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Original Article

Breast tumor cells primed by endoplasmic reticulum stress remodel macrophage phenotype

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Abstract: In the pathogenesis of breast cancer, tumor-associated macrophages have the capacity to impinge upon clinical outcomes. In light of this, reconciling mechanisms by which macrophages are primed to facilitate tumor growth and progression provide clinically relevant therapeutic targets. Given the recent linkage between activation of the endoplasmic reticulum (ER) stress response and breast cancer progression, we postulated that, similar to other carcinomas, mammary carcinoma cells undergoing ER stress re-program macrophages in order to foster both tumor cell growth and survival, and tumor angiogenesis. To test this, we modeled the interaction between ER-stressed tumor cells and macrophages in the tumor microenvironment by culturing macrophages in the conditioned medium of mammary carcinoma cells undergoing ER stress. In response to these stimuli, macrophages not only invoked a similar stress response but also adopted a pro-inflammatory phenotype. Additionally, macrophages produced the pro-angiogenic molecule, vascular endothelial growth factor (VEGF), thereby establishing the macrophage phenotype invoked by ER-stressed breast cancer cells as being pro-angiogenic. In aggregate, these findings delineate a role for ER stress-dependent cross-talk between breast tumor cells and TAMs as a potential catalyst for tumor cell growth and tumor-associated angiogenesis. Hence, by suggesting that mammary carcinoma cells cope with ER stress by influencing TAM functionality, we have partially elucidated why enhanced tumor progression and angiogenesis accompany the ER stress response in breast cancer.

Keywords: Breast cancer, macrophages, endoplasmic reticulum stress, angiogenesis

Introduction

Dating as far back as the 19th century, solid tumors have been recognized as a pathological site characterized by significant infiltrates of leukocytes. In recent years, the basis for such an influx of leukocytes, most notably macrophages, has become an area of intense investigation, particularly since tumor-associated macrophages (TAMs) have the capability of altering clinical outcomes. In fact, for certain types of cancer, i.e. breast cancer, poor prognosis correlates with TAM density [1]. In support of clinical data, genetically-modified mice and cell transfer experiments have provided direct evidence for the pro-tumor role of myeloid cells and its effector molecules. In part, macrophages create a tumor microenvironment more permissive to both tumor cell growth and invasiveness by secreting a variety of cytokines, growth factors, angiogenic factors, and proteases [2]. In fact, thanks to its effector molecules, TAMs facilitate a host of critical tumor-associated processes, including inflammation, angiogenesis, metastasis, and suppression of anti-tumor immune responses. Yet, despite advances in our understanding of the pro-tumorigenic function of TAMs, our understanding of the microenvironmental and metabolic factors which enable tumor cells to co-opt macrophages is wholly incomplete.

In an elegant study by Mahadevan *et al*., this phenomenon of cross-talk between macrophages and tumor cells is addressed by demonstrating that a well-documented facet of tumor cell biology - endoplasmic reticulum (ER) stress - can trigger tumor cells to modulate macrophage phenotype [3]. Intriguingly, ER stress can be transmitted from tumor cells to macrophages, via soluble factors, resulting in both the induction of an ER stress response and a robust pro-inflammatory phenotype. In this way, the study argues that ER-stressed tumor cells

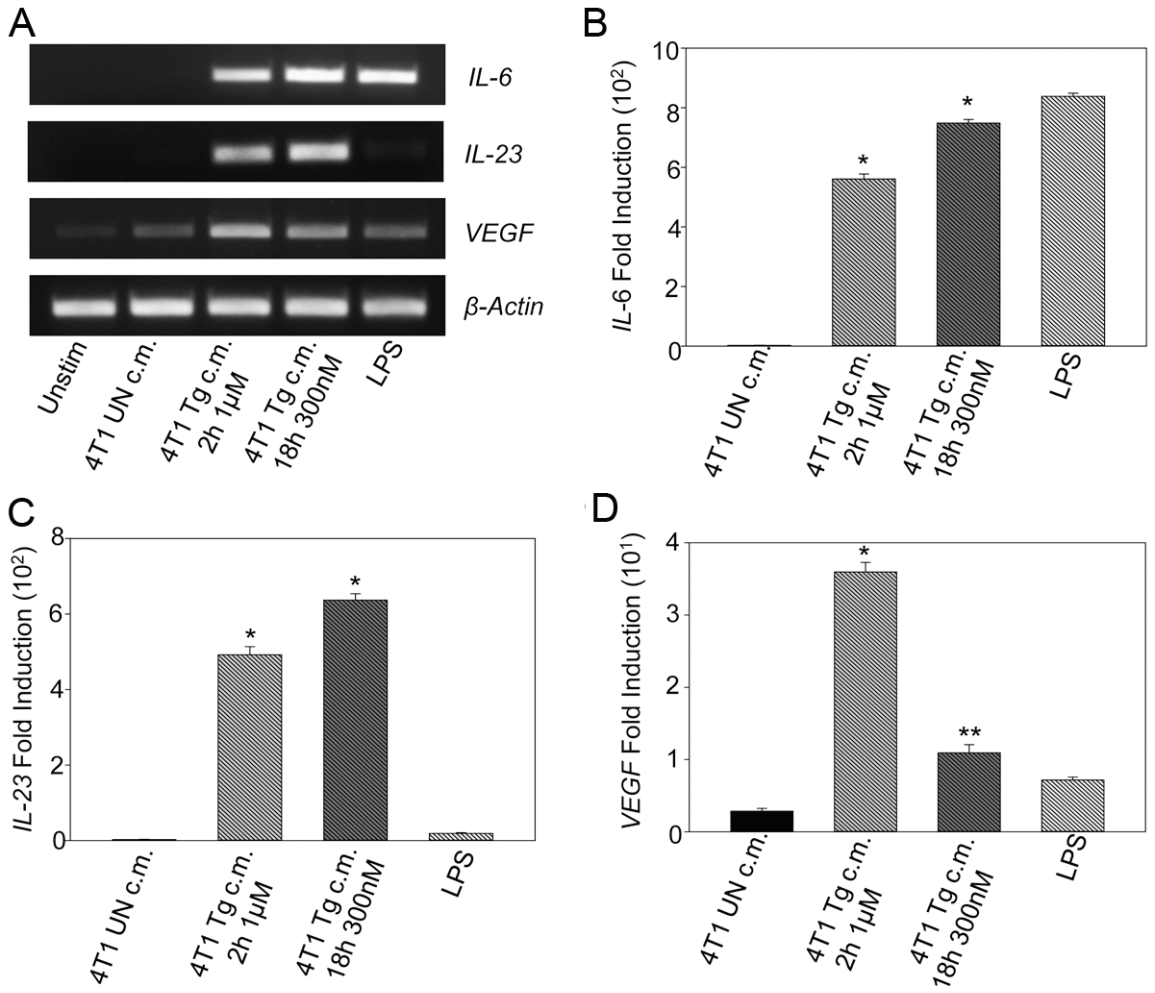

Figure 1. RAW 264.7 macrophages up-regulate gene expression of pro-inflammatory cytokines and *VEGF*, upon exposure to conditioned medium from ER-stressed 4T1 cells. RAW 264.7 cells were either left untreated (Unstim.) or treated with conditioned medium (c.m.) from 4T1 cells for 24h. Conditioned medium was harvested from 4T1 cells subjected to either no stimulation (4T1 UN c.m.) or 18h thapsigargin (Tg) treatment consisting of either a brief 2h pulse of Tg stimulation (4T1 Tg c.m. - 2h 1μM) or continuous Tg treatment for 18h (4T1 Tg c.m. - 18h 300nM). As a control for macrophage activation, RAW 264.7 cells were treated with LPS (100ng/mL) for 24h. RNA was processed and analyzed by RT-PCR for expression of genes fostering tumor growth and survival. A representative gel image (A) and gel densitometry (B), (C), & (D) of three independent experiments are depicted. Data obtained by densitometry analysis were normalized to expression of β-Actin and expressed as fold induction relative to unstimulated cells; values represent data means ± standard error. Statistically significant differences are denoted by *(p value < 0.05).

can elicit a similar stress response in macrophages and in the process, ensure tumor cell survival. Given the biological relevance of this phenomenon, this naturally begs the question: Amongst the various types of cancers, how prevalent is this ER stress-related mechanism?

To date, carcinomas such as lung and prostate cancer have been shown to regulate macrophage phenotype in an ER-stress dependent manner [3]. However, for other types of carcinomas, notably mammary carcinomas, it is unclear if ER stress can evoke a similar, pro-tumor macrophage subtype. The relevance of such a query is underscored by clinical and research data affirming a role for the ER stress response in breast cancer [4]. Like most cancers, breast cancer fosters a tumor microenvironment prone to ER stressors, i.e. hypoxia, decreased glucose and amino acid supply, low extracellular pH, and errors in biosynthesis of glycoproteins and lipids [5]. In response to these cell-extrinsic and -intrinsic insults, breast

tumor cells counteract ER stress, and the possibility of ER stress-related apoptosis, by mounting an unfolded protein response (UPR).

As its main functions, the ER regulates lipid and sterol synthesis, calcium storage, and the folding and post-translational modification of membrane and secreted proteins [6]. If perturbations in its regulatory activities occur, the resulting accumulation of misfolded and unfolded proteins triggers the ER stress response, or UPR. The UPR is mediated by three initiator/sensor molecules, namely inositol-requiring enzyme 1 (IRE1α), PKR-like ER kinase (PERK), and activating transcription factor 6 (ATF6). In the absence of stress, each is maintained in an inactive state through its association with glucose-regulated protein 78 (GRP78/BiP) [7]. Upon activation, GRP78-free PERK signals downstream effectors such as growth arrest and DNA damage gene (GADD34) and the C/EBP homologous protein (CHOP). Likewise, once unbound from GRP78, IRE1 utilizes its endoribonuclease activity to initiate unconventional splicing of the mRNA encoding X-box-binding protein 1 (XBP-1) [8]. As spliced XBP-1 is a potent transcriptional activator, up-regulation in spliced XBP-1 is accompanied by induction of its target genes involved in efficient protein folding, maturation, and degradation in the ER.

Increased expression of many of these UPR components, including XBP1, ATF6, CHOP, and GRP78, has been detected in breast cancer [9]. For example, elevated levels of GRP78 are typically associated with higher pathologic grade, recurrence, and poor patient survival. This link between GRP78 levels and clinical severity likely stems from its roles uncovered by a genetic mouse model of breast cancer which implicates the involvement of GRP78 in tumor proliferation, survival, and tumor angiogenesis [10]. In agreement with in vivo data, the angiogenic switch accompanying tumor angiogenesis has been identified as a downstream target of UPR pathways, thus underscoring the importance of the ER stress response in tumor angiogenesis [11, 12]. Clearly, compelling evidence points to a role for the ER stress response in breast cancer pathology, therefore understanding the mechanistic basis for UPR^{ER}-mediated tumor progression and angiogenesis has tremendous therapeutic value.

Given the association between UPR^{ER} and breast tumor progression and metastasis, this raises the distinct possibility that, like other carcinomas, ER-stressed mammary carcinoma cells support their survival by modulating the functionality of tumor-associated macrophages. To test this, we modeled the interaction between ER-stressed tumor cells and macrophages in the tumor microenvironment by culturing macrophages in the conditioned medium of mammary carcinoma cells undergoing ER stress. In response to these stimuli, macrophages up-regulated markers of the UPR^{ER}, as well as inducing pro-tumor, inflammatory genes, such as *IL-6* and *IL-23p19*. Additionally, as never before shown previously, ER-stress conditioned macrophages support tumor angiogenesis by producing the pro-angiogenic molecule, vascular endothelial growth factor (VEGF). In light of this, our findings highlight a potential role for ER stress-mediated cross-talk between breast cancer cells and macrophages as a driver for both tumor cell survival and progression, and tumor-associated angiogenesis. Principally, this indicates that tumor-associated macrophages likely play a critical role in the enhanced tumor progression and angiogenesis associated with UPR^{ER} in breast cancer.

Materials and methods

Antibodies and reagents

VEGF antibody was obtained from Abcam (Cambridge, MA). iNOS antibody was purchased from EMD Millipore (Billerica, MA). All other antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced Chemiluminescence reagents were from Thermo Fisher Scientific (Rockford, IL). All fine chemicals, unless otherwise mentioned, were obtained from Sigma Chemical Company (St. Louis, MO). Electrophoresis supplies and molecular weight standards were from BioRad (Richmond, CA). Thapsigargin (Tg) and bacterial LPS (L2654) were obtained from Sigma Chemical Company (St. Louis, MO).

Mice

C57BL/6 mice which were originally obtained from Jackson laboratories (Bar Harbor, ME) were housed under pathogen-free conditions. All experiments and animal care procedures

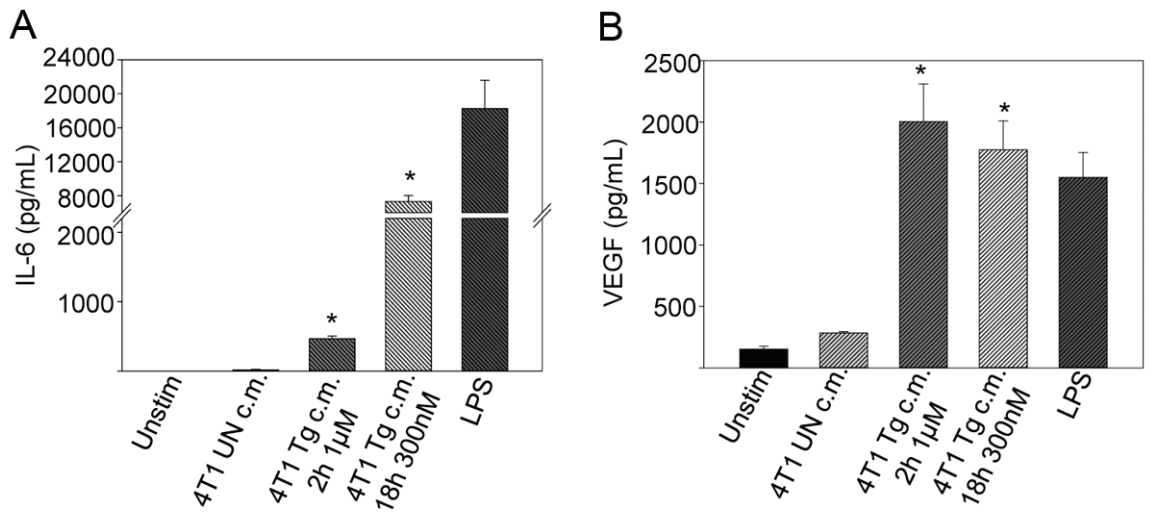

Figure 2. Secretion of IL-6 and VEGF accompanies treatment of RAW 264.7 macrophages with conditioned medium from ER-stressed 4T1 cells. RAW 264.7 cells were cultured for 24h in either conditioned medium of ER-stressed 4T1 cells (4T1 Tg c.m.) or control 4T1 cells (4T1 UN c.m.), or culture medium alone with or without LPS (100ng/mL). At the end of 24h, supernatants were collected and analyzed by murine IL-6 (A) and VEGF (B) ELISA kits per manufacturer's protocol. Values presented are mean ± standard error from three independent experiments. Statistically significant differences are denoted by *($p < 0.05$).

were performed in accordance with protocols approved by the Insititutional Animal Care and Use Committees (IACUC) at the University of Washington. To generate macrophages derived from bone marrow, bone marrow cells were flushed from the femurs of 8-12 week-old mice. Following lysis of red blood cells with ACK lysis buffer (Lonza), cells were cultured for 6 days in DMEM media (GlutaMAX-I, Life Technologies), supplemented with 10% heat-inactivated fetal bovine serum, Penicillin/Streptomycin, and 10% CMG 12-14 cell-conditioned medium as a source of CSF-1 [13] (CMG 12-14 cell line was a kind gift from J. Hamerman, Benaroya Research Institute, Seattle WA).

Cell culture

Mouse mammary tumor cell line, 4T1, was obtained from ATCC, and the monocytic/macrophage cell line, RAW 264.7, was a kind gift from A. Aderem (Seattle Biomedical Research Institute, Seattle WA). All cell lines were maintained under standard culturing conditions in DMEM media (GlutaMAX-I, Life Technologies), supplemented with Penicillin/Streptomycin and 10% heat-inactivated fetal bovine serum.

PyMT primary cell culture

In accordance with a previous report [14], we obtained primary tumor cells by employing mice with mammary gland specific overexpression of polyoma middle T antigen (PyMT) which is under the control of the mouse mammary tumor virus (MMTV) promoter. These mice were obtained by crossing FVB/N-Tg (MMTV-PyMT) 634 Mul/J transgenic males on a 100% FVB background with females on a C57/BL6 background for 10 generations. To generate primary tumor cells, mammary tumors were aseptically excised from the mammary fat pad from PyMT mice. Tumor explants were then washed with PBS and serum-free DMEM, mechanically dissociated, and cultured in DMEM (GlutaMAX-I, Life Technologies) with 20% fetal bovine serum and 1% penicillin/streptomycin (Life Technologies). Until cells reached confluence, media was replaced every 2-3 days. Thereafter, cells were trypsinized and cultured in DMEM with 10% fetal bovine serum and 1% penicillin/streptomycin.

Tumor cell conditioned medium

ER stress-conditioned medium was prepared as previously described, with slight modifications [3]. Briefly, mammary carcinoma cells were subjected to ER stress due to either brief (2h) or prolonged (18h) exposure to thapsigargin (Tg). Following Tg treatment, cells undergoing short-term treatment (2h) were washed twice with Dulbecco's PBS (Life Technologies) and then cultured in fresh medium for 16h.

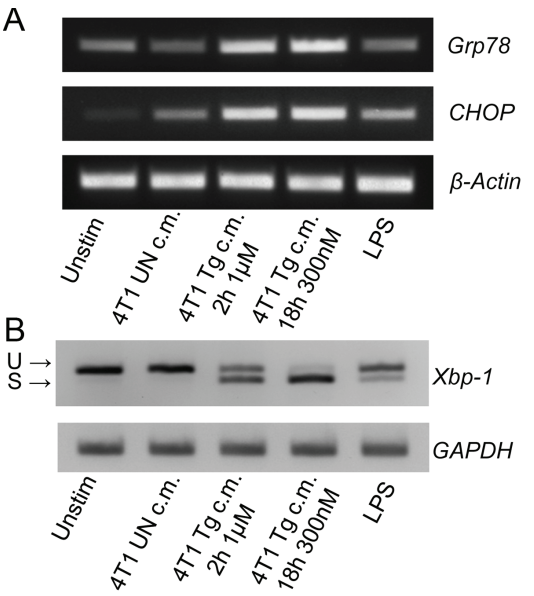

Figure 3. Raw 264.7 macrophages undergo ER stress in response to stimulation with conditioned medium from ER-stressed 4T1 cells. RAW 264.7 cells were cultured for 24h in either conditioned medium of ER-stressed 4T1 cells (4T1 Tg c.m.) or control 4T1 cells (4T1 UN c.m.), or culture medium alone with or without LPS (100ng/mL). At the end of 24h, RNA was processed and analyzed by RT-PCR for expression of genes indicative of a cell-intrinsic ER stress response. Representative gel images (n=3) of RT-PCR products depict expression of ER stress markers (A) and *Xbp-1* splicing (B).

Prior to treatment of macrophages, conditioned medium was centrifuged for 10min at 2000 rpm and then filtered through a 0.22-μm filter (VWR). As a negative control, conditioned medium was harvested from untreated tumor cells. Analysis of conditioned medium by mass spectrometry confirmed results obtained by Mahadevan *et al.* [3] - carryover of Tg was not detected in medium from cells exposed to Tg for 2h (data not shown). In contrast, analysis of conditioned medium corresponding to 18h Tg stimulation identified Tg carryover, thus positive signals obtained with continuous Tg stimulation are included in the study to merely serve as a positive control (data not shown).

Reverse transcriptase-PCR (RT-PCR)

RNA from either RAW 264.7 or primary macrophages was isolated using the Nucleospin RNA purification protocol (Machery-Nagel), with the optional DNase step included. Concentration and purity of RNA was determined by analysis on a NanoDrop spectrophotometer (Thermo Scientific). Isolated RNA was subjected to RT-PCR analysis, by employing the EZ rTth polymerase kit (Applied Biosystems) and intron-spanning primers. The resulting PCR products were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining. Densitometric analyses of gel images were performed using Image J software (NIH).

ELISA

After addition of the stimuli, cells were incubated for 24 hours and then the culture supernatants were collected. Assays for detection of murine IL-6 and VEGF were performed with an enzyme-linked immunosorbent assay kit (eBioscience) according to the manufacturer's instructions.

Lysate preparation and immunoblotting

Total cell lysates were prepared by sonicating cell extracts lysed in RIPA buffer (50mM Tris HCl pH 8, 150mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail). Protein content of total cell extracts was determined using Pierce BCA protein assay (Fisher Thermo Scientific). Cell lysates equalized for protein (25μg) were resolved by SDS-polyacrylamide gel electrophoresis (PAGE), transferred to PVDF, immunoblotted with specific antibody/s, and detected using anti-IgG followed by Enhanced Chemiluminescence, per manufacturer's instructions (SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific). To ensure equal protein loading, blots were stripped and re-probed with antibody to β-Actin.

Immunohistochemistry

Macrophages were plated at a density of 5 x 10^5 cells on tissue culture chamber slides for 24h. Following indicated treatments for an additional 24h, cells were fixed in 3% formaldehyde for 15 min. Fixed cells were permeabilized in ice cold ethanol for 5 min and then washed with PBS, including a wash containing 1% hydrogen peroxide to quench endogenous peroxidase activity. Incubation in primary antibodies occurred for 1h at room temperature, with subsequent staining performed with the rabbit ABC staining system (Santa Cruz Biotechnology),

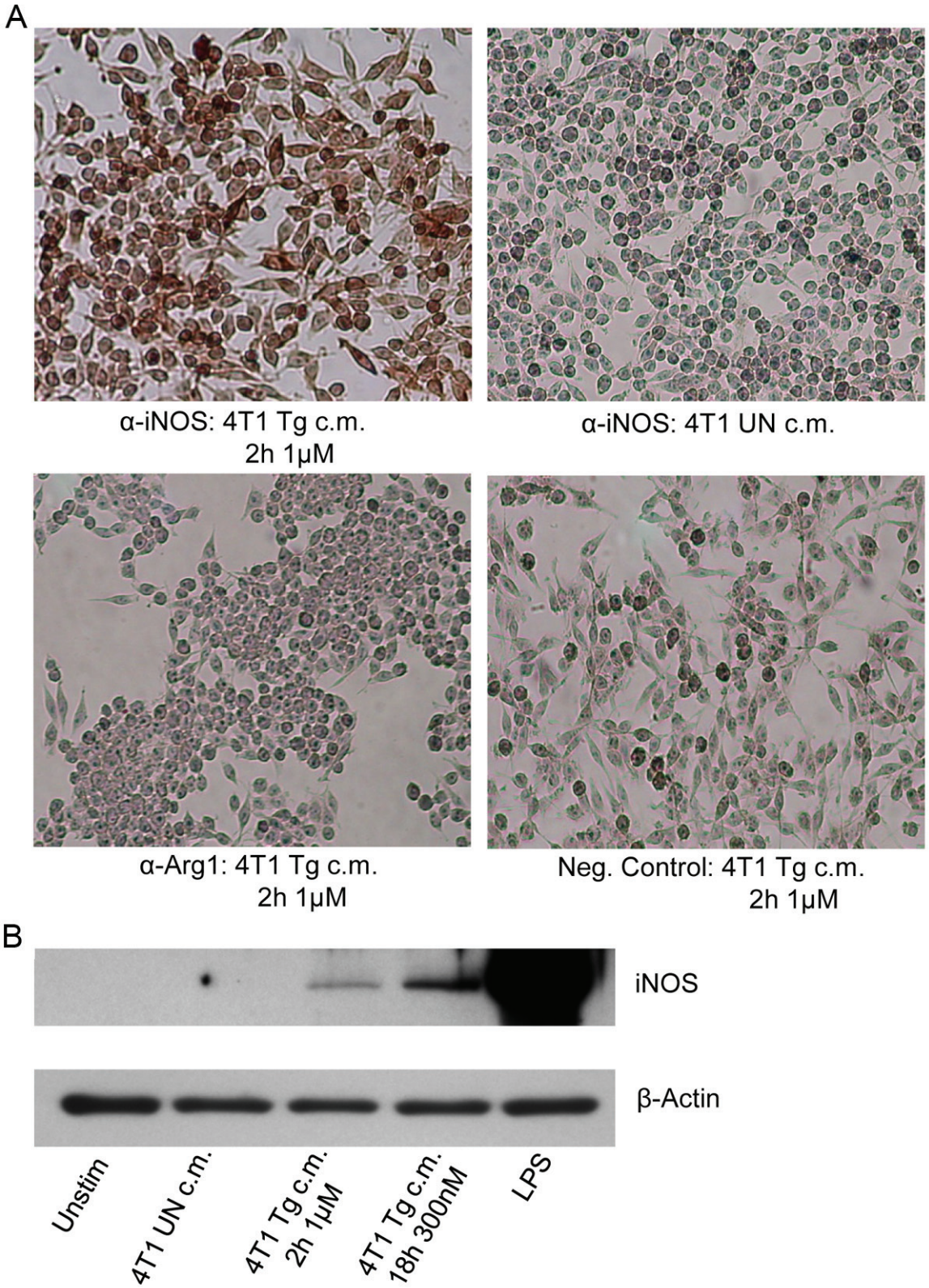

Figure 4. RAW 264.7 macrophages up-regulate iNOS expression in response to exposure to ER-stress conditioned medium. RAW 264.7 cells were cultured for 24h in either conditioned medium of ER-stressed 4T1 cells (4T1 Tg c.m.) or control 4T1 cells (4T1 UN c.m.), or culture medium alone with or without LPS (100ng/mL). A. RAW 264.7 macrophages grown on tissue culture slides were fixed and analyzed by immunostaining for iNOS and Arg-1. Repre-

sentative images are shown, including a negative control which was obtained by omitting the primary antibody from the staining procedure. B. Total cell lysates from RAW 264.7 cells were resolved by SDS-PAGE, followed by western blotting using an antibody recognizing iNOS. A representative immunoblot (n=3) wherein β-Actin served as a loading control is shown.

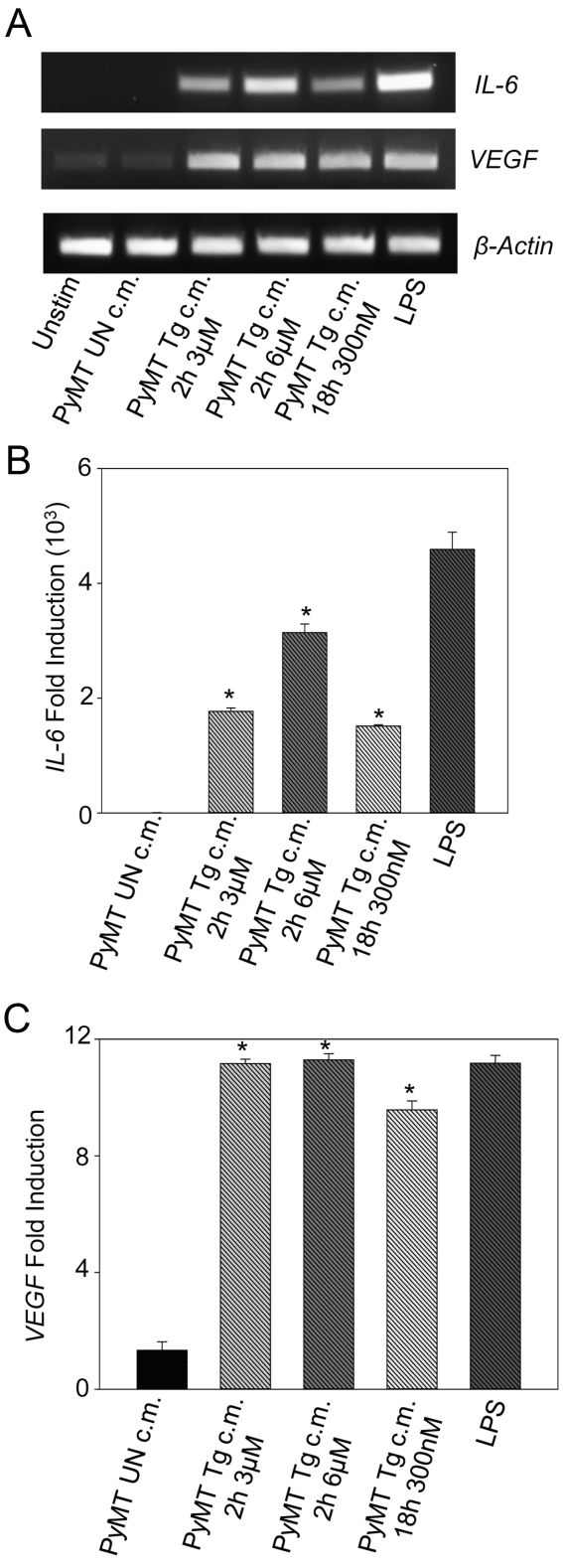

Figure 5. Conditioned medium from primary PyMT tumor cells subjected to ER stress induces *IL-6* and *VEGF* gene transcription in RAW 264.7 macrophages. RAW 264.7 cells were either left untreated (Unstim.) or treated with c.m. from primary PyMT tumor cells for 24h. Conditioned medium was harvested from tumor cells undergoing either no treatment (PyMT UN c.m.) or 18h Tg treatment (PyMT Tg c.m.) consisting of either a brief 2h pulse of Tg treatment (2h 3μM or 2h 6μM) or continuous Tg stimulation for 18h (18h 300nM). As a control for macrophage activation, RAW 264.7 cells were treated with LPS (100ng/mL) for 24h. RNA was isolated and analyzed by RT-PCR for *IL-6* and *VEGF* gene transcription. A representative gel image (A) and gel densitometry (B) & (C) of three independent experiments are depicted. Data obtained by densitometry analysis were normalized to expression of β-Actin and expressed as fold induction relative to unstimulated cells; values represent data means ± standard error. Statistically significant differences are denoted by *(p value < 0.05).

per manufacturer's instructions. Slides were counterstained with Mayer's hematoxylin and mounted with cover slips using an aqueous mounting media. As a negative control, primary antibody was omitted from the staining procedure.

Statistical analysis

Data were analyzed using student's t-test. All results are expressed as mean values plus or minus standard error from three independent experiments which produced similar results.

Results

ER-stress conditioned medium from mammary carcinoma cells elicits a pro-inflammatory and pro-angiogenic phenotype in RAW 264.7 macrophages

To test whether ER stress in the mammary carcinoma cell line, 4T1, has cell-extrinsic effects on macrophages, we first challenged 4T1 cells with thapsigargin (Tg), an inhibitor of the sarco/endoplasmic reticulum Ca^{+2} transport ATPase. To that end, mammary carcinoma cells were subjected to either brief or prolonged exposure to thapsigargin whereby cells were treated with Tg for 18h, with cells either exposed to a brief 2h pulse of Tg stimulation or cultured in Tg for

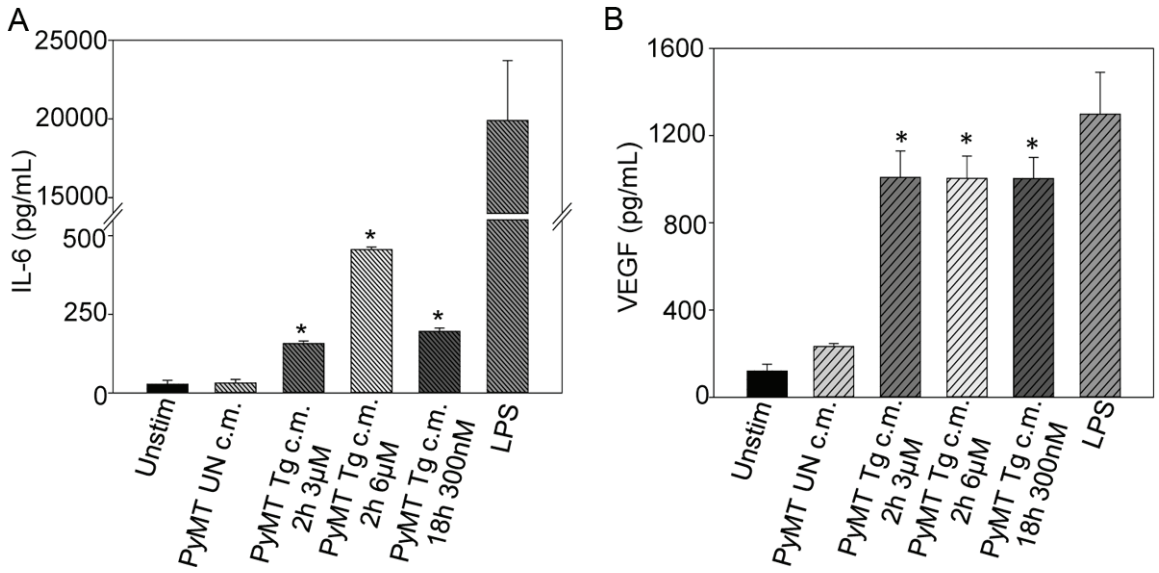

Figure 6. RAW 264.7 macrophages secrete IL-6 and VEGF upon exposure to conditioned medium from PyMT primary tumor cells subjected to ER stress. RAW 264.7 cells were cultured for 24h in either conditioned medium of ER-stressed PyMT tumor cells (PyMT Tg c.m.) or control PyMT cells (PyMT UN c.m.), or culture medium alone with or without LPS (100ng/mL). At the end of 24h, supernatants were collected and analyzed by murine IL-6 (A) and VEGF (B) ELISA kits per manufacturer's protocol. Values presented are mean ± standard error from three independent experiments. Statistically significant differences are denoted by *.

the entire 18h. As duration of Tg treatment can influence the magnitude to which cells up-regulate pro-angiogenic factors [15], continuous treatment with Tg was included in the study to ensure the induction of a pro-angiogenic phenotype in macrophages, thereby serving as a positive control. Thereafter, conditioned medium (c.m.) from the ER-stressed 4T1 cells was harvested and used as culturing medium for 24h for the monocytic/macrophage cell line, RAW 264.7.

Compared to conditioned medium from untreated 4T1 cells, RAW 264.7 macrophages responded to conditioned medium from ER-stressed 4T1 tumor cells by inducing gene transcription of the pro-inflammatory cytokines, *IL-6* and the unique p19 subunit of *IL-23 (IL-23p19)* (**Figure 1B** and **1C**). Additionally, RAW 264.7 macrophages up-regulated gene expression of the pro-angiogenic molecule, VEGF (**Figure 1D**). Consistent with these changes in gene expression, RAW 264.7 macrophages increased production of IL-6 (**Figure 2A**) and VEGF (**Figure 2B**), when exposed to conditioned medium from 4T1 cells subjected to ER stress. Further, concomitant to transcriptional activation and secretion of tumor-promoting factors, conditioned medium from ER-stressed 4T1 cells evoked an ER stress response in RAW 264.7 macrophages, as demonstrated by both the up-regulation of *CHOP* and *BiP/Grp78* and *Xbp-1* splicing (**Figure 3**). In aggregate, these findings indicate that, similar to other tumor cell lines, ER stress in mammary carcinoma cells can provoke cross-talk between tumor cells and macrophages. Moreover, these results provide mechanistic insight into cancer-associated inflammation and angiogenesis by characterizing how ER stress in breast cancer cells can give rise to a pro-tumor, pro-inflammatory subtype of macrophage.

Increased iNOS levels are observed in RAW 264.7 macrophages cultured in 4T1 ER stress-conditioned medium

To further explore the macrophage subtype obtained by exposure to ER-stress conditioned medium, we performed immunostaining with antibodies directed against the enzymes arginase I (Arg1) and the inducible NO synthase (iNOS). Divergent expression of Arg1 and iNOS has greatly defined the dichotomous nomenclature of macrophages, as M1 (or classically activated) macrophages express high levels of iNOS and low levels of Arg1, while M2 (or alternatively activated) macrophages express the

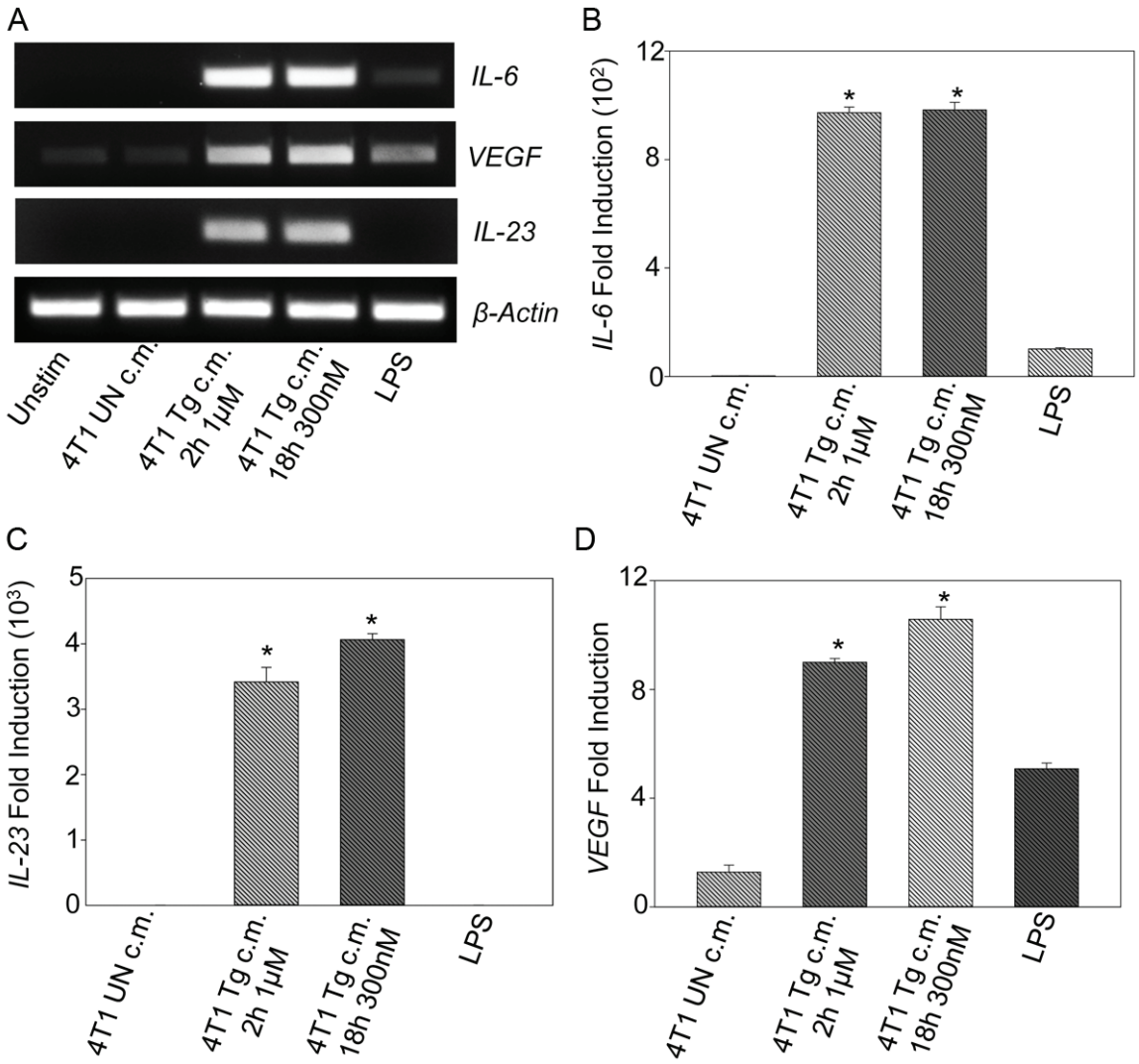

Figure 7. Bone marrow-derived macrophages up-regulate pro-tumorigenic genes in response to treatment with conditioned medium from ER-stressed 4T1 cells. BMDMs were cultured for 24h in either conditioned medium from ER-stressed 4T1 cells (4T1 Tg c.m.) or control 4T1 cells (4T1 UN c.m.), or culture medium with or without LPS (100ng/mL). RNA was isolated from BMDMs and analyzed by RT-PCR for expression of pro-inflammatory cytokines and VEGF. A representative gel image (A) and gel densitometry (B), (C), & (D) of three independent experiments are depicted. Data obtained by densitometry analysis were normalized to expression of β-Actin and expressed as fold induction relative to unstimulated cells; values represent data means ± standard error. Statistically significant differences are denoted by *(p value < 0.05).

reverse pattern (iNOS^{lo} and Arg1^{hi}). Despite the prevailing notion that tumor-associated macrophages possess an M2 phenotype [2], RAW 264.7 macrophages cultured in conditioned medium from ER-stressed 4T1 cells exhibited the hallmarks of an M1 phenotype, with high expression of iNOS and low expression of Arg1 (**Figure 4A**). As a control, RAW 264.7 cells stimulated with IL-4 exhibited high expression of Arg1 (data not shown), excluding the possibility that RAW 264.7 cells preferentially adopt an M1 phenotype regardless of stimulant. Consistent with immunostaining results, data obtained by immunoblotting confirmed that RAW 264.7 macrophages induced expression of iNOS when exposed to ER stress-conditioned medium (**Figure 4B**). Thus, taken together, these results suggest that TAMs co-opted by ER-stressed mammary carcinoma cells may more so belong to a unique macrophage subtype, as opposed to the well-established M1 or M2 classification.

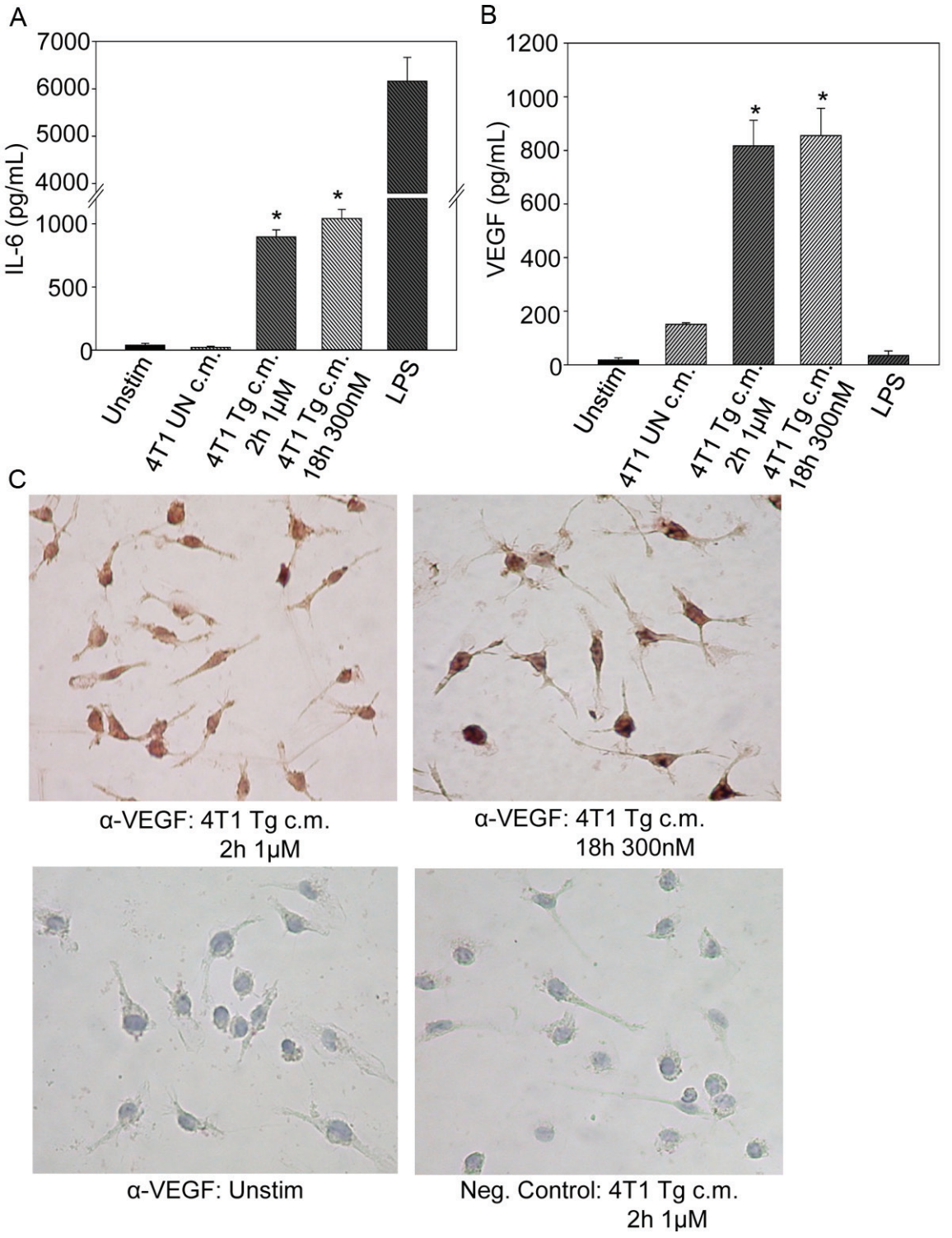

Figure 8. Bone marrow-derived macrophages develop a pro-tumor phenotype in response to treatment with conditioned medium from ER-stressed 4T1 cells. BMDMs were cultured for 24h in either ER-stressed conditioned medium from 4T1 cells (4T1 Tg c.m.) or medium from control 4T1 cells (4T1 UN c.m.), or culture medium with or without LPS (100ng/mL). At the end of 24h, supernatants were collected and analyzed by murine IL-6 (A) and VEGF (B) ELISA kits per manufacturer's protocol. Values presented are mean ± standard error from three independent experiments. Statistically significant differences are denoted by *(p value < 0.05). C. BMDMs which were cultured in tissue culture slides were stained with anti-VEGF. Representative results are shown, including a negative control obtained by omitting anti-VEGF from the staining procedure.

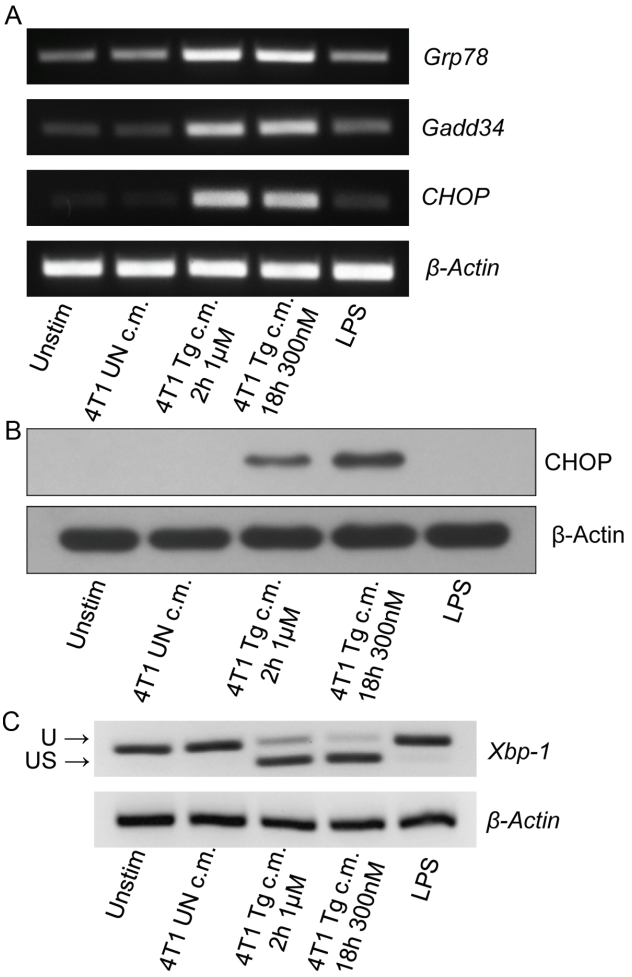

Figure 9. Primary macrophages experience ER stress upon exposure to conditioned medium from ER-stressed 4T1 tumor cells. BMDMs were cultured for 24h in either ER-stressed conditioned medium from 4T1 cells (4T1 Tg c.m.) or medium from control 4T1 cells (4T1 UN c.m.), or culture medium with or without LPS (100ng/mL). RNA from macrophages was isolated and analyzed by RT-PCR. Representative gel images (n=3) of RT-PCR products depict expression of ER stress markers (A) and *Xbp-1* splicing (B). (C) Total cell lysates from BMDMs, equalized for protein content, were resolved by SDS-PAGE, followed by western blotting using an antibody recognizing CHOP. A representative immunoblot (n=3) wherein β-Actin served as a loading control is shown.

ER-stress conditioned medium from primary tumor cells of the PyMT breast cancer model propagates ER stress, and induces IL-6 and VEGF gene expression in RAW 264.7 macrophages

To validate this phenotype with an additional mammary carcinoma cell line, we employed primary tumor cells which were derived from mammary tumor explants from MMTV-PyMT transgenic mice. As with 4T1 cells, primary PyMT tumor cells were subjected to both short-term and prolonged ER stress with thapsigargin. Thereafter, transference of the conditioned medium from ER-stressed PyMT primary tumor cells led to the transcriptional activation and secretion of IL-6 and VEGF (**Figures 5** and **6**), as well as the splicing of *Xbp-1* (data not shown). Hence, this phenomenon whereby ER stress in tumor cells elicits a pro-tumor, pro-inflammatory subtype of macrophage extends to a broad range of carcinomas, including mammary carcinomas.

In response to ER-stress conditioned medium from mammary carcinoma cells, bone marrow-derived macrophages develop a pro-angiogenic, pro-tumor phenotype

Given the responses obtained with RAW 264.7 macrophages, we next determined whether bone marrow-derived macrophages (BMDMs) developed a similar phenotype. To test this, we cultured BMDMs in conditioned medium from 4T1 cells which had undergone both short-term and prolonged ER stress. As depicted in **Figure 7**, similar to RAW 264.7 cells, BMDMs responded to ER-stress conditioned medium by not only inducing gene expression of *IL-6, IL-23p19*, and *VEGF* (**Figure 7**), but also secreting IL-6 and VEGF (**Figure 8A** and **8B**). Correspondingly, immunostaining confirmed that the up-regulation of VEGF expression occurred both at the mRNA and protein level (**Figure 8C**), thereby further substantiating the macrophage phenotype invoked by ER-stressed breast cancer cells as being pro-angiogenic. Thus, for the first time, our findings provide mechanistic insight into the previously reported linkage between ER stress in mammary carcinoma cells and the promotion of tumor angiogenesis. Furthermore, in agreement with previous reports, these results support the notion that ER stress within breast cancer cells drives tumor-associated inflammation, thereby ensuring cancer progression.

Tumor ER stress-conditioned medium transmits ER stress to primary macrophages derived from bone marrow

To characterize the signaling mechanisms underlying this phenotype in primary macrophages, we ascertained if ER stress was transmissible between Tg-treated 4T1 cells and BMDMs. As with RAW 264.7 cells, BMDMS cultured in ER-stress conditioned medium mounted an ER stress response, characterized by *Xbp-1* splicing and transcriptional up-regulation of *Grp78, Gadd34*, and *CHOP* (**Figure 9A** and **9C**). In conjunction with increased transcript levels of *CHOP*, BMDMs also expressed CHOP at the protein level, but only when exposed to conditioned medium from ER-stressed 4T1 cells (**Figure 9B**). Overall, these results indicate that ER stress signaling in mammary carcinoma cells is conveyed to macrophages, via unidentified soluble factors.

Discussion

In the pathogenesis of breast cancer, tumor-associated macrophages have the capacity to impinge upon clinical outcomes. In light of this, reconciling mechanisms by which macrophages are primed to facilitate tumor growth and progression provide potentially attractive therapeutic targets. Given the recent linkage between UPR^{ER} and breast cancer progression [4], we postulated that, similar to other carcinomas, ER stress within mammary carcinoma cells primes macrophages to foster both tumor cell growth and survival and tumor angiogenesis. Indeed, in accordance with our hypothesis, mammary carcinoma cells undergoing ER stress elicited not only a similar stress response in macrophages but also a pro-inflammatory and pro-angiogenic phenotype.

To our surprise, RAW 264.7 macrophages exposed to ER-stressed conditioned medium displayed a phenotype more closely resembling the classic, M1-polarization, i.e. $iNOS^{hi}$ and $Arg\text{-}1^{lo}$. This result challenges the popular paradigm that TAMs in established, solid tumors are a prototypic M2 population [2]. Considering a solid tumor harbors conditions conducive for metabolic ER stressors, i.e. hypoxia and nutrient deprivation, our inability to detect measurable levels of the M2 marker, Arg-1, was rather unexpected. By comparison, the original report detailing this "transmissible" ER stress phenomenon described an up-regulation in Arg-1 expression, albeit variable, which depended on the tumor cell type [3].

In relation to our data, the notion of tumor cell type being a determinant of Arg-1 expression is rather apt because advanced-stage breast tumors can feature moderate levels of iNOS [16]. This up-regulation in iNOS ostensibly stems from the ability of iNOS to generate nitric oxide (NO) which is instrumental in ensuring tumor cell adaptation and survival under hypoxic conditions. Fittingly, TAMs and stromal cells function as a key cellular source for iNOS in an advanced-stage breast tumor. In light of this, it is therefore highly plausible that TAMs which are receiving cues from hypoxic, ER-stressed breast tumor cells induce iNOS. Notably, unlike RAW 264.7 macrophages, BMDMs exposed to ER-stress conditioned medium from 4T1 cells did not up-regulate iNOS to the same extent as RAW 264.7 cells (data not shown). Nonetheless, despite this discrepancy, the lack of consistent evidence validating a prototypic M2 phenotype highlights the possibility that macrophages influenced by ER-stressed tumor cells belong in their own unique TAM subtype. In support of this idea, it has been proposed that TAMs can be classified into several distinct populations that not only share features of both M1 and M2 phenotypes but also have greater overall similarity to macrophages involved in developmental processes [17]. Thus, as more phenotypic details emerge about ER stress-regulated TAMs, we can discern how truly distinct this phenotype is.

Even though we were unable to confirm robust iNOS induction in BMDMs, the co-expression of both iNOS and inflammatory and ER stress-related genes in RAW 264.7 macrophages still has intriguing, biological significance, as this phenotype bears resemblance to the transcriptional profile of neurodegenerative diseases, such as multiple sclerosis (MS) [18]. For example, gene expression analysis of white matter of MS patients uncovered both an abundance of spliced *Xbp-1* transcripts and increased transcription of inflammatory genes, including *IL-23p19*. Moreover, efforts to model the conditions found in the white matter of MS patients led to the discovery that overexpression of a glycoprotein, Syncytin-1, in human astrocytes induced expression of inflammatory genes, ER stress markers and iNOS. Similarly, in a mouse

model of cerebellar degeneration, evidence of ER stress in cerebellar Purkinje cells was accompanied by iNOS immunoreactivity in microglia surrounding the ER-stressed Purkinje cells [19]. Principally, these studies advance a model for neurodegenerative diseases wherein ER stress response at the lesion coincides with inflammation by myeloid cells. Whether this inflammation is induced by soluble factors released by ER stressed neurons is unclear, however the similarities between this model and the ER stressed tumor cell-TAM axis are rather striking. Hence, given the parallels, broadening our mechanistic understanding of this phenomenon could have implications in other diseases, particularly neurodegenerative diseases.

Our discovery that ER stress in breast cancer cells provokes VEGF expression by macrophages further validates that this TAM phenotype is pro-angiogenic. By virtue of IL-23 production, ER stress conditioned TAMs indirectly support tumor angiogenesis, due to the ability of IL-23 to induce IL-17 in the tumor microenvironment [20]. IL-17 promotes angiogenesis in a variety of tumor models and induces matrix metalloproteinases (MMPs) which can process VEGF in the extracellular matrix into its bioactive form [21]. Aside from its role in tumor angiogenesis, IL-23 negatively regulates the function of cytotoxic $CD8^+$ T cells thereby dampening their ability to infiltrate and eliminate nascent malignancies. In this way, IL-23 dually promotes tumor angiogenesis and immunosuppression within the tumor microenvironment. Similar to IL-23, VEGF mediates immunosuppression by creating a tolerogenic tumor microenvironment which fosters immune evasion [22]. Hence, these binary roles for VEGF and IL-23 broaden the pro-tumorigenic functions of ER-stress conditioned TAMs to include both immunosuppression and pro-angiogenic.

In tandem with the original report by Mahadevan *et al*., our study highlights several areas for future research. Firstly, the precise role for UPR signaling in the macrophages requires further clarification. For instance, does UPR signaling initiate pro-tumorigenic responses or is it more so mediating the protraction of macrophage responses, particularly pro-inflammatory signaling? On a related note, the involvement of other signaling pathways, apropos to ER stress, have yet to be elucidated. NF-kappa B seems a likely mediator for reasons that are two-fold: NF-kappa B can be induced by hypoxia; plus, an upstream initiator of NF-κB signaling, toll-like receptor 4 (TLR4), is involved in mediating the cross-talk between ER-stressed tumor cells and macrophages [23]. As for other potential signaling pathways, it is difficult to predict which pathways underlie the pro-tumorigenic phenotype because there is currently a paucity of information regarding the precise mechanisms by which ER-stressed tumor cells activate macrophages. Thus far, only TLR4 has been identified as a sensor for signal(s) released by ER-stressed tumor cells [3]. Moreover, the soluble mediator(s) which serve as ligand(s) for TLR4 are unknown, although heat-shock proteins seem likely candidates, as heat-inactivation of ER stress conditioned medium has no effect on its activity as a macrophage stimulant [3]. Clearly, as highlighted above, there are gaps in our understanding of the signaling mechanisms underlying the interplay between ER-stressed tumor cells and TAMs. Yet, addressing these deficiencies has substantial clinical value because designing therapeutic interventions targeting these TAMs requires knowing precisely how UPR^{ER} affects the dynamics of the tumor cell-TAM interaction.

In summary, we have shown that, similar to other carcinomas, mammary carcinoma cells undergoing ER stress transmit this stress to macrophages. Concomitant to an ER stress response, ER stress-conditioned macrophages also up-regulate pro-tumorigenic, inflammatory cytokines and the pro-angiogenic molecule, VEGF, thereby equipping macrophages with a pro-tumorigenic, pro-inflammatory phenotype. Overall, the results presented herein provide an explanation for the poor clinical outcomes associated with UPR^{ER} in breast cancer. In fact, by demonstrating that mammary carcinoma cells cope with ER stress by hijacking TAMs, we have partially resolved why enhanced tumor progression and angiogenesis accompany UPR^{ER} in breast cancer. Hence, as future studies decipher the precise mechanisms underlying this ER stress-mediated crosstalk between tumor cells and macrophages, the therapeutic benefit could extend well beyond cancer and provide therapeutic options to other diseases with a pathogenesis involving ER stress and inflammation, i.e. neurodegenerative diseases.

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Conflict of interest statement

All the authors do not have any conflict of interest.

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