Received: 1 September 2021 | Revised: 11 January 2022 | Accepted: 27 January 2022

DOI: 10.1111/cas.15287

ORIGINAL ARTICLE

Cancer Science WILEY

Serum amyloid A 1 induces suppressive neutrophils through the Toll-like receptor 2–mediated signaling pathway to promote progression of breast cancer

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Funding information

National Natural Science Foundation of China, Grant/Award Number: 82002781; China Postdoctoral Science Foundation, Grant/Award Number: 2018M641858; Hei Long Jiang Postdoctoral Foundation, Grant/Award Number: LBH-Z18115; Science and Technology Foundation of the Finance Bureau of Heilongjiang Province, Grant/Award Number: CZKYF2021B004

Abstract

Immune inflammation plays a key role in breast cancer development, progression, and therapeutic efficacy. Neutrophils are crucial for the regulation of the suppressive tumor microenvironment and are associated with poor clinical survival. However, the mechanisms underlying the activation of suppressive neutrophils in breast cancer are poorly understood. Here, we report that breast cancer cells secrete abundant serum amyloid A 1 (SAA1), which is associated with the accumulation of suppressive neutrophils. High expression of SAA1 in breast cancer induces neutrophil immunosuppressive cytokine production through the activation of Toll-like receptor 2 (TLR2)-mediated signaling pathways. These include the TLR2/myeloid differentiation primary response 88 (MYD88)-mediated PI3K/nuclear factor-κB signaling pathway and p38 MAPK-associated apoptosis resistance pathway, which eventually promote the progression of breast cancer. Our study shows a mechanistic link between breast cancer cell secretion of SAA1 and suppressive neutrophils that potentiate tumor progression. These findings provide potential therapeutic targets for breast cancer.

KEYWORDS

breast cancer, neutrophil, serum amyloid A, suppressive, TLR2

Abbreviations: MAPK, p38 mitogen-activated protein kinase; MYD88, TLR2/myeloid differentiation primary response 88; NF-κB, nuclear factor κB; PI3K, phosphatidylinositol 3-kinase; SAA, serum amyloid A; TLR2, toll like receptor 2.

Xingjian Niu and Lei Yin contributed equally to this work.

1 | INTRODUCTION

Breast cancer is the most commonly diagnosed malignancy and the leading cause of cancer death among women worldwide.[1] Although the death rate from breast cancer has slightly declined due to advances in diagnosis and treatment, a considerable number of patients still have a poor prognosis due to breast cancer heterogeneity.[2,3] Therefore, it is critical to develop innovative strategies for breast cancer therapy.

Known as a hallmark of cancer, immune inflammation plays a predominant role in tumor development and progression. Immunotherapeutic agents, most notably those triggering immune checkpoint blockade, definitely improve the treatment benefit in various solid tumors. Therefore, these could also be useful in in breast cancer.[4–7]

Increasing evidence has shown that the immune system is constrained in the immunosuppressive tumor microenvironment (TME), which contains multiple immune cells participating in a dynamic and complex cross-talk with cancer cells.[8–10] In the TME, neutrophils are called tumor-associated neutrophils (TANs), which are important inflammatory immune cells, and have an immunosuppressive phenotype that promotes carcinogenesis and cancer cell proliferation, metastasis, and immune evasion.[11,12] Recent studies have reported the potential mechanisms by which TANs participate in tumor promotion and development.[13] We previously reported that the prognosis of luminal breast cancer correlates with immune responses.[14] Hence, neutrophils could play potential roles as predictive biomarkers and be used for therapeutic intervention in breast cancer. However, the mechanisms that govern the activation of suppressive neutrophils in breast cancer and how these cells perform immunosuppressive activities remain unclear.

During inflammation, cancer cells undergo adaptive transformation and acquire immunoediting characteristics to maintain sustained survival signals and escape the immune response.[15–17] The acquisition and maintenance of the neutrophil immunosuppressive phenotype is accompanied by the secretion of cytokines, chemokines, and adhesion molecules by coexisting tumor cells, emphasizing the complex interplay between cancers and neutrophils.[18,19] Serum amyloid A (SAA) is one of the most prominent acute phase proteins secreted by cancer cells and confers cytokine-like properties in response to inflammation.[20–22] Serum amyloid A levels are significantly elevated in several malignancies[23] and correlate with poor prognosis. Previous studies have reported that SAA is a critical mediator of pro-inflammatory cytokine production and recruitment of immune cells, and thereby promotes tumor development by facilitating angiogenesis, tumor invasion, and immunosuppression.[24,25] Recent evidence has shown that SAA mediates systemic neutrophil function in specific contexts of inflammation, suggesting that it might influence neutrophil-mediated tumor development.[26] Here, we investigated whether SAA was elevated in the breast cancer TME and its effects on neutrophils, thus unfolding the mechanism underlying neutrophil involvement in cancer, inflammation, and immunomodulatory processes.

2 | MATERIALS AND METHODS

2.1 | Bioinformatics analysis

Gene expression datasets were obtained from The Cancer Genome Atlas (TCGA) data portal (https://portal.gdc.cancer.gov). One thousand two hundred seventeen TCGA samples were grouped into breast cancer and healthy control groups. R implementation of the DESeq2 package was used for differential expression analysis. Differentially expressed genes (DEGs) are sorted according to the adjusted *p* value threshold (false discovery rate [FDR] method) of 0.05. Functional enrichment analysis was carried out using DAVID (https://david.ncifcrf.gov) with an adjusted *p* value (FDR correction) with cut-off of 0.05.

Relevance of SAA1 expression to neutrophils from the TCGA database was analyzed using the TIMER 2.0 algorithm (http://timer.cistrome.org/). The protein-protein interaction (PPI) network related to SAA1 gene and biological analyses were undertaken using the STRING online database. Finally, the network was imported to Cytoscape for visualization, and MCODE was used to identify functional submodules in PPI.

2.2 | Sample collection

This study enrolled patients pathologically diagnosed with breast cancer in Harbin Medical University Cancer Hospital and 38 healthy controls between January 1, 2017 and June 30, 2020. The characteristics of the breast cancer patients are summarized in Table 1. All patients were diagnosed by complete clinical and pathologic data, received adjuvant standard chemotherapy with or without anti-human epithelial receptor 2 (HER2) therapy. None of the patients received granulocyte/macrophage colony-stimulating factor treatment for neutropenia. Peripheral blood samples (5 ml) were collected from each breast cancer patient and healthy control. For each sample, 1 ml was centrifuged to collect plasma for SAA1 analysis by ELISA; others were used for neutrophil and leukocyte isolation and cell culture. This study protocol was approved by the Institutional Review Board of Harbin Medical University Cancer Hospital. Written informed consent was obtained from the patients and healthy controls.

2.3 | Neutrophils and leukocytes isolation

Neutrophils were separated from 3 ml peripheral blood samples using Ficoll-Hypaque density gradient centrifugation, according to the manufacturer's instructions (GE Healthcare). The purity of neutrophils was within acceptable parameters (higher than 95%). Peripheral blood samples (1 ml) were lysed twice with RBC lysis buffer (BD Biosciences) to obtain leukocytes.

TABLE 1 Characteristics of 178 breast cancer patients included in this study

Characteristic	Overall (%)	SAA1 < 7.68	SAA1 ≥ 7.68	*p* Value
Age (years)				
≤50	102 (57.3)	47	55	0.489
>50	76 (42.7)	39	37	
Tumor size (cm)				
≤3	108 (60.7)	59	49	0.036
>3	70 (39.3)	27	43	
Nodal status				
N−	79 (44.4)	46	33	0.018
N+	99 (55.6)	40	59	
Clinical stage				
I	29 (16.3)	19	10	0.002
II	106 (59.6)	54	52	
III	43 (24.2)	13	30	
Histological grade				
I	19 (10.7)	9	10	0.285
II	146 (82.0)	68	78	
III	13 (7.3)	9	4	
ER status				
ER+	57 (32.0)	26	31	0.621
ER−	121 (68.0)	60	61	
PR status				
PR+	92 (51.7)	47	45	0.444
PR−	86 (48.3)	39	47	
HER2 status				
HER2+	20 (11.2)	12	8	0.266
HER2−	158 (88.8)	74	84	
Ki-67 status				
<20%	68 (38.2)	33	35	0.964
≥20%	110 (61.8)	53	57	
P53 status				
Positive	67 (37.6)	34	33	0.614
Negative	111 (62.4)	52	59	

Abbreviations: ER, estrogen receptor; HER2, human epithelial receptor 2; PR, progesterone receptor; SAA1, serum amyloid A 1.

2.4 | Cell culture

Human breast cancer cell lines (ZR-75-1, BT-474, MCF-7, and MDA-MB-231), murine breast cancer cell line (4T1) and nontumorigenic cell line (MCF-10) were purchased from the Cell Bank for Type Culture Collection (Chinese Academy of Sciences). The cells were cultured in RPMI-1640 medium containing 10% FBS (Gibco, Thermo Fisher Scientific) at 37°C, 5% CO_2. Purified neutrophils collected from peripheral blood samples were also incubated in RPMI-1640 medium containing 10% FBS at 37°C with 5% CO_2. A portion of purified neutrophils was treated with 1 μM SAA1 (PeproTech) for 24 h. The other part was preincubated for 1 h with formyl peptide receptor-like 1 (FPRL1)-blocking Ab (1 μM, FN-1D6-AI; Genovac), Toll-like receptor 2 (TLR2)-blocking Ab (1 μM, HY-100461; MCE), nuclear factor-κB (NF-κB) inhibitor (1 μM, B5681; Sigma-Aldrich), PI3K inhibitor (1μM, LY294002; Calbiochem Merck), or p38 inhibitor (1 μM, SB203580; Calbiochem Merck), and then cultured for 24 h with or without SAA1.

2.5 | Animal models

Female Balb/c mice, aged between 8 and 10 weeks, were purchased from the Laboratory Animal Center of Harbin Medical University. All animal procedures were carried out in compliance with the protocol and guidelines for the Care and Use of Laboratory Animals by the China National Institutes of Health and approved by the Animal Care Committees of Harbin Medical University. Mice were randomly divided into four groups (n = 10/group). For each group, 1 $\times 10^4$ 4T1 cells suspended in 0.3 ml mixture was injected subcutaneously

into the fourth mammary fat pad of each mouse. For the Ab treatment, four groups of mice received the following interventions: (i) control IgG (ab37373; Abcam); (ii) anti-SAA1 Ab (LS-C150247; LifeSpan BioSciences); (iii) anti-Ly6G Ab (1A8; BioLegend); and (iv) anti-SAA1 Ab and anti-Ly6G Ab. Tumor size was measured every 4 days by a digital slide caliper, according to the formula: volume = 0.5 × length × width^2. Tumor sections were collected.

2.6 | Enzyme-linked immunosorbent assay

The expression levels of SAA1 in plasma collected from breast cancer patients or breast cancer cell lines, as well as interleukin-10 (IL-10), arginase (Arg), and inducible nitric oxide synthase (iNOS) from the supernatant of neutrophils, were quantified with the appropriate Quantikine Kit (R&D Systems) according to the manufacturer's instructions.

2.7 | Immunohistochemical staining

A 4-μm-thick tumor tissue section of paraffin-embedded block was used for immunohistochemical (IHC) staining. The sections were incubated with anti-SAA1 Ab (1:200 dilution, ab687; Abcam), anti-CD66b Ab (1:200 dilution, ab197678; Abcam), and anti-CD3 Ab (1:200 dilution, ab16669; Abcam) at 4°C overnight. The staining results were assessed by two pathologists independently, and the numbers of SAA1, CD66, and CD3 positive cells within areas of tumor were separately quantitated by counting at ×200 magnification. The results are expressed as average number of IHC positive cells per field. Ten viewing fields were randomly selected.

2.8 | Real-time PCR

Total RNA of cell lines and neutrophils were isolated with TRIzol Reagent (Invitrogen) and cDNA was obtained with the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics). Real-time RT-PCR was carried out with SYBR Premix Ex Taq II (TaKaRa). The relative mRNA levels of SAA1 and SAA2 were determined by normalizing with the mRNA level of β-actin with the $2^{-\Delta\Delta C_t}$ method.

The sequences of the primers were as follows: forward ACTIN, 5′-GCCAACCGCGAGAAGATGA-3′; reverse ACTIN, 5′-CATCAGGATGCCAGTGGT-3′; forward SAA1, 5′-TTGGCGAGGC TTTTGATGGGG-3′; reverse SAA1, 5′-AGGTCGGAAGTGATTGG GGT-3′; forward SAA2, 5′-GCTTCTTTTCGTTCCTTGGCG-3′; and reverse SAA2, 5′-GCCGATGTAATTGGCTTCTCTCA-3′.

2.9 | Flow cytometry

The leukocytes isolated from breast cancer patients and healthy controls were resuspended in 100 μl PBS and stained with master mix of Abs for cellular surface stains including FITC-conjugated CD15 and phycoerythrin-conjugated CD11b (both from BD Biosciences) for 30 min at 4°C. The stained cells were analyzed by FACSCalibur flow cytometer (Becton Dickinson).

2.10 | Immunofluorescence

For immunofluorescent staining the neutrophils treated with or without SAA1 and TLR2-blocking Ab were fixed in 4% paraformaldehyde and then permeabilized with 0.5% Triton X-100. The cells were incubated with anti-iNOS Ab (1:200 dilution, SAB14200766; Sigma), anti-Arg Ab (1:300 dilution, ab48586; Abcam), or anti-IL-10 Ab (1:300 dilution, ab34843; Abcam) at 4°C overnight. Then cells were incubated with secondary Ab for 1 h at 37°C. They were then incubated with DAPI for 15 min at room temperature. Fluorescence was imaged with fluorescence microscopy (BX40; Olympus).

2.11 | Western blot analysis

Breast cancer cells and neutrophils were each homogenized in 0.5 ml RIPA buffer. Polyacrylamide gels (10%) were used for protein testing. Membranes were incubated with anti-SAA1 Ab (1:2000 dilution, ab687; Abcam), anti-TLR2 Ab (1:3000 dilution, ab68159; Abcam), anti-myeloid differentiation primary response 88 (MYD88) Ab (1:2000 dilution, ab135693; Abcam), anti-p65/NF-κB Ab (phospho S536, 1:3000 dilution, ab86299; Abcam), anti-PI3K Ab (1:2000 dilution, ab191606; Abcam), anti-AKT Ab (phospho S473, 1:2000 dilution, ab8805; Abcam), anti-p38 (phospho T180+Y182) Ab (1:3000 dilution, ab4822; Abcam), anti-Bcl-2 Ab (1:2000 dilution, ab32124; Abcam), and anticleaved caspase-3 Ab (1:3000 dilution, ab2302; Abcam). The specific bands were visualized using ECL reagent. The bands were analyzed using Bio-Rad Chemi EQ densitometer and Bio-Rad Quantity One software (Bio-Rad Laboratories).

2.12 | Assessment of apoptosis

Apoptosis analysis was evaluated by cytofluorometry using the annexin V-FITC kit (Becton Dickinson) according to the manufacturer's instructions. Apoptosis was determined by FACSCalibur flow cytometry (Becton Dickinson).

2.13 | Statistical analysis

The correlations among clinicopathologic factors and the expression of SAA1 were analyzed by using Fisher's exact test and the χ^2 test. Overall survival (OS) and disease-free survival (DFS) curves were estimated by the Kaplan-Meier method. Statistical analyses were undertaken with the SPSS software package version 20 (SPSS, Inc.). Data are expressed as mean ± SD. p Values <0.05 were considered

statistically significant with an unpaired two-tailed Student's *t* test and one-way ANOVA test. All datasets were tested for normal distribution.

3 | RESULTS

3.1 | Serum amyloid A 1 is secreted by breast cancer cells, and its expression is increased in high-grade breast cancer patients

To investigate how gene expression alteration contributes to pathogenesis of breast cancer, we analyzed the DEGs of breast cancer patients and healthy controls using the RNA sequencing data from the TCGA database. Analysis results of gene expression profile data were visualized with volcano plots (Figure 1A) and nonsupervised hierarchical clustering (Figure 1B and Table S1). To gain more information about molecular functions and pathways that are affected by DEGs, the DAVID database was used to perform the functional enrichment analysis of all genes (Figure 1C). The DEGs identified in breast cancers were involved in a wide range of molecular functions associated with immune response, such as signaling receptor activator activity, receptor ligand activity, cytokine activity, and chemokine receptor binding (Figure 1C).

Secreted by cancer cells, SAA is an acute-phase protein involved in many malignancies.[23,25] The human SAA family comprises two SAA isoforms, SAA1 and SAA2. First, we detected the SAA1 isoform that was mainly expressed in human breast cancer cells, but not in the nontumorigenic cell line MCF-10 (Figure S1A). To evaluate whether the expression of SAA1 was upregulated in breast cancer, we collected peripheral blood samples from 178 breast cancer patients and 38 healthy controls to detect the concentrations of SAA1 in plasma. As shown in Figure 1D, SAA1 expression was significantly elevated in breast cancer patients. Furthermore, to investigate the potential role of SAA1 in breast cancer, we analyzed the clinical data of breast cancer patients (Table 1) and classified the patients into high and low SAA1 groups (optimal cut-off value, 7.68) according to the receiver operating characteristic curve (Figure S1B). We found that patients with SAA1 ≥7.68 often had a larger tumor size, lymphatic metastasis, and higher incidence of advanced clinical stages (Table 1), and found a relative higher SAA1 expression in triple-negative breast cancer patient samples (59.09%, 39/66) than luminal subtype (48.91%, 45/92) and HER-2 subtype (40.00%, 8/20). In addition, we found a high expression of SAA1 in breast cancer tissue samples and a positive correlation between SAA1 expression and disease staging (Figure 1E), which was consistent with the results for SAA1 levels in peripheral blood samples from patients with different stages of breast cancer (Figure S1C). Next, survival curves were evaluated based on the SAA1 levels and indicated that patients with higher SAA1 expression had shorter OS and DFS than those with low SAA1 levels (Figure S1D). Together, these data indicate that SAA1 expression is upregulated and is correlated with higher disease grade and poor prognosis in breast cancer patients.

3.2 | Immunosuppressive neutrophils are associated with SAA1 secretion in breast cancer

Neutrophils are the most abundant inflammatory immune cells in the TME and usually exert an immunosuppressive phenotype that promotes cancer progression;[12,27,28] neutrophils are regulated by signals produced by cancer cells.[11,29] Thus, we hypothesized that an abundant expression of SAA1 could regulate neutrophils in breast cancer. To address this possibility, we used the online platform TIMER 2.0 to analyze the correlation between SAA1 expression level and the infiltration level of neutrophils in breast cancer (Figure 2A). The results showed that the expression level of SAA1 was positively correlated with the infiltration level of neutrophils in breast cancer. To evaluate whether the level of neutrophils was increased in breast cancer, we also analyzed the positive rate of infiltration of neutrophils, which was significantly higher in breast cancers than in healthy controls (Figure 2B). The $CD15^+$ and $CD11b^+$ populations of neutrophils were more susceptible to SAA treatment as reported previously,[23] then total leukocytes were extracted from breast cancer patients and healthy controls, and $CD11b^+CD15^+$ neutrophil levels were determined. We observed that the levels of $CD11b^+CD15^+$ neutrophils were significantly increased in patients with breast cancer than in healthy controls (Figure 2C). Additionally, the immunosuppressive cytokines IL-10, Arg, and iNOS, were significantly higher in neutrophils from breast cancer patients than in those from the control group (Figure 2D), suggesting that the neutrophils in breast cancer presented an immunosuppressive phenotype. $CD66b^+$ neutrophils are reported to be an independent prognostic factor of cancer,[30,31] thus we undertook IHC staining to evaluate SAA1 and $CD66b^+$ neutrophils in tissue samples from breast cancer patients. As shown in Figure 2E, elevated SAA1 expression was associated with abundant neutrophil infiltration in breast cancer tissue samples ($p = 0.0056$). In addition, the SAA1 expression levels in plasma were positively correlated with neutrophils (Table S2) as well as the expression levels of IL-10, Arg, and iNOS, which were mainly secreted by neutrophils (Figure 2F). These results suggest that SAA1 potentially contributes to the accumulation of immunosuppressive neutrophils in breast cancer.

3.3 | Serum amyloid A 1 facilitates neutrophil secretion of immunosuppressive cytokines through the TLR2 receptor

To determine the mechanism by which SAA1 induces immunosuppressive neutrophils, STRING database was searched to extract the PPI network related to the *SAA1* gene (Figure 3A). Results showed that SAA1 was connected to genes with important functions, such as *TLR2*, *FPRL1*, *AKT2*, and *MYD88*. To further investigate the mechanisms, we detected the expression levels of SAA1 in multiple breast cancer cell lines and a nontumorigenic cell line MCF-10. As shown in Figures 3B and S1A,E, breast cancer cell lines had a significantly higher expression of SAA1 compared to MCF-10. MDA-MB-231

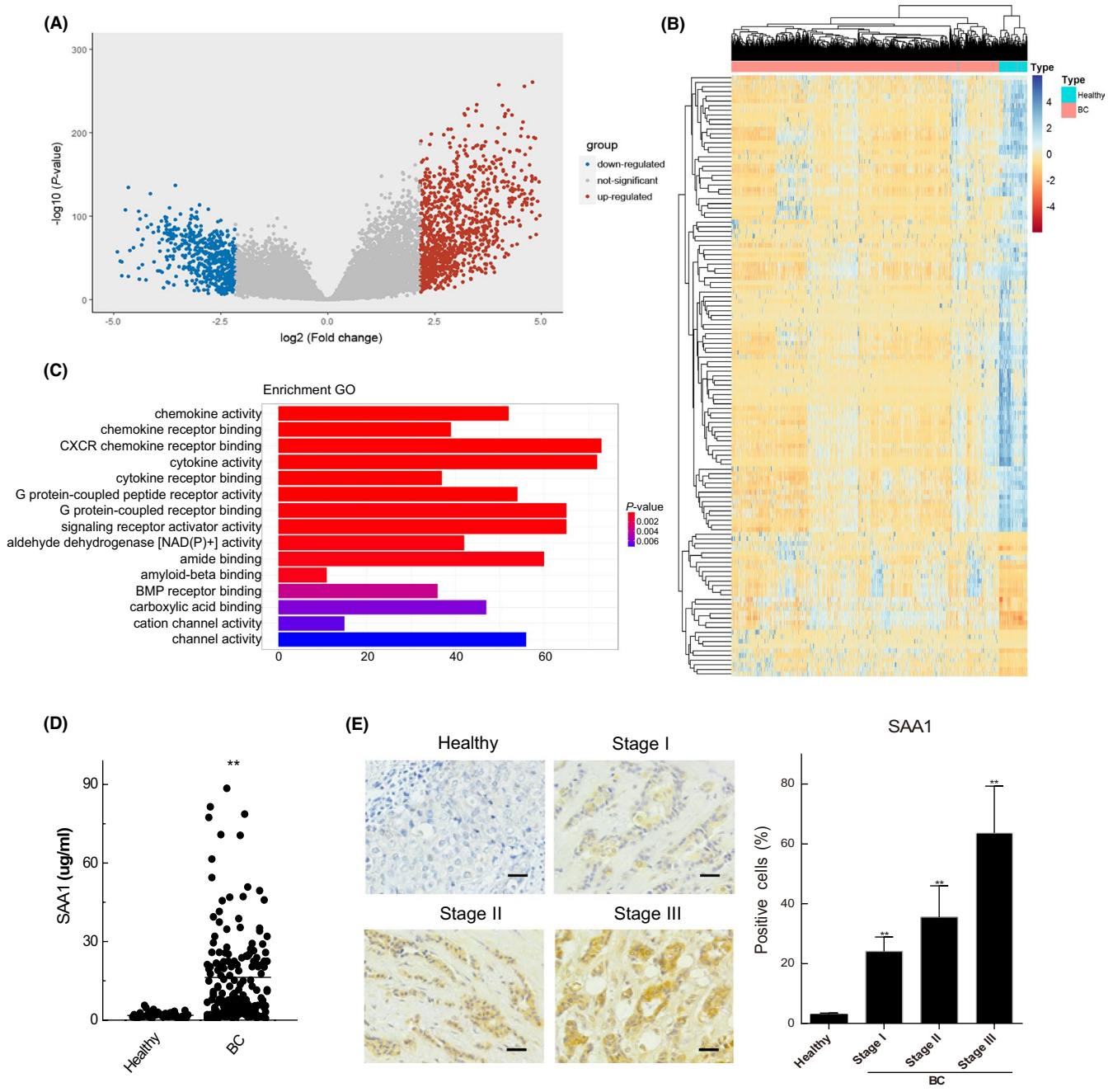


FIGURE 1 Serum amyloid A 1 (SAA1) is highly expressed in breast cancer (BC) patients and is correlated with a high tumor grade and poor prognosis. (A) Volcano map of differentially expressed genes (DEGs) between BC and healthy control groups from datasets. (B) Heatmap of DEGs between BC and healthy control groups. (C) Main enriched Gene Ontology (GO) terms of the genes in functional submodules. (D) Expression levels of SAA1 determined in plasma from BC patients and healthy controls by ELISA. (E) Representative images of immunohistochemical staining for SAA1 (200×) in BC tissues and healthy controls. Scale bar = 50 μm. Data are expressed as mean ± SD. $^{**}p < 0.01$

cells showed the highest SAA1 expression; therefore, they were used for the following experiments. In addition, to clarify whether breast cancer cells are the main source of SAA, we compared the mRNA expression levels of SAA isoforms in neutrophils and breast cancer cells and determined that neutrophils had little expression of *SAA1* (Figure S1F).

In order to investigate the effect of SAA1 on neutrophils, we isolated neutrophils from breast cancer patients and then cocultured neutrophils with either the supernatant of breast cancer cells or extrinsic SAA1. Compared with neutrophils cultured alone, neutrophils cultured with the supernatant of breast cancer cells or extrinsic SAA1 had significantly increased SAA1 expression levels (Figure 3C). In addition, we found that the secretion of IL-10, Arg, and iNOS by neutrophils was induced by SAA1 (Figure 3D), confirming the pivotal role of SAA1 in promoting the immunosuppressive phenotype and function of neutrophils. The most commonly

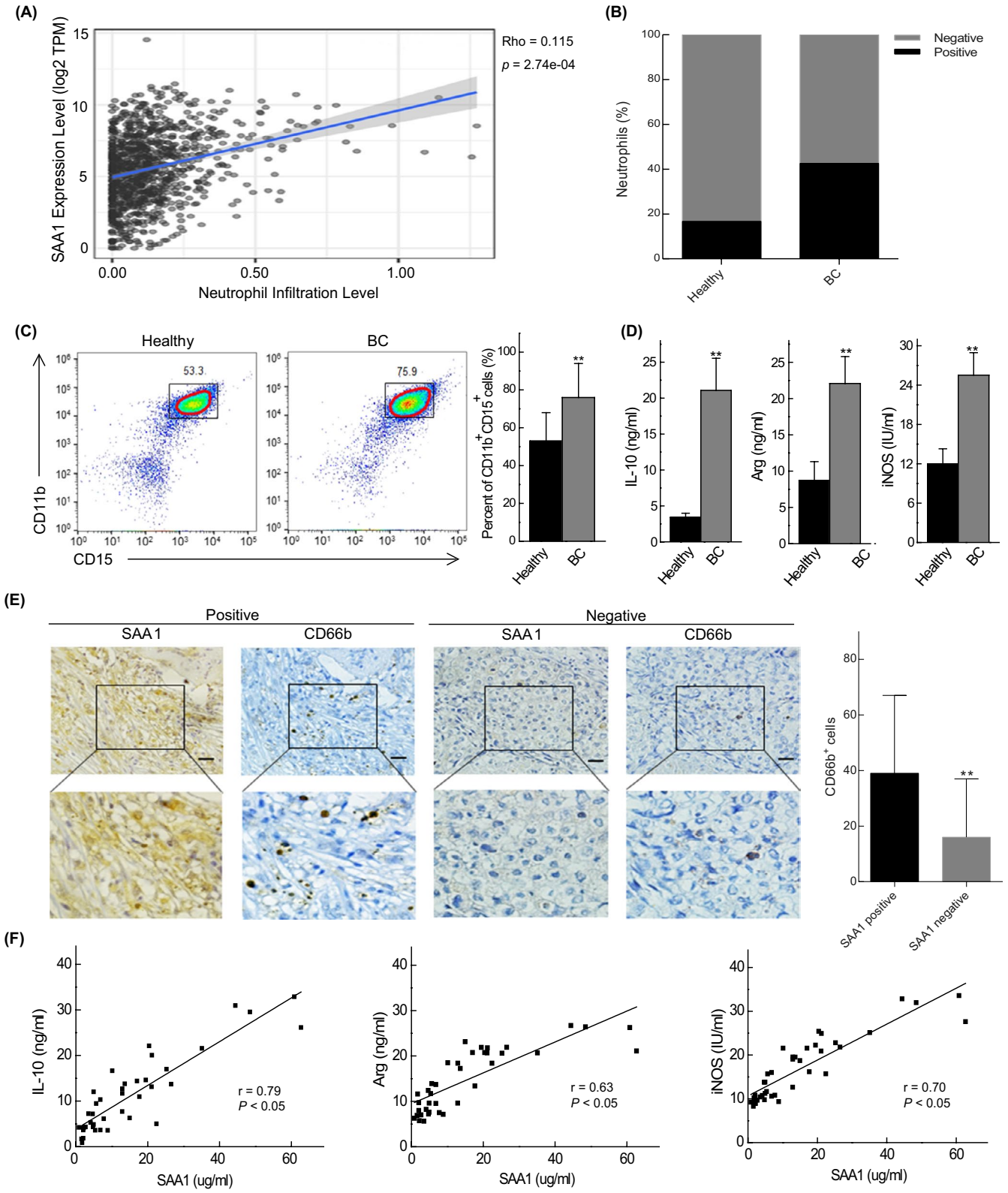


FIGURE 2 Neutrophils in breast cancer (BC) have an immunosuppressive phenotype, which is associated with serum amyloid A 1 (SAA1) expression. (A) Correlation between SAA1 expression level and infiltration level of neutrophils was analyzed by the TIMER 2.0 database. (B) Positive rates of infiltration of neutrophils in BC by TIMER 2.0 database. (C) Flow cytometry analysis of neutrophils ($CD11b^+CD15^+$) isolated from BC patients. (D) Plasma expression levels of interleukin-10 (IL-10), arginase (Arg), and inducible nitric oxide synthase (iNOS) in neutrophils from BC patients and healthy controls. (E) Representative images (left) for positive and negative expression levels of SAA1 and CD66b in BC tissues. Black boxes indicate areas shown at a higher magnification (200×). Quantification data of $CD66b^+$ cells in positive and negative expression levels of SAA1, expressed as number of immunohistochemistry positive cells per field. Scale bar = 50 μm. (F) Correlation analysis between SAA1 expression in plasma and IL-10, Arg, and iNOS expression levels in neutrophils from BC patients. Data are expressed as mean ± SD. $^{**}p < 0.01$

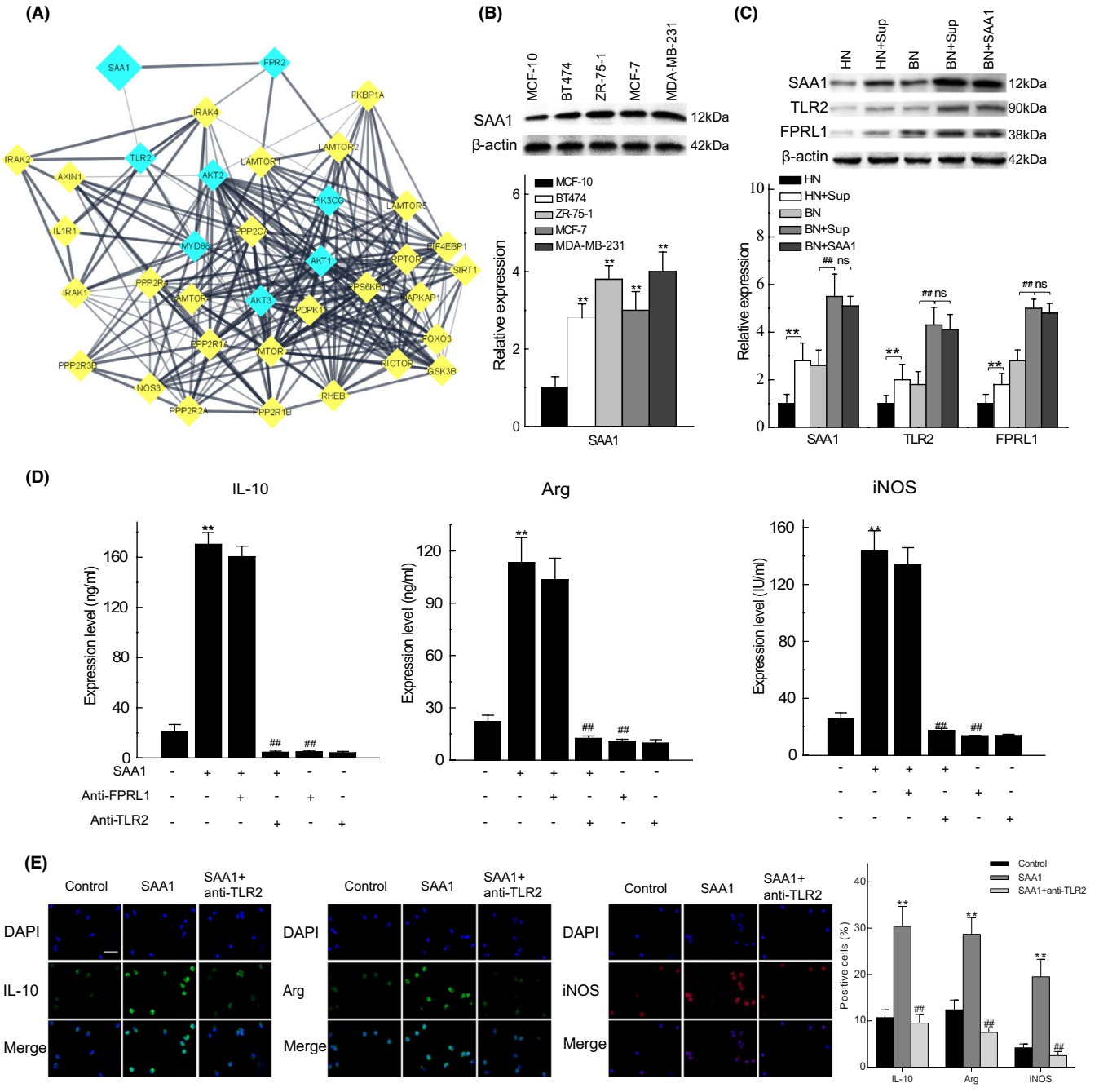


FIGURE 3 Serum amyloid A 1 (SAA1) promotes neutrophil secretion of immunosuppressive cytokines through Toll-like receptor 2 (TLR2). (A) Protein-protein interaction network relating to SAA1. (B) Representative bands and relative expression levels of SAA1 in breast cancer cell lines ZR-75–1, BT-474, MCF-7, and MDA-MB-231 and nontumorigenic cell line MCF-10 ($n = 3$). $^{**}p < 0.01$ vs. MCF-10 cell line. (C) Representative bands and relative expression levels of SAA1, TLR2, and formyl peptide receptor-like 1 (FPRL1) proteins in neutrophils isolated from breast cancer patients (BN) and healthy controls (HN), cocultured with breast cancer cell supernatant (Sup) or SAA1 ($n = 3$). $^{**}p < 0.01$ and $^{\#\#}p < 0.01$ vs. untreated neutrophils. (D) Interleukin-10 (IL-10), arginase (Arg), and inducible nitric oxide synthase (iNOS) secretion by neutrophils purified from healthy control and breast cancer patients, preincubated with SAA1 in the presence or absence of FPRL1-blocking Ab and TLR2-blocking Ab ($n = 3$) $^{**}p < 0.01$ vs. untreated neutrophils; $^{\#\#}p < 0.01$ vs. SAA1-treated neutrophils. (E) Immunofluorescence images of IL-10, Arg, and iNOS expression levels in neutrophils treated with PBS, SAA1, or SAA1 plus TLR2-blocking Ab ($n = 3$). $^{**}p < 0.01$ and $^{\#\#}p < 0.01$ vs. untreated neutrophils. Scale bar = 50 μm. Data are expressed as mean ± SD

characterized receptors for SAA1 are FPRL1 and TLR2, each of which can be expressed by neutrophils, according to previous studies.[32,33] Interestingly, we found that the expression levels of both FPRL1 and TLR2 were elevated in SAA1-treated neutrophils (Figure 3C), but whether these two receptors played a role in regulating the secretion of immunosuppressive cytokines was unknown. To identify the effects of FPRL1 and TLR2 in SAA1-treated neutrophils, the neutrophils were incubated with FPRL1- and TLR2-blocking Abs. As shown in Figure 3D, the FPRL1-blocking Ab had no effect on the levels of inflammatory cytokines. In contrast, the addition of a

TLR2-blocking Ab prevented the secretion of IL-10, Arg, and iNOS by neutrophils (Figure 3D). These results were further confirmed by immunofluorescence (Figure 3E). Together, these data indicate that SAA1 facilitates the production of immunosuppressive cytokines in neutrophils through the TLR2 receptor.

3.4 | Serum amyloid A 1 stimulates neutrophils to exert immunosuppressive functions through the TLR2/MYD88-mediated PI3K/NF-κB signaling pathway

Next, we examined the impact of TLR2 on neutrophils activated by SAA1. Toll-like receptor 2 is thought to contribute to signal transduction in a variety of immune cells, but it is not known whether TLR2 is required for the activation of downstream signals in neutrophils.[34–36] We found that the expression level of MYD88, which is an adaptor protein for TLR2, was significantly increased in SAA1-treated neutrophils (Figure 4A). In addition, SAA1 promoted the expression of PI3K, p-AKT, and p-p65/NF-κB in neutrophils, and the addition of a TLR2-blocking Ab, a PI3K inhibitor, and an NF-κB inhibitor repressed the expression of these proteins (Figure 4A). These data suggested that SAA1 could trigger the TLR2/MYD88-mediated PI3K and NF-κB pathway in neutrophils. To determine whether this signaling pathway influenced neutrophil cytokine production, we evaluated the synthesis of IL-10, Arg, and iNOS. As shown in Figure 4B, SAA1-induced IL-10, Arg, and iNOS expression was inhibited by the TLR2-blocking Ab, PI3K inhibitor, and NF-κB inhibitor, which indicates that the variations in immunosuppressive functions of neutrophils promoted by SAA1 occurred through the TLR2/MYD88-mediated PI3K/NF-κB signaling pathway.

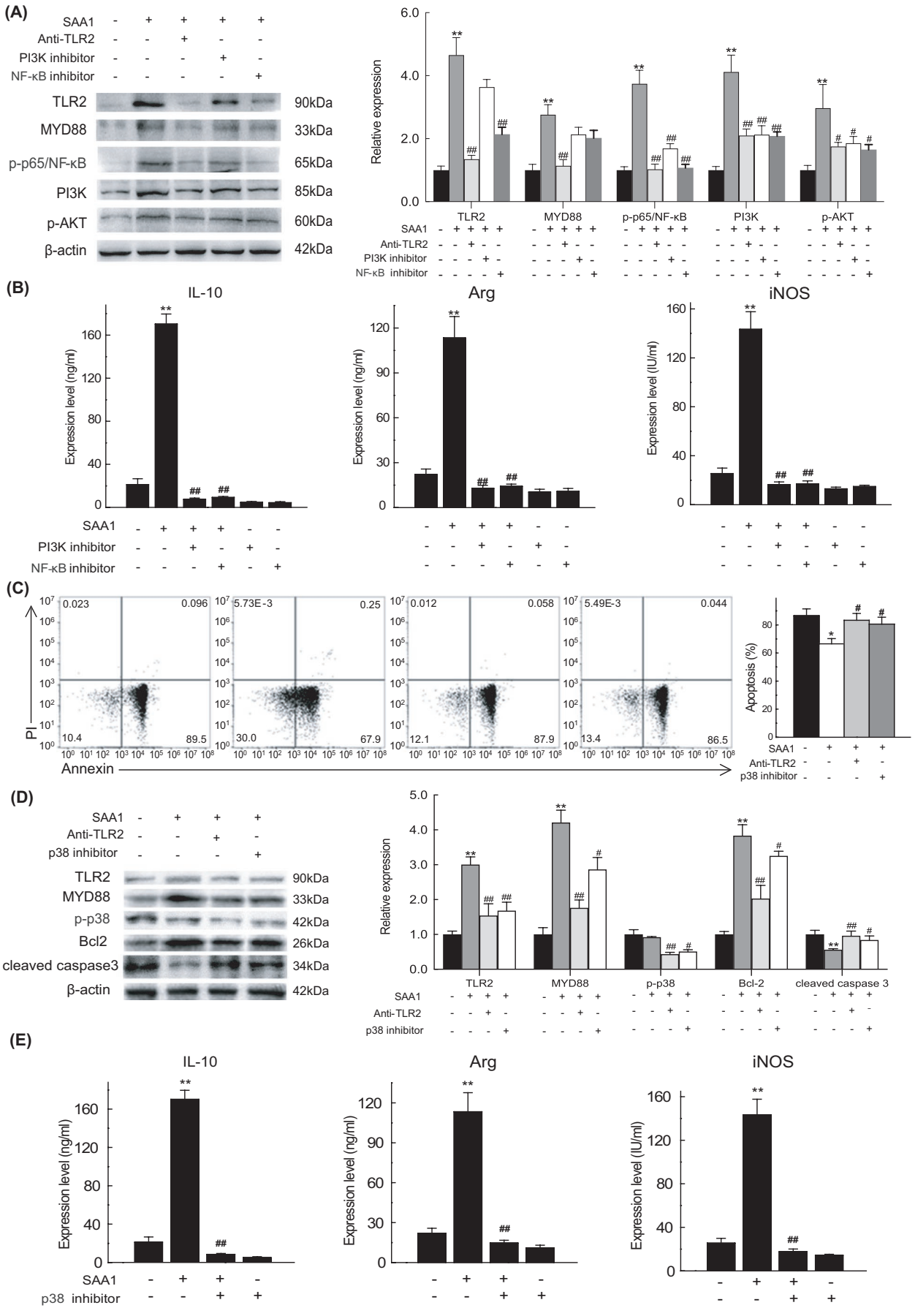
3.5 | Serum amyloid A 1 induces neutrophil apoptosis resistance through the p38 MAPK pathway

In addition to the effects of SAA1 on neutrophils mentioned above, we also observed that SAA1-treated neutrophils showed appreciably lower apoptotic levels than untreated cells (Figure 4C), indicating that SAA1 plays a crucial role in mediating neutrophil apoptosis. It has been reported that the p38 MAPK pathway is involved in TLR2-mediated apoptosis.[37,38] Therefore, the ratio of apoptotic cells in SAA1-treated neutrophils was investigated with and without a TLR2-blocking Ab and p38 inhibitor. We observed that the suppression of the TLR2 and p38 MAPK pathways stimulated the apoptotic process (Figure 4C), suggesting that SAA1 might induce neutrophil apoptosis resistance through the p38 MAPK pathway. To further evaluate potential apoptotic signals, we detected the expression of several apoptotic proteins in SAA1-treated and untreated neutrophils. As shown in Figure 4D, SAA1 promoted the expression of p-p38, Bcl-2, and cleaved caspase-3 and activated TLR2 and MYD88; this promotion was inhibited by TLR2 and p38 MAPK suppression, further confirming the ability of SAA1 to promote neutrophil apoptosis resistance. We next determined whether SAA1-mediated apoptosis resistance could influence the production of IL-10, Arg, and iNOS in neutrophils. As expected, the addition of a p38 inhibitor prevented SAA1-treated cytokine secretion by neutrophils from patients with breast cancer and healthy controls (Figure 4E).

3.6 | Growth-promoting effects of SAA1 expression and immunosuppressive neutrophils in breast cancer cells are recapitulated in vivo

Finally, we sought to validate the correlation of SAA1 expression and immunosuppressive neutrophils in breast cancer cells in vivo. Towards this, we established implanted xenografts of 4T1 cells and compared the growth of mice treated with or without anti-SAA1 and anti-Ly6G Ab to block SAA1 and neutrophils (Figure 5A). Tumor growth in animals with anti-SAA1 or anti-Ly6G blocking was significantly inhibited (Figure 5A,B). The lowest rate of growth was observed in animals treated with anti-SAA1 and anti-Ly6G Ab (Figure 5A,B). Additionally, mice treated with control IgG showed a notable increase in SAA1 and CD66b expression, whereas animals treated with anti-SAA1 Ab showed decreased SAA1 as well as CD66b (Figures 5C and S1H). Furthermore, to investigate that immunosuppressive function of neutrophils is regulated by the SAA1/neutrophil blockade, infiltration of $CD3^+$ T cells were also evaluated (Figure S1G,H). The results showed that after anti-SAA1 and anti-Ly6G Ab treatment, the average numbers of $CD3^+$ cells increased, which is probably due to the release of neutrophil inhibition, supporting our belief that SAA1 might be associated with immunosuppressive neutrophils in breast cancer. Taken together, these findings indicate that SAA1 could promote neutrophils to produce immunosuppressive

FIGURE 4 Serum amyloid A 1 (SAA1) induced neutrophils to produce immunosuppressive cytokines and apoptosis resistance. (A) Representative bands and relative expression levels of Toll-like receptor 2 (TLR2), myeloid differentiation primary response 88 (MYD88), p-p65/nuclear factor-κB (NF-κB), PI3K, and p-AKT in neutrophils treated with SAA1 and anti-TLR2, PI3K inhibitor, or NF-κB inhibitor ($n = 3$) $^{**}p < 0.01$ vs. untreated neutrophils; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. SAA1-treated neutrophils. (B) Release of interleukin-10 (IL-10), arginase (Arg), and inducible nitric oxide synthase (iNOS) by neutrophils isolated from breast cancer patients treated with various combinations of SAA1, PI3K inhibitor, and NF-κB inhibitor ($n = 3$). $^{**}p < 0.01$ vs. untreated neutrophils; $^{\#\#}p < 0.01$ vs. SAA1-treated neutrophils. (C) Apoptosis of neutrophils treated with SAA1, anti-TLR2, and p38 inhibitor ($n = 3$) $^{*}p < 0.05$ vs. untreated neutrophils; $^{\#}p < 0.05$ vs. SAA1-treated neutrophils. (D) Representative bands and relative expression levels of TLR2, MYD88, p-p38, Bcl-2, and cleaved caspase-3 in neutrophils treated with SAA1, anti-TLR2, and p38 inhibitor ($n = 3$). $^{**}p < 0.01$ vs. untreated neutrophils; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. SAA1-treated neutrophils. (E) Release of IL-10, Arg, and iNOS by neutrophils isolated from breast cancer patients treated with SAA1 and p38 inhibitor. Data are expressed as mean ± SD


(A)
SAA1
Anti-TLR2
PI3K inhibitor
NF-κB inhibitor
TLR2
MYD88
p-p65/NF-κB
PI3K
p-AKT
β-actin
90kDa
33kDa
65kDa
85kDa
60kDa
42kDa
Relative expression
(B)
IL-10
Arg
iNOS
Expression level (ng/ml)
Expression level (IU/ml)
(C)
PI
Annexin
0.023
0.096
10.4
89.5
5.73E-3
0.25
30.0
67.9
0.012
0.058
12.1
87.9
5.49E-3
0.044
13.4
86.5
Apoptosis (%)
p38 inhibitor
(D)
p-p38
Bcl2
cleaved caspase3
cleaved caspase 3
Bcl-2
(E)

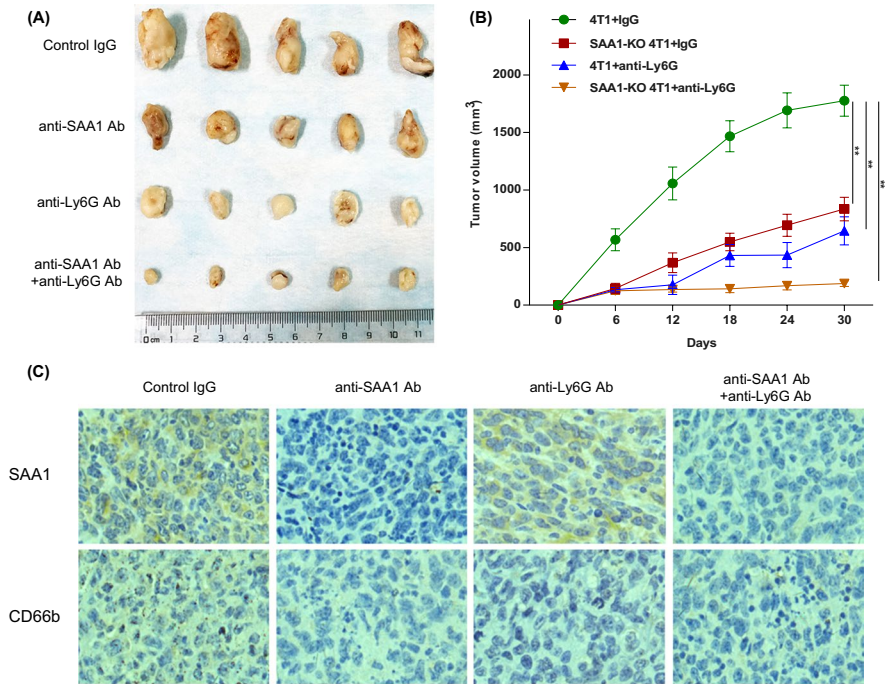


FIGURE 5 Tumor-promoting effects of serum amyloid A 1 (SAA1) on neutrophils in vivo. (A) Images of dissected xenografted tumors implanted subcutaneously with 4T1 cells, which were treated with or without anti-SAA1 and anti-Ly6G Ab after 30 days. (B) Tumor growth curves determined by caliper measurements shown as mean (mm^3) ± SEM. $N = 10$/group. (C) Representative immunohistochemistry staining of SAA1 and CD66b in xenografted tumors implanted subcutaneously with 4T1 cells, which were treated with or without anti-SAA1 and anti-Ly6G Ab. Scale bar = 50 μm

cytokines, not only by activating the PI3K/NF-κB signaling pathway, but also by mediating apoptosis resistance (Figure 6).

4 | DISCUSSION

In this study, we detected the high level of SAA expression in breast cancer and investigated its effects on the regulation of immunosuppressive neutrophils in the TME. Our data showed that SAA1 induced immunosuppression of neutrophils through the TLR2/MYD88-mediated PI3K/NF-κB signaling pathway and triggered p38 MAPK pathway-associated apoptosis resistance to promote the progression of breast cancer. Our study provides the first evidence that SAA induces immunosuppressive neutrophils in breast cancer, thus suggesting potential targets for novel therapeutic strategies for breast cancer patients.

Inflammation is an important risk factor for cancer. Despite great advances in breast cancer immunotherapy, a large proportion of patients do not benefit from this treatment. There is increasing evidence that immune tolerance of solid tumors is probably modulated by immune inflammatory cells that are induced by developing tumors.[39] For most human solid tumors, the host immune inflammatory response is associated with prognosis,[40,41] and we and other researchers have confirmed a causal relationship between host immune cells and breast tumor progression.[14] Neutrophils in the TME have been regarded as key myeloid-derived suppressor cells, which have immunosuppressive properties that induce tumor progression.[42] Neutrophil immunosuppression can occur by the release of a variety of suppressive inflammatory cytokines in solid tumors.[43] Several mechanisms have been proposed to explain how neutrophils are a substantial obstacle to successful immunotherapy.[13] Neutrophils elicited from cancer cells can prevent T cell proliferation through PD-1/PD-L1 signaling and inhibit natural killer cell functions, leading to tumor cell proliferation, survival, and metastasis.[44] Several suppressive factors, such as Arg and iNOS, produced by neutrophils can inhibit immune responses and infiltration of $CD8^+$ T lymphocytes into the TME.[45] There are many markers that can be expressed by neutrophils, such as CD11b, CD66b, CD15, and MPO as reported previously.[31] Consistent with previous observations, we found here that $CD11b^+CD15^+$ neutrophils was more susceptible to SAA treatment[23] and had an increased ability to secrete immunosuppressive molecules, including IL-10, Arg, and iNOS, confirming the immunosuppressive role of neutrophils in breast cancer. However, the intrinsic mechanisms that mediate the acquisition of immunosuppressive characteristics by neutrophils remain unknown.

There is emerging experimental and clinical evidence indicating that the regulation of the phenotype and function of neutrophils requires the participation of a series of inflammatory mediators. Serum amyloid A is an apolipoprotein considered to be a biomarker of inflammation and tumor progression. The levels of SAA appeared to increase in malignancies, such as breast cancer,[46] lung cancer,[25] and melanoma.[23] As SAA regulates the immune system and immunosuppressive neutrophils accumulate in the breast cancer TME, we hypothesized that there might be a strong link between SAA1 and immunosuppressive neutrophils. As expected, SAA1 secreted by cancer cells was found to contribute to the promotion of the immunosuppressive phenotype of neutrophils, resulting in tumor progression. These data suggest a possible feedback loop between breast cancer cells and immunosuppressive neutrophils through SAA. Inhibition of neutrophils or SAA1-associated TLR2 on neutrophils by blocking Abs thus provided us a potential intervention approach in breast cancer treatment.

We then sought to determine the potential mechanisms by which SAA induces immunosuppression of neutrophils. Toll-like

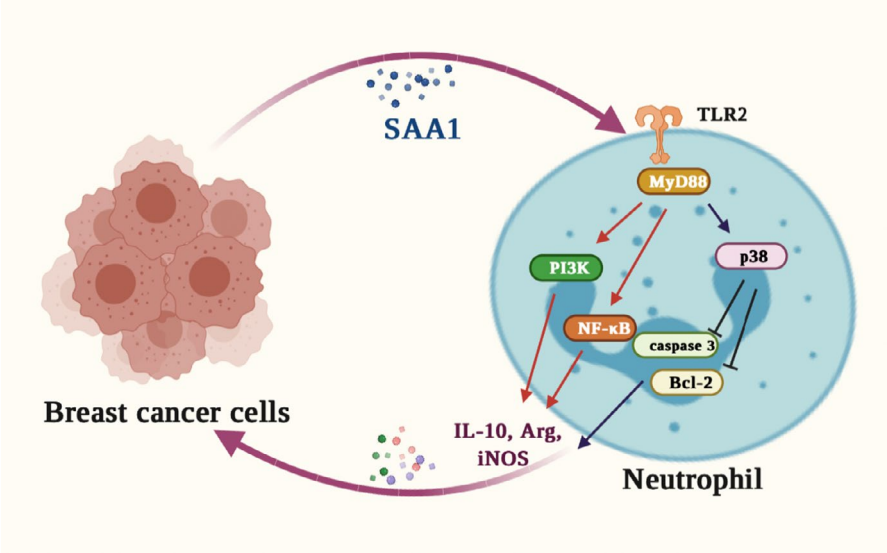


FIGURE 6 Proposed model for the role of serum amyloid A 1 (SAA1) in promoting immunosuppressive neutrophils in breast cancer. Secreted by breast cancer cells, SAA1 activates the Toll-like receptor 2 (TLR2)/myeloid differentiation primary response 88 (MYD88) regulatory pathway in neutrophils. Activation of TLR2/MYD88 stimulates the PI3K/nuclear factor-κB (NF-κB) signaling pathway; however, SAA1 bound to TLR2 receptor induces neutrophil apoptosis resistance through the p38 MAPK pathway. Both effects of SAA1 on neutrophils promote the secretion of interleukin-10 (IL-10), arginase (Arg), and inducible nitric oxide synthase (iNOS) from neutrophils, which exert an immunosuppressive phenotype in breast cancer. Thus, there is a potential positive feedback loop between breast cancer cells and immunosuppressive neutrophils through SAA1

receptor 2 and FPRL1 are the main receptors of SAA1[47,48] and were both expressed on SAA1-treated neutrophils in our study. Although both of these receptors are responsible for SAA-mediated biological activities, TLR2 seems to play a more prominent role in SAA-treated neutrophils and is significant in recognizing a wide range of molecules required for downstream signal transduction.[49,50] We found that MYD88, which was reported to induce the Ras-mediated signaling pathway and engage in protumorigenic function in many immune cells[51–53] was the most important adaptor protein for TLR2 in neutrophils. We confirmed that cytokine production by immunosuppressive neutrophils was facilitated by the TLR2/MYD88-dependent PI3K/NF-κB signaling pathway and led to an immunosuppressive TME in breast cancer. In addition, SAA1 triggered the apoptosis resistance of neutrophils through the TLR2/MYD88-mediated p38 MAPK pathway, further demonstrating the important role of SAA in regulating neutrophil function and survival. Collectively, our findings indicate that SAA induces the secretion of immunosuppressive cytokines in neutrophils through two means: (a) SAA activates the TLR2/MYD88-mediated PI3K/NF-κB signaling pathway; and (b) SAA promotes neutrophil apoptosis resistance, which decreases the life span of neutrophils in the TME. Both of these effects of SAA on immunosuppressive neutrophils result in the progression of breast cancer (Figure 6).

There are some limitations of our study. For example, the substantial experiment should be conducted to support SAA1 derived from cancer cell, and the other cytokines released from tumor cell or TME could have somewhat impact on immunosuppressive neutrophils, the exploration for interaction between SAA1 and other cytokines would meaningful in the future.

In conclusion, our study describes a previously unknown mechanism underlying the effect of SAA on immunosuppressive neutrophils in breast cancer. These findings provide new insights into the mechanisms by which neutrophils are regulated in the TME and provide potential therapeutic targets for breast cancer patients.

ACKNOWLEDGMENTS

This study was funded by the National Natural Science Foundation of China (Grant No. 82002781), China Postdoctoral Science Foundation (Grant No. 2018M641858), the Hei Long Jiang Postdoctoral Foundation (Grant No. LBH-Z18115), and the Science and Technology Foundation of the Finance Bureau of Heilongjiang Province (Grant No. CZKYF2021B004).

DISCLOSURE

The authors have no conflict of interest.

AUTHOR CONTRIBUTIONS

Study conception and design: X. Niu, Q. Zhang, and H. Ji. Development of methodology: L. Yin and X. Yang. Acquisition of data (patient data acquisition and management, facilities, etc.): Y. Yang, Y. Gu, and Y. Sun. Analysis and interpretation of data (eg, statistical analysis, biostatistics, computational analysis): M. Yang and Y. Wang. Writing, reviewing, and/or revision of the manuscript: X. Niu, L. Yin, and H. Ji. Study supervision: H. Ji and Q. Zhang.

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How to cite this article: Niu X, Yin L, Yang X, et al. Serum amyloid A 1 induces suppressive neutrophils through the Toll-like receptor 2–mediated signaling pathway to promote progression of breast cancer. *Cancer Sci*. 2022;113:1140–1153. doi:10.1111/cas.15287