TITLE:
iNOS expression in CD4+ T cells limits T-reg induction by repressing TGFβ-1: combined iNOS inhibition and T-reg depletion unmask endogenous anti-tumor immunity

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iNOS in CD4+ T cells limits T-reg induction by repressing TGFβ

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TRANSLATIONAL RELEVANCE:

A detailed understanding of mechanisms regulating cancer-associated immunoregulatory cells, including myeloid derived suppressor cells (MDSC) and regulatory T cells (Treg) can lead to more effective strategies for cancer immunotherapy. We found that inducible nitric oxide synthase (iNOS) expression has divergent effects on myeloid- and lymphoid-derived regulatory cells, stimulating induction of the former and suppressing the later. Whereas iNOS inhibition suppresses intratumoral infiltration of MDSC, inhibition or knockout of iNOS in host CD4+ T cells upregulates FOXP3+ expression via a TGF-β1-dependent mechanism, thus limiting the beneficial immunologic effects of iNOS inhibition. Simultaneously targeting MDSC with an iNOS inhibitor and T-reg with low dose cyclophosphamide dramatically increased intratumoral accumulation of CD8+ T cells and enhanced immune-mediated control of melanoma in a syngeneic mouse model. Both cyclophosphamide and small molecule inhibitors of iNOS are clinically available, making this approach translationally relevant and suitable for human clinical trials in melanoma and other solid tumors.
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 anti-tumor immunity.

**Experimental design**: WT or iNOS KO mice bearing established MT-RET-1 melanoma were treated with the small molecule iNOS inhibitor L-NIL and/or cyclophosphamide (CTX) 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 TGF-β1 -depleting antibodies, and TGF-β1 levels assessed by ELISA.

**Results**: Whereas intratumoral 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 TGF-β1-dependent manner. While either iNOS inhibition or Treg depletion with low-dose CTX 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 TGF-β1 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 (T-reg) 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 immunosurveillance 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 T-reg cells in cancer. T-reg can be induced from naïve CD4+ T cells by molecules expressed by tumor or tumor-infiltrating cells (1-4), and by TGF-β1, which is often expressed at high levels in the tumor microenvironment. These so-called inducible T-reg (iT-reg) are supplemented by pre-existing natural T-reg cells (nT-reg) generated in the thymus and subsequently directed to the tumor by soluble molecules such as TGF-β1 and PGE-2 (5). T-reg 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 induction 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 T-reg induction beyond a handful of studies showing that nitric oxide (NO) supplied by chemical donors or iNOS-expressing myeloid cells can suppress T-reg induction(9, 10), and nothing about the potential role of iNOS in regulating T-reg 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 nitric oxide and T cell-expressed iNOS regulate uncommitted CD4 T cell fate and could play a role in regulation of T-reg induction by cancer cells.

We hypothesized that host-expressed iNOS may play a negative regulatory role in T-reg induction by cancer cells, and that the beneficial effect of iNOS inhibition on MDSC is
antagonized by simultaneous T-reg 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 T-reg by antagonizing release of TGF-β1. Concurrent treatment of MT-RET1 melanoma-bearing mice with the iNOS inhibitor L-NIL and T-reg depletion with low-dose cyclophosphamide simultaneously suppressed intratumoral accumulation of MDSC and T-reg, 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 T-reg depleting agents.
MATERIALS AND METHODS:

**Mice and Tumor models:** C57BL/6, iNOS−/−(B6.129P2-Nos2tm1Lau/J), C57BL/6 Foxp3tm1Flv/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) 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, TX). Since 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 T-reg cells:** 10⁶ splenocytes 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 (0.5µg/ml) and anti-CD28 (0.5µg/ml) antibodies in the presence or absence of exogenous L-NIL (1mM) or TGFβ-1 (10ng/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 hr at 4°C after they were washed with perm wash and re-suspended in 300 µl of staining buffer for FACS analysis using LSR Fortessa (BD Biosciences). Data was analyzed using flowjo 7.6 software.

**Nitric oxide measurement:** Unfractionated splenocytes derived from C57BL/6 mice were activated with soluble anti-CD3 and anti-CD28 antibodies in the presence or absence of LNIL (1mM). 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 µM) containing PBS ++ media.
for 15 minutes and incubated at $37^\circ$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 $3 \times 10^5$ MT-RET tumor cells in PBS suspension. Tumors were measured every 2-4 days with electronic calipers. Tumor sizes were determined according to the bi-dimensional product of the longest measurement x its perpendicular. Once tumors became established (>30 mm$^2$, 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 tumor bearing 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 micron mesh cup (Fisherbrand) placed on top of a 50 ml tube, using a syringe plunger. 10ml of RPMI containing 1% FBS was added to the mesh cup to elute the splenocytes, and cells were centrifuged at 1400 rpm for 10 minutes at $4^\circ$ C. The pellet was resuspended in 2ml of ACK lysing 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 2ml 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).

**T-reg 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 TGF-B1 (10ng/ml) cells or L-NIL (1mM) for 5 days after which RFP+ cells were sorted using a MoFlo sorter (Becton-Coulter). Sorted RFP+ T-reg were added to CFSE (Carboxyfluorescein diacetate succinimidy ester) labeled wild type splenocytes at a different ratios in 96 well plates and activated with soluble anti-CD3 (0.5µg/ml) + anti-CD28 (0.5µg/ml) antibodies for 72 hours at $37^\circ$ C. Cells were harvested in 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).

**TGF-β1 ELISA:** Both total and bio-active (free) TGF-β1 in serum from MT RET tumor bearing mice, culture supernatants from activated splenocytes or CD4 T cells was determined by ELISA (PromegaTGFβ1 E$_{max}$® ImmunoAssay Systems) as per the manufacturer’s instructions.
RNA isolation and quantitative real-time RT–PCR: Total RNA was extracted from WT or iNOS KO C57/BL6 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:

- TGFβ-1 fwd: CCC GAA GCG GAC TAC TAT GC
- TGFβ-1 rev: CGA ATG TCT GAC GTA TTG AAG AAC A
- ubiquitin fwd: TGG CTA TTA ATT ATT CGG TCT GCA
- ubiquitin rev: GCA AGT GGC TAG AGT GCA GAG TAA

Human PBMC isolation and T-reg generation: Blood was collected from healthy control individuals under an approved ISMMS IRB protocol (GCO# 10-1219) and 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 x 10^6 cells per well to a 48 well plate coated with OKT3 antibody (1µg/ml) for 18 hours overnight prior to cell culture. The CD4+ T cells were activated for 5 days in the presence or absence of L-NIL (1mM). Cells were harvested and surface stained for CD4 and intracellularly stained for FOXP3 as per manufacturer’s instructions and analyzed with a BD Fortessa flow cytometer.

TGF-β1 promoter luciferase reporter assay. HEK293 cells were transiently transfected with a TGF-β1 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 h and harvested in reporter lysis buffer (Promega) for determination of luciferase activity. Cells were co-transfected with a -galactosidase reporter plasmid to normalize experiments for transfection efficiency.
RESULTS:

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

Since TGF-β1 is a well-described regulator of T-reg differentiation and function, we determined the effect of iNOS inhibition/knockout on serum levels of free (bioactive) and total TGF-β1 by ELISA. We found that absolute levels of free (bioactive) and total TGF-β1 were significantly increased in L-NIL-treated or iNOS KO mice (Fig 1E), without a significant change in the ratio of free to total TGF-β1 (Fig. 1F). This suggests that negative regulation of TGF-β1 production by iNOS may is a potential mechanism limiting T-reg accumulation in iNOS-expressing mice.

**iNOS expression is upregulated in CD4+ T cells in tumor-bearing mice, and activated CD4+ T cells.** Since the data presented above clearly implicate host iNOS as a regulator of FOXP3 expression in T-reg, 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). While 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 was significantly upregulated in tumor-bearing mice (Fig. 2Ai). 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. Further, we found that NO levels were increased in CD4+ T cells (measured by DAF-DA staining) after anti-CD3 + anti-CD28 activation (Fig. 2Ci), 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 nitric oxide levels measured by DAF-DA in all treatment groups (Fig 2Cii). 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 nitric oxide 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 T-reg accumulation without altering their suppressive function.** In order to understand the potential role of T cell-expressed iNOS in T-reg induction and function, we analyzed the effect of iNOS inhibition/knockout on T-reg generated from unfractionated splenocytes and purified CD4+ T cells in ex vivo culture. Unfractionated splenocytes or purified CD4+ T cells from WT or iNOS KO naïve (tumor-free) mice were activated with soluble anti-CD3 and anti-CD28 antibodies in the presence or absence of L-NIL or under T-reg-polarizing conditions in the presence of exogenous TGF-β1 as described in the Materials and Methods. Activation of either purified CD4+ T cells (Fig 3A,Ci) or unfractionated splenocytes (Fig 3B,E) in the presence of TGF-β1 induced 3-4-fold increase in FOXP3+ T-reg over baseline levels. Surprisingly, for both purified CD4+ cells (Fig 3A,Ci) and unfractionated splenocytes (3B), WT cells cultured in the presence of L-NIL and cells isolated from iNOS KO mice generated FOXP3+ T-reg at levels comparable to culture in the presence of TGF-β1. While 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-γ levels consistent with altered Th1 polarization (Fig 3Ciii). We did observe significantly increased expression of RORγT with both L-NIL treatment and iNOS knockout (Fig 3Cii), consistent with our previously published observations, however increased RORγT
expression was not accompanied by enhanced Th17 function, as measured by IL-17 ELISA (Fig 3Cii).

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-β1 as described above. RFP+ cells were then sorted and co-cultured 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+ T-reg on a per-cell basis was unaffected by treatment with L-NIL (Fig 3D).

Since 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 Ova 323-347 in the presence or absence of L-NIL or exogenously added TGF-β1 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+ T-reg cultured from OT-2 splenocytes, and T-reg numbers were comparable to those observed when cells were activated in the presence of TGF-β1. The above data demonstrate that iNOS expression by CD4+ T cells themselves acts to repress differentiation of these cells to FOXP3+ T-reg, without altering the suppressive function of FOXP3+ cells.

**iNOS expression inhibits T-reg induction by repressing TGF-β1 production at the transcriptional level.** We hypothesized that iNOS controls T-reg induction indirectly by modulating TGF-β1 production. To test this hypothesis, anti-CD3/anti-CD28-activated unfractionated splenocytes or purified CD4 T cells from naïve WT or iNOS KO mice were cultured ex vivo in the presence or absence of L-NIL and supernatants were collected and assayed for bioactive and total TGF-β1 levels by ELISA. iNOS knockout or inhibition with L-NIL significantly increased both free and total TGF-β1 levels in culture supernatants from splenocytes or purified CD4+ T cells (Fig 4A). To further confirm that iNOS controls TGF-β1 production by CD4+ T cells themselves, we performed intracellular staining for TGFβ-1 in CD4+ T cells (Fig 4B) and other immune cell populations including MDSC, macrophages, and dendritic cells (DC) (supplemental fig S4) We observed a >2-fold increase in the number of TGF-β1 expressing CD4 T cells; (Fig 4Bi &ii) interestingly, this increase was not seen in myeloid cells (MDSC, macrophages, and DC), where if anything we observed a reciprocal decrease in
the number of TGF-β1 expressing cells, although these changes did not achieve statistical
significance.

Since both free and total TGF-β1 levels increased with iNOS inhibition, we hypothesized
that iNOS controls TGF-β1 production at the transcriptional level. We performed quantiative
RT-PCR to determine TGF-β1 mRNA levels in unfractionated splenocytes or purified CD4 T
cells from WT or iNOS KO 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 4Ci). Also the basal levels of TGF-β1 were
significantly higher in splenocytes and CD4 T cells derived from iNOS KO mice compared to
their WT counterparts, further confirming the role of iNOS as a regulator of TGFβ-1
transcription. In order to further confirm if iNOS controls TGF-β1 promoter activity, HEK293 cells
were transfected with human full length TGF-β1 promoter in the presence or absence of the nitric
oxide donor SNAP at various concentrations (Fig 4Cii). It was seen that nitric oxide significantly
decreases TGF-β1 promoter activity as read out by luciferase assay in a dose-dependent
fashion. This data provides strong evidence of nitric oxide's role in affecting TGF-β activity. The
increase in T-reg induction by iNOS inhibition was shown to depend upon elevated TGF-β1
production, since the ability of L-NIL to enhance accumulation of T-reg in ex vivo culture was
abrogated by neutralizing antibody to TGF-β1 (Fig. 4D). Collectively, the above data
demonstrate that iNOS expression inhibits induction of FOXP3+ T-reg from CD4+ T cells by
repressing TGF-β1 transcription in CD4+ T cells themselves.

Combination therapy with L-NIL and cyclophosphamide effectively suppresses
MDSC and T-reg 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 T-reg numbers observed
in Figure 1, and that simultaneous targeting of MDSC with L-NIL and T-reg with low-dose
cyclophosphamide would have a greater anti-tumor 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 (CTX). Parallel experiments were conducted in WT C57/BL6 mice; iNOS KO
mice; and syngeneic RAG KO mice, which lack adaptive immune cells, to determine whether anti-tumor 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+ T-reg. iNOS inhibition with L-NIL and/or knockout of host iNOS led to the expected decrease in intratumoral MDSC and elevated number of T-reg in spleen and tumor. A single dose of CTX alone had little effect on T-reg levels in tumor and spleen (Fig. 5A) but caused an unexpectedly sharp decrease in the number of tumor-infiltrating MDSC (Fig. 5A, lower right panel). Combination therapy with L-NIL and CTX reversed the increase in T-reg seen with L-NIL monotherapy, driving T-reg levels in spleen and tumor below baseline, while strongly reducing the number of intratumoral MDSC.

While low-dose CTX has been shown to efficiently deplete T-reg 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, CTX 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 + CTX treatment, which strongly increased the number of tumor-infiltrating CD8+ T cells, and sharply elevated the ratios of intratumoral CD8:T-reg and CD8:MDSC (Fig 5C). We conclude that combination therapy with L-NIL and CTX has beneficial effects on the tumor immune microenvironment, including a relative reduction in T-reg 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 CTX) beginning day 14 to determine whether the beneficial immunologic effects of combination therapy result in enhanced anti-tumor efficacy. While tumor growth in mice treated with either L-NIL or CTX monotherapy was not significantly different from that in untreated mice (Fig. 5D), combination therapy markedly inhibited tumor growth, which plateaued after 7 days treatment with 6/9 mice still alive on day 25. However the beneficial effect of combination treatment on tumor growth was not observed in RAG KO mice, demonstrating that the antitumor effect of L-NIL and CTX is dependent on adaptive immune cells (T and/or B cells). Thus we conclude that combination therapy with iNOS inhibition and low dose CTX reverses both the L-NIL-mediated increase in T-reg, and the CTX-mediated ablation of CD8+ T cells, leading to strongly enhanced immune-mediated tumor control.
iNOS regulates TGFβ-1 production and T-reg induction in human CD4+ T cells.

Since 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 T-reg from naïve human CD4+ T cells in ex vivo culture. PBMC were harvested from healthy donors, and naïve (CD45RO-) CD4+ cells sorted by flow cytometry. Naïve CD4+ T cells were activated with plate-bound OKT3 antibody for 5 days in the presence or absence of L-NIL or TGF-β1 before quantification of T-reg by flow cytometry. Baseline levels of T-reg (identified by intracellular FOXP3+ or surface CD127lo staining of CD4+ cells) were consistently 3-4% of cultured cells, and increased by roughly two-fold after culture with TGF-β1 or L-NIL (Fig 6A-B.). The increase in T-reg number was associated with a striking increase in TGF-β1 production by CD4+ T cells, although the increase in free (bioactive) TGF-β1 was more modest (Fig. 6C). Thus, we conclude that the regulation of FOXP3 expression and T-reg 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 towards immunity or immunosuppression depending on the cell types in which they are expressed. The present 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 T-reg. This antagonistic balance of MDSC and T-reg limits the beneficial effects of iNOS inhibition on anti-tumor immunity, but can be overcome by concurrently targeting T-reg 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 T-reg accumulation and function have focused on the effects of exogenously-produced NO on CD4+ T cells. One study by Lee, et al (9)found that exogenous NO suppressed FOXP3+ T-reg accumulation and skewed T cell cultures towards a Th1 phenotype; they proposed that NO produced by TIP-DC/conventional DC co-clusters could serve as a physiological 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 which acted to suppress FOXP3+ T-reg proliferation,
since FOXP3+ T-reg accumulation was enhanced by iNOS inhibition or use of iNOS KO 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 T-reg by adjacent NO-producing APCs) in another paper proposing that IL-12p40 acted on APC to induce NO capable of modulating T-reg induction(17). While 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 T-reg 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 TGF-β1, a “master regulator” of CD4+ T cell differentiation to the T-reg phenotype. This model is supported by our findings that 1) CD4+ FOXP3+ T cells express iNOS; 2) T-reg 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 KO splenocytes; and 3) TGFβ levels are significantly increased by iNOS inhibition in vivo and in vitro, and TGFβ-depleting antibody is sufficient to abolish iNOS inhibition-induced T-reg accumulation in ex vivo culture. Control of TGF-β1 release appears to act primarily at the transcriptional level, since both INOS inhibition and knockout upregulate TGF-β1 mRNA without consistently altering the ratio of total to free TGF-β1. According to this model, expression of iNOS in CD4+ T cells is sufficient to suppress their differentiation to T-reg 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+ T-reg production. In both cases, iNOS acts by positively (VEGF/MDSC) and negatively (TGF-β1/T-reg) 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 TGF-β1 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 T-reg accumulation in tumor-bearing mice led to a combination treatment strategy that simultaneously targets MDSC and T-reg, causing a dramatic restoration of immune function. While each agent used in isolation has beneficial effects on host immunity, these effects are limited by paradoxical induction of T-reg (in the case of L-NIL) and ablation of tumor-infiltrating CD8+ T cells (in the case of CTX). Combination therapy neutralizes both of these limitations, and leads to efficient and durable immune-mediated tumor control. This is a strategy which could be readily tested in human clinical cancer trials: low dose CTX 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 which inhibit iNOS, such as sildenafil and doxycycline could also be tested in combination with CTX.

A limitation of this study is that the molecular mechanisms through which iNOS regulates TGF-β1 expression are not described. In fact, far more is known about regulation of TGF-β1 signaling downstream of TGF-β1 receptor activation than about regulation of TGF-β1 expression itself. The TGF-β1 promoter contains several binding sites for transcription factors which 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 TGF-β1 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 TGF-β1 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 T-reg. We have translated this observation into a therapeutic approach with significant anti-tumor 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 cancer patients.
FIGURE LEGENDS:

FIGURE 1: The effect of iNOS inhibition on splenic and intratumoral T-reg accumulation in tumor-bearing mice. Wild type or iNOS KO C57/BL6 mice were injected s.c. with MT-RET-1 melanoma cells, and on day 14 treated with the selective iNOS inhibitor L-NIL or plain drinking water control for 7-9 days before sacrifice and harvest of spleen and tumors. A-B) Representative FACS plots of A) CD4+FOXP3+ T-reg cells or B) GR1+CD11b+ MDSC from tumors. C) Relative percentages of CD4+FOXP3+ T-reg or GR1+CD11b+ MDSC in tumor and spleen – data aggregated from 3 independent experiments. D) Tumor volumes on day 21 after MT-RET-1 injection, following 7 days treatment with L-NIL or plain drinking water control. Neither L-NIL nor iNOS KO significantly affects growth of established (14 day) tumors. E) Absolute total and free (bio-active) TGFβ-1 levels measured on day 21-23 from serum of MT-RET-1 tumor bearing mice treated as described above. F) Ratio of free to total TGFβ-1 in serum of tumor bearing mice. Each graph summarizes data from at least 3 experiments, with at least 5 mice per group.

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/BL6 mice and intracellular staining was performed for FOXP3 and iNOS expression in CD4+ cells. A-i) Representative FACS plots of iNOS expression in CD4+ T cells from tumor-free and tumor-bearing mice and graphical representation of aggregated data. A-ii) 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/BL6 mice cultured 5 days ex vivo in the presence or absence of anti-CD3/anti-CD28 mAb with or without exogenous TGFβ-1. B-i) 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. B-ii) iNOS expression by CD4+FOXP3+ cells represented as mean fluorescence intensity (MFI) and graphical representation of aggregated data from non-tumor and tumor bearing mice. All graphs represent pooled data from at least 2 experiments with at least 5 mice per group. C) Intracellular nitric oxide 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 LNIL 48 hours after activation. Tables shows
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 T-reg suppressive function** A) Representative FACS plots of CD4+FOXP3+ T-reg after 5 day ex-vivo culture of purified splenocytes harvested from wild type or iNOS KO mice activated with soluble anti-CD3/CD28 antibodies in the presence or absence of L-NIL or TGF-β1. B) Relative percentage of CD4+FOXP3+ T-reg generated from aCD3+aCD28 activated unfractionated splenocytes after ex-vivo culture in the presence or absence of TGFβ-1. C) CD4 T cells were purified from WT or iNOS KO 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 IL-17 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 TGF-β1 to generate CD4+FOXP3+ T-reg. T-regs 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 TGF-β1, control and LNIL groups) normalized to T cells activated with anti-CD3/anti-CD28 alone. E) Relative percentage of CD4+FOXP3+ T-reg 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 3 experiments with at least 5 mice per experiment.

**FIGURE 4: TGFβ-1 production in activated splenocytes and CD4 T cells from control and LNIL treated mice**
WT or iNOS KO CD4 T cells or splenocytes were activated with soluble anti-CD3/CD28 antibodies ex vivo in the presence or absence of exogenous L-NIL for 5 days as described in Figure 2. A) Culture supernatants were assayed for bio-active and total TGF-β1 levels. The graphs depict -fold change of TGF-β1 release relative to untreated splenocytes or CD4 T cells from WT mice. B) CD4 T cells from splenocyte cultures were stained for intracellular TGF-β1; i. representative FACS plots and ii. data expressed as –fold increase relative to untreated cells are shown. Ci) cDNA from purified CD4 T cells or unfractionated splenocytes from untreated
and L-NIL-treated cultures were analyzed for TGFβ-1 transcript levels by quantitative PCR. Graph shows relative TGFβ-1 mRNA levels normalized to ubiquitin levels. ii) The promoter activity in HEK293 cells transfected with full-length TGF-β promoter construct in the presence or absence of SNAP are depicted in the graph. D) Dose-dependent decrease of FOXP3+ T-reg after neutralization of TGFβ-1 with anti-TGF-β1 antibody in splenocyte cultures activated in the presence of L-NIL. Each graph represents data from at least 2 independent experiments.

**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 SC with 3x10⁵ 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+ T-reg and MDSC populations in spleen and tumor. B) Relative (top graphs) and absolute (bottom graphs) 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+ T-reg cells or MDSC. D) 3x10⁵ MT-RET-1 cells were injected into WT or RAG KO mice. Once the tumors reached approximately 30mm³ 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 thrice weekly. Each graph above shows pooled data from at least 3 experiments with 5 mice per treatment group.

**FIGURE 6: The effect of iNOS inhibition on TGF-β1 release from human CD4 T cells and generation of T-reg in ex-vivo culture**

A) Representative FACS plots of T-reg generated ex vivo from naïve CD4 T cells sorted from human PBMCs. Sorted CD45RO- CD4 T cells were cultured 5 days with plate bound OKT3 antibody in the presence or absence of L-NIL or TGF-β1. T-reg are identified both as CD25+FOXP3+ (upper panels) and CD25+CD127lo (lower panels) cells, gated on CD4. B) Graphical representation of numbers of CD4+CD25+FOXP3+ and CD4+CD25+CD127lo T-reg expressed as -fold change with respect to control/untreated (OKT3 only) group. C) Free and total TGF-β1 levels in supernatants from control or L-NIL or TGF-β1 -treated human CD4 T cell cultures. Each experiment represents cumulative data obtained from 2 or more healthy control subjects.
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iNOS expression in CD4+ T cells limits T-reg induction by repressing TGFb-1: combined iNOS inhibition and T-reg depletion unmask endogenous anti-tumor immunity

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