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Tumor-Expressed Inducible Nitric Oxide Synthase Controls Induction of Functional Myeloid-Derived Suppressor Cells through Modulation of Vascular Endothelial Growth Factor Release

Padmini Jayaraman,*1,† Falguni Parikh,*1,‡ Esther Lopez-Rivera,*1,# Yared Hailemichael,§ Amelia Clark,*1,‡ Ge Ma,§ David Cannan,*1,‡ Marcel Ramacher,‖ Masashi Kato,⁎ Willem W. Overwijk,§ Shu-Hsia Chen,⁎ Viktor Y. Umansky,‖ and Andrew G. Sikora*1,†,‡

Inducible NO synthase (iNOS) is a hallmark of chronic inflammation that is also overexpressed in melanoma and other cancers. Whereas iNOS is a known effector of myeloid-derived suppressor cell (MDSC)-mediated immunosuppression, its pivotal position at the interface of inflammation and cancer also makes it an attractive candidate regulator of MDSC recruitment. We hypothesized that tumor-expressed iNOS controls MDSC accumulation and acquisition of suppressive activity in melanoma. CD11b+GR1+ MDSC derived from mouse bone marrow cells cultured in the presence of MT-RET-1 mouse melanoma cells or conditioned supernatants expressed STAT3 and reactive oxygen species (ROS) and efficiently suppressed T cell proliferation. Inhibition of tumor-expressed iNOS with the small molecule inhibitor L-NIL blocked accumulation of STAT3/ROS-expressing MDSC, and abolished their suppressive function. Experiments with vascular endothelial growth factor (VEGF)-depleting Ab and recombinant VEGF identified a key role for VEGF in the iNOS-dependent induction of MDSC. These findings were further validated in mice bearing transplantable MT-RET-1 melanoma, in which L-NIL normalized elevated serum VEGF levels; downregulated activated STAT3 and ROS production in MDSC; and reversed tumor-mediated immunosuppression. These beneficial effects were not observed in iNOS knockout mice, suggesting L-NIL acts primarily on tumor- rather than host-expressed iNOS to regulate MDSC function. A significant decrease in tumor growth and a trend toward increased tumor-infiltrating CD8+ T cells were also observed in MT-RET transgenic mice bearing spontaneous tumors. These data suggest a critical role for tumor-expressed iNOS in the recruitment and induction of functional MDSC by modulation of tumor VEGF secretion and upregulation of STAT3 and ROS in MDSC.

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Materials and Methods

Mice and tumor models

C57BL/6, iNOS<sup>−/−</sup> (B6.129P2-Nos2tm1Lau/J) and RAG<sup>−/−</sup> (B6.129S7-Rag1tm1Mom/J) mice were obtained from The Jackson Laboratory and housed in the Mount Sinai animal facility under pathogen-free conditions. All animal experiments were performed in accordance with the regulations of the local Mount Sinai School of Medicine institutional animal care and use committee. The B16 melanoma cell line was obtained from the American Type Culture Collection. 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 W. Overwijk, University of Texas MD Anderson Cancer Center).

Transgenic mice. C57BL/6 mice expressing human ret oncogene in melanocytes under the control of mouse metallothionein promoter-enhancer were provided by L. Nakashima (Chibu University, Aichi, Japan). All mice were crossed and kept under specific pathogen-free conditions in the animal facility of the German Cancer Research Center. Experiments were performed in accordance with government and institute guidelines and regulations. Spontaneous tumor development was assessed macroscopically and, when appropriate, histologically.

Immunoblotting

Cells were lysed with Nonidet P-40 lysis buffer in the presence of protease and phosphatase inhibitors. Protein lysates were subjected to 10% SDS-PAGE electrophoresis and transferred to nitrocellulose membranes. Membranes were probed with appropriate primary Abs and incubated overnight at 4°C. Membranes were washed and incubated for 1 h with secondary Ab conjugated with peroxidase. Results were visualized by chemiluminescence detection using a commercial kit (Millipore). Cells were stained with GR1 FITC, CD11b allophycocyanin, and mouse anti-VEGF-R1 (eBioscience; clone Avas12a1) or VEGF-R2 (eBioscience; clone APL4) or isotype Ab for 30 min in the dark at 4°C. Cells were then washed, spun, and resuspended in staining buffer containing PE-conjugated goat anti-mouse IgG and incubated further for 30 min in the dark at 4°C. Cells were stained with washing buffer and resuspended in 300 μl staining buffer for FACS analysis using the FACSCalibur.

Detection of ROS levels in MDSC

Cells were stained with GR1 FITC, CD11b allophycocyanin, and mouse anti-VEGF-R1 (eBioscience; clone Avas12a1) or VEGF-R2 (eBioscience; clone APL4) or isotype Ab for 30 min in the dark at 4°C. Cells were then washed, spun, and resuspended in staining buffer containing PE-conjugated goat anti-mouse IgG and incubated further for 30 min in the dark at 4°C. Cells were washed with staining buffer and resuspended in 300 μl staining buffer for FACS analysis using the FACSCalibur.

Detection of pSTAT3 levels in MDSC

Cells were stained with GR1 PE and CD11b allophycocyanin, as mentioned above. They were fixed with BD fixation buffer and incubated for 10–30 min at 37°C. Cells were washed with staining buffer and permeabilized by adding BD perm wash buffer III. The plate was incubated for 30 min on ice. Cells were then washed again with staining buffer and stained with anti-pSTAT3 PE (BD Biosciences) for 1 h at room temperature. Cells were washed with staining buffer and resuspended in 300 μl staining buffer for FACS analysis using the FACSCalibur. Data were analyzed using Flowjo 7.6 software.

BioPlex Multiplex assay for cytokine analysis

GM-CSF, G-CSF, M-CSF, IL-1, IL-6, MIP-1α, MCP-1, and VEGF levels were measured in serum obtained from MT-RET or B16 tumor-bearing mice from both control and L-NIL-treated groups or tumor-conditioned supernatant-derived MT-RET cell lines cultured in the presence or absence of L-NIL using CTOT01 BioPlex Multiplex assay kit (BD Biosciences) run on a Luminex (BD Biosciences) processor using the manufacturer’s instructions.

Transplantable tumor models

Mice were injected s.c. with 3 × 10<sup>6</sup> MT-RET or B16 tumor cells in suspension. Mice were manually restrained, and the tumors were measured twice per week with calipers. Tumor sizes were determined according to the bidimensional product of the longest measurement multiplied by its perpendicular. Once tumors became established (>30 mm<sup>2</sup>, ~2 wk), half of the mice received L-NIL (0.2%) in drinking water for 7 d, and the other half received plain drinking water. Water bottles were replaced promptly when the water level was low. After completing the course of L-NIL, all mice were sacrificed and different organs were collected.

Serum collection

Blood was collected by cardiac puncture and allowed to clot at room temperature prior to centrifugation to obtain serum.
Cell harvesting/purification
Spleens were mashed on a filter mesh cup (Fisher) that is placed on top of a 50-ml tube, using a syringe plunger. A total of 10 ml 1640 containing 1% FBS was added on mesh cup, and cells were centrifuged at 1400 rpm for 10 min at 4˚C. The pellet was resuspended in 2 ml ACK lysing buffer (Life Technologies) and incubated at room temperature for 5 min to remove red cells. Cells were washed with RPMI 1640 containing 1% FBS, and the pellet was resuspended in 2 ml complete RPMI medium. Femur was collected, and the ends of the femur were cut to expose the cavity containing bone marrow. The bone marrow cells were flushed out with RPMI 1640 using a 27.5-G syringe. Yields were collected, and single-cell suspensions were prepared, as described above for spleen. Tumor-infiltrating lymphocytes (TIL) were isolated using Ficoll gradient centrifugation.

MDSC suppression assay
Purified MDSC (CD11b+GR1+ cells) were sorted from spleens or tumor of untreated MT-RET-bearing mice, L-NIL–treated, or iNOS knockout (KO) mice using a MoFlo XDP cell sorter (Beckman-Coulter, Brea, CA). Additional CD11b+ cells were sorted from tumor-infiltrating leukocytes from the tumors of untreated or L-NIL–treated MT-RET–bearing mice. CFSE 5 mM stock solution was diluted to 20 μM in PBS and added immediately to 2× cell-concentrated cell suspension (5×10^6 cells/ml) of splenocytes of wild-type C57BL/6 mouse. The mixture was incubated for 10 min at 37˚C. Five volumes of ice-cold RPMI 1640/10% FBS were added and incubated for 5 min on ice. Cells were washed three times with RPMI-CM. Sorted MDSC (CD11b+GR1+ from spleen or CD11b+ from TIL) from each condition were added to CFSE-labeled wild-type splenocytes at different ratios and activated with soluble anti-CD3 (0.3 μg/ml) plus anti-CD28 (0.5 μg/ml) Abs for 72 h at 37˚C.

CTL assay
Splenocytes or TIL harvested from tumor-bearing mice were activated with gp100/90 peptides for 72 h to enrich for tumor-specific T cells. The activated cells were then cultured with varying ratios of gp100/90-pulsed EL-4 target cells, and CTL assay was performed according to manufacturer’s instructions (Cytotox 96 nonradioactive cytotoxicity assay from Promega).

Results
Inhibition of tumor-expressed iNOS decreases accumulation and suppressive function of CD11b+GR1+ MDSC in ex vivo bone marrow cultures
To assess the role of tumor-derived soluble factors in generation of MDSC, we used a modification of the ex vivo bone marrow progenitor culture method described by Youn et al. (18), in which MDSC are differentiated from bone marrow cells in tumor/myeloid cell coculture, or in the presence of tumor-conditioned supernatants. Whereas the percentage of CD11b+GR1+ cells in 6-d culture remained stable to decreasing in medium alone, either coculture with MT-RET-1 (Supplemental Fig. 1A) cells across a permeable membrane or addition of tumor-conditioned supernatants was sufficient to induce ex vivo accumulation of functional CD11b+GR1+ MDSC (Fig. 1A, 1B). Like in vivo-derived MDSC, these cells were capable of dose-dependent suppression of CD4 and CD8 T cell proliferation in suppression assay (Fig. 1A, 1C, and data not shown). MDSC accumulation from bone marrow cells cocultured with MT-RET-1 tumor cells in a Transwell chamber was significantly decreased in the presence of 1 mM L-NIL (Fig. 1D), demonstrating that accumulation is iNOS dependent.

To determine the relative importance of iNOS expression in the tumor and myeloid compartments, MT-RET-1 supernatant transfer experiments were performed (Fig. 1E). Incubation with the NO donor diethylenetriamine NONOate enhanced accumulation of MDSC (Supplemental Fig. 2), and addition of L-NIL to bone marrow cultures partially suppressed tumor supernatant-induced MDSC accumulation, suggesting that there is some direct regulatory effect of NO on bone marrow cells. However, addition of L-NIL to the tumor cells during production of supernatants was more effective and almost completely reduced MDSC accumulation to baseline levels. The combination of L-NIL applied to both the tumor and myeloid cultures was most effective of all, reducing MDSC accumulation to below baseline levels. The effect of L-NIL in tumor cell culture was not due to carryover of L-NIL into the myeloid culture, because L-NIL is labile in aqueous solution, and equivalent concentrations of L-NIL incubated at 37˚C in medium alone without tumor cells had no effect (data not shown). Thus, we conclude that inhibition of tumor-expressed iNOS is sufficient to suppress accumulation of functional MDSC induced by tumor-derived soluble mediators in ex vivo culture.

L-NIL downregulates STAT3 activation and inhibits ROS production in MDSC
The transcription factor STAT3 is a major regulator of MDSC functional activity that is upregulated in both human and murine-derived MDSC (19). VEGF and STAT3 can interact in a positive feedback loop in which STAT3 activation drives VEGF expression, and signaling through VEGF-R activates STAT3 in many cell types (20, 21). One consequence of STAT3 activation in MDSC is upregulation of NADPH oxidase subunits p47^phox and gp91^phox, leading to production of ROS (22, 23). Thus, we examined the effect of iNOS inhibition on STAT3 activation and ROS production by MDSC in ex vivo bone marrow culture.

Bone marrow-derived CD11b+GR1+ cells expressed low levels of resting ROS in the absence of tumor cells. However, ROS levels were dramatically upregulated in Transwell culture with MT-RET-1 cells (Fig. 2A, 2B), and upregulation of ROS was suppressed to levels below baseline by addition of L-NIL to coculture. Coculture with MT-RET-1 cells also strongly upregulated levels of activated phospho-STAT3 in bone marrow-derived MDSC, and STAT3 activation was suppressed by addition of L-NIL (Fig. 2C). Parallel experiments examining the effect of L-NIL on T cell-suppressive activity of MT-RET-1 supernatant–derived MDSC demonstrated that addition of L-NIL to the tumor cell culture was sufficient to completely abolish MDSC-mediated T cell suppression (Fig. 2D). Together, these data demonstrate that STAT3 activation and ROS production are induced in MDSC by soluble mediators and downregulated by iNOS inhibition.

iNOS-dependent VEGF secretion is required for tumor-induced MDSC accumulation
Tumor-derived factors such as VEGF, GM-CSF, G-CSF, and MCP-1 and inflammatory cytokines such as IL-1β and IL-6 play a pivotal role in eliciting MDSC from bone marrow as well as directing their accumulation in the spleen, tumor, and other peripheral sites (1). VEGF is a major angiogenic growth factor that is secreted by many murine cancer models, and has been shown to play an important role in the induction of MDSC by both mouse and human cancers (24). Release of VEGF from tumor cells has also been shown to be upregulated by iNOS expression. The potential for VEGF to be a direct modulator of MDSC differentiation and functional activity in our system is supported by the robust secretion of VEGF by MT-RET-1 tumor cells, and the expression of VEGF receptors VEGF-R1 and VEGF-R3 on ex vivo-derived...
MDSC (Fig. 3A, 3B). VEGF release by MT-RET-1 cells was reduced 2-fold by L-NIL in vitro (Fig. 3A) without evidence of direct toxicity to tumor cells (Supplemental Fig. 1B).

We used Luminex to profile cytokine expression levels in MT-RET-1 tumor-bearing untreated and L-NIL–treated wild-type and iNOS-deficient mice (Fig. 3C). We observed nonsignificant trends toward decreased levels of IL-6, G-CSF, and IL-1β in serum from L-NIL–treated wild-type and iNOS KO mice. However, elevated serum VEGF levels in tumor-bearing mice were significantly reduced by treatment with L-NIL (Fig. 3C, Supplemental Fig. 4); the magnitude of this decrease was similar in L-NIL–treated mice bearing both early and late MT-RET tumors (data not shown). Although L-NIL also significantly decreased VEGF levels in iNOS-deficient mice, untreated iNOS KO mice showed a paradoxical trend toward elevated serum VEGF levels with respect to wild-type mice, suggesting that it is tumor- rather than host-expressed iNOS that is responsible for enhanced VEGF production in tumor-bearing mice.

A functional role for VEGF in the accumulation of MDSC cultured with MT-RET-1 supernatants was confirmed by the ability of anti-VEGF–neutralizing Ab to suppress accumulation of CD11b+GR1+ cells in a dose-dependent fashion (Fig. 3D). Conversely, addition of exogenous rVEGF to L-NIL–treated MT-RET-1 supernatants was sufficient to restore the accumulation of MDSC in ex vivo culture, again in a dose-dependent fashion (Fig. 3E). These data suggest that upregulation of VEGF production is a key mechanism through which tumor-expressed iNOS regulates the induction of MDSC.

**FIGURE 1.** Tumor-expressed iNOS is required for accumulation and functional activity of ex vivo-derived CD11b+GR1+ MDSC. (A) Representative FACS plots of T cell (CD4 and CD8) proliferation as measured by CFSE dilution. (B) Time course of accumulation of bone marrow-derived CD11b+GR1+ cells cultured in medium alone, in the presence of MT-RET–conditioned supernatant, and in Transwell coculture with MT-RET cells. (C) Dose-response curve of ability of sorted ex vivo MT-RET supernatant-derived CD11b+GR1+ MDSC (harvested on day 4 in culture) to inhibit CD4 and CD8 T cell proliferation. (D) Relative accumulation (days 5–6) of CD11b+GR1+ MDSC in MT-RET Transwell coculture ± 1 mM L-NIL. (E) Relative accumulation of MDSC (day 5) in the presence of MT-RET–conditioned supernatants alone, or MT-RET supernatants + 1 mM L-NIL added to the tumor cells (L-NIL-tumor) and/or to the bone marrow cell culture (L-NIL-BM). Each graph summarizes data from at least three experiments. *p ≤ 0.05.
iNOS inhibition leads to decreased induction and accumulation of MDSC in vivo

It has been demonstrated in numerous tumor models that CD11b+GR1+ MDSC are produced in the bone marrow in response to tumor-derived soluble mediators that also direct their accumulation in spleen and tumor (25, 26). Because MDSC accumulate in increasing numbers as tumors grow in size (27), we studied accumulation and functional activity of MDSC in tumors treated beginning on day 14, at which point L-NIL had no effect on tumor growth (see Fig. 4A). We demonstrated efficacy of iNOS inhibition by staining for nitrotyrosine, a stable product formed by reaction of NO with ROS species. Nitrotyrosine levels in splenic MDSC from both iNOS-deficient and L-NIL–treated mice were both significantly lower than in MDSC from untreated wild-type mice (Fig. 5C), demonstrating efficacy of pharmacologic iNOS inhibition with L-NIL, and suggesting that compensatory upregulation of other NOS isoforms does not occur in iNOS KO mice.

Mice bearing transplantable MT-RET-1 (Fig. 5A, 5B) or B16 melanoma (Supplemental Fig. 4) had increased percentages of MDSC populations in bone marrow and substantial accumulation of intratumoral MDSC. Treatment with L-NIL decreased total MDSC in bone marrow and tumor by 2- to 3-fold in MT-RET-1–bearing mice, and significantly decreased total MDSC in tumor and spleen of B16-bearing mice (Supplemental Fig. 4). Dolcetti et al. (28) have recently classified CD11b+GR1+ cells into three distinct populations of GR1high, GR1int (intermediate), and GR1low
CD11b⁺ cells, each differing in their expression of surface markers and mechanisms of immune suppression, but all of which have suppressive activity. However, the overall magnitude of MDSC decrease with L-NIL treatment was similar for GR1⁹⁹ and GR1⁹⁻⁰⁻ MDSC (Fig. 5A, Table I). The number of tumor-infiltrating MDSC was significantly decreased in MT-RET-1–bearing (Fig. 5B) and B16-bearing (Supplemental Fig. 4) iNOS-deficient mice; however, numbers of CD11b⁺GR1⁺ cells in the bone marrow were not significantly reduced in iNOS KO mice. iNOS is required for acquisition of T cell-suppressive function in MDSC

We used ex vivo suppression assay to confirm that purified CD11b⁺GR1⁺ cells derived from the spleens and tumor of MT-RET-1–bearing mice were competent to suppress T cell proliferation, and to assess the effect of L-NIL treatment on their suppressive function. Total CD11b⁺GR1⁺ cells consisting of both GR1⁹⁹ and GR1⁹⁻⁰⁻ populations were sorted from control and L-NIL–treated splenocytes from MT-RET-1 tumor-bearing mice and cultured with CFSE-labeled splenocytes activated with anti-CD3 and anti-CD28 Abs (Fig. 6A). Whereas spleen- or tumor-derived MDSC from tumor-bearing untreated mice could efficiently suppress proliferation of both CD4 and CD8 T cells in a dose-dependent fashion, MDSC from tumor-bearing untreated mice failed to suppress proliferation even at a 1:1 ratio (Fig. 6A, 6B, Supplemental Fig. 3). No suppression was observed when T cells were cocultured with CD11b⁻GR1⁻ cells from tumor-bearing mice, or CD11b⁺GR1⁺ cells from tumor-free mice (Fig. 6B). Because iNOS is a known effector mechanism of MDSC-mediated T cell inhibition, we performed additional experiments to determine whether suppression of ex vivo MDSC function by L-NIL was caused by direct inhibition of iNOS expressed by MDSC. We found that the addition of even high doses (3 mM) of L-NIL to purified MDSC prior to washing and plating does not reverse their

**FIGURE 3.** Tumor-secreted VEGF mediates iNOS-dependent cross-talk required for MDSC accumulation. (A) Relative VEGF levels in 48-h supernatants derived from MT-RET-1 cells cultured in the presence or absence of 1 mM L-NIL. (B) Expression of VEGF-R1 and VEGF-R3 in CD11b⁺GR1⁺ MDSC derived ex vivo from wild-type bone marrow cultured with MT-RET-1 supernatants for 5 d. (C) In vivo concentration of VEGF, IL-6, G-CSF, and IL-1β in serum of untreated or L-NIL–treated wild-type mice on day 21 after MT-RET-1 tumor injection. L-NIL treatment decreases VEGF in both serum from RET tumor-bearing mice and RET tumor supernatants compared with their untreated counterparts (p ≤ 0.05). VEGF concentration in serum was compiled from at least three experiments with n = 5 mice per group. (D) Ability of the indicated concentrations of anti-VEGF–neutralizing Ab to block MT-RET-supernatant-induced MDSC accumulation ex vivo at 5 d in culture. Neutralization of VEGF leads to significantly decreased accumulation of MDSC in BM cultures (p ≤ 0.05). (E) Ability of the indicated concentrations of murine rVEGF to reverse L-NIL–mediated suppression of MT-RET-1 supernatant-induced MDSC accumulation ex vivo at 5 d in culture. Each graph summarizes data from at least three experiments. *p ≤ 0.05.
ability to suppress T cell proliferation (Fig. 6B, L-NIL wash), demonstrating that the effects of L-NIL are not due to carryover of trace quantities of inhibitor into ex vivo cultures. We also found that MDSC from iNOS-deficient mice, which cannot use iNOS as an effector mechanism, retained 50% CD8+ T cell-suppressive capacity when compared with MDSC from L-NIL–treated mice that had no suppressive capacity (Fig. 6B, iNOS KO). Thus, we conclude that the primary effect of L-NIL treatment is suppression of the tumor-directed acquisition of functional activity by MDSC in vivo.

iNOS inhibition in vivo also had an effect on MDSC ROS and activated p-STAT3 levels similar to that observed in the ex vivo model. ROS levels in MDSC from spleen of L-NIL–treated tumor-bearing mice were significantly reduced as compared with untreated mice (Fig. 6C). As was the case for VEGF levels, MDSC from tumor-bearing iNOS KO mice had ROS levels comparable to wild-type mice. Western blot analysis of total and phospho-STAT3 levels in MT-RET-1 tumors demonstrated robust phosphorylation of serine 727 and tyrosine 705 in tumors from untreated mice. STAT3 activation was downregulated by ∼2-fold in L-NIL–treated mice (Fig. 6D), suggesting that iNOS inhibition in vivo downregulates STAT3 activation in the tumor, in tumor-infiltrating myeloid cells, or both. However, we did not observe a similar downregulation of STAT3 phosphorylation in spleens of L-NIL–treated mice (data not shown).

Tumor-expressed iNOS drives cancer-associated immunosuppression, and iNOS inhibition enhances immune-mediated tumor control

We studied the effect of L-NIL on growth of transplantable MT-RET-1 tumors in syngeneic mice, including wild-type C57BL/6 mice, iNOS KO mice, and RAG KO mice that lack mature T cells and B cells. In immunocompetent wild-type mice, 7- to 10-d L-NIL treatment modestly inhibited growth of early (day 4), but not established (day 14) tumors (Fig. 4A, Supplemental Fig. 4A).

FIGURE 4. Pharmacologic iNOS inhibition reverses systemic T cell decline in tumor-bearing mice and enhances intratumoral accumulation of CD4 and CD8 T cells. (A) Growth of MT-RET–untreated and early (day 4) L-NIL–treated melanoma in wild-type and RAG KO mice demonstrates modest T/B cell-dependent antitumor effect. Treatment of established (day 14) tumors does not lead to decreases in tumor growth. (B) Absolute and relative number of tumor-infiltrating CD4 and CD8 T cells in untreated and L-NIL–treated (WT or iNOS KO) MT-RET–bearing mice on day 21. Each graph shown represents pooled data from at least three experiments with n = 5 mice per group. (C) Cytotoxicity assay against EL-4 target cells with effector cells from splenocytes or TIL of control or L-NIL–treated mice (harvested on day 21 after MT-RET injection, enriched for gp100/trp2-specific T cells). CTL efficacy is expressed as percentage of target cell populations lysed. Graph is representative of two experiments with n = 5 mice per group.
growth in immunocompromised mice, inhibition of tumor growth by L-NIL in syngeneic melanoma models appears to depend on adaptive immune mechanisms, because L-NIL had no effect on tumor growth in RAG KO mice (Fig. 4A), and we observed no direct effect on growth of MT-RET-1 tumor cells in vitro (Supplemental Fig. 1B).

**Table I.** Absolute number of cells (10^6) and CD11b^+GR1^+^, GR1^high^, and GR1^int^ cells from TIL, spleen, and bone marrow

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<th>Sample</th>
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<th>CD11b^+GR1^+</th>
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*p ≤ 0.05.
Because the lack of effect in RAG-deficient mice strongly suggested that L-NIL acts through immune mechanisms, we examined the effect of L-NIL on CD4 and CD8 T cell accumulation in tumor-bearing wild-type and iNOS-deficient mice. Treatment of both 4-d (data not shown) and 14-d MT-RET-1 tumors with L-NIL increased numbers of tumor-infiltrating and splenic CD4 and CD8 T cells in wild-type mice (Fig. 4B). Whereas splenic T cell levels were reduced in the tumor-bearing state, L-NIL treatment restored numbers of splenic CD4 and CD8 T cells to numbers comparable to those seen in tumor-free mice (3 × 10^6 CD4 and 2.5 × 10^6 CD8 T cells) and reversed the splenomegaly observed in many tumor-bearing mice (data not shown). A similar trend toward recovery of splenic T cell numbers was observed in mice bearing transplanted B16 melanoma (Supplemental Fig. 4). Increased T cell numbers in L-NIL–treated mice were also associated with enhanced per-cell cytolytic activity of CTL from spleen and TIL (Fig. 4C) against EL4 cells pulsed with peptides for the gp100 and Trp2 melanoma Ags expressed by MT-RET-1 (Supplemental Fig. 1C and data not shown). Thus, pharmacologic inhibition of iNOS with L-NIL reverses both quantitative and qualitative tumor-mediated T cell dysfunction.

Unlike L-NIL–treated mice, iNOS KO mice did not show enhanced numbers of splenic or tumor-infiltrating CD4 or CD8 T cells (Fig. 4B), or altered tumor growth (Fig. 4A). A similar lack of effect of host iNOS KO on tumor growth and T cell numbers was observed in the B16 model (data not shown). L-NIL treatment

FIGURE 6. CD11b^+Gr1^+ MDSC sorted from spleens of untreated MT-RET-1–bearing mice, but not L-NIL–treated mice, inhibit CD4 and CD8 T cell proliferation. (A) Relative proliferation of CD3/CD28-stimulated CD4 and CD8 T cells in the presence of different ratios of sorted CD11b^+Gr1^+ MDSC from spleens (left) or TIL (right) of untreated and L-NIL–treated mice on day 21 after MT-RET-1 injection, or (B) relative proliferation of CD8 (top) and CD4 (bottom) T cells in the presence of 1:1 CD11b^+Gr1^+ MDSC or CD11b^+Gr1^- cells sorted from splenocytes of iNOS KO mice, L-NIL–treated mice, or untreated mice in the presence or absence of 1 nM L-NIL added to the sorted MDSC prior to the final wash step. Each graph shown above represents pooled data from at least three experiments with n = 5 mice per group. L-NIL–treated MDSC lose their ability to suppress T cell proliferation compared with wild type (*p < 0.05). (C) Effect of L-NIL or genetic ablation of host iNOS on ROS production in vivo by CD11b^+Gr1^+ MDSC from spleen on day 21 after MT-RET injection. L-NIL treatment leads to decreased production of ROS from splenic CD11b^+Gr1^+ MDSC. (D) L-NIL treatment in vivo downregulates activated (phosphorylated) STAT3 expression and pSTAT3/total STAT3 ratio in MT-RET-1 tumors by Western blot. Each graph represents pooled data from at least three experiments.
of iNOS-deficient mice led to robust recovery of splenic CD4 and CD8 T cell numbers (Fig. 4B), demonstrating that the failure of host iNOS ablation to restore T cell numbers in tumor-bearing mice is not due to upregulation of iNOS-independent compensatory mechanisms in iNOS KO mice. These data confirm a role for tumor-expressed iNOS in mediating tumor-mediated T cell dysfunction and suppression of antitumor immunity, most likely by driving accumulation and activation of MDSC.

iNOS inhibition enhances intratumoral CD8+ T cell infiltration and suppresses growth of spontaneous melanoma in MT-RET transgenic mice

To test the effect of iNOS inhibition on spontaneously arising tumors, we compared melanoma growth in untreated and L-NIL–treated MT-RET transgenic mice. Mice were allowed to develop palpable tumors, and then randomized to 10-d treatment with L-NIL (0.2%) or control. Spontaneous melanomas tended to arise in the face, head, and neck (Fig. 7A), although they also involved other parts of the body. These tumors contained substantial numbers of CD11b+GR1+ MDSC (10–20% of all CD45+ leukocytes), and MDSC were also detected in the metastatic (tumor-infiltrated) lymph nodes, although at much lower levels (<1%; Fig. 7B). L-NIL treatment significantly suppressed tumor growth (Fig. 7C), as was shown previously with treatment of early MT-RET-1 transplantable tumors (Fig. 4A), and this was associated with a trend toward increased numbers of CD8+ T cells in the primary tumor (Fig. 7D). We also observed a modest, but significant decrease in the number of NO-producing MDSC in both tumor and lymph node (Fig. 7D). A model of iNOS-mediated control of MDSC accumulation and functional maturation based on the above data is shown in Fig. 8.

Discussion

In the current study, we demonstrate that tumor-expressed iNOS plays a key role in recruitment and activation of MDSC in the tumor-bearing state, and that iNOS inhibition reverses tumor-mediated immune suppression in mouse melanoma. The beneficial effect of iNOS inhibition on accumulation of functional MDSC, CD8 T cell numbers, and tumor growth is seen in both transplantable and spontaneous melanoma models. This role for iNOS as a mediator of recruitment of functional MDSC is distinct from its previously described role as an effector mechanism of immunosuppression via the direct effects of high-output NO production (9) and formation of the reaction product peroxynitrite (11) on T cell activation, proliferation, and survival. Rather, we found that iNOS inhibition suppressed VEGF release, STAT3 activation, and ROS upregulation required for induction of functional MDSC (see Fig. 8 for working model). These results are consistent with the known role of NO as a signal transduction mediator capable of controlling gene expression and cellular differentiation and development (31).

Treatment with the selective iNOS inhibitor L-NIL reversed tumor-associated immunosuppression by decreasing numbers of tumor-infiltrating MDSC, abolishing the ability of MDSC to suppress T cell proliferation, restoring systemic and tumor-infiltrating CD4 and CD8 T cell numbers, and boosting Ag-specific cytotoxicity of CTL from spleen and TIL. Because MDSC are known to potently inhibit antitumor T cell responses (6, 9, 11), we focused on the effects of iNOS inhibition on MDSC activity and distribution in tumor-bearing mice as the likely primary mechanism of immune restoration in L-NIL–treated mice. However, it is also possible that iNOS inhibition has direct or indirect effects on T cell migration and function independent of MDSC. In fact, a recent publication by Molon et al. (32) describes a novel mechanism of chemokine nitration that prevents T cells from migrating intratumorally in several different tumor models. This could potentially account for the enhanced intratumoral CD4 and CD8 T cell numbers we observed in L-NIL–treated mice, but not the...
increased number of T cells observed in the spleen. It also does not account for the higher per-cell cytotoxicity observed in splenocytes isolated from spleen and tumor of L-NIL–treated mice. Rather, these effects are consistent with the nearly absolute loss of T cell-suppressive capacity we observed in MDSC from L-NIL–treated mice.

The loss of functional activity in MDSC from L-NIL–treated mice suggests a failure of MDSC to upregulate or maintain mechanisms of T cell suppression that are normally induced by cancer. iNOS upregulation and production of NO is itself a direct effector mechanism of T cell suppression, particularly by monocytic MDSC (3, 18). However, it is unlikely that sufficient amounts of L-NIL, a reversible competitive antagonist of iNOS, were carried over into our ex vivo suppressor assay to affect iNOS-dependent T cell suppression (see Fig. 5B). Also, suppressive activity was only partly impaired in MDSC derived from iNOS KO mice, suggesting that other suppressive mechanisms predominate in our system. These potential mechanisms include expression of the enzyme arginase (7, 10) and production of ROS by MDSC (33). We did not observe a significant change in arginase expression levels on MDSC after in vivo L-NIL treatment (data not shown). However, we saw a significant decrease in ROS production after iNOS inhibition in both ex vivo-derived MDSC and MDSC isolated from tumor-bearing mice. Thus, rather than a direct effect on reactive nitrogen-mediated T cell inhibition, iNOS inhibition acts primarily by interfering with tumor-mediated upregulation of ROS production by MDSC.

ROS production in MDSC has been shown to be controlled by STAT3 activation and concomitant expression of ROS-producing NADPH oxidase subunits (30). STAT3 upregulation correlates with suppressive function in many studies of MDSC in both mice and humans, and has been shown to regulate the expression of NADPH oxidase subunits and production of high-output ROS (30). We observed that pharmacologic iNOS inhibition with L-NIL downregulates both STAT3 activation and ROS production by MDSC in ex vivo culture and in RET-bearing mice. Because signaling through VEGF-R has been shown to induce activation of suppressive activity in MDSC and STAT3 activation in myeloid cells (34, 35), VEGF is an attractive candidate soluble mediator of MDSC activation in our system. In fact, we found that iNOS-mediated release of VEGF is strongly implicated in accumulation of functional MDSC, because L-NIL suppresses VEGF levels in tumor-bearing mice in vivo and in RET culture, and because exogenous VEGF is sufficient to restore MDSC accumulation in L-NIL–treated ex vivo culture. Similarly, in ex vivo culture, anti-VEGF mAb alone was capable of mimicking the effect of iNOS inhibition by suppressing accumulation of CD11b+GR1+ MDSC, suggesting that iNOS-dependent VEGF production is required for MDSC induction. This mechanism is supported by the observation that VEGF levels are elevated in both tumor and serum of mice bearing spontaneous RET melanomas, and have been shown to correlate with tumor size and progressive immunosuppression (36). This is consistent with prior literature describing a key role for VEGF in MDSC induction and tumor-mediated immunosuppression in melanoma and other solid tumors (24, 37, 38). The ability of iNOS and NO expression in tumor cells to induce VEGF release has also been well described (39, 40), and provides a logical mechanism through which modulation of iNOS could control the induction and activation of MDSC and—potentially—other immunosuppressive cells (41).

Because both tumor and host cells can express VEGF (as well as other soluble inflammatory mediators, such as IL-6), the relative importance of these compartments in the iNOS-mediated induction of MDSC is as yet incompletely defined. Our ex vivo supernatant transfer results, in which treatment of tumor cells with L-NIL causes robust suppression of MDSC accumulation, are consistent with a model in which tumor-expressed iNOS enhances VEGF release, and thus supports MDSC accumulation and activation. These results closely parallel the in vivo accumulation of CD11b+GR1+ MDSC in bone marrow of tumor-bearing mice, in which only a slight trend toward decrease of MDSC is observed in iNOS KO mice, but inhibition of iNOS in both the tumor and host compartments with L-NIL significantly reduces the number of MDSC to that seen in tumor-free mice. This is accompanied by a significant decrease (VEGF) or trend toward decrease (IL-6, IL-1β) of key inflammatory mediators in serum. Thus, the iNOS-dependent production of tumor-derived VEGF seems to play a pivotal role in MDSC induction from myeloid precursors. This is quite consistent with what is known about the RET gene driving oncogenesis in this model, a so-called inflammatory oncogene that induces the expression of numerous proinflammatory mediators, including VEGF, IL-6, and IL-1β (36, 42).

The pivotal role of tumor-expressed iNOS in regulation of MDSC is supported by several points of evidence in our study. Despite efficient reduction of MDSC NO levels, as demonstrated by nitrotyrosine staining, ablation of host iNOS fails to decrease the number of MDSC-infiltrating tumor and spleen, reverse immunosuppression, or suppress tumor growth. This is in stark contrast to the effect of pharmacologic iNOS inhibition, which affects iNOS in both tumor- and host-expressed compartments and has beneficial effects on MDSC function, T cell numbers, and tumor growth. In tumor-bearing iNOS KO mice, we observed neither ROS down-regulation (Fig. 6) nor downregulation of activated STAT3 (data not shown), despite robust downregulation of these molecules with pharmacologic iNOS inhibition. These findings are supported by experiments with ex vivo-derived MDSC, in which the suppression of MDSC accumulation and function is strongest with inhibition of tumor-expressed iNOS. Together, these data suggest a pivotal role for tumor-expressed iNOS in control of MDSC-mediated immunosuppression through modulation of VEGF release and VEGF-driven signaling events in the target MDSC.

Reversal of immune dysfunction by L-NIL was associated with modest immune-dependent suppression of growth of early transplantable MT-RET-1 tumors; a more profound decrease in spontaneous tumor growth was also observed in L-NIL–treated MT-RET transgenic mice, possibly related to the much slower initial growth rate of spontaneous tumors. Thus, iNOS inhibition is sufficient to restore endogenous immunity in melanoma-bearing mice, but not to levels sufficient to mediate tumor regression or eradication. Combining iNOS inhibition with other immunomodulatory approaches, such as antitumor vaccination or adoptive transfer of antitumor T cells, is a logical next step because this would simultaneously increase the number of high-affinity tumor-specific CTL while disarming mechanisms that lead to their death or inactivation upon entry into the tumor. Thus, iNOS inhibition may function as relatively simple and nontoxic strategy for conditioning the immunosuppressive tumor microenvironment, and could potentially be combined in a modular fashion with any number of other immunotherapeutic approaches.

The aberrant expression of iNOS and other inflammatory molecules has been recently identified as a critical tipping point in progression of many different cancers (43, 44), and a logical focus for targeted molecular therapy (29). iNOS and NO have already been shown to promote tumor growth, survival, and treatment resistance in melanoma and other cancers. Understanding the regulation of MDSC recruitment and function by tumor-expressed iNOS will provide important insights into the link between inflammatory signaling and development of the immunosuppressive
tumor microenvironment, and new opportunities for therapeutic intervention.

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Disclosures
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