


# The STAT3 inhibitor pyrimethamine displays anti-cancer and immune stimulatory effects in murine models of breast cancer

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**Abstract** The transcription factor signal activator and transducer or transcription (STAT3), which regulates genes controlling proliferation, survival, and invasion, is activated inappropriately in many human cancers, including breast cancer. Activation of STAT3 can lead to both malignant cellular behavior and suppression of immune cell function in the tumor microenvironment. Through a chemical-biology screen, pyrimethamine (PYR), an FDA approved anti-microbial drug, was identified as an inhibitor of STAT3 function at concentrations known to be achieved safely in humans. We report that PYR shows therapeutic activity in two independent mouse models of breast cancer, with both direct tumor inhibitory and immune stimulatory effects. PYR-inhibited STAT3 activity in TUBO and TM40D-MB metastatic breast

cancer cells in vitro and inhibited tumor cell proliferation and invasion into Matrigel basement membrane matrix. In tumor-transplanted mice, PYR had both direct and indirect tumor inhibitory effects. Tumor-bearing mice treated with PYR showed reduced STAT3 activation in tumor cells, attenuated tumor growth, and reduced tumor-associated inflammation. In addition, expression of Lamp1 by tumor infiltrating CD8<sup>+</sup> T cells was elevated, indicating enhanced release of cytotoxic granules. These findings suggest that PYR may have beneficial effects in the treatment of breast cancer.

**Keywords** Breast cancer · Metastasis · Immunity · Pyrimethamine · STAT3

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## Abbreviations

BSA	Bovine serum albumin
BTBM	BALBL/c TUBO transplant breast cancer mouse model
CTLs	Cytotoxic T-lymphocytes
FDA	United States food and drug administration
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HER2	Epidermal growth factor receptor 2
IL-6	Interleukin 6
JAK	Janus kinase
NMP	1Methyl-2-pyrrolidinone
PBS	Phosphate buffered saline
Poly	Polyethylene glycol
pSTAT	Phospho-STAT
PYR	Pyrimethamine; 2, 4-diamino-5- <i>p</i> -chlorophenyl-6-ethyl-pyrimidine
ROR $\gamma$ T	Retinoic acid-related orphan receptor gamma T
STAT	Signal activator and transducer of transcription
TAMs	Tumor associated macrophages
TH17	T-helper 17
Tregs	Regulatory T cells

## Introduction

Signal transducer and activator of transcription (STAT3) is a member of a family of seven closely related transcription factors. STAT3 is present in the cytoplasm of cells under basal conditions. In response to cytokines or other extracellular stimuli, STAT3 becomes phosphorylated by Jaks or other kinases on carboxy-terminus tyrosine residues. It then translocates to the nucleus as a dimer, where it can bind to nine base pair regions in the promoters of target genes to regulate transcription [1]. In addition to regulating the genes controlling proliferation, survival, and pluripotency, STAT3 activity can contribute to inflammation and immune suppression [2]. In all subtypes of breast cancer, STAT3 can become constitutively activated. In particular, it is commonly upregulated in the tumor-initiating cell population, where it regulates cancer initiation, maintenance, and relapse [3, 4].

Given the central role played by STAT3 in the pathogenesis of breast cancer, and other human cancers, efforts have been made to identify clinically accessible inhibitors of this protein. From a screen of a chemical library biased to drugs already known to be safe in humans, pyrimethamine (2,4-diamino-5-*p*-chlorophenyl-6-ethyl-pyrimidine) (PYR), an anti-microbial that is effective in the prevention and treatment of malaria and toxoplasmosis, was found to inhibit STAT3 transcriptional function at concentrations known to be safely achieved in patients [5, 6].

While previous studies have focused on the direct impact of PYR on tumor cells in cell culture systems [7–10], there is little information on the *in vivo* action and immunological impact of PYR in cancer models. STAT3 is activated downstream of cytokine signaling in hematopoietic cells and plays a dual role in tumor inflammation and immunity by stimulating pro-oncogenic inflammatory pathways, including nuclear factor- $\kappa$ B (NF- $\kappa$ B) and interleukin-6 (IL-6)–GP130–Janus kinase (JAK) pathways, as well as by antagonizing STAT1- and NF- $\kappa$ B-regulated T<sub>H</sub>1 anti-tumor immune responses [11]. Therefore, the inhibition of STAT3 activity by PYR may have both tumor cell autonomous and immune modulatory effects.

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer-related death among women worldwide [12]. The *HER2* gene, which encodes a tyrosine kinase receptor, is a strong mediator of cellular growth and proliferation in normal as well as malignant epithelial cells [13]. *HER2/neu* overexpression occurs in 20–30% of breast cancer cases and is associated with inflammatory tumors and poor prognosis [13]. MMTV-promoter-*NeuT* transgenic mice faithfully reproduce the tumor and immune pathology of *HER2+* breast cancer and breast cancer metastasis [14–16]. The TM40D-MB is a highly metastatic and

well-characterized breast tumor cell line that does not express *HER2* [17].

We report protective effects of PYR through inactivation of STAT3 in tumor cells and enhanced tumor immunity in mouse models of breast cancer. In these studies, we used the BALBc-*NeuT* transgenic mice as well as mice transplanted with TUBO cells that were originally derived from a lobular carcinoma that arose spontaneously in a BALBc-*neuT* mouse [18]. The efficacy of PYR as a therapeutic drug was validated in mice transplanted with TM40D-MB, an unrelated breast cancer cell line with high metastatic potential. We show that the protective effects of PYR are mediated in part through suppressing cancer-associated inflammation and reducing the immunosuppressive tumor microenvironment, thereby leading to the activation of anti-tumor immunity and effector functions of cytotoxic T cells.

## Materials and methods

### Cell culture

TUBO cells [19] were cultured in DMEM (ThermoFisher) supplemented with 5% fetal bovine serum. TM40D-MB cells were grown in DMEM/F12 medium with 2% ABS, epidermal growth factor, and insulin, as described previously [20].

### BALB/c-TUBO transplant breast cancer mouse model (BTBM)

TUBO cells at 40–50% confluence were trypsinized, washed 2× with phosphate buffered saline (PBS), and triturated into a single cell suspension in PBS. TUBO cells ( $1 \times 10^6$ ) were injected in the left mammary fat pad of 6-week-old virgin BALB/c females and were monitored for 1 week for visible tumor growth. One week following implantation, the mice were treated by gavage with either PYR [60 mg/kg/day in NMP (1-methyl-2-pyrrolidinone):Poly (ethylene glycol)-300 in 1:9 ratio] or vehicle [NMP (1Methyl-2-pyrrolidinone) and Poly (ethylene glycol)-300 in 1:9 ratio] for 41 days.

### BALB-*neuT* transgenic mice

Inbred BALB/c mice overexpressing the transforming rat *Her-2/neu* oncogene (*neuT<sup>+</sup>/neuT<sup>-</sup>*) driven by the mouse mammary tumor virus promoter (BALB-*neuT*) and transgene negative (*neuT<sup>-</sup>/neuT<sup>-</sup>*) (BALB/c) were produced and screened for the presence of the transgene as previously described [18]. Mice were treated with either PYR (60 mg/kg/day) or carrier for 48 days.

For all murine experiments, mice were maintained under specific pathogen-free conditions at the Northwestern University Animal Care Facility, and the Animal Care Usage Committee of Northwestern University approved all experiments. Tumors were measured with calipers in two perpendicular diameters.

### Western blotting

Whole-cell extracts were prepared in RIPA buffer [10 mM Tris–HCl (pH 7.5), 500 mM NaCl, 0.1% SDS, 1% NP-40, 1% sodium deoxycholate, 2 mM EDTA, and 1% protease, phosphatase I and II inhibitor cocktail (Sigma)]. Cellular protein (30 µg) was resolved by SDS-PAGE and transferred following standard protocols. Immunoreactive proteins were detected with antibodies to phospho-STAT3 (Tyr705), total STAT3 (Cell Signaling), and GAPDH (Sigma) using appropriate HRP-conjugated secondary antibodies and SuperSignal chemiluminescent reagent (Thermo Scientific).

### Immunohistochemistry and immunofluorescence

4 µm thick paraffin-embedded tissue sections were deparaffinized, and antigen-retrieval was performed using target retrieval solution (Dako, Carpinteria, CA) in decloaking chamber. Sections were then blocked with 1%BSA and incubated with anti-pSTAT3-Y705 (Abcam), anti-F4/80 (Abcam) or anti-Ki-67 (Abcam) overnight at 4 °C. The sections were then incubated with anti-rabbit–horseradish peroxidase-labeled polymer (Dako) for 1 h at room temperature, followed by DAB substrate (Dako), counterstaining with hematoxylin, and mounting with permount mounting medium (for immunohistochemistry). For double-immunofluorescence, after antigen-retrieval and blocking with 1%BSA, staining was performed with anti-Foxp3 (ebiosciences) and anti-RORγT (Abcam), or anti-CD8 (Abcam) and anti-Lamp1 (Abcam). Antibodies were applied on tissue sections overnight at 4 °C, followed by anti-rat Alexaflor-594 and anti-rabbit Alexaflor-488 for 1 h in the dark at room temperature followed by DAPI (Invitrogen), and mounted as described [21].

### Preparation of conditioned medium

For the production of TUBO conditioned medium (TUBO CM), TUBO cells were grown to 100% confluency in DMEM and treated with either 100 µM S3I-201 (inhibitor of STAT3 activity, Calbiochem) or vehicle [22], washed three times with PBS, and incubated with fresh DMEM medium for 48 h. The conditioned medium (designated either “TUBO CM (+S3I-201)” or “TUBO CM (+DMSO)”) was removed, filtered, and used for cell migration experiments.

### Cell proliferation assay

$1 \times 10^4$  TM40D-MB cells were seeded in triplicate in 100 µl per well in a 96 well plate with a standard medium for 24 h at 37 °C and 5% CO<sub>2</sub>. The next day, the medium was removed and 100 µl of either DMEM/F12 with carrier or DMEM/F12 with 10 µM PYR or DMEM/F12 with 20 µM PYR was added and incubated for an additional 24, 48, or 72 h at 37 °C and 5% CO<sub>2</sub>. 0.5 mCi of [<sup>3</sup>H]-thymidine was added to each well and incubated for 6 h after the 24, 48, and 72 h time points. [<sup>3</sup>H]-thymidine incorporation was quantified using a scintillation counter (LKB RackBeta; Wallac) as described [21].

### Cell invasion assay

$3 \times 10^4$  TM40D-MB cells were treated with either vehicle or 10 µM PYR or 20 µM PYR, and  $3 \times 10^4$  TUBO cells were either treated with either carrier or 100 µM S3I-201 or 20 µM PYR. Subsequently, these cells were washed three times with PBS and seeded in triplicate in 500 µl per well of their respective serum-free standard media in the top wells (inserts) in a 24 well (12) insert plate (BD Biocoat). The bottom well contained the appropriate serum-containing standard media for each cell line. After 48 h of incubation at 37 °C, the non-invading cells were removed; after fixing the membrane and staining with Diffquick, invading cells were counted using a bright-field microscope as described [21].

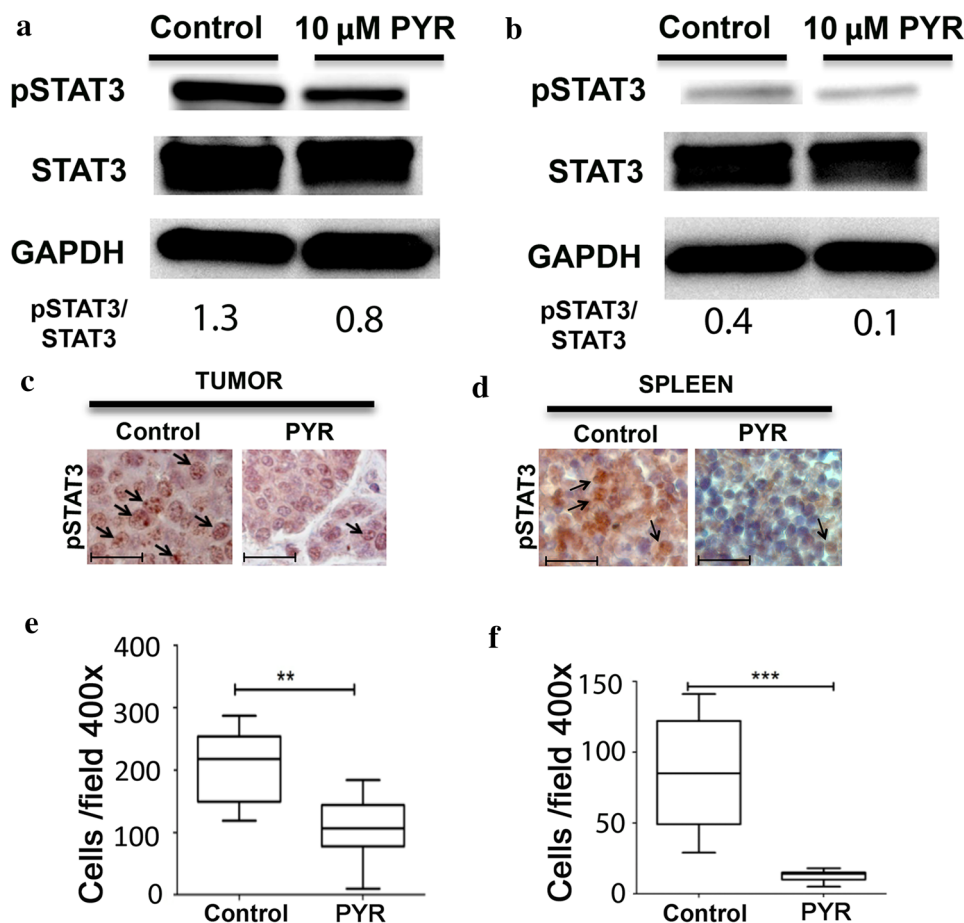
### Statistical analysis

Statistical analysis between study groups was performed using the Student *t* test, One-way ANOVA, or Two-way ANOVA. *P* values less than 0.05 were considered statistically significant.

## Results

### PYR inhibits phosphorylation of STAT3 in breast cancer cell lines and tumors

In both TUBO and TM40D-MB cells, STAT3 was phosphorylated on Y705, reflecting functional activation (Fig. 1a, b). Following treatment with PYR, phosphorylation of STAT3 decreased in both cell types, though it was not completely abrogated. This is consistent with the finding that PYR decreases STAT3 transcriptional function through both phosphorylation-dependent and phosphorylation-independent mechanisms. Furthermore, *in vivo* treatment of BTBM mice with PYR via gavage significantly inhibited the STAT3 phosphorylation in both mammary tumors (Fig. 1c) and spleen (Fig. 1d), as assessed by immunohistochemistry.



**Fig. 1** PYR decreases the phosphorylation of STAT3 in vitro and in vivo. **a** TUBO and **b** TM40D-MB breast cancer cells were treated for 1 h with vehicle or 10  $\mu$ M PYR, after which the indicated proteins were detected by western blot. The ratios of pSTAT3 to STAT3 normalized relative to GAPDH are shown (quantified using ImageJ). **c**, **d** BTBM mice were treated with vehicle or PYR, and pSTAT3 was detected by immunohistochemistry in tumors (**c**) or spleen (**d**); representative immunohistochemistry images (400X magnification; scale

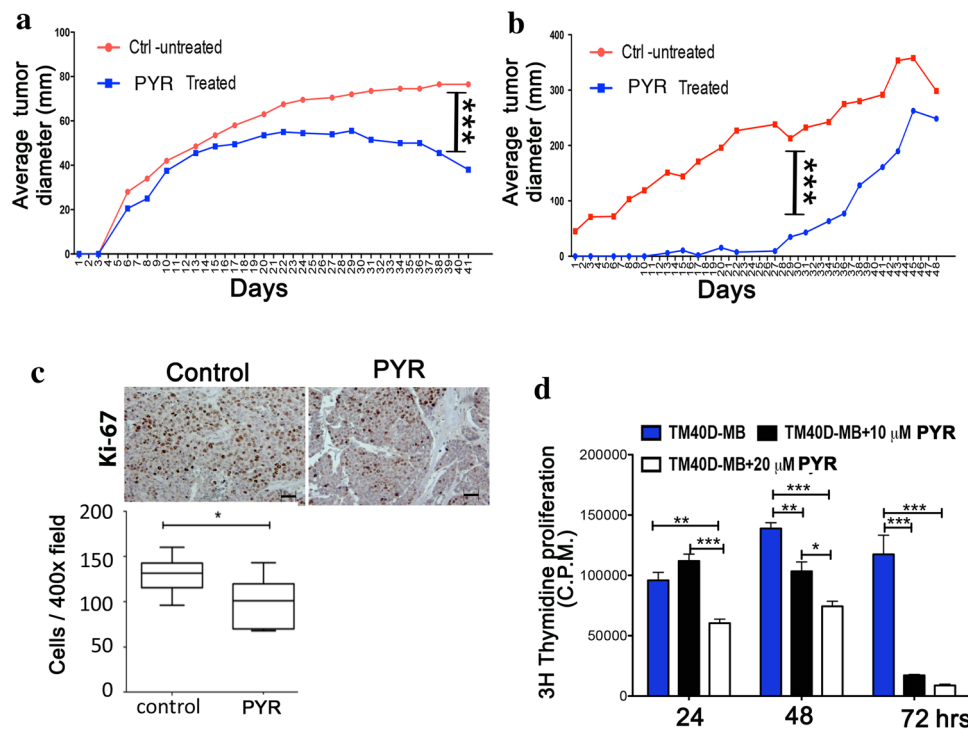
bar 20  $\mu$ m, arrows indicate nuclear pSTAT3 staining). **e**, **f** Quantification of pSTAT3 expressing cells in tumor and spleen, respectively (mean  $\pm$  standard error;  $n = 7$ ). Tumor sections from a minimum of 3 mice per group were stained for pSTAT3. A minimum of 3 high-power field ( $\times 400$ ) images were taken per section (areas of duplication were avoided) and pSTAT3 positive cells per high-power field were counted in each group.  $**P < 0.001$  and  $***P < 0.0001$ , by Student's  $t$  test

Taken together, these data show that PYR inhibits STAT3 in breast cancer cell lines, mammary tumors, and immune organs.

### Inhibition of pSTAT3 by PYR suppresses breast cancer cell proliferation

Enhanced expression of HER2 protein is associated with more rapid tumor growth [23, 24]. Increased tumor cell proliferation has also been directly associated with poor clinical outcome of breast cancer patients [25, 26]. HER2 protein tyrosine kinase activity can lead to phosphorylation of STAT3 by both direct and indirect mechanisms. We first assessed the effect of PYR on HER2-overexpressing cell lines in vitro. PYR led to a dose-dependent decrease in relative viable cell number, with IC50 values in the low

micromolar range (Supplementary Fig. 1). Furthermore, at sub-lethal concentrations, PYR decreased expression of a panel of STAT3 target genes in both cell types (Supplementary Fig. 2). Micromolar concentrations of PYR are routinely achieved in patients taking this drug for infectious diseases continuously for months without undue toxicity. Therefore, we next studied the in vivo impact of STAT3 inhibition by PYR on tumor growth in BTBM mice and BALB-NeuT mice. PYR treatment significantly reduced tumor growth in BTBM mice as well as BALB-NeuT mice in comparison with vehicle-treated mice (Fig. 2a, b). Since STAT3 target genes, such as cyclin D1, regulate cell cycle progression, we determined the effect of PYR on proliferation, using Ki-67 staining. We found that tumors from PYR-treated BTBM mice display significantly lower proliferation rates (measured by Ki-67 expression) in comparison with the tumors



**Fig. 2** PYR treatment in vitro and in vivo decreases growth and proliferation of breast cancer cells. **a** BTBM mice with transplanted TUBO mammary tumors ( $n = 14$ ) and **(b)** BALB/c-NeuT transgenic mice ( $n = 14$ ) with palpable tumors were randomized into arms of treatment with vehicle (Control; red lines) or PYR (blue lines) and tumor size was quantified. **c** Representative images ( $\times 200$  magnification, scale bar 50  $\mu\text{m}$ ) of TUBO tumors on day 41 post transplant, and quantification (brown nuclear staining, mean  $\pm$  standard error)

from vehicle-treated BTBM mice (Fig. 2c). To complement this finding, we also studied the effect of inhibition of STAT3 on TM40D-MB cell proliferation rates in an in vitro thymidine incorporation assay. We observed that 10  $\mu\text{M}$  PYR significantly lowered the proliferation rates of TM40D-MB cells in comparison with the control group 48 and 72 h post treatment (Fig. 2d). Furthermore, 20  $\mu\text{M}$  PYR significantly lowered the proliferation rates of TM40D-MB cells at 24, 48 and 72 h time points in comparison with control as well as 10  $\mu\text{M}$  PYR treatment group (Fig. 2d), indicating dose-dependent inhibition of proliferation by PYR.

These findings indicate that PYR-mediated inhibition of STAT3 leads to a reduction in the growth and proliferation of HER2/*neu* overexpressing TUBO breast tumor cells and TM40D-MB metastatic breast cancer cells, and the corresponding tumors in mice.

### PYR attenuates in vitro invasion of TUBO and TM40D-MB cells

In several types of cancer, including breast cancer, the HER2-dependent signaling pathway aberrantly accelerates

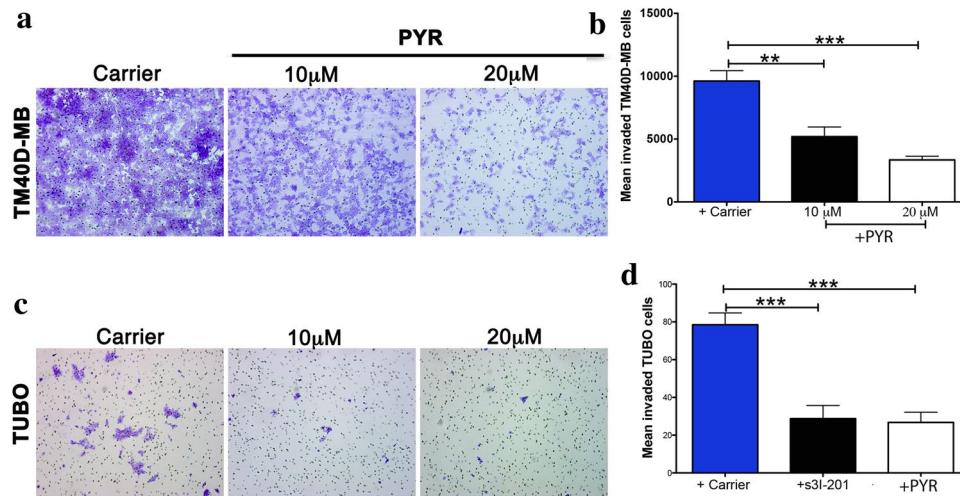
of Ki-67 immunohistochemistry of tumor sections. **d** TM40D-MB cells were treated starting from 1 day post transplant for the indicated times with vehicle or the indicated concentration of PYR, and proliferation was measured by thymidine incorporation; data represent mean  $\pm$  standard error.  $*P < 0.05$ ,  $**P < 0.001$  and  $***P < 0.0001$ , with two-way ANOVA used in (a, b), Student's *t* test used for (c), and one-way ANOVA used in (d)

cell invasion and proliferation of tumor cells and stimulates the epithelial–mesenchymal transition (EMT) necessary for metastases [27–29]. Hence, we studied the effect of STAT3 inhibition on invasiveness of TUBO and TM40D-MB cells. We found that PYR significantly attenuated the invasion of TM40D-MB cells in a dose-dependent manner (Fig. 3a, b). In addition, we found that PYR significantly inhibited invasion by TUBO cells (Fig. 3c, d). To determine if other STAT3 inhibitors shared these properties of PYR, we performed a parallel experiment using S3I-201 (100  $\mu\text{M}$ ). This compound produced a similar decreased invasion of TUBO cells (Fig. 3c, d). We conclude that inhibition of STAT3 function blocks invasion by HER2/*neu* overexpressing TUBO breast tumor cells as well as the TM40D-MB metastatic breast cancer cells.

### Blocking STAT3 inhibits tumor infiltration by F4/80<sup>+</sup> macrophages

Over the past few decades, compelling evidence has emerged demonstrating that the immune system participates both in tumor development (via chronic inflammation orchestrated





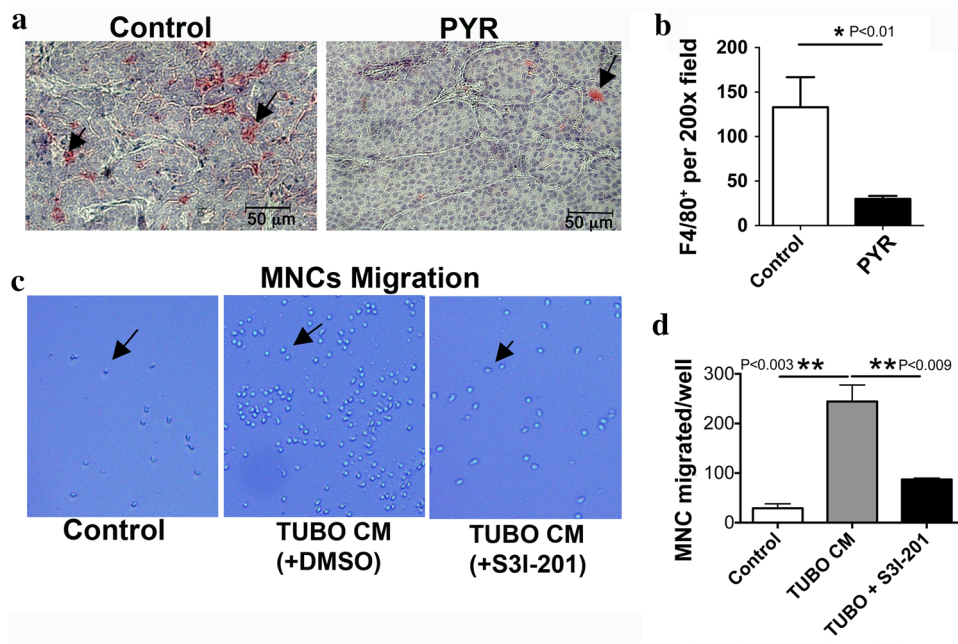
**Fig. 3** STAT3 inhibitors decrease in vitro breast cancer cell invasion. **a, b** TM40D-MB cells were treated for 1 h with vehicle or the indicated concentration of PYR and membrane invasion was analyzed by microscopy ( $\times 200$  magnification, *scale bar* 50  $\mu\text{m}$ ) (**a**) and quantified (mean  $\pm$  standard error) (**b**). **c, d** TUBO breast cancer cells were

treated for 1 h with vehicle or the indicated STAT3 inhibitor, washed, and membrane invasion was analyzed by microscopy ( $\times 200$  magnification, *scale bar* 50  $\mu\text{m}$ ) (**c**) and quantified (mean  $\pm$  standard error) (**d**).  $**P < 0.001$  and  $***P < 0.0001$ , by one-way ANOVA

by the innate immune system) and in tumor elimination and control (through the actions of the adaptive immune system). Tumor-associated macrophages (TAMs) consist mainly of a polarized M2 (F4/80<sup>+</sup>/CD206<sup>+</sup>) macrophage population with little cytotoxicity for tumor cells, because of their restricted production of nitric oxide and pro-inflammatory cytokines [30]. TAMs also have poor antigen-presenting capability and effectively suppress T cell activation. Moreover, TAMs promote tumor cell proliferation and metastasis by secreting a wide range of growth factors, angiogenic factors, and metalloproteinases, and by their involvement in signaling circuits regulating the function of fibroblasts in the tumor stroma [31]. Hence, we investigated the population of F4/80<sup>+</sup> TAMs in BTBM treated with PYR. We found that in vivo STAT3 inhibition significantly attenuated F4/80<sup>+</sup> TAM infiltration of transplanted TUBO tumors (Fig. 4a, b). Next, we examined the migration of spleen-derived mononuclear cells (MNC) in response to conditioned medium from TUBO tumor cells. We found that S3I-201 treatment of TUBO cells significantly inhibited migration of MNC in response to TUBO cell conditioned medium (Fig. 4c, d). Taken together, these findings indicate that PYR treatment lowers the density of MNC/TAMs in breast cancer tumors and that inhibition of STAT3 blocks migration of HER2/*neu* overexpressing TUBO breast tumor cells.

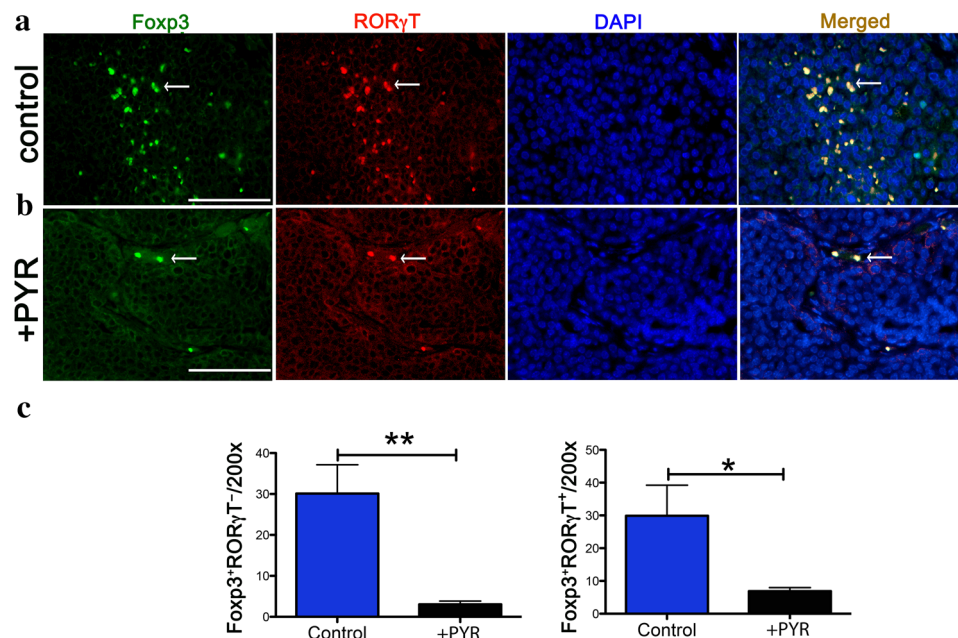
### Inhibition of STAT3 by PYR mitigates regulatory T cell/T-helper-17 immune markers and enhances exocytosis of cytotoxic granules from cytotoxic T-lymphocytes in BALB-NeuT mice

Regulatory T cells have a dual role in cancer. They are a negative prognostic marker in breast cancer in part due to their ability to suppress anti-tumor immunity [32]. However, they can also protect against cancer by suppressing tumor-associated inflammation [33]. Our earlier studies showed that regulatory T cells (Tregs) that gain expression of the transcription factor retinoic acid related orphan receptor gamma T (ROR $\gamma$ T) lose their ability to suppress cancer-associated inflammation [34] and are potently tumor promoting [33]. Hence, our next goal was to study the in vivo impact of PYR-mediated STAT3 inhibition on regulatory T cell/T-helper-17 (Treg/TH17) cells as well as CD8<sup>+</sup> T cell-mediated cytotoxicity. We found that PYR treatment significantly lowered the frequencies of Foxp3<sup>+</sup>ROR $\gamma$ T<sup>-</sup> Tregs (Fig. 5a, b) as well as Foxp3<sup>+</sup>ROR $\gamma$ T<sup>+</sup> T cells (Fig. 5a, c) in the BALB-NeuT mice. This reflects a decrease in both pro-inflammatory and anti-inflammatory Tregs, which yield an overall effect favoring anti-tumor T cell cytotoxicity. By contrast, there was no difference in the frequency of CD8<sup>+</sup> T cells infiltrating the tumors with PYR treatment (data not shown). However, we found that PYR treatment significantly elevated the numbers of CD8<sup>+</sup> T cells that were releasing their cytotoxic granules by exocytosis as measured by Lamp1 staining (Fig. 6a, b,  $P < 0.0001$ ). Hence, it can be concluded that PYR-mediated inhibition of STAT3 restricts Tregs as well as TH17-associated immune responses and



**Fig. 4** PYR treatment inhibits migration of F4/80<sup>+</sup> TAMs in vivo and in vitro. **a, b** Infiltration of transplanted breast tumors by TAMs in vivo. BTBM mice (*n* = 7) were treated with vehicle or PYR for 41 days. Formalin fixed tumor sections were immunostained for infiltrating F4/80 TAMs. Representative images (×100 magnification) are shown in (a) and quantification (mean ± standard error) is shown in (b). **c, d** Migration of leukocytes in response to tumor-conditioned

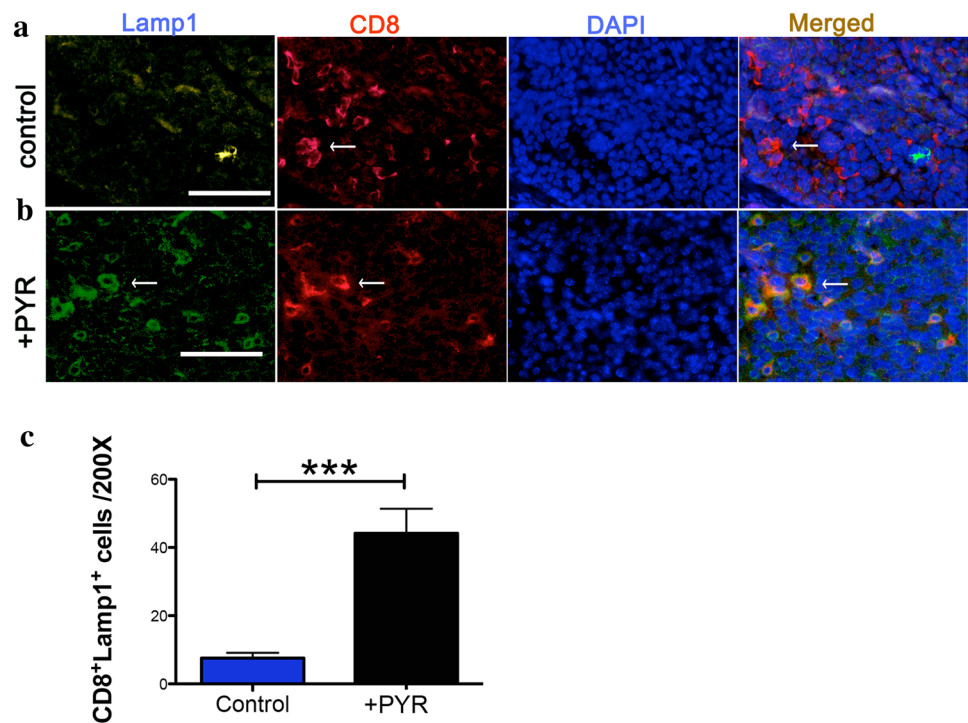
medium in vitro. Splenocyte MNCs were treated with conditioned medium from vehicle- or S31-201-treated TUBO cells, and migration was determined compared to serum-free DMEM medium as control. Representative images (×100 magnification) are shown in (c) and quantification (mean ± standard error) is shown in (d). *P* values shown were calculated with Student’s *t* test



**Fig. 5** PYR treatment lowers the levels of Foxp3<sup>+</sup> and Foxp3<sup>+</sup>RORγT<sup>+</sup> cells in mammary tumors. BALB-NeuT mice (*n* = 7) were (a) treated with vehicle or (b) PYR, and Foxp3<sup>+</sup> (green), RORγT<sup>+</sup> (red) and Foxp3<sup>+</sup>RORγT<sup>+</sup> (yellow/merged) cells were visualized by immunofluorescence. White arrow indicates Foxp3<sup>+</sup>RORγT<sup>+</sup> cells; scale bar 50 μm. (c) Quantifica-

tion (mean ± standard error) is shown for the Foxp3<sup>+</sup>RORγT<sup>-</sup> and Foxp3<sup>+</sup>RORγT<sup>+</sup> cells in the mammary tumors (*n* = 7). A minimum of 5 high-power field images per mouse (×200) were acquired and Foxp3<sup>+</sup>RORγT<sup>-</sup> or Foxp3<sup>+</sup>RORγT<sup>+</sup> cells per high-power field were counted. Areas of overlap or duplication were avoided. \*\**P* < 0.001 and \**P* < 0.05, by Student’s *t* test

**Fig. 6** PYR treatment increases densities of tumor infiltrating CD8<sup>+</sup>Lamp1<sup>+</sup> cells. BALB-NeuT mice ( $n = 7$ ) were treated with (a) vehicle or (b) PYR, and CD8<sup>+</sup> (red), Lamp1<sup>+</sup> (green) and CD8<sup>+</sup>Lamp1<sup>+</sup> (yellow/merged) cells were visualized by immunofluorescence. A minimum of 5 high-power field images per mouse ( $\times 200$ ) were acquired and CD8<sup>+</sup>Lamp1<sup>-</sup> or CD8<sup>+</sup>Lamp1<sup>+</sup> cells per high-power field were counted. Areas of overlap or duplication were avoided. Scale bar 50  $\mu\text{m}$ . c Quantification of CD8<sup>+</sup>Lamp1<sup>+</sup> cells (mean  $\pm$  standard error). \*\*\* $P < 0.0001$ , by Student's  $t$  test



promotes CD8<sup>+</sup> T cell-mediated cytotoxicity, thus attenuating tumor growth.

## Discussion

Constitutive activation of STAT3 occurs in a majority of breast cancers and many other human tumors. That STAT3 directly contributes to oncogenesis has been shown by a number of techniques, including tissue-specific deletion of STAT3, dominant negative STAT3 constructs, and anti-sense and short hairpin RNA [35, 36]. Since loss of STAT3 can be tolerated in normal tissues, STAT3 inhibition has the potential to have a high therapeutic index. Using a high-throughput, cell-based assay for specific inhibition of STAT3, PYR was identified as an inhibitor of STAT3 transcriptional function at concentrations known to be routinely achieved in humans without toxicity. HER2+ breast cancer and mouse models of mammary tumors driven by HER2 amplification are critically dependent on the HER2-IL-6-STAT3 signaling pathway [37]. Thus, inhibiting STAT3 activity is likely to be an important therapeutic modality for these cancers. STAT3 activation has also been shown to support cancer stem cells in triple negative breast cancer [38–41]. Thus, the therapeutic significance of PYR and other STAT3 inhibitors may expand beyond the HER2 positive group. Testing the effect of PYR on a triple negative breast cancer model could potentially further enhance the clinical importance of the compound.

In the present study, we investigated the efficiency of PYR [6], an FDA approved anti-microbial drug, for inhibiting STAT3 activity, tumor-associated inflammation and immunity, and tumor growth in mouse models of breast cancer. We focused on HER2+ breast cancer in view of the existing evidence linking HER2 to a systemic inflammatory mechanism that regulates tumor growth [37]. IL-6 secretion induced by HER2 overexpression turns on STAT3 and alters gene expression, enforcing an autocrine loop of IL-6/STAT3 expression. To validate our findings and extend these to HER2-negative breast cancer, we used the TM40D-MB metastatic breast cancer model.

Here, we show that PYR inhibits STAT3 activity in HER2/neu overexpressing TUBO cells, as well as in the tumor and spleen of mice transplanted with TUBO breast cancer cells. STAT3 is activated in response to cytokines and growth factors, through phosphorylation of tyrosine 705 (Y705) by receptor-associated Janus kinases (JAK) and non-receptor tyrosine kinases of the Src family [42, 43]. Dimers of phosphorylated STAT3 translocate to the cell nucleus where they activate transcription of genes mediating cell proliferation and survival, as well as genes that encode immune modulators and growth factors [44]. PYR treatment inhibited the proliferation of TM40D-MB metastatic breast cancer cells in vivo and invasion of both TM40D-MB and TUBO cells into Matrigel in vitro, in a dose-dependent manner. Furthermore, we observed reduced numbers of pSTAT3 positive cells in the tumors and spleen of tumor-bearing mice that had been transplanted with TUBO cells. These findings demonstrate the ability of PYR to inhibit STAT3



activity in breast cancer cells in vitro as well as in breast tumor cells in vivo.

The tumor-promoting properties of STAT3 have been attributed to regulation of cell proliferation. We showed in mouse models of breast tumor transplant and spontaneous tumor growth that PYR had notable inhibitory effects on tumor growth. This was in part related to reduced tumor cell mitotic activity in vivo, and could be reproduced by treatment of either TUBO or TM40D-MB cells in ex vivo culture. Further, down regulating STAT3 activity reduced tumor cell proliferation in vitro and decreased breast tumor growth in vivo. These findings are consistent with earlier reports on the effects of PYR on apoptosis, cell cycle distribution, and cell proliferation in human metastatic melanoma cell lines. In those experiments, PYR used at a clinically relevant concentration induced apoptosis in metastatic melanoma cells by activating cathepsin B and the caspase cascade (i.e., caspase-8 and caspase-9) and subsequent mitochondrial depolarization. Moreover, PYR caused S-phase cell cycle arrest and inhibited cell growth. In addition, results obtained in severe combined immunodeficiency (SCID) mice injected subcutaneously (SC) with human metastatic melanoma cells and treated with PYR, showed an inhibitory effect on tumor growth [9]. PYR treatment was also reported to inhibit telomerase activity [45]. Hence, inhibition of tumor cell growth may be due to multiple mechanisms.

JAK-STAT3 signaling promotes invasion and metastasis by activating several key genes such as WASF3 [46] and MMP-2 [47]. WASF3 [46] is involved in actin cytoskeleton dynamics [48] by recruiting the ARP2/3 complex [49, 50], which subsequently upregulates actin polymerization. We found that PYR inhibits invasion of breast cancer in in vitro assays, consistent with a role of STAT3 in tumor invasion.

It has become increasingly clear that tumor cells thrive in a chronically inflamed microenvironment and evade immune recognition [51]. Interventions to shift the balance from pro-tumor to anti-tumor immunity offer novel opportunities to control cancer development and progression. Pro-tumor inflammation marked by increased tumor-associated macrophages (TAMs) can foster local invasion by supplying matrix-degrading enzymes such as metalloproteinases and cysteine cathepsin proteases [52]. We showed that PYR-inhibited TAMs in vivo and suppressed migration of monocytes in ex vivo assays. Therefore, PYR has anti-inflammatory action, and inhibition of cancer-associated inflammation is likely to contribute to the anti-tumor effects of PYR.

Tregs are considered to be a major obstacle to tumor rejection by cytotoxic T-lymphocytes (CTLs) and suppress the action of CTLs by inhibiting granule exocytosis (marked by Lamp1) [53]. We reported earlier that Tregs also have anti-tumor properties related to their ability to suppress cancer-associated inflammation, in an IL-10 dependent manner. However, we also showed that in progressive cancer, Tregs

lose their ability to suppress inflammation while maintaining their T cell suppressive properties [33, 54–56]. This change in function was attributed to the upregulation of the canonical TH17 transcription factor ROR $\gamma$ T in Tregs. STAT3 activity contributes to the upregulation of ROR $\gamma$ T, expression of IL17, and to the TH17 response, which includes the recruitment of pro-tumor TAMs. In the current study, we demonstrated inhibition of both ROR $\gamma$ T<sup>-</sup> and ROR $\gamma$ T<sup>+</sup> Tregs in tumor-bearing mice upon treatment with PYR. The drop in Treg frequency correlates with increased densities of LAMP1<sup>+</sup> CD8 T cells. These findings are consistent with the notion that PYR antagonizes Treg function and favors tumor cell lysis by release of cytotoxic granules from CTLs. These findings suggest that by decreasing cancer-associated inflammation and immunosuppression while enhancing effector activity of CTLs, a STAT3 inhibitor would be a particularly attractive and promising therapeutic strategy for treating breast cancer and other malignancies.

In conclusion, we have presented data indicating that PYR has STAT3-inhibitory activity in breast cancer cells and inhibits tumor cell growth in vitro and in vivo. Furthermore, we have shown that treatment of tumor-bearing mice with PYR leads to a reduction in the number of TAMs, Foxp3<sup>+</sup> Tregs, and Foxp3<sup>+</sup>ROR $\gamma$ T<sup>+</sup> T cells, and an increase in the Lamp1 marker on CD8<sup>+</sup> T cells. Thus, PYR inhibits the growth of breast tumors directly by acting on the cancer cells as well as indirectly by shifting a pro-tumor immune response to an anti-tumor immune response. These findings suggest that it may be worthwhile to conduct a clinical trial of PYR, alone or in conjunction with other therapies, for the treatment of advanced breast cancer.

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#### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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