（様式 10）

（海外特別研究員事業）

平成 **30** 年 **9** 月 **30** 日

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（氏名は必ず自署すること）

海外特別研究員としての派遣期間を終了しましたので，下記のとおり報告いたします。

なお，下記及び別紙記載の内容については相違ありません。

記

1. 用務地（派遣先国名）用務地： **ボストン**　（国名： **アメリカ合衆国**　）
2. 研究課題名（和文）※研究課題名は申請時のものと違わないように記載すること。

誘導型一酸化窒素合成酵素による腫瘍微小環境リプログラミングを利用した乳癌治療戦略

3. 派遣期間：平成 **30** 年 **4** 月 **1** 日 ～ 平成 **30** 年 **8** 月 **31** 日
4. 受入機関及び部局名

ハーバード医科大学　スティール研究所

SUMMARY / ABSTRACT

Breast cancer (BC) is the leading cause of cancer-associated death in women worldwide (2nd in US). Despite recent improvements in early detection and effective adjuvant chemotherapies, about one-third of patients with early disease will relapse with distant metastasis. Advances in cancer immunotherapy i.e., immune checkpoint blockers (ICBs) are revolutionizing cancer treatment by offering a hope for a cure. However, the benefit of ICBs in BCs, especially triple-negative BC (TNBC) is yet to be demonstrated. For immunotherapy to be successful, it is essential to understand the tumor immune contexture. Tumor immune microenvironment (TIME) abnormalities, such as poor vascular perfusion, increased extracellular matrix, and hypoxia, create conditions that impair immunotherapy in BCs. We have demonstrated that normalizing structure and function of abnormal tumor vasculature improves tumor microenvironment, delivery and function of immune cells. We have also shown that tumor-derived nitric oxide (NO) induces abnormal features of the tumor vasculature and that selective blockade of NO production from tumor cells, while maintaining physiological production of NO from vascular endothelial cells, restore perivascular gradients of NO and blood vessel maturation and function. We also found high inducible NO synthase (iNOS) expressing BCs exhibit significantly worse outcome using the TCGA data base. Here, we will first test the hypothesis that blockade of BC cell-derived NO normalizes tumor vasculature and reprograms the tumor immune microenvironment (TIME) using orthotopic murine TNBC models (**Aim 1**). We will also establish downstream angiogenic factor signaling which mediates this process. We then will demonstrate that NO-mediated TIME reprogramming enhances ICB immunotherapy in metastatic BCs by tumoral iNOS blockade (**Aim 2**).

Hypothesis 1: NO production by BC cells induces abnormal tumor vasculature and TME.

Blockade of tumor cell NO production normalizes breast cancer vessel morphology and function

We monitored microvascular parameters during E0771, which is TNBC model, growth using intravital multiphoton microscopy (MPM) through a mammary window model in C57BL/6 mice (47). To knock down iNOS expression, we transduced the *iNOS-shRNA* construct in E0771 cells. While the vasculature in control E0771 tumors became progressively abnormal (i.e., dilated, irregular, tortuous) during tumor growth, iNOS knockdown (KD) E0771 tumors maintained smooth and normal size vessels with organized network structure and sufficient density (**Fig. 1A**). Furthermore, intravital MPM with *aSMA-DsRed* mice (43) revealed that blood vessels in iNOS KD E0771 tumors had significantly higher perivascular cell coverage (i.e., more mature vessels) as compared to the control tumors (**Fig. 1B**). These vessels also had significantly higher perfusion rate (**Fig. 1C-left**) indicating that iNOS silencing induces structural and functional normalization of the tumor vasculature resulting in reduced hypoxia (**Fig. 1C-right**). Hypoxia creates a highly

immunosuppressive TEM (39). We also determined the effect of the selective iNOS inhibitor (1400W) in EMT6 murine TNBC model (34), and found that iNOS inhibitor treated tumors had significantly higher perivascular cell coverage as compared to the control tumors as determined by immunohistochemistry (**Fig. 1D**).

Blockade of tumor cell NO production regulates CXCL1, 3-CXCR2 signaling and anti-CXCR2 normalizes breast cancer vessel morphology and function.

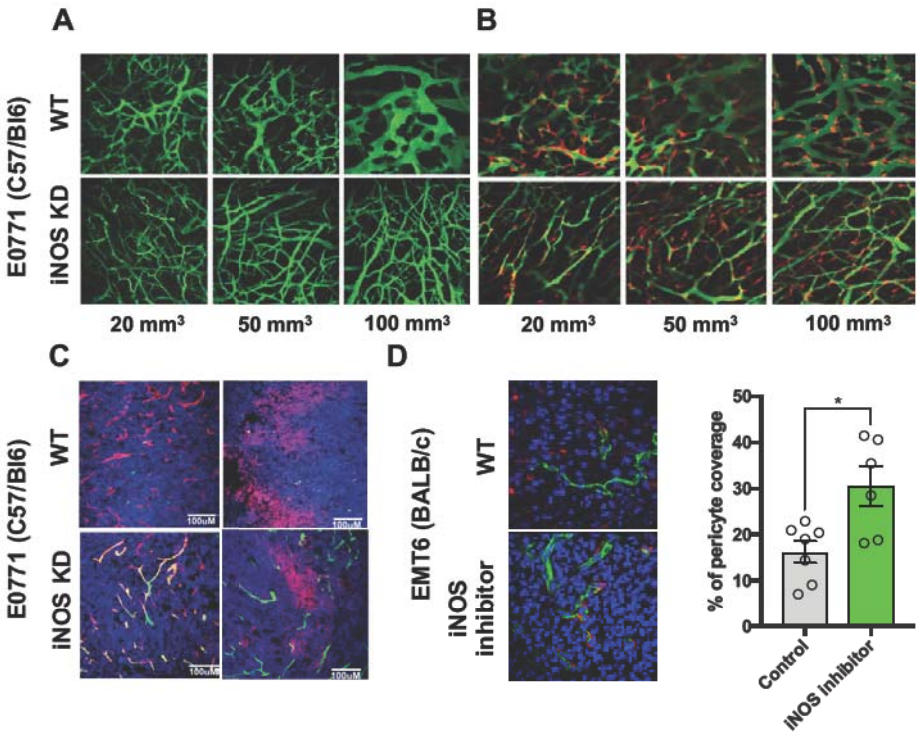

Fig. 1. Vascular normalization by iNOS blockade in breast cancer cells (**A-C**: E0771, **D**: EMT6). **A-B**: Multiphoton microangiographies (green). **B**: Pericyte cell distribution determined with αSMA promotor driven DsRed reporter mice. **C**: Confocal laser scanning microscopy images. **Left**: CD 31+ cells (red), lectin-bound perfused blood vessels (green) and DAPI stained nuclei (blue). **Right**: Hypoxyprobe™-1 adducts stained hypoxic cells (red), lectin-bound perfused blood vessels (green) and DAPI stained nuclei (blue). **D**: Effect of iNOS inhibition (1400W) on EMT6 tumor vasculature. Left: Confocal laser scanning microscopy images of CD 31+ cells (green), NG2 (red) and DAPI stained nuclei (blue). Right: % pericyte coverage, N=8, *p<0.05. t-test.

Hypothesis 2: Reprogramming TIME by iNOS blockade enhances ICB immunotherapy in mBC.

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iNOS KD in tumor cells leads to increased infiltration of CD8+ T cells in breast cancers. We have previously shown that low-dose anti-vascular endothelial growth factor receptor 2 (VEGFR2) treatment normalizes the tumor vasculature and microenvironment that in turn facilitates recruitment and function of cytotoxic T lymphocytes (CTLs), the key player of adaptive immunity, and improves the efficacy of immunotherapy (39). Thus, we hypothesized that the improvement of vessel morphology and function by knocking down iNOS in tumor cells could increase the infiltration of CTLs *i.e.,* CD8+ T cells in BCs. Immunohistochemistry revealed significantly increased infiltration of CD8+ T cells in iNOS KD E0771 tumors as compared to WT tumors (**Fig. 2A**). Furthermore, flow cytometry analysis confirmed the increase in CD8+ T cells in iNOS KD tumors (**Fig. 2B**) consistent with growth delay in these tumors (**Fig. 3**). However, we also found higher fraction of PD-1+

Tim3+ exhausted CD8+ T cells in iNOS KD tumors (**Fig. 2B**). These data suggest that a combination with

ICBs enhances anti-tumor immunity in iNOS KD tumors (**Aim 2b**).

iNOS KD in tumor cells leads to reduced infiltration of PMN-MDSCs in breast cancers. Myeloid-derived suppressor cells (MDSCs) are implicated in various aspects of immune regulation in diseases that involve chronic inflammation, especially cancer(56). Recently, evidence of the clinical significance of MDSC in cancer has emerged (81). MDSCs consist of two large groups of cells termed granulocytic or polymorphonuclear (PMN-MDSCs), which are phenotypically and morphologically similar to neutrophils, and monocytic (M-MDSCs), phenotypically, which are morphologically similar to monocytes. Besides immune-suppressive mechanisms, MDSCs promote tumor progression by affecting the remodeling of the tumor microenvironment and tumor angiogenesis, via production of VEGF, bFGF, Bv8, and MMP9 (10, 23, 80). Consistent with our findings in CD8+ T cells, we found reduced PMN-MDSC infiltration in iNOS KD BCs in our preliminary studies (**Fig. 2C**). These preliminary data support our hypothesis that targeting iNOS turns the TIME to support anti-tumor immunity.

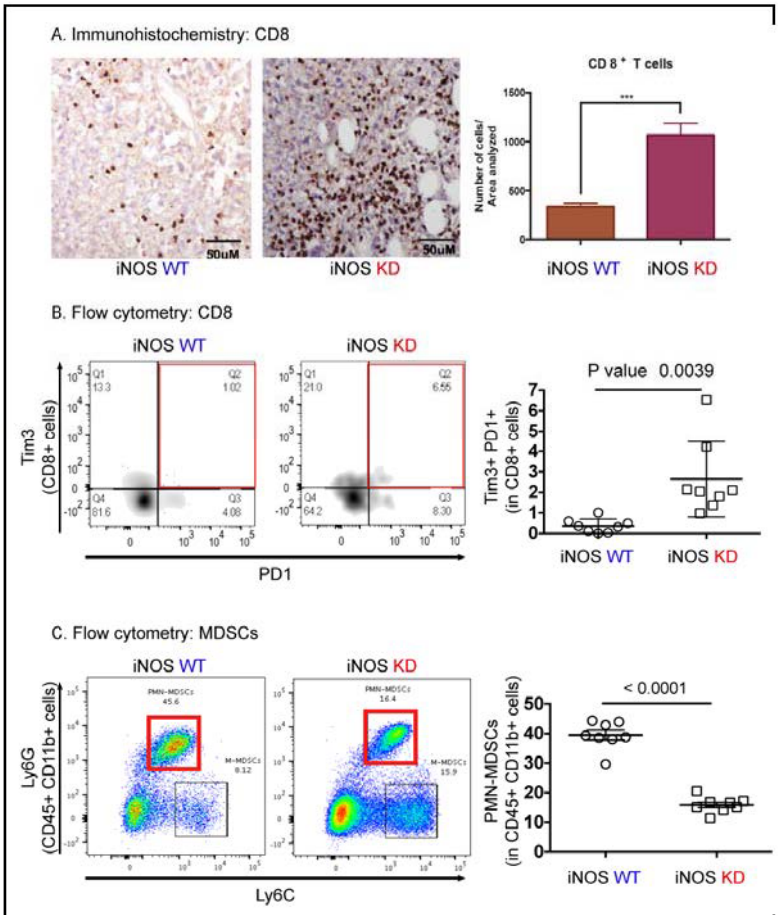

Fig. 2 iNOS KD in tumor cells leads to increased infiltration of CD8+ and CD8+ PD-1 T cells in breast cancers. A: CD8 expression in E0771 tumor by immunohistochemistry, and analysis of number of CD8+ cells/area was shown. B: Flow cytometry revealed that iNOS KD enhance CD8+ Tim3 + PD-1+ T cells accumulation, C: and decrease PMN-MDSCs infiltration * p<0.05, ** p<0.01, *** p<0.001, student-t test.

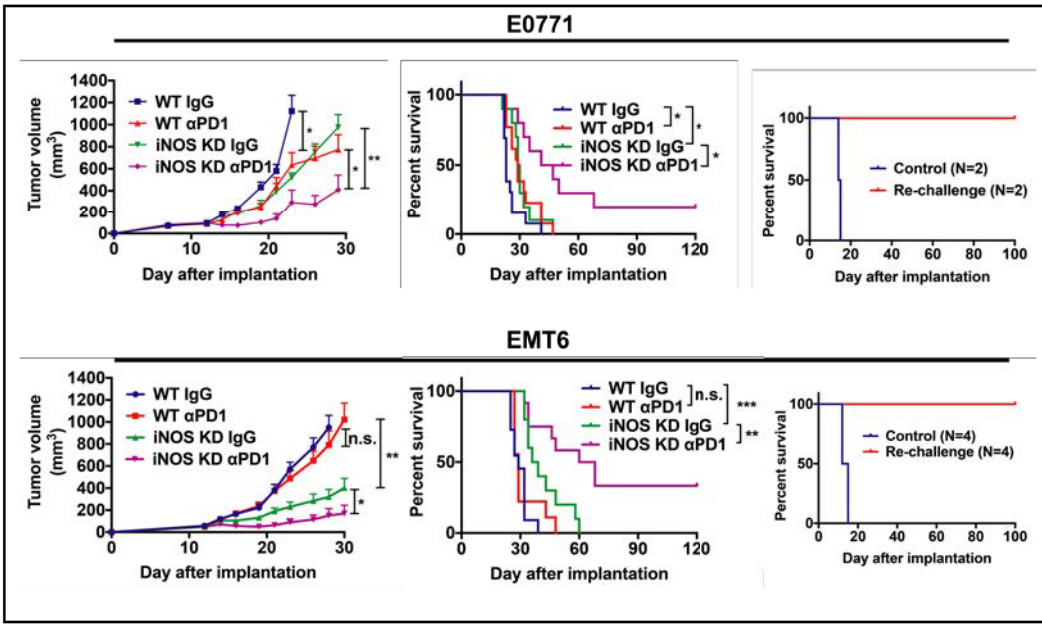

Fig. 3 The effect of iNOS KD and PD-1 blockade on tumor growth and survival of orthotopic murine breast cancer models. treated by anti- PD1 therapy. **Upper**, E0771 in C57Bl6; **Lower**, EMT6 in BALB/c; **Left**, Tumor growth; **Middle**, Survival; **Right**, Re-challenging of surviving animals from the middle.

iNOS KD in tumor cells enhances efficacy of ICB in murine breast cancer models. Following our preliminary

studies, which demonstrated reprograming of tumor microenvironment including anti-tumor immunity in BC models by targeting iNOS, we assessed if iNOS regulation improves ICB therapy in murine breast cancer models. We found that iNOS KD in tumor cells improved efficacy of anti-PD-1 antibody treatment dramatically both in E0771 and EMT6 models (**Fig. 3**). Furthermore, 2 out of 10 mice in iNOS KD E0771 and 4 out of 10 mice in iNOS KD EMT6 demonstrated complete response to anti-PD-1. These data suggest that iNOS may be an effective target to enhance immunotherapy in breast cancer.

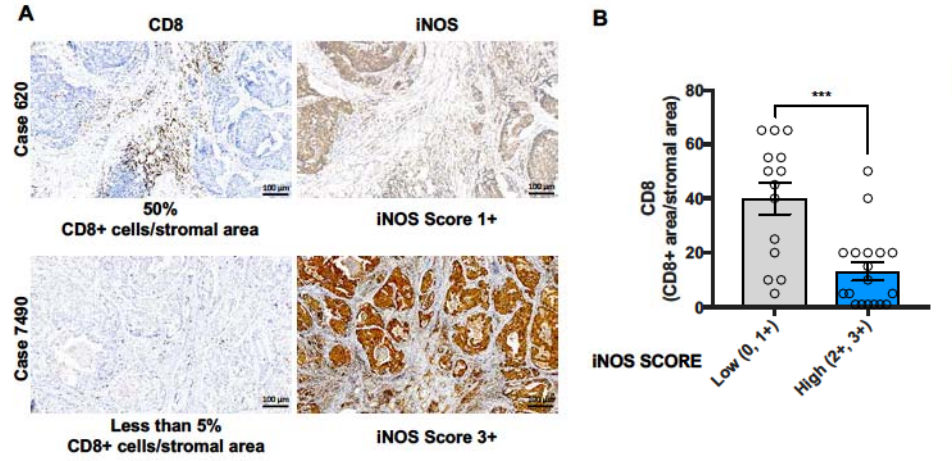

Fig. 4 Correlation between CD8+ TILs and iNOS expression in patients with TNBC. A. representative IHC staining by CD8 and iNOS in TNBC. The immunohistochemical analyses of iNOS expression in the tumor was evaluated by the intensity of the staining and received a score of 0, 1, 2, or 3 if the staining was negative, weak, moderate, or strong, respectively, as previously described (78). The dataset was scored by two independent pathologist and breast oncologist. **B.** Analysis of %CD8 TILs iNOS Low (0, 1+) vs. iNOS High (2+, 3+). ***p<0.001, student t-test,

iNOS expression inversely correlates with CD8 infiltration in patients with breast cancer. In an effort to translate our hypothesis to human breast cancer, we evaluated iNOS expression and CD8+ TILs in tissues obtained from patients with operable TNBC. Representative figures are shown in **Fig. 4A**. Case 620 had low iNOS expression and the number of TILs was high in the stromal area, whereas Case 7490 displayed high iNOS expression and very limited number of TILs. Interestingly, we found that the iNOS score was inversely correlated with infiltrating CD8+ T cells (**Fig. 4A**). CD8+ TILs were significantly ($p<0.001$) higher in low iNOS scoring group (0, 1+) compared to the high iNOS scoring group (2+, 3+) suggesting that iNOS regulates CD8+ T cells infiltration in TNBC patients (**Fig. 4B**).

Publication

1. Incio J, Ligibel JA, McManus DT, Suboj P, Jung K, **Kawaguchi K**, *et al*. Obesity promotes resistance to anti-VEGF therapy in breast cancer by up-regulating IL-6 and potentially FGF-2, ***Sci Transl Med,*** 10(432), 2018

2. Jung K, Incio J, Huang Y, Beech E, Pinter M, Ho W, **Kawaguchi K**, et al. Targeting CXCR4-dependent immunosuppressive Ly6Clow monocytes improves anti-angiogenic therapy in colorectal cancer. ***Proc Natl Acad Sci U S A***, **114**(39): 10455-10460, 2017

Presentation

1. **Kawaguchi K**, Kosuke Tsukada, Rakesh K Jain, Dai Fukumura: Reprogramming the Tumor Microenvironment to Sensitize Breast Cancers to Immunotherapy, Japan Breast Cancer Symposium, 2018, Kyoto

2. **Kawaguchi K**, Kosuke Tsukada, Rakesh K Jain, Dai Fukumura: Normalizing tumor vasculature and immune microenvironment by targeting iNOS in breast cancer cells, Ludwig cancer meeting, 2018, Boston