Inducible Nitric Oxide Synthase and CD11b⁺Gr1⁺ Cells Impair Lymphatic Contraction of Tumor-Draining Lymphatic Vessels

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Abstract

Background: Metastatic tumor cells spread through lymphatic vessels and colonize draining lymph nodes (LNs). It is known that tumors induce lymphangiogenesis to enhance lymphatic metastasis and that metastatic cancer cells are carried by lymph flow to LNs.

Methods and Results: Here, we investigated the molecular and cellular regulation of collecting lymphatic vessel contraction in vessels draining a metastatic tumor using intravital microscopy. In tumor-draining collecting lymphatic vessels, we found vessel contraction was suppressed. The infiltration of peritumor tissue by inducible nitric oxide synthase positive and CD11b⁺Gr1⁺ myeloid cells played a critical role in the suppression of lymphatic contraction. Depletion of Gr1⁺ cells with an anti-Gr1 antibody improved contraction of tumor-draining lymphatic vessels. In addition, inducing tumor cell death restored lymphatic contraction in nude mice.

Conclusions: These findings indicate that tumors contribute to regulation of lymphatic transport in a reversible manner, warranting further investigation into the role of impaired lymphatic transport in cancer progression.

Keywords: lymph node metastasis, lymphatic contraction, tumor draining, lymphatic vessels, inducible nitric oxide synthase, nitric oxide

Introduction

DURING CANCER PROGRESSION, metastatic cancer cells invade peritumor lymphatic vessels and travel to tumor-draining lymph nodes (TDLNs) to form lymph node (LN) metastasis.¹ Tumors induce peritumor and TDLN lymphangiogenesis, which provides an expanded lymphatic vessel network to promote LN metastasis.^{2,3} After metastatic cells enter initial lymphatic vessels, they travel through collecting lymphatic vessels to reach the TDLN. Although normal LNs allow flow to pass through the LN (mostly through the subcapsular sinus with some fluid moving through LN parenchyma),⁴ TDLNs can become full of cancer cells causing flow to be diverted and drainage patterns altered.⁵

Unlike initial lymphatic vessels, collecting lymphatic vessels are invested in organized lymphatic muscle cells (LMCs) that drive lymphatic contraction and force lymph toward LNs.⁶ Lymph transport is driven by both passive forces from tissue movement and contractions from LMCs.⁶

However, proper LMC coverage is required to maintain lymphatic function, demonstrating the importance of these cells.⁷ Nitric oxide (NO) is a key modulator of LMC activity and, therefore, lymph transport.^{8,9}

Previously, it was demonstrated that tumor cell supernatant reduces lymphatic contraction *ex vivo* through an NO-mediated mechanism.¹⁰ Recently, noninvasive methods to study lymphatic pumping with near infrared fluorescence imaging showed reduced tumor-draining lymphatic contraction.¹¹ However, how tumors regulate lymphatic contraction is not clear. In this study, we investigate alterations of tumor-draining collecting lymphatic vessel contraction through intravital imaging to assess the molecular and cellular regulation of this process.

In previous studies, we found that CD11b⁺Gr1⁺ cells suppressed lymphatic contraction by expression of inducible nitric oxide synthase (iNOS) during sterile inflammation.¹² CD11b⁺Gr1⁺ cells are a heterogeneous population of immature myeloid cells—including precursors of granulocytes, macrophages/monocytes, and dendritic cells—with powerful

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FIG. 1. Lymphatic vessels are dilated and lymphatic contraction is inhibited in vessels draining B16F10 melanomas. B16F10 melanoma was implanted into the hind footpad of C57BL/6 mice. Lymphatic contraction analysis of the afferent lymphatic vessel to the popliteal lymph node was performed in nontumor-bearing control animals (**A**, top) and in animals when the footpad reached 6–8 mm in thickness after B16F10 tumor implantation (**A**, bottom). Representative lymphatic diameter measurements of control and tumor-bearing mice are shown (**B**).

immune suppressive function in cancer progression.^{13,14} CD11b⁺Gr1⁺ cells also suppress T cell responses through multiple mechanisms, including inhibiting CD3-zeta chain expression as well as producing arginase 1 and NO (through iNOS).^{15–17} In this study, we investigate whether tumor-associated CD11b⁺Gr1⁺ cells impair lymphatic contraction during cancer progression.

Materials and Methods

Mice

In all experiments, 8- to 10-week-old mice were used. Before imaging, mice were anesthetized using a ketamine–xylazine mixture at 100 mg:10 mg per kg body weight. The protocol for the animal experiments was reviewed and approved by Institutional Animal Care and Use Committee of the Massachusetts General Hospital. The procedures were performed in accordance with the approved guidelines.

Statistical analysis

Significance was determined by a *t*-test with a Welch's correction for comparison between two groups or an analysis of variance with a Tukey's *post hoc* test for comparison

between more than two groups. *p*-Values <0.05 were considered significant. Data analysis was performed in Prism (GraphPad Software, La Jolla, CA).

Tumor implantation

A total of 2×10^6 tumor cells were implanted in the lower leg close to the dorsal side of the footpad. The lymphatic contraction was assessed when paw thickness reaches 6–8 mm. At this stage of B16F10 tumor growth, there was spontaneous LN metastasis in ~40% of the mice. We used four different tumor cell lines in their syngeneic mouse strains: B16F1 and B16F10 (melanoma in C57BL/6 mice), P0008 (breast cancer in FVB mice), and SCCVII (squamous cell carcinoma in C3H mice) to study alterations of tumor-draining lymphatic vessel contraction.

Imaging collecting lymphatic vessels

In brief, mice were anesthetized and 2 μL of 2% FITC-Dextran (2×10^6 kDa) was injected into the footpad, as previously described.¹⁸ The leg skin and underlying connective tissue near the popliteal lymphatic vessel were carefully removed using sterile microsurgical dissection, thereby exposing the collecting lymphatic vessels. Time-lapse images were taken with an inverted fluorescence

TABLE 1. REDUCED LYMPHATIC CONTRACTION STRENGTH IN MULTIPLE TUMORS

Mouse strain or tumor	Tumor size (mm ³)	Minimum diameter (μm)	Maximum diameter (μm)	Frequency (contractions/minute)	Amplitude (%)	Ejection fraction (%)	n
C57BL/6	—	26.8 ± 1.1	35.7 ± 1.3	9.5 ± 1.0	15.7 ± 1.0	51.5 ± 3.2	7
B16F1	225 ± 23	56.2 ± 5.8 [†]	63.3 ± 4.2 [†]	24.4 ± 6.5*	8.2 ± 2.5*	20.9 ± 10.3*	10
B16F10	371 ± 171	69.8 ± 4.7 [†]	75.1 ± 4.4 [†]	19.5 ± 6.8	4.3 ± 1.1 [†]	14.7 ± 4.0 [†]	7
C3H	—	36 ± 2.5	45.9 ± 2.5	21.3 ± 2.9	12.3 ± 1.6	49.2 ± 11.8	7
SCCVII	341 ± 105	62.4 ± 8.3 [†]	66 ± 6.7*	21.1 ± 9.9	4.0 ± 2.3 [†]	12.7 ± 8.4*	10
FVB	—	27.4 ± 6.7	35.7 ± 5.1	30.6 ± 6.6 [‡]	17.6 ± 4.4	53.0 ± 12.4	8
P008	241 ± 75	95.0 ± 28.0	105 ± 24*	58.8 ± 27.3	9.3 ± 1.8	24.7 ± 7.3	5
Nude	—	25.0 ± 2.0	36.0 ± 4.1	13.1 ± 1.5	23.1 ± 5.6	64.1 ± 9.0	8
HST26T	427 ± 81	89.9 ± 4.9 [§]	98.2 ± 5.2 [§]	10.1 ± 0.9	5.7 ± 0.8 [§]	17.4 ± 2.4 [§]	11
HST26T-DT	250 ± 59	51.5 ± 5.7**	62.0 ± 6.5**	12.4 ± 0.9	11.7 ± 1.5	34.3 ± 4.8	11

Mean ± standard error of the mean.

**p* < 0.05 of tumor compared with nontumor-implanted control tested with an unpaired *t*-test with a Welch's correction.

[†]*p* < 0.01 of tumor compared with nontumor control tested with an unpaired *t*-test with a Welch's correction.

[‡]*p* < 0.01 compared with C57BL/6 by an ANOVA with a Tukey's *post hoc* test.

[§]*p* < 0.01 compared with nude by an ANOVA with a Tukey's *post hoc* test.

***p* < 0.01 compared with HST26T by an ANOVA with a Tukey's *post hoc* test.

ANOVA, analysis of variance; DT, diphtheria toxin.

microscope. Images of the lymphatic vessels were analyzed using in-house Matlab code. Ejection fraction—the percentage of total volume ejected per contraction—was used to quantify the strength of lymphatic contraction.¹²

Gr1⁺ cell depletion

Control immunoglobulin G or anti-Gr1 antibody (clone RB6-8C5) 100 μ g/injection was injected 7 days after

tumor implantation and every 3 days until the time of imaging.

HSTS26T implantation and diphtheria toxin treatment

HSTS26T human sarcoma tumor chunks (1–2 mm) were implanted on dorsal side of the footpad in nude mice, and when the paw thickness reached 6 mm, mice were randomly

FIG. 2. Lymphatic contraction is maintained in *iNOS*^{-/-} mice. B16F1 or B16F10 melanoma was implanted into the hind footpad of C57Bl/6 WT or *iNOS*^{-/-} mice before undergoing lymphatic contraction analysis. There is no difference in tumor size between WT and *iNOS*^{-/-} mice implanted with B16F1 or B16F10 melanoma (**A** and **B**, left). Ejection fraction is increased in *iNOS*^{-/-} mice versus WT (**A** and **B**, right). TDLNs were collected to assess LN metastasis. Representative H&E sections of a nonmetastatic LN and an LN with a B16F1 metastasis are shown (**C**, **D**). Scale bar = 40 μ m. There is no significant difference in the percentage of LN metastasis between *iNOS*^{-/-} and WT mice with B16F1 or B16F10 melanoma (**E**, **F**). Bars shown $\pm$ SEM, $n=5-14$, * $p<0.05$ by unpaired *t*-test with a Welch's correction. H&E, hematoxylin and eosin; *iNOS*, inducible nitric oxide synthase; LN, lymph node; SEM, standard error of the mean; TDLNs, tumor-draining lymph nodes; WT, wild-type.

divided into two groups [phosphate-buffered saline (PBS) or diphtheria toxin (DT)]. DT (2 μ g/mouse) or PBS was injected i.p. once after randomization and again 3 days later. Five days after the first injection, lymphatic contraction was measured.

Imaging LN metastases

Hematoxylin and eosin (H&E) staining was performed on multiple 10 μ m frozen sections from individual TDLNs. TDLNs were imaged and examined by two independent reviewers to determine the presence of metastatic tumor cells.

Results

Multiple tumor types suppress tumor-draining lymphatic vessel contraction

Cancer-induced lymphangiogenesis is correlated with increased dissemination of tumor cells to regional LNs and poor prognosis in a variety of human cancers.^{19–27} Although tumor-draining lymphatic vessels have been shown to have impaired contraction,¹¹ it is not clear how cancer modulates lymphatic contraction strength. We measured the lymphatic contraction strength when hind paws bearing tumors reached a thickness of 6–8 mm. At this stage, there were only sporadic cancer cells or very small LN metastases in the TDLN. Lymphatic vessels were dilated, and there was a reduction in contraction strength across all tumor types tested (Fig. 1, Table 1, Supplementary Movies 1 and 2). Interestingly, lymphatic ejection fraction and frequency under normal conditions in nontumor-bearing mice are dependent on the mouse strain (Table 1).

iNOS suppresses lymphatic contraction during tumor metastasis

Previously, it was found that *iNOS*-expressing cells suppress lymphatic contraction during sterile inflammation through NO.¹² Therefore, we tested whether *iNOS* is involved in suppressing lymphatic contraction during cancer progression by using *iNOS*^{−/−} mice available on the C57Bl6 background. We observed maintenance of lymphatic contraction strength (ejection fraction) in *iNOS*^{−/−} mice bearing syngeneic B16F1 and B16F10 tumors (Fig. 2A, B), comparable with nontumor-bearing controls. To determine whether altered lymphatic contraction affects LN metastasis, H&E staining was performed on the TDLNs from wild-type (WT) and *iNOS*^{−/−} mice implanted with B16F1 and B16F10 (Fig. 2C, D). We found that the LN metastasis rate was not different in WT versus *iNOS*^{−/−} in both B16F1 and B16F10 models/tumors (Fig. 2E, F), even though lymphatic contraction was maintained in *iNOS*^{−/−} mice compared with WT mice.

Myeloid-derived cells suppress lymphatic contraction in cancer-draining lymphatic vessels

Previously, it was found that CD11b⁺Gr1⁺ cells are the major contributors of iNOS-mediated suppression of lymphatic function in sterile inflammation.¹² To confirm the presence of this cell type in tumor models, flow cytometry was performed on B16F10 tumor tissue and it was found that 82.8 $\pm$ 2.1% of infiltrated CD45⁺ cells were CD11b⁺. Within this population, 40 $\pm$ 7% of CD45⁺CD11b⁺ were also Gr1⁺ cells. We further showed that a fraction of these CD11b⁺ cells

were also expressing iNOS and could be responsible for inhibiting lymphatic contraction (Supplementary Fig. S1). Next, we assessed whether depleting Gr1⁺ cells with an anti-Gr1 antibody restored lymphatic contraction after B16F1 or B16F10 tumor implantation. We found that depleting Gr1⁺ cells had no effect on tumor growth (Fig. 3A, B), but the strength of lymphatic contraction was greater than that in placebo-treated animals (Fig. 3C). These data show that Gr1⁺ cells inhibit lymphatic contraction in tumor-bearing animals.

Induced cancer cell death restores lymphatic contraction

Previously, we found that intratumor lymphatic vessels are collapsed.²⁸ Therefore, we tested the hypothesis that inducing tumor cell death may open these compressed vessels within the tumor and stimulate lymphatic contraction. To prevent any off-target effects on murine host cells, we took advantage of the differential sensitivity of human and murine cells to DT.²⁹ HSTS26T human sarcoma tumor chunks were implanted into the footpad in nude mice and were treated with DT to kill the human cancer cells. DT does not cause death of murine cells.²⁹ PBS injections were used as control. Mice treated with DT had a reduced maximum lymphatic diameter

FIG. 3. Lymphatic contraction is increased by depleting Gr1 cells. WT C57Bl/6 mice were implanted with B16F10 melanoma and underwent treatment with anti-Gr1 IgG or nonspecific rat IgG (control) to determine whether depletion of Gr1⁺ cells would restore lymphatic contraction. Anti-Gr1 IgG treatment had no significant effect on B16F10 melanoma growth or tumor size (A, B). The ejection fraction is significantly higher in anti-Gr1 IgG-treated animals (C). Bars shown $\pm$ SEM, $n = 12$, * $p < 0.05$ by unpaired *t*-test with a Welch's correction. IgG, immunoglobulin G.

and restored lymphatic ejection fraction (Fig. 4A–C, Table 1), demonstrating that tumor burden itself plays a role in inhibiting lymphatic contraction. This is further confirmed by a correlation between tumor size and ejection fraction (Fig. 4D). Furthermore, these data show that the impairment in lymphatic contraction induced by tumors is reversible; however, studying the contribution of immune cell infiltrates in this model may be compromised because of the use of an immunodeficient mouse strain.

Conclusion

In this study, we demonstrate that iNOS is a regulator of tumor-draining lymphatic vessels. We found that multiple tumor types reduce contraction in tumor-draining collecting lymphatic vessels (Fig. 1, Table 1), and that *iNOS*^{−/−} mice were able to maintain a higher ejection fraction than WT mice in melanoma models (Fig. 2). This finding suggests that NO produced by *iNOS* expressing cells suppresses lymphatic contraction in both sterile inflammation¹² and the tumor setting.

It is well known that tumors recruit various myeloid cells, including Gr1⁺ cells, that support tumor growth by inducing angiogenesis, lymphangiogenesis, and immune suppression.^{13,30,31} Although *iNOS* is expressed in many cells, Gr1⁺

cells commonly express *iNOS* in the tumor setting. Therefore, we determined whether depleting Gr1⁺ cells had an effect on lymphatic contraction. We found that, although Gr1⁺ cell depletion did not have an effect on tumor growth or size, lymphatic contraction was improved (Fig. 3C). This finding suggests that Gr1⁺ cells reduce lymphatic contraction during tumor growth, likely through an iNOS-mediated mechanism.

Previously, it was found that tumors induce lymphatic hyperplasia.^{32,33} We used DT treatment of a human cell line to demonstrate that direct tumor cell death can restore lymphatic contraction (Fig. 4B, C). A correlation was found between tumor size and lymphatic ejection fraction, establishing that tumor burden contributes to a reduction in lymphatic pumping (Fig. 4D). This cumulative data lead to the proposed mechanism (Fig. 4E), wherein both the tumor and the tumor-associated myeloid cells suppress lymphatic contraction. Furthermore, these data show that lymphatic dysfunction in tumor-draining lymphatic vessels is reversible, in contrast to lymphatic vessel dysfunction during and after bacterial infections.³⁴ Whether the reduction in tumor-draining lymphatic contraction leads to reduced lymph flow and lymphatic drainage requires further study, as no change was measured in lymphatic drainage from perilesion regions of melanomas compared with normal skin in patients.³⁵

FIG. 4. Depleting tumor cells increase lymphatic contraction in HSTS26T human sarcoma. HSTS26T was implanted in the footpad of nude mice and DT was used to reduce tumor burden. DT treatment directly killed tumor cells, reducing tumor size (A). Maximum lymphatic diameter and ejection fraction were measured after depletion of tumor cells (B, C). Correlation between tumor size and ejection fraction is shown (D). This cumulative data support the model that iNOS expressing CD11b⁺Gr1⁺ cells (green) associated with tumors (blue cells) contribute to the inhibition of lymphatic contraction (E). Bars shown $\pm$ SEM, * $p < 0.05$ by unpaired *t*-test with a Welch's correction. DT, diphtheria toxin.

Lymphatic vessels deliver not only tumor cells to the LN but also tumor antigen tumor-derived regulatory factors and dendritic cells. Thus, lymphatic vessels are critical for generating anticancer immune responses in TDLNs. This research highlights the need to further study the relationship between lymphatic function and immune regulation during cancer progression.³⁶ Future work is needed to understand the complex relationship between lymphatic contraction, metastasis, and antitumor immunity.

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Author Disclosure Statement

No competing financial interests exist.

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