Biomedicine & Pharmacotherapy 144 (2021) 112274

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

ERα-independent NRF2-mediated immunoregulatory activity of tamoxifen

Giovanna Pepe [a,1], Chiara Sfogliarini [a,2], Loris Rizzello [a,b,3], Giuseppe Battaglia [c,d,e,f,g,4], Christian Pinna [a,5], Gianenrico Rovati [a,6], Paolo Ciana [h,7], Electra Brunialti [h,8], Federica Mornata [i,9], Adriana Maggi [a,10], Massimo Locati [i,j,11], Elisabetta Vegeto [a,12,*]

[a] Department of Pharmaceutical Sciences, University of Milan, 20133 Milan, Italy
[b] National Institute of Molecular Genetics (INGM) Milan, 20122 Milan, Italy
[c] Department of Chemistry and
[d] The EPSRC/Jeol Centre for Liquid Phase Electron Microscopy, University College London, WC1H 0AJ London, U.K
[e] Institute for Bioengineering of Catalonia (IBEC), The Barcelona Institute of Science and Technology, 08028 Barcelona, Spain
[f] Institute for Physics of Living System, University College London, WC1E 6BT London, U.K
[g] Catalan Institution for Research and Advanced Studies (ICREA), 08010 Barcelona, Spain
[h] Department of Health Sciences University of Milan, 20142 Milan, Italy
[i] IRCCS Humanitas Research Hospital, via Manzoni 56, 20089 Rozzano, Milan, Italy
[j] Department of Medical Biotechnologies and Translational Medicine, University of Milan, 20133 Milan, Italy

ARTICLE INFO

Keywords:
tamoxifen
macrophage
inflammation
drug repurposing
Nrf2

ABSTRACT

Sex differences in immune-mediated diseases are linked to the activity of estrogens on innate immunity cells, including macrophages. Tamoxifen (TAM) is a selective estrogen receptor modulator (SERM) used in estrogen receptor-alpha (ERα)-dependent breast cancers and off-target indications such as infections, although the immune activity of TAM and its active metabolite, 4-OH tamoxifen (4HT), is poorly characterized. Here, we aimed at investigating the endocrine and immune activity of these SERMs in macrophages. Using primary cultures of female mouse macrophages, we analyzed the expression of immune mediators and activation of effector functions in competition experiments with SERMs and 17β-estradiol (E2) or the bacterial endotoxin LPS. We observed that 4HT and TAM induce estrogen antagonist effects when used at nanomolar concentrations, while pharmacological concentrations that are reached by TAM in clinical settings regulate the expression of VEGFα and other immune activation genes by ERα- and G protein-coupled receptor 1 (GPER1)-independent mechanisms that involve NRF2 through PI3K/Akt-dependent mechanisms. Importantly, we observed that SERMs potentiate cell phagocytosis and modify the effects of LPS on the expression of inflammatory cytokines, such as TNFα and IL1β, with an overall increase in cell inflammatory phenotype, further sustained by potentiation of IL1β secretion through caspase-1 activation.

Abbreviations: Akt, protein kinase B; LPS, lipopolysaccharide; NRF2, Nuclear Factor erythroid-2-related factor 2; PI3K, phosphoinositide 3-kinase; TNFα, tumor necrosis factor-α; IL1β, interleukin 1β; VEGFα, vascular endothelial growth factor-α.

* Correspondence to: via Balzaretti, 9, 20133 Milan-Italy.
E-mail address: elisabetta.vegeto@unimi.it (E. Vegeto).
[1] https://orcid.org/0000-0003-4752-4349
[2] https://orcid.org/0000-0002-3151-4210
[3] https://orcid.org/0000-0002-8230-853X
[4] https://orcid.org/0000-0003-3349-6770
[5] https://orcid.org/0000-0001-8701-1316
[6] https://orcid.org/0000-0002-8788-9783
[7] https://orcid.org/0000-0001-5771-5638
[8] https://orcid.org/0000-0002-0247-3819
[9] https://orcid.org/0000-0002-8282-7866
[10] https://orcid.org/0000-0001-5700-5273
[11] https://orcid.org/0000-0003-3077-590X
[12] https://orcid.org/0000-0003-4351-3650

https://doi.org/10.1016/j.biopha.2021.112274

Available online 12 October 2021
0753-3322/© 2021 The Authors. Published by Elsevier Masson SAS. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Altogether, our data unravel a novel molecular mechanism and immune functions for TAM and 4HT, sustaining their repurposing in infective and other estrogen receptors-unrelated pathologies.

1. INTRODUCTION

Sexual dimorphism in innate immunity mainly manifests by lower susceptibility and better outcomes against infections in females, who also display better vaccination responses and transplantation outcomes as well as higher incidence of autoimmune diseases compared to males [12,16,21,44]. Estrogen hormones contribute to the disparity in immunity by regulating immune cells. Estrogens bind to the estrogen receptors (ERs), ERα and ERβ, which are ligand-activated transcription factors that modulate gene transcription by binding to target gene promoter or by interfering with the activity of other transcription factors [24,32]. Estrogens also induce rapid cytoplasmic responses, such as calcium influx and cAMP formation, mediated by estrogen-activated ERs and G protein-coupled estrogen receptor 1 (GPER1), a membrane ER [39]. ER-selective antagonists have been developed to counteract the transcriptional effects of estrogens. These antagonists are defined as selective estrogen receptor modulators (SERMs) for their tissue-selective pharmacological activity; one relevant example is tamoxifen (TAM) which triggers ER-antagonist and agonist responses in the mammary tissue and bone, respectively (-[55]). TAM is widely employed in ERα-positive breast cancers as a precursor drug of the active metabolite, 4-hydroxytamoxifen (4HT), that inhibits cancer cells proliferation through ERα-antagonistic mechanism. In fact, ERs binding affinity of 4HT is similar to the endogenous ligand 17β-estradiol (E2), while TAM affinity is 100-fold lower than E2 [40,6]. Interestingly, TAM is currently used in repurposing strategies as recent clinical evidence proved its efficacy in ER-independent cancers and infections by intracellular pathogens [14,30,33,45,5,56]. In these circumstances, higher doses of TAM are used, reaching plasma and tissue drug concentrations in the micromolar range.

Macrophages are key players in innate immunity and carry out effector and protective functions through the acquisition of distinct phenotypes [31]. The classic inflammatory phenotype (also referred to as M1) is activated by inflammatory cytokines and pathogen-derived signals, such as the bacterial endotoxin lipopolysaccharide (LPS), and results in the expression of effector functions including production of inflammatory mediators, such as TNFα and IL1-β, and reactive molecular species that are pivotal for pathogens and cancer cells killing. On the other hand, the macrophage alternative phenotype (also referred to as M2) is stimulated by Th2 cytokines blunts inflammation and promotes tissue remodeling. These two phenotypes simplistically represent the extremes of a spectrum of intermediate functions acquired by macrophages under the combined influence of different endogenous molecules, including estrogens or xenobiotics [36]. The transcription factor NRF2 has been recently identified as a molecular player involved in macrophage phenotypic conversion. In resting conditions, NRF2 is bound to Keap1 in an inhibitory complex that leads NRF2 to proteasomal degradation, while an oxidative burst induces Keap1 dissociation and NRF2 migration to the nucleus, where it regulates gene expression upon binding to antioxidant responsive elements (ARE) in the promoter regions of NRF2 target genes [15]. During inflammation or infections, NRF2 activation in macrophages increases bacterial clearance by phagocytosis and modulates the expression of inflammatory mediators, in parallel with the production of antioxidant proteins that buffer the reactive oxygen species (ROS) generated by macrophages for pathogen killing [13,22,3,53].

Through ERα-mediated mechanisms, estrogens have been shown to induce cell expansion and phenotypic switch in macrophages, leading to a faster activation and conversion towards an M2-like phenotype [36,37,51]. This immune activity may explain the better performance of females in some physio-pathological conditions, such as vaccination, infections or neurodegenerative pathologies, and may turn detrimental in others, such as tumors or endometriosis, both conditions sustained by the M2 macrophage phenotype [37,48–50]. The clinical use of ER antagonists may thus offer therapeutic benefit or adverse effects, depending on the specific pathogenic role of macrophages and drug efficacy in these cells. Despite the wide use of TAM in estrogen-dependent and off target indications, its activity in immune cells is still poorly defined.

The present study was envisioned to understand the hormonal and immunomodulatory activity of SERMs in macrophages. We found that pharmacological levels of 4HT and, more importantly, TAM (i) induce ERα and GPER1-independent immunomodulatory effects in macrophages, that modify cell polarization through NRF2 activation, (ii) increase phagocytosis and (iii) potentiate the ability to respond to LPS. Our results shed new light on the pharmacological potential and immune regulatory activity of TAM and 4HT, sustaining the use of SERMs in repurposing strategies against infections and other ERα-unrelated pathologies.

2. METHODS

2.1. Animals

Animal care and experimental protocols were approved by the Italian Ministry of Research. Animal studies were conducted according to the EU Directive 2010/63/EU and they are reported in compliance with the ARRIVE 2.0 guidelines [18]. The experiments were designed based on the 3R principles of replacement, refinement and reduction. Animals were allowed to food and water access *ad libitum* and kept in temperature-controlled facilities on a 12-hour light and dark cycle. C57BL/6J mice were supplied by Charles River Laboratories. ARE-luc2 reporter mice were generated by Paolo Ciana, as already reported [41]. ERαKO female mice were obtained from P. Chambon, IGBMC, Strasbourg, France [57]. RosaTdTomato mice (#B6.Cg-Gt(ROSA) 26Sor$^{tm9(CAG\text{-}tdTomato)Hze}$/J, #007909) carrying the ubiquitous expression of the red fluorescent protein Td-Tomato were obtained from The Jackson Lab (The Jackson Laboratory, BarHarbor, Maine, USA); heterozygous mice were used in our study, obtained by crossing RosaTdTomato with C57BL/6J mice. Only female mice of all mice models were used in the present study and sacrificed at 4 months of age by carbon dioxide inhalation.

2.2. Primary cultures of mouse peritoneal macrophages

Peritoneal macrophages were recovered as previously described [36]. Briefly, 5 ml of pre-chilled 0.9% NaCl were injected in the peritoneal cavity using a 21G needle, recovered and centrifuged at 1500 rpm for 8 minutes; cells were incubated with ACK solution (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM EDTA; pH 7.3) for 5 minutes at 4°C and seeded at the concentration of 1 x 10^6 cells/well in 12-wells plate with RPMI (Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with 10% endotoxin-free FBS, 1% penicillin/streptomycin and 1% Na pyruvate. After 45 minutes, cells were intensively washed with PBS and incubated in RPMI w/o phenol red with 10% dextran coated charcoal-FBS. Cell numbers were analyzed by counting viable cells after harvesting with StemPro Accutase (Thermo Fisher Scientific) and staining with Trypan Blue (Sigma-Aldrich, St. Louis, Missouri, USA).

2.3. RNA preparation and real time PCR

Total RNA was purified using Direct-zol RNA Miniprep (Zymo Research, Irvine, California, USA), according to the manufacturer's instructions, including a step with deoxyribonuclease incubation. For real time PCR, RNA (150 ng) was reverse transcribed to cDNA with 8 U/μg RNA of Moloney murine leukemia virus reverse transcriptase (Promega, Milan, Italy) and random hexamer primers in a final volume of 25 μl; the reaction was performed at 37°C for 1 h, and the enzyme inactivated at 75°C for 5 min. Control reactions without the addition of the reverse transcription enzyme were performed (data not shown). Triplicates of 1:4 cDNA dilutions were amplified using GoTaq®qPCR Master Mix technology (Promega) according to the manufacturer's protocol, with QuantStudio®3 real time PCR system (Applied Biosystems, Waltham, Massachusetts, USA) with the following thermal profile: 2 min at 95°C; 40 cycles, 15 sec at 95°C, 1 min at 60°C. Primer sequences are reported in Supplementary Table 1. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method.

2.4. Western Blotting analysis

Cells were lysed using ice-cold lysing buffer (20 mM HEPES pH 7.9, 5 mM $MgCl_2$, 420 mM NaCl, 0.1 mM EDTA, and 20% glycerol) containing protease and phosphatase inhibitors according to the manufacturer's protocols (Phosphatase and Protease Inhibitor Mini Tablets, Pierce). After three repeated cycles of freezing and thawing, cell homogenates were centrifuged at 16,100 g for 15 min at 4 °C. Protein concentration was determined by Bradford assay (Pierce). Equal amounts of cell extracts (20 μg) were loaded with Laemmli sample buffer, boiled for 5 min, run on 7.5%–12% SDS-polyacrylamide gels and then transferred to a nitrocellulose membrane. After incubation with with blocking solution containing 5% (w/v) non-fat milk in Tris-buffered saline membranes were incubated with the specific primary antibodies overnight at 4 °C and then with the appropriate secondary antibody conjugated with peroxidase for 1 h, at RT. Immunoreactivity was detected with a chemiluminescence assay detection system according to the manufacturer's instructions (Amersham™ ECL™ Western Blotting Analysis System, GE Healthcare, Milan, Italy). For semi-quantitative analyses, the densities of the protein bands were measured by densitometric scanning of the membrane with Gel Doc™ XR Imaging Densitometer (Bio-Rad, Hercules, California, USA) and a computer program (Image Lab™ Software, Bio-Rad).

The primary antibodies used in western blotting are listed in Supplementary Table 2.

2.5. Luciferase assay

Cells were lysed with Luciferase Cell Culture Lysis Reagent (Promega); after protein concentration determination (Bradford assay) luciferase quantification was assessed in luciferase assay buffer (470 μM luciferin, 20mM Tricine, 0.1 Mm EDTA, 1.07 Mm $(MgCO_3)_4{\cdot}Mg(OH)_2{\times}5H_2O$; 2.67 mM $MgSO_4{\times}7H_2O$ in H_2O, pH 7.8, with 33.3 mM DTT and 530 μM ATP), by measuring luminescence emission with a Veritas luminometer (Promega). The relative luminescence units (RLU), determined during a measurement of 10 s time and expressed as luciferase units/μg protein, were calculated as compared with blank control samples.

2.6. Enzyme-Linked Immunosorbent Assay (ELISA)

Cell supernatants were centrifuged at 450×g for 5 mins and stored at -20°C until usage. Cytokine concentrations were determined using ELISA Duoset kits for mouse IL-1β, IL-6 and TNF-α according to the manufacturer's protocol (#DY401, #DY406 and #DY410, respectively; R&D System, Minneapolis, Canada). Absorbance at the wavelength of 450 nm was measured using a plate reader (SpectraMax 190; Molecular Devices, San Jose, California, USA) as a correction wavelength of 540 nm. Concentrations were calculated using eight-parameter fit curve.

2.7. Cell viability Assays

Cell viability was measured using the MTT assay according to manufacturer's instruction. Briefly, the MTT solution was added to cells at the final concentration of 1 mg/mL and incubated at 37°C for 2 hours. Then, an equal volume of the extraction buffer (20% (w/v) of SDS was dissolved in 50% DMF – 50% H_20 solution) was added to each well and incubated at 37°C for 20 hours. The absorbance at 595 nm wavelength was measured using a microplate reader (Bio-Rad). The results were expressed as percentage of viability versus vehicle (absorbance sample/absorbance vehicle x 100).

To evaluate cell toxicity, SYTOX Green (#S7020 Invitrogen, Eugene, Oregon, USA) staining or evaluation of LDH release were used. Briefly, 24h after the appropriate treatment cells washed in PBS and incubated with SYTOX Green dye (stock solution 5 mM, dil. 1:500'000) for 20 min at 37°C. 3% DMSO was used as positive control. Cells were fixed in 4% paraformaldehyde (#15710 Electron Microscopy Sciences, Hatfield, Pennsylvania, USA) for 10 min at 37°C. Fluorescence was determined by fluorescence microscopy (ZEISS Axiovert 200M). For the LDH assay, supernatants were collected 6h after treatments, with 100 ng/mL LPS+1 mM ATP used as positive control, and the amount of LDH was determined using CyQUANT LDH Cytotoxicity Assay kit by Thermo Fisher Scientific (#20300) according to the manufacturer's instructions.

2.8. Polymersomes preparation

PMPC−PDPA Copolymers, useful for the assembling of the polymeric vesicles, were kindly provided by Prof. Giuseppe Battaglia and synthetized as described elsewhere [10]. PMPC−PDPA self-assembly of polymersomes was carried out using the thin film rehydration method. In particular, the polymers were first dissolved in a chloroform/methanol solution (2:1), in a 5% molar ratio between Cy5-labeled and unstained PMPC−PDPA polymers. The solvent was then evaporated, and the film was rehydrated with endotoxin/LPS-free Dulbecco's PBS (Sigma-Aldrich) for a period of 4 weeks under vigorous stirring, to have a final polymer concentration of 10 mg/mL. After this period, the formed polymersomes were purified from the formed tubular structures, and only spherical nanoparticles were isolated, according to sucrose-based density gradient centrifugation [42]. These pre-purified samples were then further purified by size exclusion chromatography for isolating the Cy5-labelled nanovesicles and removing any unreacted free fluorescent dye.

2.9. Polymersomes uptake

Peritoneal macrophages from heterozygous RosaTdTomato female mice were seeded at the density of 10^5 cells/well on 8 microwell glass-bottom petri dishes (ibidi). After 24 h, cells were treated for 3 h as specified and then incubated with Cy5-labelled nanoparticles for 1 and 3 hours. After medium discharge, cells were washed 3 times in PBS to remove any excess of nanoparticles and fixed in formaldehyde (4%) for 10 minutes, then washed and analysed by confocal microscopy (ZEISS Axio Observer). Fluorescence intensity of polymersomes inside macrophages was scored by ImageJ. The number of polymersomes-positive cells was counted in 4 fields/well, with an average of 200 total cells counted in each well.

2.10. Statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology [7]. Experimental group size of n = 5 was predetermined based on prior experience in evaluating for statistical significance. One-way ANOVA followed by Bonferroni post hoc test for comparison of multiple independent groups

were used for all statistical analyses with the GraphPad Prism version 8.0 for Windows. Statistical analysis was undertaken only when group size was at least n = 5, where *n* represents the number of independent *in vitro* experiments, not technical replicates. Differences are considered significant at the values of *p<0.05, **p<0.01 and ***p<0.001.

2.11. Materials

TAM (#T5648), 4HT (#H7904), E2 (#E8875) and LPS (from *Escherichia Coli* O111:B4; #L4130) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). TAM, 4HT and E2 were dissolved in EtOH (#20821.321, VWR, Radnor, Pennsylvania, USA) to a stock concentration of 10^{-2} M. LPS was dissolved in sterile H_2O to a stock concentration of 1 mg/mL. G1 (#3577) was purchased from Tocris (Bristol, UK) and dissolved in DMSO (#D8779, Sigma-Aldrich) to a stock concentration of 10^{-2} M. Ly294,002 (#278-038) was obtained from Alexis Biochemicals (Axxora LLC, San Diego, USA). Phosphatase (#A32957) and Protease (#A32953) Inhibitor Mini Tablets, Blue Coomassie Plus Bradford Assay Reagent (#23238), Bovine Serum Albumin Standard 2 mg/mL (#23209) were purchased from Pierce (Waltham, Massachusetts, USA). MTT (Thiazolyl Blue Tetrazolium Bromide, #M5655) was purchased from Sigma-Aldrich. Primary antibodies used in Western blotting are listed in Supplementary Table 2. Secondary HRP-conjugated antibodies used as 1:2000 dilutions were purchased from Vector Laboratories (Burlingame, California, USA), specifically goat anti-rabbit IgG (#PI-1000) for primary antibodies against NRF2, HMOX-1, pAKT and AKT, and HRP horse anti-mouse IgG (#PI-2000,) for Caspase-1 and β-actin.

3. RESULTS

3.1. Immunomodulatory effects of 4HT and TAM in macrophages

Since E2 induces a pro-resolving and anti-inflammatory phenotype in macrophages through ERα-mediated effects on gene expression [36, 37], we asked whether E2 transcriptional activity could be mimicked or antagonized by SERMs. Primary cultures of peritoneal macrophages were used to more faithfully mimic the reactivity and function of resident macrophages. Cells were treated with increasing concentrations of 4HT, ranging from 0.001 to 10 μM, and assayed for the expression of *Vegfα*, a prominent E2-responsive gene in macrophages [36]. In parallel, cells were treated with E2, used as positive control, and TAM, the inactive precursor of 4HT endowed with 100-fold lower binding affinity for ERα as compared with estrogens [6], which indeed reaches micromolar concentrations in target tissues and plasma of patients in various clinical settings. As shown in Fig. 1, dose-dependent positive effects were observed with E2, as expected, while *Vegfa* mRNA levels were significantly increased by 4HT only at 5 μM and further upregulated at 10 μM. Surprisingly, also TAM induced Vegfα expression with positive effects already observed at 2.5 μM and with a 11-fold induction with 10 μM TAM. We excluded that these drug concentrations could be toxic to the cells, as cell viability was not affected (see Supplementary Figure 1). We then extended our observation to other E2-target genes, namely *Il1b* and arginase 1 (*Arg1*), which are also involved in immune polarization [36]. Fig. 1 shows that 4HT and TAM decreased both *Il1b* and *Arg1* mRNA levels, with TAM showing a stronger effect as compared with 4HT. These results corroborate the evidence on the ability of SERMs to modulate immune gene expression. Unexpectedly, the effects of 4HT and TAM were opposite to those observed with E2, which suggests that SERMs, at least at high concentrations, may activate E2-unrelated mechanisms of action.

Altogether, these results demonstrate that 4HT and TAM are immunomodulatory agents, that induce similar or divergent effects compared with E2, depending on their concentration.

3.2. Role of the estrogen receptors in the immunomodulatory effects of 4HT and TAM

In order to better understand the activity of 4HT and TAM, we performed competition experiments with E2, expecting to observe increased hormonal effects in case of agonist activity. Instead, the results of Fig. 2A show that E2 action on *Vegfa* expression was significantly reduced by 4HT when this drug was tested in a 10:1 or higher 4HT/E2 ratio and in combination with low concentrations of E2 (0.001 μM E2 + 0.1 or 1 μM 4HT; 0.1 μM E2 + 1 μM 4HT). When equimolar to 0.1 μM E2, the inhibitory activity of 4HT was lost, in accordance with the higher ERα binding affinity of E2 compared with 4HT [40]. On the other hand, the positive effect observed with 10 μM 4HT was still present and, notably, additive with 10 μM E2, reaching a 13-fold induction as compared with the 5-fold inductions of each individual treatment. We observed analogous antagonistic effects of TAM when tested in a 100-fold excess to E2 (0.001 μM E2 + 1 μM 4HT), as shown in Fig. 2B. Interestingly, TAM and E2 induced additive effects when 10 μM TAM was used in combination with 0.1 and 10 μM E2, resulting in a 15 and 19-fold induction of *Vegfa*, respectively, with a higher potency and efficacy as compared to the 4HT+E2 combination.

In order to study the molecular mechanism underlying the transcriptional activity of high-dose 4HT and TAM, we analyzed the involvement of ERα using macrophages isolated from ERαKO animals.

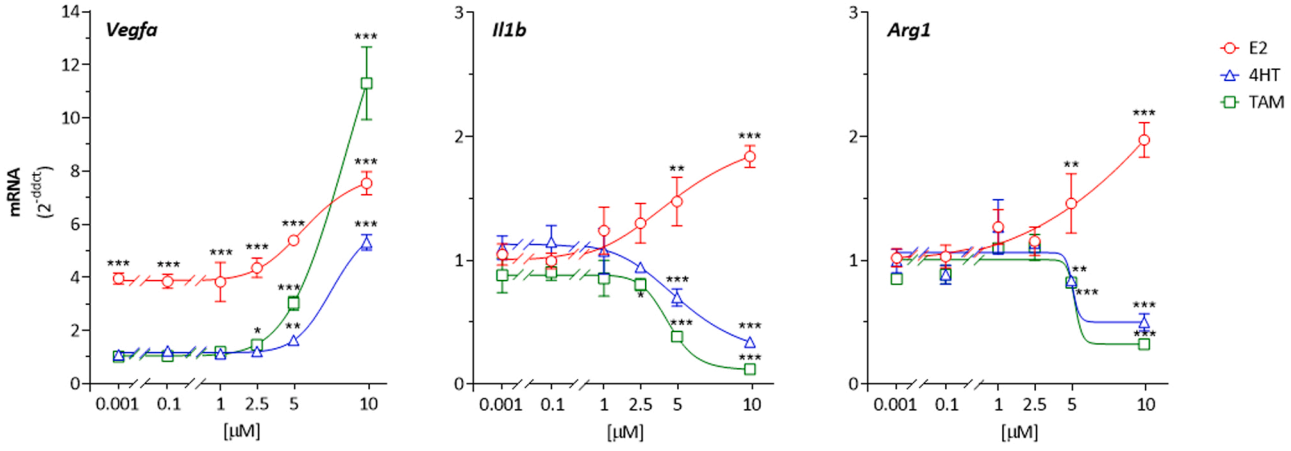

Fig. 1. Dose-dependent effects of 4HT and TAM on immune gene expression in macrophages. *Vegfa, Il1b* and *Arg1* mRNA levels were analyzed by real time PCR in primary cultures of peritoneal macrophages treated for 3h with increasing concentrations of 4OH-Tamoxifen (4HT), Tamoxifen (TAM) or 17β-estradiol (E2), as specified. Data sets were calculated using the 2^{-ddCt} method with respect to the mean value of vehicle-treated cells (=1). Bars represent mean values ± SEM (n=5). One-way ANOVA followed by Bonferroni post hoc test, *p<0.05, **p<0.01, ***p<0.001 *versus* vehicle.

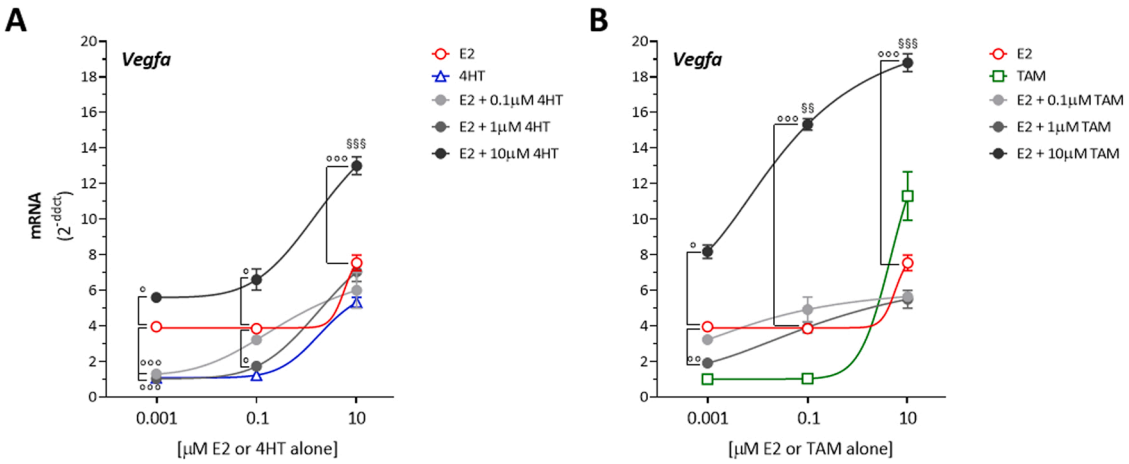

Fig. 2. Estrogen-competition effects of 4HT and TAM on *Vegfa* expression in macrophages. Primary cultures of peritoneal macrophages from WT mice were treated for 3h with increasing concentrations (0.001, 0.1, 1, 10 μM) of E2 and A) 4HT or B) TAM were used alone or in combination, as specified, and analyzed for *Vegfa* mRNA levels by real time PCR. Data sets were calculated using the 2^{-ddCt} method with respect to the mean value of vehicle-treated cells (=1). Bars represent mean values ± SEM (n=5). One-way ANOVA followed by Bonferroni post hoc test, *p<0.05, **p<0.01, ***p<0.001 *versus* vehicle; °p<0.05, °°p<0.01 °°°p<0.001 *versus* E2; §§p<0.01, §§§p<0.001 *versus* 10 μM 4HT or 10 μM TAM.

Despite the lack of this receptor, the immune activity of TAM and 4HT remained unaltered. In fact, data in Fig. 3A show that 10 μM 4HT induced a 6-fold increase in *Vegfa* mRNA in ERαKO macrophages similarly with wild-type cells. As expected, E2 failed to modulate gene transcription in the absence of ERα and, as consequence, also 4HT synergistic effects with E2 could not detected. Superimposable results were obtained with TAM, which induced *Vegfa* mRNA at similar levels and with higher efficacy as 4HT in ERαKO as in wild-type macrophages. We extended these observations to *Il1b* and *Arg1*, which we observed to be modulated by 10 μM E2 (see Fig.1). Using this concentration in competition experiments, the results show that the effects of 4HT and TAM on the expression of these immune genes were not modified by E2 in wild type cells and remained unaltered in ERαKO macrophages, as reported in Fig. 3B.

We previously showed that macrophages also express GPER1, and not ERβ [36]. We therefore used the GPER1 selective ligand, G1, at similar concentrations as those used in the previous experiments to compare the effects of GPER1 activation with those induced by 4HT and TAM on immune gene expression. As shown in Fig. 3C, the highest G1 concentration induced positive effects on *Vegfa* expression, similar to 4HT and TAM, while strong differences were detected on *Il1b* and *Arg1* mRNAs, which were significantly induced by G1 and strongly inhibited by 4HT and TAM. Thus, the different responses induced by the GPER1 ligand suggest that 4HT and TAM act by distinct, GPER1-unrelated mechanisms of action. Moreover, the different transcriptional activity of the ligands did not associate with alterations in estrogen receptor expression, since mRNAs coding for ERα and GPER1 did not vary in response to any treatment used (Supplementary Figure 2A-2B).

Altogether, these results demonstrate that 4HT and TAM when used at low concentrations act as E2-antagonists in macrophages, while higher amounts induce unanticipated ERα and GPER1-independent immunomodulatory responses, that may add on the effects of E2 in a gene-specific manner.

3.3. Role of NRF2 and PI3K/Akt in the immunoregulatory effects of 4HT and TAM

It is known that NRF2 activation regulates the expression of immune genes, increasing *Vegfa* and reducing *Il1b* and *Arg1* mRNAs [34]. We thus asked whether NRF2 was involved in 4HT and TAM activity and used the ARE-luc2 reporter mice, which carry the luciferase gene linked to an ARE-containing promoter [41], to readily acquire evidence for this hypothesis. Notably, 4HT increased the luciferase activity in peritoneal macrophages obtained from ARE-luc2 mice (Fig. 4A), while similar concentrations of E2 were inactive. NRF2 activation generally derives from the reduced proteasomal degradation and consequent increased stability of NRF2. Consistent with this, Western blot analyses showed increased NRF2 protein levels following 4HT treatment, while E2 was again ineffective (Fig. 4B). These effects were not mediated by increased NRF2 expression, as *Nrf2* mRNA levels did not change in response to 4HT (see Supplementary Figure 2C). We then assessed the expression of endogenous NRF2-target genes and observed that 4HT and TAM increased the mRNA levels coding the antioxidant enzyme HMOX1, both in wild-type and ERαKO macrophages (Fig. 4C). Moreover, a strong increase in HMOX1 protein levels was observed following TAM and 4HT treatments (Fig. 4D), providing a biological evidence for NRF2 mediated effects of these drugs. Positive effects were also observed for other NRF2 target genes, namely NAD(P) quinone dehydrogenase 1 and the metabolic enzyme Transaldolase-1 (See Supplementary Figure 2D). These results support the hypothesis that 4HT and TAM induce macrophage responses through the activation of NRF2 and NRF2-mediated regulation of gene expression.

The PI3K/AKT pathway has been associated with induction of NRF2 stability and transcriptional activity. We thus assessed the involvement of this pathway in NRF2 activation by SERMs. We observed that the positive effect of 4HT and TAM on *Hmox1* mRNA was completely abolished when assayed in the presence of the PI3K inhibitor, LY294,002, used at 10 μM concentration for 30 min before SERMs addition (Fig. 4E). The effects on *Vegfa* expression were also significantly decreased. To further sustain the involvement of the PI3K pathway, we evaluated the presence of the phosphorylated form of Akt (pAkt), a downstream mediator of PI3K signaling. Indeed, pAkt was detected shortly after 4HT and TAM treatments (see Fig. 4F). On the other hand, PI3K inhibition did not modify E2 action on *Vegfa* mRNA. Moreover, Akt phosphorylation was not detected in macrophages treated with this hormone, thus supporting the evidence that PI3K and NRF2 activation are specific events induced by 4HT and TAM in macrophages. Altogether, these results demonstrated that the transcriptional response of macrophages to 4HT and TAM is mediated, at least in part, by NRF2 activation and involves the PI3K/Akt signaling pathway.

3.4. Role of TAM in immune and inflammatory responses of macrophages

Beyond ERα-positive mammary cancer, TAM is used at high dosages for off-target indications, such as infections. Thus, we asked whether the immune activity of TAM and 4HT could potentiate the ability of

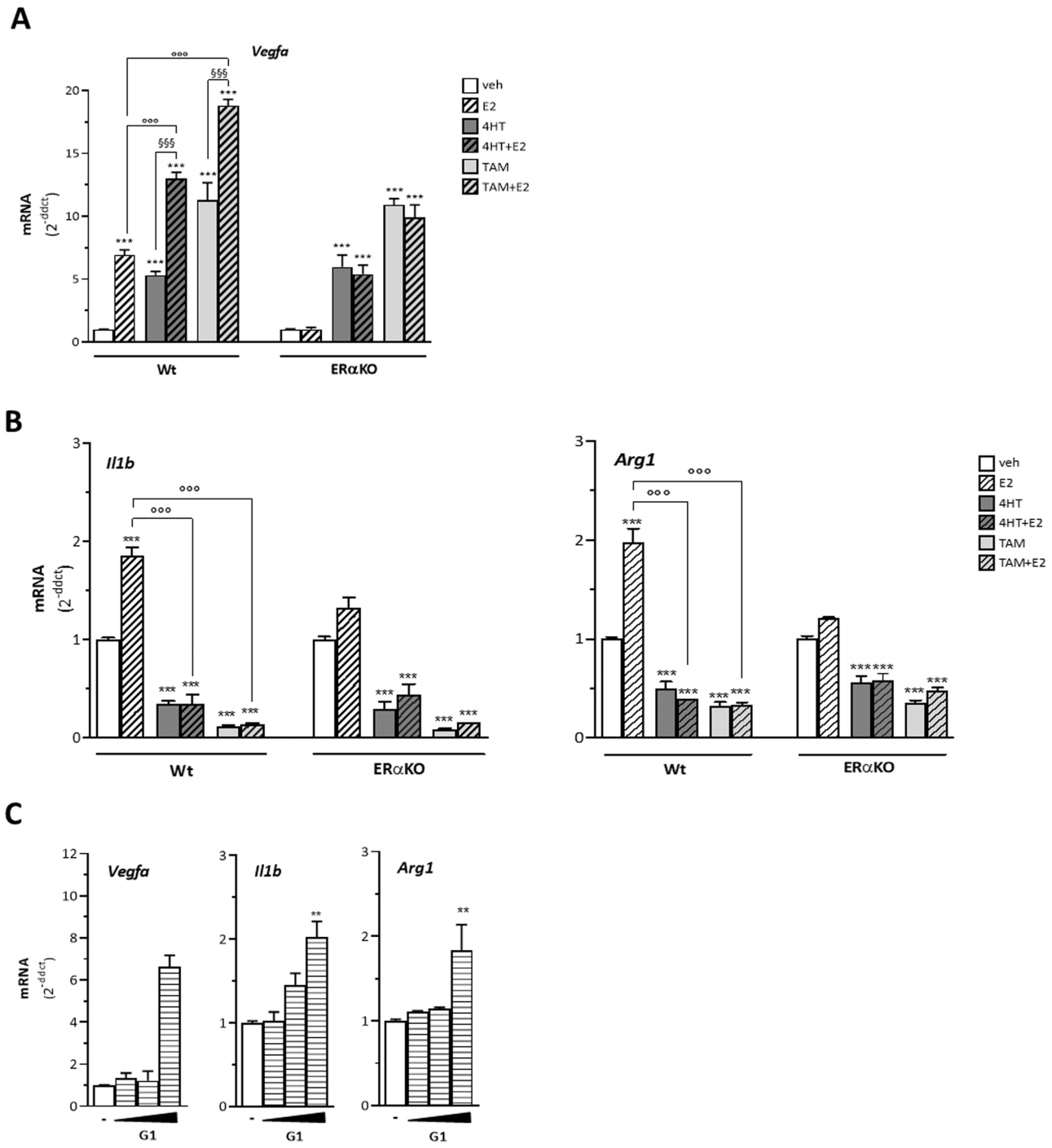

Fig. 3. Role of estrogen receptors in the immunomodulatory activity of 4HT and TAM. A) *Vegfa* or B) *Il1b* and *Arg1* mRNA levels were assayed in WT and ERαKO macrophages treated with 10 μM 4HT, TAM and E2 alone or in combination, as specified. C) Macrophages from WT mice were treated with increasing concentrations (0.001, 0.1, 10 μM) of G1 and analyzed for *Vegfa*, *Il1b* and *Arg1* expression. (A-C) mRNA levels were quantified by real time PCR. Data sets were calculated using the 2^{-ddCt} method with respect to the mean value of vehicle-treated cells (=1). Bars represent mean values ± SEM (n=5). One-way ANOVA followed by Bonferroni post hoc test, **p<0.01, ***p<0.001 *versus* vehicle; °°°p<0.001 *versus* E2; §§§p<0.001 *versus* 4HT or TAM.

macrophages to eliminate pathogens. We first assessed the uptake ability of macrophages, a key step in microbe elimination by these cells. Peritoneal macrophages collected from RosaTdTomato mice were treated with fluorescent polymeric nanoparticles, named polymersomes, that were taken up by cells in a time-dependent manner. 4HT and TAM induced a faster accumulation of nanoparticles and a higher number of cells involved in the phagocytosis process (Fig. 5).

Next, we evaluated whether the inflammatory response of M1 macrophages induced by bacterial invasion could be modulated by 4HT and TAM. We thus analyzed SERMs activity on the expression of the inflammatory mediators, TNFα and IL1β, after short and long-term treatments with LPS. As expected, the bacterial endotoxin increased *Tnfa* and *Il1b* mRNAs, with a more potent effect observed shortly after stimulation (see Fig. 6A). Interestingly, when assayed alone, 4HT induced a 5-fold increase in *Tnfa* mRNA shortly after its addition, while a positive effect could be observed for TAM at the later time point analyzed. Notably, both 4HT and TAM potentiated the LPS effects on *Tnfa* mRNA. At 3 h treatment, a higher activity was observed for 4HT as compared with

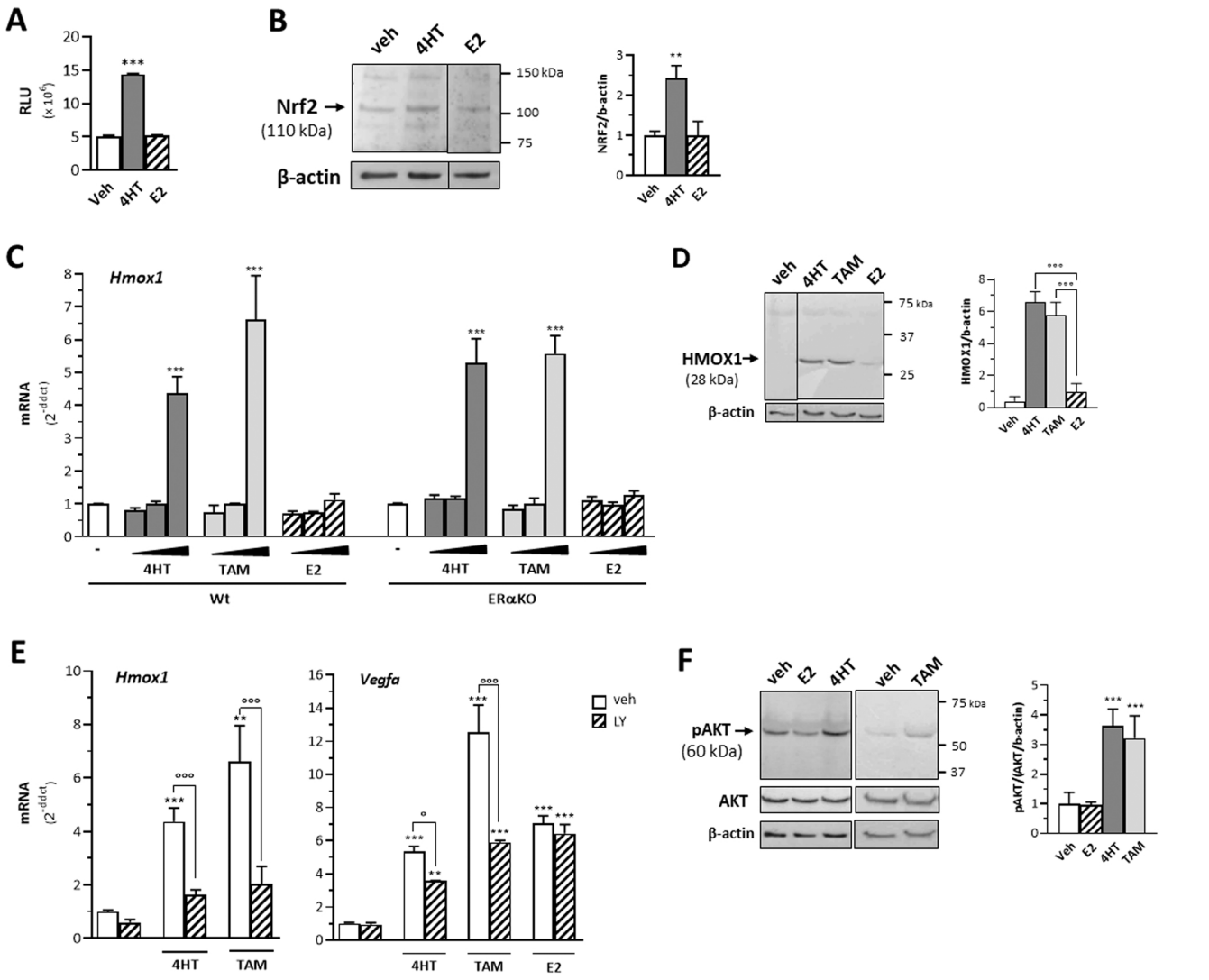

Fig. 4. Role of NRF2 and PI3K/Akt in the immunomodulatory activity of 4HT and TAM. A) Primary cultures of macrophages from ARE-Luc2 mice were treated with vehicle (veh), 10 uM 4HT or 10 uM E2; luciferase activity was assayed and reported as relative luciferase units (RLU). B) Macrophages from WT mice, treated as in A) for 30 min, were analyzed for NRF2 protein expression; a representative Western blot of NRF2 and β-actin proteins is shown. In the right panel, the quantification of band signals was calculated from 3 independent experiments, normalized by the corresponding b-actin signal and reported as fold induction *versus* veh. C and D) Expression of the NRF2-target protein HMOX1 was analyzed in WT and ERαKO macrophages (C) by real time PCR after 3h treatment with 0.001, 0.1 and 10 μM 4HT, TAM and E2 or (D) by Western blot after 16h treatment with 10 μM TAM, 4HT or E2; a representative Western blotting of HMOX1 and β-actin proteins is shown. In the right panel, the quantification of band signals was calculated from 3 independent experiments, normalized by the corresponding b-actin signal and reported as fold induction *versus* E2. E) Macrophages were treated with 10 μM LY294,002 (LY) prior to 10 μM 4HT, TAM or E2 and analyzed for *Hmox1* and *Vegfa* mRNA levels. F) phospho-Akt (pAkt) protein levels was analyzed in WT cells treated for 30 min with 10 μM E2, 4HT and TAM. A representative Western blotting of pAKT, AKT and β-actin is shown; in the right panel, the quantification of band signals was calculated from 3 independent experiments, normalized by the corresponding b-actin signal and reported as fold induction *versus* veh. C-E) Data sets for each gene were calculated using the 2^{-ddCt} method with respect to the mean value of the vehicle. Bars represent mean values ± SEM (n=5). One-way ANOVA followed by Bonferroni post hoc test, **p<0.01, ***p<0.001 versus vehicle; °p<0.05; °°p<0.01, °°°p<0.001.

TAM, which instead is more efficient than 4HT on *Tnfa* mRNA levels after 16 h. On the other hand, the induction of Il1b by LPS is significantly reduced by 4HT and TAM, with similar time-dependent effects of these molecules observed for *Tnfa*. This suggests that 4HT provides faster, while TAM more long-lasting effects in M1 macrophages. In order to substantiate the biological relevance of these results, we analyzed the amounts of these inflammatory cytokines that are secreted by macrophages in response to the above-mentioned signals. As expected, TNFα protein levels were modulated in accordance with the mRNA data, as 4HT and TAM induced a 3 and 2-fold increase, respectively, in the cytokine levels obtained in response to LPS (Fig. 6B). Strikingly, the effect of LPS on IL1β protein levels was significantly increased by SERMs at all time points analyzed, with time-dependent differences in drug efficacy that are similar to what observed above, as 4HT induced a higher response at the short time point analyzed, while TAM provided a long-lasting effect on IL1β induction.

After its biosynthesis, IL1β is secreted by macrophages through the activity of the NLRP3 inflammasome complex and the transformation of pro-caspase-1 into the active caspase-1 enzyme, allowing the active form of IL1β to be produced and secreted by cells. Thus, we performed western blot analyses to assay the formation of active caspase-1 in response to SERMs. In line with this assumption, we observed the appearance of active caspase-1 in macrophages treated with 4HT or TAM. This effect occurred shortly (30 min) after SERMs addition and increased with a longer (6 h) treatment. Gene expression analyses did not show any effect of 4HT or TAM on NLRP3 inflammasome

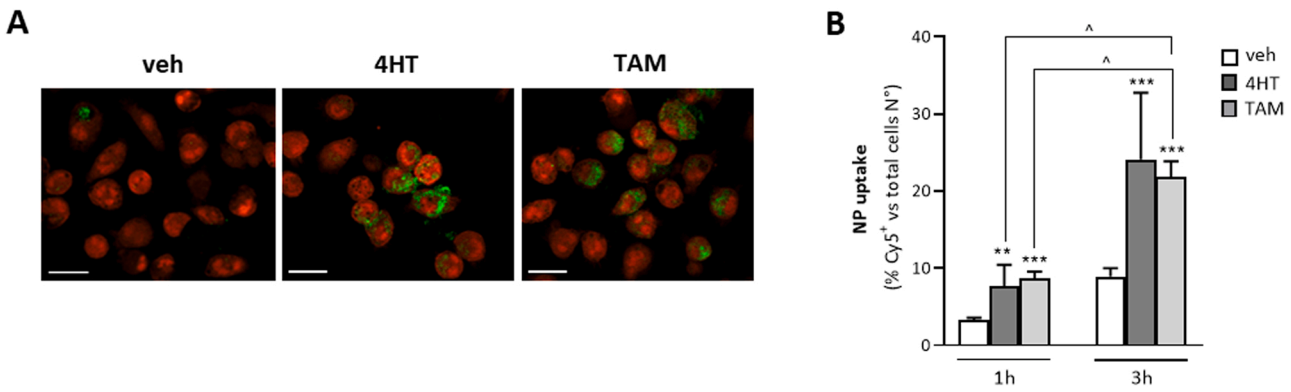

Fig. 5. Nanoparticles uptake by macrophages following 4HT and TAM treatment. Primary cultures of macrophages from RosaTdTomato mice were treated with 10 μM 4HT or TAM for 30 min and then with nanoparticles (NP) labelled with Cy5; following the indicated periods of time, cells were analyzed by confocal microscopy. A) Representative images of cells (red signal) and nanoparticles (green signal) after 3h treatment. Scale bars, 20 μm. B) Fluorescence quantification was obtained by counting the number of green and red (total) cells and represented as %. Bars represent mean values ± SEM (n=5). One-way ANOVA followed by Bonferroni post hoc test, **p<0.01, ***p<0.001 *versus* vehicle; ^p<0,05 *versus* 4HT or TAM 1h.

components (*Nlrp3*, *Pycard* and *Caspase-1*; see Supplementary Figure 3). Since the PI3K/Akt system has been reported to regulate inflammasome activation, and considering our data of Fig. 4 on a role of these enzymes in 4HT and TAM activity, we asked whether caspase-1 activation by 4HT/TAM involved the PI3K/Akt pathway. However, treatment with LY294,002 did not modify the effect of TAM and 4HT (see Fig. 6C), suggesting that PI3K/Akt activation is not required for TAM and 4HT-induced caspase-1 activation. These data show that 4HT and TAM-induced caspase-1 activation may thus potentiate the secretion of LPS-induced IL1β protein expression, helping to explain the increase in IL1β protein levels induced by 4HT or TAM with a concomitant reduction in *Il1b* mRNA. Interestingly, the inflammatory burst induced by the combined SERMs + LPS treatments did not result in cell toxicity, while 4HT or TAM were able to attenuate the proliferative effects of LPS on macrophages (See Supplementary Figure 4).

Taken together, these results demonstrate that pharmacological concentrations of 4HT and TAM are able to alter the immune functions of macrophages and promote an inflammatory phenotype that may counteract pathogen infections.

4. DISCUSSION

TAM is widely prescribed in breast cancer patients due to ERα-mediated anti-proliferative and pro-apoptotic effects on tumor cells. TAM cytotoxic activity is also exploited in off-target indications, such as infections, in accordance with repurposing strategies. We here demonstrate that TAM triggers macrophage immune activation, without inducing macrophage cell death, and potentiates cell responses to inflammatory signals by ERs-independent mechanisms that involve NRF2 and inflammasome activation. These results extend our knowledge on the molecular and biological activity of TAM and indicate the immune system as a pharmacological target for this drug, with relevant therapeutic implications for human diseases, such as cancer and infections, that may benefit from TAM-induced immune activation.

The limited number of reports published so far on TAM activity in inflammatory cells mainly focused on lipid trafficking and outlined ERα-independent effects of high TAM concentrations, mediated by the interference with transcription factors, such as GR, PPARγ, and STAT1 [17,2,26,27,4]. We here extend this knowledge and indicate novel mechanism and activity of TAM in macrophages. In fact, we show that TAM regulates the expression of VEGFα, IL1β and ARG1, that are related to cell immune activation, and increases phagocytosis. Moreover, TAM alters the macrophage response to inflammatory signals, by increasing the effects of LPS on IL1β protein secretion and altering the endotoxin-induced mRNA levels encoding inflammatory mediators, such as TNFα and IL1β. The response to TAM is still detected using ERαKO macrophages and differs from that induced by the GPER1-specific ligand, G1. Altogether, this evidence led us to exclude the involvement of estrogen receptors in the molecular mechanism of TAM action, also considering that ERβ is not expressed in macrophages [37,51]. Conversely, we ascribed TAM transcriptional response in macrophages to the activation of NRF2 by using NRF2-reporter and target gene expression assays. Indeed, classic NRF2 activators act in macrophages by regulating the expression of antioxidant, phagocytic and immune mediators with superimposable results as those here described for TAM, such as the increase in *Vegfa* and reduction in *Il1b* and *Arg1* mRNAs, inhibition of the LPS-induced expression of *Il1b* and increase in LPS-positive effects on *Tnfa* [13,22,3,34,53]. Activation of NRF2 by TAM has been previously described in epithelial cells [11]. Thus, we demonstrate that NRF2 is a key molecular mediator of TAM immunomodulatory activity and suggests NRF2 to be a candidate target for novel therapeutic interventions aimed at regulating macrophage responses and TAM therapeutic efficacy.

Consistent evidence has previously reported that TAM induces cell apoptosis in non-macrophagic cells, such mammary epithelial cells, hepatocytes and retinal cells. This effect has been reconciled with the induction of oxidative stress, formation of active caspase-1 and transcription of NRF2-target genes [2,26,27,4]. Instead, we here show that TAM does not induce cell death in macrophages despite our data indicate oxidative stress as a primary event in TAM activity, as revealed by caspase-1 activation and induction of ARE-driven and NRF2-target gene expression. The reasons for this different outcome are unknown. However, macrophages contain regulatory systems that limit oxidative and inflammasome activation, which are highly activated in macrophages and essential for pathogen killing and inflammation, from damaging macrophages themselves and may also be involved in uncoupling the oxidative and inflammatory responses induced by TAM in macrophages from cell death programs.

Conventional dosages of TAM in breast cancer patients lead to drug concentrations within the mammary gland that are similar to those used in the present study [20]; higher dosages are used for off-target indications, reaching micromolar drug concentrations in patients serum [20]. Our data show that these pharmacological doses of TAM trigger immunomodulatory effects, which may also be potentiated by high E2 concentrations (>1-100nM) that are reached in the breast adipose tissue or in the peritoneal fluid following ovulation [23,28,29]. This leads us to hypothesize that TAM immune activity may contribute to its clinical outcome. Indeed, the antitumor efficacy of TAM is also observed in ERα-negative cancers and appears not to be limited to tumor cells. On the other hand, macrophages are key players in the defense against

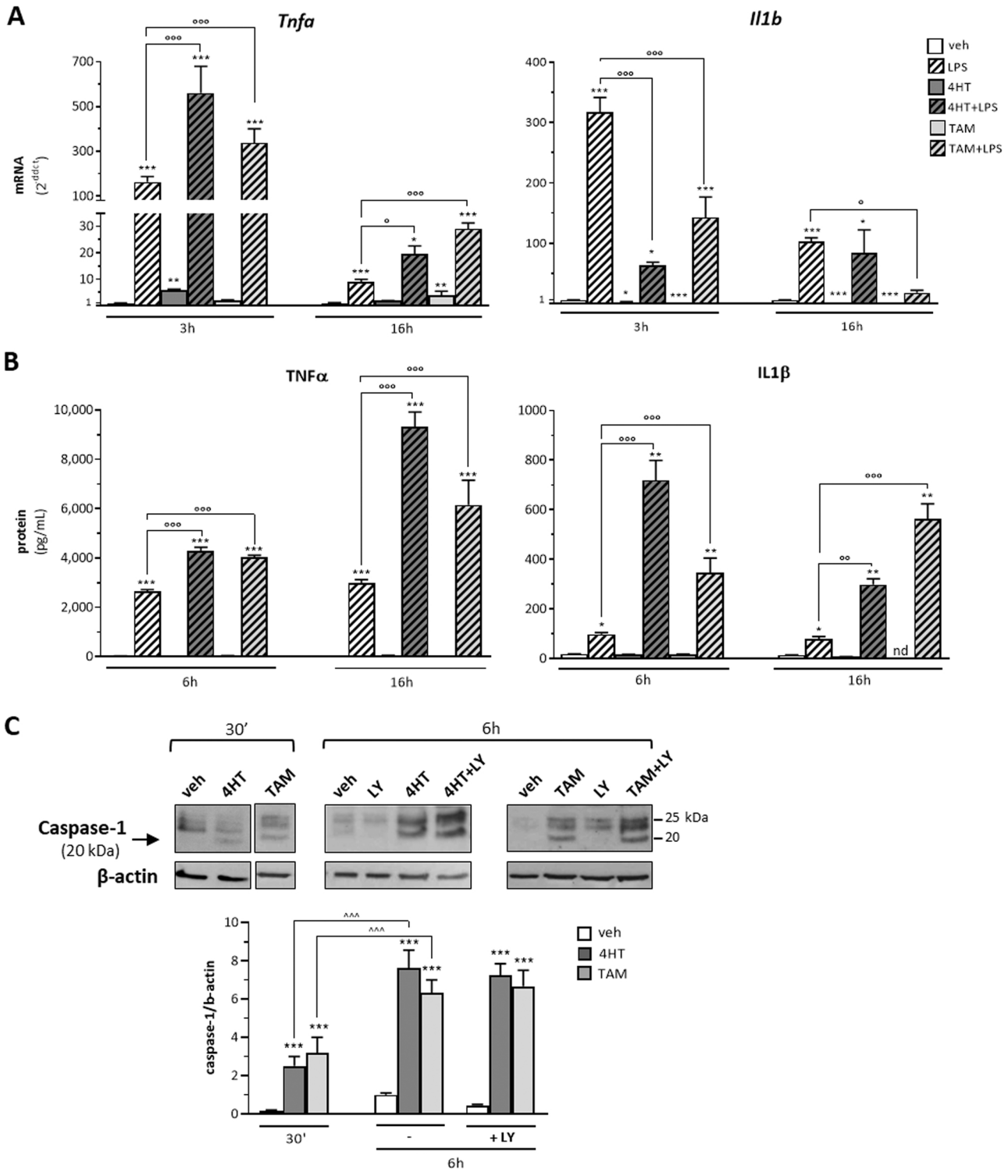

Fig. 6. Immunomodulatory effects of 4HT and TAM on the LPS-induced inflammatory response. A-B) Primary cultures of macrophages were treated with vehicle (veh), 100 ng/ml LPS, 10 μM 4HT or 10 μM TAM, alone or in combination as specified in the legend, and analyzed for TNFα and IL1β expression after the indicated periods of time. A) mRNA levels were evaluated by real time PCR. B) protein levels were determined in cell supernatants by ELISA assay. C) Cells were treated with 10 μM 4HT, 10 μM TAM or 10 μM Ly294,002 (LY), alone or in combination as specified in the legend, and cell lysates analyzed for caspase-1 and β-actin protein levels by Western blotting, after the indicated periods of time. Images are from a single, representative experiment; in the lower panel, the quantification of band signals was calculated from 3 independent experiments, normalized by the corresponding b-actin signal and reported as fold induction *versus* veh 6h. A) Data sets for each gene were calculated using the 2^{-ddCt} method with respect to the mean value of the vehicle 3h. A-B) Bars represent mean values ± SEM (n=5). One-way ANOVA followed by Bonferroni post hoc test, *p<0.05, **p<0.01, ***p<0.001 *versus* vehicle; °p<0.05; °°p<0.01, °°°p<0.001 *versus* LPS; ^^^p<0,001 *versus* 4HT or TAM 30'.

cancer, with M1 macrophages exerting direct cytotoxic effects on tumor cells, while the M2 phenotype is considered pro-tumorigenic. Interestingly, TAM therapy has been shown to revert the enrichment in myeloid-derived suppressor cells in the peripheral blood of breast cancer patients, supporting a contribution of the immune system to drug efficacy [2,25]. From the data shown here, we speculate that the TAM-induced potentiation of the inflammatory response and extracellular material uptake by tumor-associated macrophages may contribute to TAM therapeutic effects, inducing stronger immune responses against tumor cells and a more efficient disposal of apoptotic cancer cells. Nevertheless, long-term TAM therapy eventually leads to the acquisition of drug resistance and disease relapse. Interestingly, TAM resistance has been associated with non-cell autonomous processes that may involve NRF2, as this transcription factor has been implicated in TAM resistance in epithelial cells [19,2,43]. Since some evidence has linked tumor-associated macrophages with TAM resistance in ER+ breast cancer patients [38,8] we speculate that macrophages may cooperate in TAM resistance through the acquisition of a dominant immune phenotype that favors cancer cell survival. Reprogramming events that inhibit or override TAM signaling or the accumulation of selected TAM-induced tumor-promoting factors, such as VEGFα, may have long-lasting detrimental effects on therapeutic outcome that are mainly influenced by neighboring tumor cells and local E2 levels. Finally, the endocrine therapy is also associated with side effects, such as retinopathy, in which the role of macrophages might be relevant, although until now neglected. Interestingly, the oxidative toxicity of TAM, which leads to ERα-independent degeneration of retinal cells, has recently been shown to be counterbalanced by TAM action on retinal microglia, which can rescue retinal cell loss in murine models of photoreceptor degeneration [52]. Thus, the role of inflammatory cells in mediating both the therapeutic as well as adverse effects of TAM in breast cancer needs to be more deeply investigated by future studies.

Due to its chemical scaffold, low cost and safety profile, TAM is a highly challenging molecule for repurposing strategies. At higher than standard anti-estrogen doses, it has been used as ERα-independent, off target therapeutic option for a wide range of immune-dependent pathologic conditions, although its mechanism of action on immunity remains unknown [1,46,9]. One of its major exploitation pertains a broad range of human infections [33,35,45,47,54]. We here suggest that TAM may potentiate the macrophage response to infective agents, particularly in the case of pathogens that persist inside macrophages, and improve the microbicidal activity by activating phagocytosis and potentiating the induction of M1 macrophages. Indeed, TAM has been shown to increase intracellular killing of *Mycobacterium tuberculosis* in macrophages [45] and it has been used with clinical success in association with classic antifungal drugs which, differently from TAM, do not diffuse through the macrophage cell membrane. Pertaining TAM activity in host immune cells, we here demonstrate the activation of distinct mechanisms: regulation of proinflammatory cytokine expression, formation of active caspase-1 and increase in cell uptake of extracellular material. Beyond the specific involvement of PI3K in the transcriptional activity of TAM, it is still not clear whether these are independent or integrated events. Based on previous reports that studied TAM in different cell types, we suggest that the increase in intracellular oxidative species, that are known to induce active capsase-1 formation and NRF2 activation through Keap1 binding, may mediate TAM and 4HT immune responses.

5. CONCLUSIONS

In summary, our study demonstrates that pharmacological concentrations of TAM act in macrophages independently of ERα and are able to skew macrophage polarization induced by inflammatory conditions. These results provide novel hypothesis for TAM pharmacology in breast cancer and other off-target clinical indications and provide molecular targets for future drug development strategies.

Funding

European Union's Framework Program (FP7/2007–2013; grant N° 278850 INMiND, to AM); PRIN 2017 (grant N° #20174T7NXL, to ML) from Ministero Italiano dell'Istruzione Università e Ricerca; LEUP project from Volkswagen Stiftung (to ML); ERC-2019-STG (grant N° 850936, to LR); Fondazione Cariplo (grant N° 2019-4278, to LR).

CRediT authorship contribution statement

G.P, C.S., L.R., G.R., P.C., M.L. and E.V. designed the experiments. G. P., C.S., C.P., E.B. and F.M. performed the experiments; G.P., M.L. and E. V. wrote the manuscript, with contribution from G.R., L.R., C.P., P.C. and A.M.; M.L. and E.V. supervised the project. All authors read and edited the final manuscript.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgements

Authors thank Nicoletta Rizzi, Clara Meda and Monica Rebecchi for technical assistance and Arianna Dolce and Alessia Troilo for experimental support. Authors acknowledge support from the University of Milan through the APC initiative.

Conflict of interest statement

Authors declare no conflict of interest

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2021.112274.

REFERENCES

[1] S. Behjati, M. Frank, The Effects of Tamoxifen on Immunity, Curr. Med. Chem. *16* (2009) 3076–3080.
[2] R.T. Bekele, G. Venkatraman, R.Z. Liu, X. Tang, S. Mi, M.G. Benesch, J.R. Mackey, R. Godbout, J.M. Curtis, T.P. McMullen, D.N. Brindley, Oxidative stress contributes to the tamoxifen-induced killing of breast cancer cells: Implications for tamoxifen therapy and resistance, Sci. Rep. *6* (2016) 21164.
[3] M.A. Bewley, R.C. Budd, E. Ryan, J. Cole, P. Collini, J. Marshall, U. Kolsum, G. Beech, R.D. Emes, I. Tcherniaeva, G. Berbers, S.R. Walmsley, G. Donaldson, J. A. Wedzicha, I. Kilty, W. Rumsey, Y. Sanchez, C.E. Brightling, L.E. Donnelly, P. J. Barnes, D. Singh, M. Whyte, D.H. Dockrell, COPDMAP., Opsonic phagocytosis in chronic obstructive pulmonary disease is enhanced by Nrf2 agonists, Am. J. Respir. Crit. Care Med. *198* (2018) 739–750.
[4] M.L. Bowie, E.C. Dietze, J. Delrow, G.R. Bean, M.M. Troch, R.J. Marjoram, V. L. Seewaldt, Interferon-regulatory factor-1 is critical for tamoxifen-mediated apoptosis in human mammary epithelial cells, Oncogene *23* (2004) 8743–8755.
[5] A. Butts, K. Koselny, Y. Chabrier-Roselló, C.P. Semighini, J.C. Brown, X. Wang, S. Annadurai, L. DiDone, J. Tabroff, Jr Childers WE, M. Abou-Gharbia, M. Wellington, M.E. Cardenas, H.D. Madhani, J. Heitman, D.J. Krysan, Estrogen receptor antagonists are anti-cryptococcal agents that directly bind EF hand proteins and synergize with fluconazole in vivo, MBio *5* (1) (2014) e00765-13.
[6] R. Clarke, M.C. Liu, K.B. Bouker, Z. Gu, R.Y. Lee, Y. Zhu, T.C. Skaar, B. Gomez, K. O'Brien, Y. Wang, L.A. Hilakivi-Clarke, Antiestrogen resistance in breast cancer and the role of estrogen receptor signaling, Oncogene *22* (2003) 7316–7339.
[7] M.J. Curtis, S. Alexander, G. Cirino, J.R. Docherty, C.H. George, M.A. Giembycz, D. Hoyer, P.A. Insel, A.A. Izzo, Y. Ji, D.J. MacEwan, C.G. Sobey, S.C. Stanford, M. M. Teixeira, S. Wonnacott, A. Ahluwalia, Experimental design and analysis and their reporting II: updated and simplified guidance for authors and peer reviewers, Br. J. Pharmacol. *175* (2018) 987–993.
[8] D. Li, H. Ji, X. Niu, L. Yin, Y. Wang, Y. Gu, J. Wang, X. Zhou, H. Zhang, Q. Zhang, Tumor-associated macrophages secrete CC-chemokine ligand 2 and induce tamoxifen resistance by activating PI3K/Akt/mTOR in breast cancer, Cancer Sci. *111* (2020) 47–58.
[9] H. Dellê, J.R.C. Rocha, R.C. Cavaglieri, J.M. Vieira, D.M.A.C. Malheiros, I. L. Noronha, Antifibrotic effect of tamoxifen in a model of progressive renal disease, J. Am. Soc. Nephrol. *23* (2012) 37–48.

[10] F. Fenaroli, J.D. Robertson, E. Scarpa, V.M. Gouveia, C. Di Guglielmo, C. De Pace, P.M. Elks, A. Poma, D. Evangelopoulos, J.O. Canseco, T.K. Prajsnar, H.M. Marriott, D.H. Dockrell, S.J. Foster, T.D. McHugh, S.A. Renshaw, J.S. Martí, G. Battaglia, L. Rizzello, Polymersomes Eradicating Intracellular Bacteria, ACS Nano *14* (2020) 8287–8298.

[11] L. Feng, J. Li, L. Yang, L. Zhu, X. Huang, S. Zhang, L. Luo, Z. Jiang, T. Jiang, W. Xu, X. Wang, H. Jin, Tamoxifen activates Nrf2-dependent SQSTM1 transcription to promote endometrial hyperplasia, Theranostics *7* (2017) 1890–1900.

[12] J. Fischer, N. Jung, N. Robinson, C. Lehmann, Sex differences in immune responses to infectious diseases, Infection *43* (2015) 399–403.

[13] C.J. Harvey, R.K. Thimmulappa, S. Sethi, X. Kong, L. Yarmus, R.H. Brown, et al., Targeting Nrf2 Signaling Improves Bacterial Clearance by Alveolar Macrophages in Patients with COPD and in a Mouse Model HHS Public Access, Sci Transl Med *13* (2011) 78–110.

[14] G. Hasegawa, K. Akatsuka, Y. Nakashima, Y. Yokoe, N. Higo, M. Shimonaka, Tamoxifen inhibits the proliferation of non-melanoma skin cancer cells by increasing intracellular calcium concentration, Int. J. Oncol. *53* (2018) 2157–2166.

[15] K. Itoh, N. Wakabayashi, Y. Katoh, T. Ishii, K. Igarashi, J.D. Engel, M. Yamamoto, Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain, Genes Dev. *13* (1999) 76–86.

[16] S. Jaillon, K. Berthenet, C. Garlanda, Sexual Dimorphism in Innate Immunity, Clin. Rev. Allergy Immunol. *56* (2019) 308–321.

[17] M. Jiang, L. Zhang, X. Ma, W. Hu, Y. Chen, M. Yu, Q. Wang, X. Li, Z. Yin, Y. Zhu, X. Gao, D.P. Hajjar, Y. Duan, J. Han, Tamoxifen inhibits macrophage FABP4 expression through the combined effects of the GR and PPARγ pathways, Biochem. J 454 (2013) 467–477.

[18] C. Kilkenny, W. Browne, I. Cuthill, M. Emerson, D. Altman, Improving bioscience research reporting: The ARRIVE guidelines for reporting animal research, J. Pharmacol. Pharmacother. *1* (2010) 94–99.

[19] S.K. Kim, J.W. Yang, M.R. Kim, S.H. Roh, H.G. Kim, K.Y. Lee, H.G. Jeong, K. W. Kang, Increased expression of Nrf2/ARE-dependent anti-oxidant proteins in tamoxifen-resistant breast cancer cells, Free Radic. Biol. Med. *45* (2008) 537–546.

[20] E.R. Kisanga, J. Gjerde, A. Guerrieri-Gonzaga, F. Pigatto, A. Pesci-Feltri, C. Robertson, D. Serrano, G. Pelosi, A. Decensi, E.A. Lien, Tamoxifen and Metabolite Concentrations in Serum and Breast Cancer Tissue during Three Dose Regimens in a Randomized Preoperative Trial, Clin. Cancer Res. *10* (2004) 2336–2343.

[21] S.L. Klein, K.L. Flanagan, Sex differences in immune responses, Nat. Rev. Immunol. *16* (2016) 626–638.

[22] E.H. Kobayashi, T. Suzuki, R. Funayama, T. Nagashima, M. Hayashi, H. Sekine, N. Tanaka, T. Moriguchi, H. Motohashi, K. Nakayama, M. Yamamoto, Nrf2 suppresses macrophage inflammatory response by blocking proinflammatory cytokine transcription, Nat. Commun. *7* (2016) 11624.

[23] P.R. Koninckx, S.H. Kennedy, D.H. Barlow, Endometriotic disease: The role of peritoneal fluid, In Human Reproduction Update, (Hum Reprod Update) 4 (1998) 741–751.

[24] S. Kovats, Estrogen receptors regulate innate immune cells and signaling pathways, Cell. Immunol. *294* (2015) 63–69.

[25] Larsson, A.M., Roxå, A., Leandersson, K., and Bergenfelz, C. (2019). Impact of systemic therapy on circulating leukocyte populations in patients with metastatic breast cancer. Sci. Rep. *9*:(1):13451.

[26] Y.S. Lee, Y.S. Kang, S.H. Lee, J.A. Kim, Role of NAD(P)H oxidase in the tamoxifen-induced generation of reactive oxygen species and apoptosis in HepG2 human hepatoblastoma cells, Cell Death Differ *7* (2000) 925–932.

[27] L. Liu, P. Zou, L. Zheng, L.E. Linarelli, S. Amarell, A. Passaro, D. Liu, Z. Cheng, Tamoxifen reduces fat mass by boosting reactive oxygen species, Cell Death Dis. *6* (1) (2015), e1586.

[28] P.E. Lønning, B.P. Haynes, A.H. Straume, A. Dunbier, H. Helle, S. Knappskog, M. Dowsett, Exploring breast cancer estrogen disposition: The basis for endocrine manipulation, Clin. Cancer Res. *17* (2011) 4948–4958.

[29] P.E. Lønning, B.P. Haynes, A.H. Straume, A. Dunbier, H. Helle, S. Knappskog, M. Dowsett, Recent data on intratumor estrogens in breast cancer, In Steroids, (Steroids) 76 (2011) 786–791.

[30] G. Ma, J. He, Y. Yu, Y. Xu, X. Yu, J. Martinez, D.M. Lonard, J. Xu, Tamoxifen inhibits ER-negative breast cancer cell invasion and metastasis by accelerating twist1 degradation, Int. J. Biol. Sci. *11* (2015) 618–628.

[31] A. Mantovani, M. Locati, Orchestration of macrophage polarization, Blood *114* (2009) 3135–3136.

[32] D.P. McDonnell, J.D. Norris, Connection and regulation of the human estrogen receptor, Science *296* (80) (2002) 1642–1644.

[33] M.C. Montoya, D.J. Krysan, Repurposing estrogen receptor antagonists for the treatment of infectious disease, MBio *9* (6) (2018) e02272-18.

[34] F. Mornata, G. Pepe, C. Sfogliarini, E. Brunialti, G. Rovati, M. Locati, A. Maggi, E. Vegeto, Reciprocal interference between the NRF2 and LPS signaling pathways on the immune-metabolic phenotype of peritoneal macrophages, Pharmacol. Res. Perspect. *8* (4) (2020), e00638.

[35] M.C. Nicolao, M.C. Elissondo, G.M. Denegri, A.B. Goya, A.C. Cumino, In Vitro and in Vivo effects of tamoxifen against larval stage Echinococcus granulosus, Antimicrob. Agents Chemother. *58* (2014) 5146–5154.

[36] G. Pepe, D. Braga, T.A. Renzi, A. Villa, C. Bolego, F. D'avila, C. Barlassina, A. Maggi, M. Locati, E. Vegeto, Self-renewal and phenotypic conversion are the main physiological responses of macrophages to the endogenous estrogen surge, Sci. Rep. *7* (2017) 44270.

[37] G. Pepe, M. Locati, S. Della Torre, F. Mornata, A. Cignarella, A. Maggi, E. Vegeto, The estrogen-macrophage interplay in the homeostasis of the female reproductive tract, Hum. Reprod. Update *24* (2018) 652–672.

[38] Q. Xuan, J. Wang, A. Nanding, Z. Wang, H. Liu, X. Lian, Q. Zhang, Tumor-associated macrophages are correlated with tamoxifen resistance in the postmenopausal breast cancer patients, Pathol. Oncol. Res. *20* (2014) 619–624.

[39] C.M. Revankar, D.F. Cimino, L.A. Sklar, J.B. Arterburn, E.R. Prossnitz, A transmembrane intracellular estrogen receptor mediates rapid cell signaling, Science *307* (80) (2005) 1625–1630.

[40] R.L. Rich, L.R. Hoth, K.F. Geoghegan, T.A. Brown, P.K. Lemotte, S.P. Simons, P. Hensley, D.G. Myszka, Kinetic analysis of estrogen receptor/ligand interactions, Proc. Natl. Acad. Sci. U. S. A. *99* (2002) 8562–8567.

[41] N. Rizzi, E. Brunialti, S. Cerri, G. Cermisoni, G. Levandis, N. Cesari, A. Maggi, F. Blandini, P. Ciana, In vivo imaging of early signs of dopaminergic neuronal death in an animal model of Parkinson's disease, Neurobiol. Dis. *114* (2018) 74–84.

[42] J.D. Robertson, L. Rizzello, M. Avila-Olias, J. Gaitzsch, C. Contini, M.S. Magoń, S. A. Renshaw, G. Battaglia, Purification of Nanoparticles by Size and Shape, Sci. Rep. *6* (2016) 27494.

[43] V.R. Sanghvi, J. Leibold, M. Mina, G. Ciriello, R.C. Hendrickson, H.-G. W. Correspondence, The Oncogenic Action of NRF2 Depends on De-glycation by Fructosamine-3-Kinase In Brief Fructosamine-3-kinase promotes hepatocellular carcinoma by mediating deglycation of NRF2, a protein modification process previously understudied for cellular proteins, Cell *178* (2019) 807–819.

[44] R. Shepherd, A.S. Cheung, K. Pang, R. Saffery, B. Novakovic, Sexual Dimorphism in Innate Immunity: The Role of Sex Hormones and Epigenetics, Front. Immunol. *11* (2021), 604000.

[45] W.S. Jang, S. Kim, B. Podder, M.A. Jyoti, K.-W. Nam, B.-E. Lee, H.Y. Song, Anti-Mycobacterial Activity of Tamoxifen Against Drug-Resistant and Intra-Macrophage Mycobacterium tuberculosis, J. Microbiol. Biotechnol *25* (2015) 946–950.

[46] A. Vaglio, A. Palmisano, F. Alberici, U. Maggiore, S. Ferretti, R. Cobelli, F. Ferrozzi, D. Corradi, C. Salvarani, C. Buzio, Prednisone versus tamoxifen in patients with idiopathic retroperitoneal fibrosis: An open-label randomised controlled trial, Lancet *378* (2011) 338–346.

[47] J.A. Vargas-Villavicencio, C. Larralde, M.A. León-Nava, De, G. Escobedo, J. Morales-Montor, Tamoxifen treatment induces protection in murine cysticercosis, J. Parasitol. *93* (2007) 1512–1517.

[48] E.R. Vázquez-Martínez, E. García-Gómez, I. Camacho-Arroyo, B. González-Pedrajo, Sexual dimorphism in bacterial infections, Biol. Sex Differ. *9* (1) (2018) 27.

[49] E. Vegeto, S. Cuzzocrea, C. Crisafulli, E. Mazzon, A. Sala, A. Krust, A. Maggi, Estrogen Receptor-as a Drug Target Candidate for Preventing Lung Inflammation, Endocrinology *151* (2010) 174–184.

[50] E. Vegeto, A. Villa, S. Della Torre, V. Crippa, P. Rusmini, R. Cristofani, M. Galbiati, A. Maggi, A. Poletti, The Role of Sex and Sex Hormones in Neurodegenerative Diseases, Endocr. Rev. *41* (2) (2020) 273–319.

[51] A. Villa, N. Rizzi, E. Vegeto, P. Ciana, A. Maggi, Estrogen accelerates the resolution of inflammation in macrophagic cells, Sci. Rep. *5* (2015) 15224.

[52] X. Wang, L. Zhao, Y. Zhang, W. Ma, S.R. Gonzalez, J. Fan, F. Kretschmer, T. C. Badea, H. Qian, W.T. Wong, Tamoxifen provides structural and functional rescue in murine models of photoreceptor degeneration, J. Neurosci. *37* (2017) 3294–3310.

[53] Z. Wang, S. Guo, J. Wang, Y. Shen, J. Zhang, Q. Wu, Nrf2/HO-1 mediates the neuroprotective effect of mangiferin on early brain injury after subarachnoid hemorrhage by attenuating mitochondria-related apoptosis and neuroinflammation, Sci. Rep. *7* (1) (2017) 11883.

[54] A. Weinstock, J. Gallego-Delgado, C. Gomes, J. Sherman, C. Nikain, S. Gonzalez, E. Fisher, A. Rodriguez, Tamoxifen activity against Plasmodium in vitro and in mice, Malar J *18* (2019) 378.

[55] Y. Maximov, P. M. Lee, T., V. Craig Jordan, The Discovery and Development of Selective Estrogen Receptor Modulators (SERMs) for Clinical Practice, Curr. Clin. Pharmacol. *8* (2013) 135–155.

[56] W. Zheng, W. Sun, A. Simeonov, Drug repurposing screens and synergistic drug-combinations for infectious diseases, Br. J. Pharmacol. *175* (2018) 181–191.

[57] Dupont S, Krust A, Gansmuller A, Dierich A, Chambon P, Mark M. Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse reproductive phenotypes. Development. 2000 Oct;127(19):4277-91. PMID: 10976058.