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14. ABSTRACT
Dissemination of breast cancer (BCa) to distant organs is the main cause of patient death and treatment failure, thereby representing the major barrier for cure of BCa. Scavenger receptor A (SRA/CD204) is an innate pattern recognition receptor primarily expressed on the host cells of myeloid origin (e.g., macrophage) and displays pleiotropic functions in immune homeostasis. Using a genetic BCa model (i.e., MMTV-PyMT), we have shown that lack of SRA suppresses spontaneous mammary tumorigenesis and that the partial loss of SRA considerably reduces BCa metastasis in lungs and lymph nodes. We further established a critical role of SRA for promoting BCa metastasis using an orthotopic BCa implantation model. The SRA-enhanced BCa metastasis appears to involve functional modulation of macrophages in the metastatic niche, indicated by polarization of alveolar macrophages toward a M1-like phenotype in the absence of SRA. Additionally, the loss of SRA conferred macrophages increased cytolytic capacity during interactions with BCa cells, supporting that SRA function acts as an important determinant of macrophage-mediated innate immune surveillance of BCa metastasis. Given the critical role of SRA in BCa tumorigenesis and metastasis, these findings may offer new opportunities to develop novel SRA-targeting approaches for treatment of metastatic BCa.

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1. INTRODUCTION

Breast cancer (BCa) is one of the most common cancers and the leading cause of mortality in women. Most of the deaths from BCa are mostly caused by metastases in the body rather than the tumor in the primary site. [1-3] Despite advances in screening, diagnosis, and treatment, nearly 12% of patients with a diagnosis of BCa eventually develop metastatic disease. Unfortunately, no single or combination therapy are currently available to control disease progression or to reduce morbidity in patients with metastatic or recurrent BCa.[4,5]

Despite a well-established role of oncogenic landscape of tumor in cancer progression and metastasis, accumulating evidences indicates that constitutive and multilayered crosstalk between tumor cells and non-cancerous stromal cells (e.g., cancer-associated fibroblasts, leukocytes) tailors the tumor microenvironment (TME) to support development, invasion, and metastasis of malignancies including BCa.[6-8] A large body of evidence shows that tumor-associated macrophages (TAMs), one of the most abundant cellular components in the TME, play a critical role in BCa progression and invasion. Clinical and experimental research has demonstrated that high levels of infiltrating TAMs positively correlate with poor patient prognosis and cancer resistance to therapies.[9] Studies using MMTV-PyMT mice, the most commonly used model for mammary tumor progression and metastasis, demonstrated that macrophages associated with primary mammary adenocarcinomas accelerate late-stage carcinogenesis by facilitating the angiogenesis.[10] Moreover, TAMs in the mammary tumor environment also foster the pulmonary metastasis by production of epidermal growth factor to alter the invasiveness of malignant mammary epithelial cells (MECs).[11,12] Therefore, understanding the key steps of the metastatic process associated with phenotype or function of TAMs and identifying key pro-metastatic environmental factors during cancer-immune interactions may provide unique therapeutic opportunities for effective management of this fatal disease.

The current project is built on our discovery that scavenger receptor A (SRA), an immune receptor that is abundantly expressed on macrophages but not on cancer cells, exhibits a previously unreported tumor-promoting feature, particularly in the metastatic process of BCa. The proposed studies aim to understand how this gene facilitates BCa growth and increases their invasiveness using multiple experimental models and approaches. It is anticipated that the findings from this research will advance our knowledge on SRA as a critical stromal factor in driving BCa growth and metastasis and establish the SRA as a novel therapeutic target for blocking BCa metastasis.

2. KEYWORDS

Breast cancer, metastasis, scavenger receptor A, tumor microenvironment, tumor associated macrophages.

3. ACCOMPLISHMENTS

The major goals of this Idea Development Award for the period from Apr 1st, 2022 through Mar 31st, 2023 are to provide important evidence of SRA function in governing BCa progression and metastasis. We have demonstrated that the partial loss of SRA in heterozygous SRA$^{+/-}$ MMTV-PyMT mice results in significantly decreased SRA expression on tumor infiltrating myeloid cells, which correlated with reduced metastatic burden in the lungs and lymph nodes. Mechanistic studies suggest that SRA may promote metastatic process through functional modulation of macrophage polarization as well as their tumor-destructive activity in the distant organs.

Specific Aim 1: **Establish the critical role of SRA for governing BCa progression and metastasis.**

Major Task 1: Determine SRA expression during mammary tumor progression.

Subtask 2: Evaluate SRA expression during mammary tumor progression.

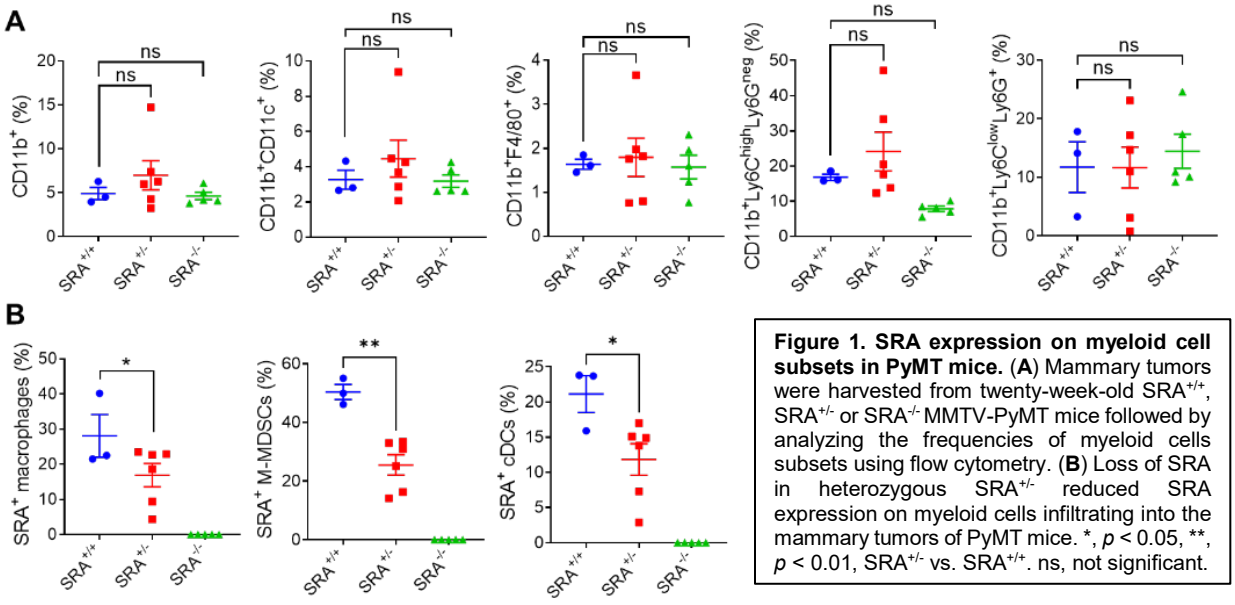

Figure 1. SRA expression on myeloid cell subsets in PyMT mice. (**A**) Mammary tumors were harvested from twenty-week-old SRA$^{+/+}$, SRA$^{+/-}$ or SRA$^{-/-}$ MMTV-PyMT mice followed by analyzing the frequencies of myeloid cells subsets using flow cytometry. (**B**) Loss of SRA in heterozygous SRA$^{+/-}$ reduced SRA expression on myeloid cells infiltrating into the mammary tumors of PyMT mice. *, $p < 0.05$, **, $p < 0.01$, SRA$^{+/-}$ vs. SRA$^{+/+}$. ns, not significant.

Our previous studies showed that complete ablation of SRA in homozygous SRA$^{-/-}$ MMTV-PyMT mice significantly delayed the formation of mammary tumors, prolonged animal survival, and reduced pulmonary metastasis. Although a lack of one SRA allele did not affect primary tumor development, there was significantly reduced pulmonary metastases, indicating that SRA acts as a critical environmental regulator of mammary tumor development and metastasis. Given that SRA is an innate pattern recognition molecule primarily expressed on the cells of myeloid origin, including macrophages,[13] we examined the expression of SRA on different myeloid cell subsets that were infiltrating into mammary tumors using multi-color FACS analysis after staining with antibodies against cell surface markers, including total myeloid cells (CD11b$^+$), convetional dendritic cells (cDCs, CD11b$^+$CD11c$^+$), macrophages (CD11b$^+$F4/80$^+$), monocytic myeloid-derived suppressor cells (MDSCs, CD11b$^+$Ly6C^{high}Ly6G$^-$) and granulocytic MDSCs (CD11b$^+$Ly6C^{low}Ly6G$^+$) in 20-week old SRA$^{+/+}$, SRA$^{+/-}$ or SRA$^{-/-}$ MMTV-PyMT mice. We showed that absence of SRA did not affect the levels of tumor-infiltrating myeloid cell populations (**Fig. 1A**). Intriguingly, loss of one allele of SRA gene caused significant reduction in SRA expression levels on macrophages, M-MDSCs, or cDCs in the mammary tumors (**Fig. 1B**), suggesting that partial loss of SRA activity could contribute to the phenotic changes (i.e., reduced pulmonary metastases) seen in the heterozygous strain. We have completed 50% of the proposed work in Specific Aim 1/Major Task 1.

Major Task 3: Define the critical role of SRA in mammary tumor metastasis using MMTV-PyMT mice, orthotopic E0771 model and orthotopic MCF-7 human breast cancer model.

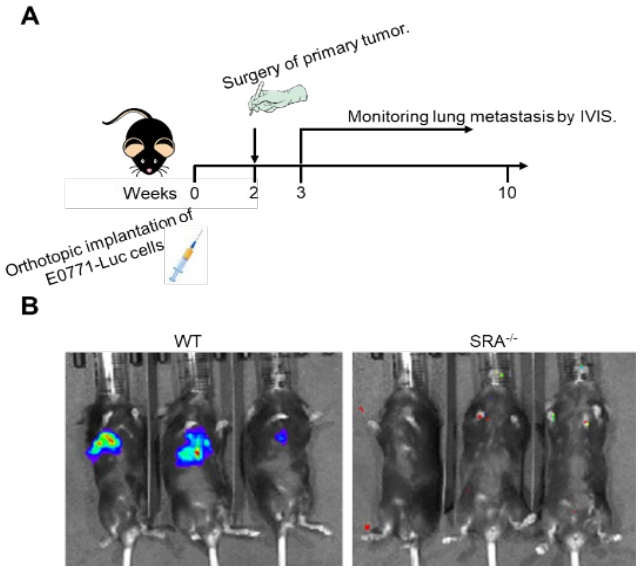

Figure 2. SRA facilitates spontaneous lung metastasis of BCa in in vivo. E0771 mouse mammary cells (2×10^5 in 100 μL PBS) were injected into the fourth mammary gland of WT or SRA$^{-/-}$ mice. Lung metastasis formation was followed by bioilluminant imaging after surgical removal of primary tumors.

We used multiple BCA metastasis models to determine the role of SRA in the metastatic process. Orthotopic injection of mouse mammary tumor E0771 cells to the mammary fat pad of mouse is a widely used model for studying BCa metastasis, in which lung metastases are typically detectable 2-4 weeks after surgical removal of primary mammary tumors.[14-16] To study SRA-mediated host effects on BCa metastasis *in vivo*, we implanted luciferase-expressing E0771 cells into the mammary fat pad of wild-type (WT) or SRA$^{-/-}$ mice. When the primary tumors reached an area of 200 mm^3, tumors were surgically removed followed by monitoring lung metastases using live imaging (**Fig. 2A**). At 7 weeks post-surgery, substantial metastatic spreading to the lungs had occurred in WT mice. However, the lung metastasis was largely absent in SRA$^{-/-}$ mice (**Fig. 2B**), indicating that SRA on myeloid cells promotes distant metastasis of BCa.

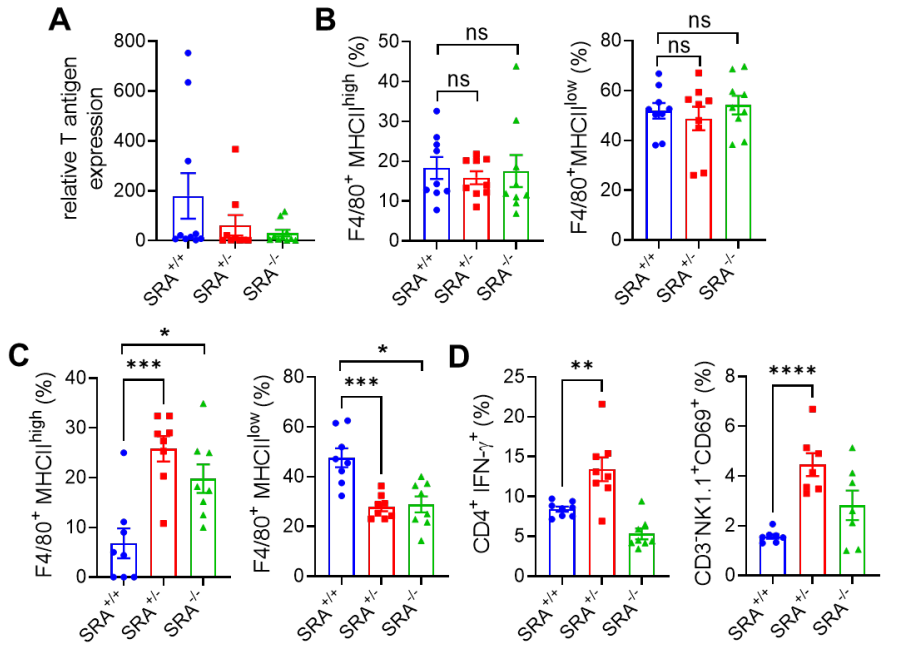

Figure 3. SRA loss inhibited lung metastasis of BCa through functional modulation of lung macrophages and lymphocytes. (**A**) Transcription of T antigen in the lungs of SRA$^{+/+}$, SRA$^{+/-}$ or SRA$^{-/-}$ MMTV-PyMT mice was determined by realtime PCR. (**B**) Single cell suspensions were prepared from the lungs of 90-day-old SRA$^{+/+}$, SRA$^{+/-}$ or SRA$^{-/-}$ MMTV-PyMT mice (**B**) or 120-day-old SRA$^{+/+}$, SRA$^{+/-}$ or SRA$^{-/-}$ MMTV-PyMT mice (**C** and **D**). Macrophage polarization, CD4$^+$ T cell and NK cell activation were analyzed by flow cytometry. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$, ns, not significant.

In MMTV-PyMT mice, the expression of the oncogene middle T antigen in mammary epithelium cell (MEC) results in primary mammary tumorigenesis as well as secondary metastatic tumors (e.g., lungs and lymph nodes).[17,18] To further address the involvement of SRA in BCa metastasis, we collected lungs from age-matched SRA$^{+/+}$, SRA$^{+/-}$ or SRA$^{-/-}$ MMTV-PyMT mice during BCa development to analyze lung metastases. Quantification of middle

T antigen expression level in lymph nodes using real-time PCR assays showed that loss of one SRA allele reduced T antigen expression in the lung tissue, further underscoring a critical role of SRA for regulating mammary tumor metastasis (**Fig. 3A**). In BCa, alveolar macrophages (AMs) can help create a metastatic niche for suppressing tumor-specific T cell responses to promote tumor establishment.[19] We examined the potential polarization of macrophages within the lungs of PyMT mice during disease onset (90-day of age) or progressive state (120-day of age) by FACS analysis of MHCII expression. There were no differences in $MHCII^{high}$ or $MHCII^{low}$ lung macrophages between 90-day old $SRA^{+/+}$, $SRA^{+/-}$ or $SRA^{-/-}$ MMTV-PyMT mice when primary tumors started to form in mammary glands (**Fig. 3B**). Strikingly, loss of even one SRA allele resulted in a sharp increase in $MHCII^{high}$ M1-like alveolar macrophages known to inhibit metastatic niche formation as well as significant reduction in the $MHCII^{low}$ or tumor-supporting M2-like macrophages (**Fig. 3C**).[20] Consistent with the phenotypic changes in these lung macrophages, there was an elevation of activated $CD4^+$ T cells and natural killer (NK) cells in $SRA^{+/-}$ MMTV-PyMT mice as compared with that in $SRA^{+/+}$ MMTV-PyMT mice (**Fig. 3D**), which is supported by a previous report that interplay between helper T cells and macrophage defines the invasive phenotype of mammary tumor cells.[21] However, the functional activation of $CD4^+$ T cells and NK cells in the lungs of $SRA^{-/-}$ MMTV-PyMT mice needs to be further investigated, since it did not correlated with the reduced metastatic burden seen in these mice.

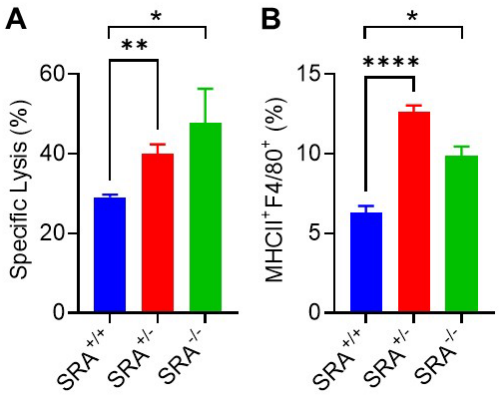

Figure 4. SRA suppresses the capability of macrophages to kill mammary tumor cells. (**A**) E0771-Luc cells were co-cultured with BMMΦ as effector cells (2:1) from $SRA^{+/+}$, $SRA^{+/-}$ or $SRA^{-/-}$ mice for 24 h. Percent specific lysis is calculated. MHCII expression on macrophages was determined by flow cytometry (**B**). *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$.

It is well recognized that macrophages have the potential to destroy cancer cells or induce their necrosis.[22,23] To test the hypothesis that SRA enhanced BCa metastasis involves reduced macrophage capability in recognizing and eliminating tumor cells, we performed a luciferase-based killing assay to examine the SRA effect on the tumor-lytic activity of macrophages. E0771 tumor cells expressing luciferase were co-cultured with bone marrow-derived macrophages from $SRA^{+/+}$, $SRA^{+/-}$ or $SRA^{-/-}$ mice for 24 hours, followed by calculation of percent specific lysis based on luciferase signal relative to that of tumor cells alone. As shown in **Fig. 4A**, $SRA^{+/-}$ or $SRA^{-/-}$ macrophages displayed enhanced cytotoxicity against E0771 cells, which correlated positively with MHCII expression and phenotypic polarization of macrophages (**Fig. 4B**). 70% of the work proposed in Specific Aim 1/Major Task 3 has been completed during the past funding period.

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Opportunities for training and professional development has the project provided:

This funding provided Post-doc training opportunity for Drs. Jinyang Cai and Halie Blevins.

The results disseminated to communities of interest:

Nothing to Report

Plan to do during the next reporting period:

We will dissect SRA-dependent myeloid cell activity required for BCa metastasis in MMTV-PyMT model by using FACS, histology analysis, real-time PCR, immunoblotting, and colongenic assay. SRA-mediated reprogramming of BCa microenvironment will be interrogated by RNA sequencing. Cell sorting and organoid differential centrifugation will also be performed to isolate immune cells and mammary epithelial cells in the mammary tumors for global transcription changes in the presence or absence of SRA. The therapeutic efficacy of anti-SRA antibodies will be evaluated using the MMTV-PyMT transgenic mice.

4. IMPACT

We have successfully demonstrated that genetic ablation of SRA strongly inhibits spontaneous BCa metastasis using both transgenic mice developing mammary tumors and orthotopic mammary tumor implantation model. Our findings establish SRA as a crucial environmental regulator that can define the macrophage function in polarizing the immune landscape and eliminating mammary tumor cells in metastatic niche. SRA therefore represents a novel therapeutic target for potential treatment of BCa metastasis.

The impact on the development of the principal discipline(s) of the project:

Nothing to Report

The impact on other disciplines:

Nothing to Report

The impact on technology transfer:

Nothing to Report

The impact on society beyond science and technology:

Nothing to Report

5. CHANGES/PROBLEMS

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6. PRODUCTS:

PUBLICATIONS:

Nothing to Report

ABSTRACTS AND PRESENTATIONS:

Nothing to Report

INVENTIONS, PATENT, AND LICENSES

Nothing to Report

OTHER PRODUCTS

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name	Xiang-Yang Wang	Chunqing Guo	Jinyang Cai	Halie Blevins	
Project Role	Principal Investigator	Co-Investigator	Post-doc	Post-doc	
Nearest person month worked	0.84	1.2	8	4	
Contribution to Project	Oversee the overall project and executed the research plan. Supervised Dr. Cai to carry out proposed studies.	Work with Dr. Wang to oversee the progress of the project and supervise Dr. Cai to perform the proposed studies.	Performed work in mouse colony maintenance, tumor monitoring, histology and pathology analysis of breast cancer invasion and metastasis in PyMT mice.	Performed work in macrophage-mammary tumor cell interaction using in vitro culture system.	
Funding Support	NIH/DOD	NIH/DOD	DOD	NIH/DOD	

COLLABORATING ORGANIZATIONS

Nothing to Report

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report

What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

N/A

9. APPENDICES