS expression inhibits Treg induction by repressing TGFβ1 production at the transcriptional level**

We hypothesized that iNOS controls Treg induction indirectly by modulating TGFβ1 production. To test this hypothesis, anti-CD3/anti-CD28–activated unfractionated

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**Figure 2.** iNOS expression in CD4+ T cells from tumor-bearing mice and activated CD4+ and CD4+ FOXP3+ T cells. Splenocytes were isolated from tumor-free or tumor-bearing (day 21) C57/B6 mice and intracellular staining was performed for FOXP3 and iNOS expression in CD4+ cells. Ai, representative FACS plots of iNOS expression in CD4+ T cells from tumor-free and tumor-bearing mice and graphical representation of aggregated data. Aii, iNOS expression by CD4+ FOXP3+ cells as mean fluorescence intensity (MFI) and graphical representation of aggregated data from non-tumor and tumor-bearing mice. B, purified CD4+ T cells were isolated from C57/B6 mice cultured 5 days ex vivo in the presence or absence of anti-CD3/anti-CD28 mAb with or without exogenous TGFβ1. Bi, representative FACS plots of iNOS expression in CD4+ T cells, and graphical representation of aggregated data showing a significant increase in iNOS expression in activated CD4+ T cells. Bii, iNOS expression by CD4+ FOXP3+ cells represented as MFI and graphical representation of aggregated data from non-tumor and tumor-bearing mice. All graphs represent pooled data from at least two experiments with at least 5 mice per group. C, intracellular NO levels as measured by (i) DAF-DA fluorescence and (ii) nitrotyrosine immunostaining of CD4+ T cells from splenocytes stimulated with anti-CD3 and anti-CD28 antibodies in the presence of absence of L-NIL 48 hours after activation. Tables show cumulative DAF-DA or nitrotyrosine data derived from n = 6 samples in two independent experiments. *, P < 0.05 significance.
Figure 3. The effect of iNOS inhibition on CD4+ T cell commitment and Treg-suppressive function. A, representative FACS plots of CD4+ FOXP3+ Treg after 5-day ex vivo culture of purified splenocytes harvested from WT or iNOS knockout mice activated with soluble anti-CD3/CD28 antibodies in the presence or absence of L-NIL or TGFβ1. B, relative percentage of CD4+ FOXP3+ Treg generated from aCD3+aCD28-activated unfractionated splenocytes after ex vivo culture in the presence or absence of TGFβ1. C, CD4 T cells were purified from WT or iNOS knockout splenocytes and activated with anti-CD3/CD28 as previously described for 5 days in the presence or absence of L-NIL. Foxp3 (i), RORγt (ii), and IFNγ (iii) expression of CD4 T cells was determined by intracellular staining. Levels of IL17 were also measured from culture supernatants (ii). D, splenocytes from RFP-FOXP3 reporter mice were activated with anti-CD3/CD28 as previously described and cultured in the presence of exogenous L-NIL or TGFβ1 to generate CD4+ FOXP3+ Treg. Tregs were sorted and cocultured with various ratios of CFSE-labeled WT CD4+ or CD8+ T cells, in the presence of soluble anti-CD3/CD28 antibodies for 3 days. CD4+ and CD8+ T-cell proliferation was evaluated by CFSE dilution. The graph depicts percentage of T cells (CD4 or CD8 from TGFβ1, control, and L-NIL groups) normalized to T cells activated with anti-CD3/anti-CD28 alone. E, relative percentage of CD4+ FOXP3+ Treg generated ex vivo from WT C57BL/6 splenocytes (activated with soluble anti-CD3/CD28) or OT-2 splenocytes (activated with Ova323) for 5 days in the presence or absence of L-NIL. All graphs represent pooled data from at least three experiments with at least 5 mice per experiment.
splenocytes or purified CD4 T cells from naive WT or iNOS knockout mice were cultured ex vivo in the presence or absence of L-NIL for 5 days as described in Fig. 2. A, culture supernatants were assayed for bioactive and total TGFβ1 levels. The graphs depict fold change of TGFβ1 release relative to untreated splenocytes or CD4 T cells from WT mice. B, CD4 T cells from splenocyte cultures were stained for intracellular TGFβ1; (i) representative FACS plots and (ii) data expressed as fold increase relative to untreated cells are shown. C, i, cDNA from purified CD4 T cells or unfractionated splenocytes from untreated and L-NIL–treated cultures were analyzed for TGFβ1 transcript levels by quantitative PCR. Graph shows relative TGFβ1 mRNA levels normalized to ubiquitin levels. C, ii, the promoter activity in HEK293 cells transfected with full-length TGFβ1 promoter construct in the presence or absence of SNAP are depicted in the graph. D, dose-dependent decrease of FOXP3+ Treg after neutralization of TGFβ1 with anti-TGFβ1 antibody in splenocyte cultures activated in the presence of L-NIL. Each graph represents data from at least two independent experiments.

where if anything we observed a reciprocal decrease in the number of TGFβ1-expressing cells, although these changes did not achieve statistical significance.

Because both free and total TGFβ1 levels increased with iNOS inhibition, we hypothesized that iNOS controls TGFβ1 production at the transcriptional level. We performed quantitative RT-PCR to determine tgf-β1 mRNA levels in unfraccionated splenocytes or purified CD4 T cells from WT or iNOS knockout mice activated in the presence or absence of L-NIL as previously described. iNOS inhibition markedly increased tgf-β1 message levels in both unfractionated splenocytes and purified CD4 T cells (Fig. 4C, i). Also the basal levels of tgf-β1 were significantly higher in splenocytes and CD4 T cells derived from iNOS knockout mice compared with their
WT counterparts, further confirming the role of iNOS as a regulator of tgf-β1 transcription. To further confirm whether iNOS controls TGFβ promoter activity, HEK293 cells were transfected with human full-length TGFβ promoter in the presence or absence of the NO donor SNAP at various concentrations (Fig. 4C, ii). It was seen that NO significantly decreases TGFβ promoter activity as read out by luciferase assay in a dose-dependent fashion. These data provide strong evidence of NO’s role in affecting TGFβ activity. The increase in Treg induction by iNOS inhibition was shown to depend upon elevated TGFβ1 production, because the ability of L-NIL to enhance accumulation of Treg in ex vivo culture was abrogated by neutralizing antibody to TGFβ1 (Fig. 4D). Collectively, the above data demonstrate that iNOS expression inhibits induction of FOXP3+ Treg from CD4+ T cells by repression of TGFβ1 transcription in CD4+ T cells themselves.

**Combination therapy with L-NIL and cyclophosphamide effectively suppresses MDSC and Treg levels while boosting tumor-infiltrating T cells, leading to robust immune-mediated inhibition of tumor growth**

We have previously shown that iNOS inhibition in tumor-bearing mice suppresses intratumoral accumulation and functional activity of MDSC, resulting in a modest reduction in tumor growth (8). We hypothesized that the beneficial effects of iNOS inhibition on MDSC were offset by the reciprocal rise in Treg numbers observed in Fig. 1, and that simultaneous targeting of MDSC with L-NIL and Treg with low-dose cyclophosphamide would have a greater antitumor effect than either agent alone. We tested this hypothesis in the syngeneic MT-RET-1 melanoma model that we previously described (8); however, while in our previous tumor growth experiments we treated mice on day 3 after tumor injection, for this study we treated more resistant 14-day tumors. MT-RET-1 tumor-bearing mice were treated for 7 days with L-NIL in drinking water, and/or a one-time i.p. injection of low-dose cyclophosphamide. Parallel experiments were conducted in WT C57/BL6 mice; iNOS knockout mice; and syngeneic RAG knockout mice, which lack adaptive immune cells, to determine whether antitumor effects of the treatment regimen were immune-mediated.

Splenocytes and TIL were harvested on day 21 after initial tumor inoculum (day 7 of treatment) as previously described and analyzed for MDSC, CD4, and CD8 T cells, and CD4+ FOXP3+ Treg. iNOS inhibition with L-NIL and/or knockout of host iNOS led to the expected decrease in intratumoral MDSC and elevated number of Treg in spleen and tumor. A single dose of cyclophosphamide alone had little effect on Treg levels in tumor and spleen (Fig. 5A) but caused an unexpectedly sharp decrease in the number of tumor-infiltrating MDSC (Fig. 5A, lower right). Combination therapy with L-NIL and cyclophosphamide reversed the increase in Treg seen with L-NIL monotherapy, driving Treg levels in spleen and tumor below baseline, while strongly reducing the number of intratumoral MDSC.

Although low-dose cyclophosphamide has been shown to efficiently deplete Treg in both mouse and human studies, it can also have immunoablative effects on effector T-cell populations, particularly tumor-infiltrating CD8+ CTL (12, 13). In the MT-RET-1 model, cyclophosphamide strongly depleted intratumoral CD4+ and CD8+ T cells, as well as CD8+ T cells in spleen (Fig. 5B). On the contrary, L-NIL boosted CD4+ and CD8+ T-cell numbers in tumor, and to a lesser degree in spleen, as we have previously described (8). However, the most favorable results were seen with combined L-NIL + cyclophosphamide treatment, which strongly increased the number of tumor-infiltrating CD8+ T cells, and sharply elevated the ratios of intratumoral CD8+ Treg and CD8+MDSC (Fig. 5C). We conclude that combination therapy with L-NIL and cyclophosphamide has beneficial effects on the tumor immune microenvironment, including a relative reduction in Treg and MDSC and strongly enhanced infiltration of CD8+ CTL.

We examined growth of MT-RET-1 tumors in mice treated with these different regimens (L-NIL in drinking water given continuously until the end of the experiment or a single i.p. dose of cyclophosphamide) beginning day 14 to determine whether the beneficial immunologic effects of combination therapy result in enhanced antitumor efficacy. Although tumor growth in mice treated with either L-NIL or cyclophosphamide monotherapy was not significantly different from that in untreated mice (Fig. 5D), combination therapy markedly inhibited tumor growth, which plateaued after 7 days of treatment with 6 of 9 mice still alive on day 25. However, the beneficial effect of combination treatment on tumor growth was not observed in RAG knockout mice, demonstrating that the antitumor effect of L-NIL and cyclophosphamide is dependent on adaptive immune cells (T and/or B cells). Thus, we conclude that combination therapy with iNOS inhibition and low-dose cyclophosphamide reverses both the L-NIL-mediated increase in Treg, and the cyclophosphamide-mediated ablation of CD8+ T cells, leading to strongly enhanced immune-mediated tumor control.

**iNOS regulates TGFβ1 production and Treg induction in human CD4+ T cells**

Because iNOS and NO production can be regulated differently in human and mouse immune cells, we sought to determine whether iNOS inhibition enhances the induction of Treg from naive human CD4+ T cells in ex vivo culture. PBMCs were harvested from healthy donors, and naive (CD45RO-) CD4+ T cells sorted by flow cytometry. Naive CD4+ T cells were activated with plate-bound OKT3 antibody for 5 days in the presence or absence of L-NIL or TGFβ1 before quantification of Treg by flow cytometry. Baseline levels of Treg (identified by intracellularFoxp3+ or surface CD127lo staining of CD4+ cells) were consistently 3% to 4% of cultured cells, and increased by roughly 2-fold after culture with TGFβ1 or L-NIL (Fig. 6A and B). The increase in Treg number was associated with a striking increase in TGFβ1 production by CD4+ T cells, although the increase in free (bioactive) TGFβ1 was more...
Figure 5. Effect of combination therapy with L-NIL and cyclophosphamide on the immunosuppressive tumor microenvironment and tumor growth. WT or iNOS knockout mice were injected s.c. with 3 × 10⁵ syngeneic MT-RET-1 melanoma cells and treated starting on day 14 with L-NIL (provided continuously in drinking water) and/or cyclophosphamide (single 20 mg/mL i.p. injection on day 14). Mice were harvested on day 21 after initial tumor injection and analyzed for: A, CD4⁺ FoxP3⁺ Treg and MDSC populations in spleen and tumor. B, relative (top) and absolute (bottom) numbers of CD4 and CD8 T cells in spleen and tumor. C, the ratio of the absolute number of intratumoral CD8⁺ T cells to intratumoral Foxp3⁺ Tregs or MDSC. D, 3 × 10⁵ MT-RET-1 cells were injected into WT or RAG knockout mice. Once the tumors reached approximately 30 mm³ in diameter, mice were randomized into treatment groups, and treated with a one-time i.p. dose of cyclophosphamide and/or L-NIL administered in drinking water for 7 days (treatment start indicated by arrow). Tumor sizes were recorded three times weekly. Each graph above shows pooled data from at least three experiments with 5 mice per treatment group.
modest (Fig. 6C). Thus, we conclude that the regulation of FOXP3 expression and Treg induction in human CD4+ T cells by iNOS occurs through a mechanism fundamentally similar to that which we have described in mice.

**Discussion**

The role of NO in immunologic functions can appear contradictory, such as in cancer where NO is an effector mechanism of tumor killing by M1-polarized macrophages (14, 15) but is also found to drive tumor-mediated immunosuppression in many cancer models (16). Such seeming contradiction suggests that compartmentalized expression of NO and NO-producing molecules, such as iNOS, could tip the balance toward immunity or immunosuppression depending on the cell types in which they are expressed. This study describes one such example, where iNOS expression in the myeloid compartment drives MDSC-mediated immunosuppression, while expression in CD4+ T cells acts to limit FOXP3 expression and accumulation of Treg. This antagonistic balance of MDSC and Treg limits the beneficial effects of iNOS inhibition on antitumor immunity, but can be overcome by concurrently targeting Treg with an iNOS inhibitor and low-dose cyclophosphamide, resulting in robust immune-mediated tumor control in the MT-RET-1 model used in this study.

Prior studies of the role of NO in regulating Treg accumulation and function have focused on the effects of exogenously produced NO on CD4+ T cells. One study by Lee and colleagues (9) found that exogenous NO suppressed FOXP3+ Treg accumulation and skewed T-cell cultures toward a Th1 phenotype; they proposed that NO produced by TIP-DC/conventional DC coclusters could serve as a physiologic source of NO in vivo. Another study, by Brahmachari and Pahan (10), also found that ex vivo restimulation of splenocytes from MBP-immunized mice...
led to NO production that acted to suppress FOXP3+ Treg proliferation, because FOXP3+ Treg accumulation was enhanced by iNOS inhibition or use of iNOS knockout splenocytes. They proposed that iNOS expression by APC’s modulated FOXP3 expression in T cells, but did not identify the cell types in which iNOS expression was important in their experimental system. They further explored this concept (modulation of CD4+ T-cell differentiation to Treg by adjacent NO-producing APCs) in another article proposing that IL12p40 acted on APC to induce NO capable of modulating Treg induction (17). Although these mechanisms are all plausible, these authors did not consider the possibility that iNOS expression in CD4+ T cells themselves may be an important source of NO capable of regulating Treg induction in a cell-autonomous fashion, such as we have previously described for regulation of Th17 differentiation.

We propose that iNOS expression in CD4+ T cells themselves acts to limit FOXP3+ expression by suppressing release of TGFβ1, a “master regulator” of CD4+ T-cell differentiation to the Treg phenotype. This model is supported by our findings that (i) CD4+ FOXP3+ T cells express iNOS; (ii) Treg accumulation is enhanced when purified CD4+ T cells are cultured ex vivo in the presence of an iNOS inhibitor, or when CD4+ T cells are sorted from iNOS knockout splenocytes; and (iii) TGFβ levels are significantly increased by iNOS inhibition in vitro and in vivo, and TGFβ-depleting antibody is sufficient to abolish iNOS inhibition-induced Treg accumulation in ex vivo culture. Control of TGFβ1 appears to act primarily at the transcriptional level, because both iNOS inhibition and knockout upregulate (gf-b1) mRNA without consistently altering the ratio of total to free TGFβ1. According to this model, expression of iNOS in CD4+ T cells is sufficient to suppress their differentiation to Treg independent of iNOS expression in myeloid-derived APC, although exogenous NO may act as an additional regulator of FOXP3 expression in CD4+ cells.

This model suggests that iNOS expression is independently controlled in myeloid and lymphoid regulatory cell compartments: promoting MDSC-mediated immunosuppression while “putting the brakes” on FOXP3+ Treg production. In both cases, iNOS acts by positively (VEGF/MDSC) and negatively (TGFβ1/Treg) modulating release of soluble mediators responsible for driving induction of immunosuppressive cell types. This could potentially provide an additional level of fine regulatory control that can rheostatically adjust myeloid and lymphoid regulatory compartments independently and maintain the balance of regulatory and effector immune function across a wide range of inflammatory states. Importantly, we observed negative regulation of TGFβ1 and FOXP3 expression by iNOS in both human and mouse CD4+ T cells, suggesting that this model is potentially relevant to human disease states.

A detailed understanding of the regulatory mechanisms contributing to cancer-induced immunosuppression can lead to improved therapeutic approaches. In this case, the observation that iNOS inhibition enhances Treg accumulation in tumor-bearing mice led to a combination treatment strategy that simultaneously targets MDSC and Treg, causing a dramatic restoration of immune function. Although each agent used in isolation has beneficial effects on host immunity, these effects are limited by paradoxical induction of Treg (in the case of L-NIL) and ablation of tumor-infiltrating CD8+ T cells (in the case of cyclophosphamide). Combination therapy neutralizes both of these limitations, and leads to efficient and durable immune-mediated tumor control. This is a strategy that could be readily tested in human clinical cancer trials: low-dose cyclophosphamide is already an integral part of many immunotherapy regimens, and L-NIL has been previously tested in humans in a clinical asthma study (18). Other clinically available drugs that inhibit iNOS, such as sildenafil and doxycycline, could also be tested in combination with cyclophosphamide.

A limitation of this study is that the molecular mechanisms through which iNOS regulates TGFβ1 expression are not described. In fact, far more is known about regulation of TGFβ1 signaling downstream of TGFβ1 receptor activation than about regulation of TGFβ1 expression itself. The TGFβ1 promoter contains several binding sites for transcription factors that have previously been reported to be regulated by NO, including AP-1 (19–21) and FOXO1 (22). Similarly, while we have described here and elsewhere the upregulation of iNOS expression in CD4+ T cells after activation, the mechanism by which this occurs is similarly unclear. An attractive potential mediator of activation-induced iNOS upregulation is the transcription factor NF-kB, which is activated after TCR stimulation (23), and which has been shown to drive iNOS expression (24). Future investigations will examine the potential role of NF-kB activation in upregulation of iNOS expression in CD4+ T cells, and the effect of NO and iNOS overexpression on TGFβ1 promoter activity and transcription factor binding as a possible mechanism of regulatory control. A more detailed understanding of the signal transduction pathways connecting the TCR with iNOS, and iNOS with altered TGFβ1 expression may identify additional opportunities for therapeutic modulation of T-cell fate and activity.

In summary, we describe a novel mechanism in which iNOS expression by CD4+ cells FOXP3+ expression and limit their differentiation into Treg. We have translated this observation into a therapeutic approach with significant antitumor activity against an in vivo mouse model of melanoma. These data significantly enhance our understanding of the regulatory mechanisms directing the fate of activated CD4+ T cells, and suggest a clear pathway for translation of these findings to human clinical trials in patients with cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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