ERα-independent NRF2-mediated immunoregulatory activity of tamoxifen

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Abstract

Background and Purpose: 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. Experimental Approach: Using primary cultures of female mouse macrophages, we analyzed the expression of immune mediators and activation of effectors functions in competition experiments with SERMS and 17β-estradiol (E2) or the bacterial endotoxin LPS. Key results: 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, IL-6 and IL1-β, with an overall increase in cell inflammatory phenotype, further sustained by potentiation of IL1-β secretion through caspase-1 activation. Conclusion and Implications: 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.

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 (Fischer et al., 2015; Jaillon et al., 2019; Klein & Flanagan, 2016; Shepherd et al., 2021). 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 genes promoter or by interfering with the activity of other transcription factors (McDonnell and Norris, 2002; Kovats, 2015). 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 (Revankar et al., 2005). 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 (Y. Maximov et al., 2013). 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 (Rich et al., 2002;·Clarke et al., 2003). Interestingly, TAM is currently used in repurposing strategies as recent clinical evidence proved its efficacy in ER-independent cancers and infections by intracellular pathogens (Butts et al., 2014; Ma et al., 2015; Sik Jang et al., 2015; Hasegawa et al., 2018; Montoya and Krysan, 2018; Zheng et al., 2018). 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 (Mantovani and Locati, 2009). 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 (Pepe et al., 2017). 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 ARE responsive elements in the promoter regions of NRF2 target genes (Itoh et al., 1999). 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 (Harvey et al., 2011; Kobayashi et al., 2016; Wang et al., 2017b; Bewley et al., 2018).

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 (Villa et al., 2015; Pepe et al., 2017, 2018). 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 (Vegeto et al., 2010, 2020; Pepe et al., 2018; Vázquez-Martínez et al., 2018). 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.

METHODS

Animals

Animal care and experimental protocols were approved by the Italian Ministry of Research. Animal studies are reported in compliance with the ARRIVE 2.0 guidelines (Kilkenny et al., 2010) and with the recommendations made by the *British Journal of Pharmacology*(Lilley et al., 2020). 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 (Rizzi et al., 2018). ERαKO female mice were obtained from P. Chambon, IGBMC, Strasbourg, France. RosaTdTomato mice (#B6.Cg-Gt(ROSA)26Sor$^{tm9(CAG-tdTomato)Hze}$/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.

Primary cultures of mouse peritoneal macrophages

Peritoneal macrophages were recovered as previously described (Pepe et al., 2017). 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).

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 $2^{-\Delta\Delta^{\wedge}\tau}$ method.

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 semiquantitative 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.

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.

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.

MTT Assay

Cell viability was measured using 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).

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 (Fenaroli et al., 2020). 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 (Robertson et al., 2016). 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.

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.

Statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2018). 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. The post hoc test was run only if F value achieved $P < 0.05$ and there was no significant variance in homogeneity. A P value less than 0.05 was considered as statistically significant.

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.

RESULTS

Immunomodulatory effects of 4HT in macrophages.

Since E2 induces a pro-resolving and anti-inflammatory phenotype in macrophages through ERα-mediated effects on gene expression (Pepe et al., 2017, 2018), we asked whether E2 transcriptional activity could be mimicked or antagonized by 4HT. Primary cultures of peritoneal macrophages from female mice, chosen as experimental model to more faithfully mimic the reactivity and function of resident macrophages, were treated with increasing 4HT concentrations and assayed for the mRNA levels of *ĕγφα* , a prominent E2-responsive gene in macrophages (Pepe et al., 2017). Only 10 μM 4HT elicited *ĕγφα* expression, while dose-dependent positive effects were observed with E2, as expected (Figure 1A). We excluded that high drug concentrations could be toxic to the cells, as cell viability was not affected (see Supplementary Figure 1). These results suggest that 4HT may behave as a mild agonist, with estrogen-like effects emerging only at the highest concentrations. We therefore tested the combined activity of 4HT and E2, expecting an increase of hormonal effects in case of 4HT agonist activity. Unexpectedly, the positive effects triggered by E2 were significantly reduced by 4HT (Figure 1B), when this drug was tested in a 10:1 or higher 4HT/E2 ratio and in combinations with 0.001 and 0.1 μM 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 (Rich et al., 2002). On the other hand, the positive effects observed with 10 μM 4HT were still present when assayed with E_2 and, notably, were additive with 10 μM E2, reaching a 14-fold induction as compared with the 6-fold inductions of each individual treatment (Figure 1B). Altogether, these results demonstrate that 4HT acts as an ERα-antagonist in macrophages when used in the nanomolar concentrations range, while higher amounts act by different mechanisms to induce unanticipated immunomodulatory macrophage responses, which can be added on the effects of high estrogenic levels.

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To study the molecular mechanism of high-dose 4HT responses, we analyzed the involvement of ERα in 4HT transcriptional activity using macrophages isolated from ERαKO animals. 10 μM 4HT induced a 6-fold increase in*ĕγφα* mRNA in ERα-KO macrophages (Figure 2), similarly with wild-type cells, while smaller concentrations of 4HT did not induce any effect (data not shown). As expected, E2 did not trigger transcriptional responses in the absence of ERα and, as consequence, also 4HT interference and synergistic effects with E2 were not detected. These data strongly indicated that the transcriptional activity of high 4HT levels is ERα-independent.

Immunomodulatory effects of TAM in macrophages

TAM has a 100-fold lower binding affinity for ERα as compared with estrogens (Clarke et al., 2003) and may

be administered in specific clinical conditions at dosages that lead to micromolar concentrations of TAM in plasma and breast target tissue. Based on the results of Figures 1 and 2, we explored whether TAM elicits ERα-independent, immunoregulatory effects similar to 4HT. 10 μM TAM induced a 7-fold induction of Vegfα expression (Figure 3A), while lower concentrations were ineffective. Moreover, TAM antagonized E2 action when tested in a 100-fold excess (0.001 μM E2 + 1 μM 4HT). Importantly, additive effects were observed when 10 μM TAM was used in combination with 0.1 and 10 μM E2. TAM activity is not mediated by ERα, as it is still observed in ERαKO macrophages, where the additive effects to E2 were also not observed (see Figure 3B). Cell viability assays did not provide evidence for cytotoxic effects of TAM at any concentration used (See Supplementary Figure 1). These data show that pharmacological concentrations of TAM exert immune-regulatory effects through off-target, ERα-independent mechanism of action.

GPER1-unrelated immune activity of 4HT and TAM

Macrophages express GPER1 and not ERβ (Pepe et al., 2017). We therefore evaluated the involvement of GPER1 in 4HT and TAM activity. The expression of Vegfα and other E2-target genes (i.e. IL1β and arginase 1 (Arg1), was analyzed upon treatment with increasing concentrations of GPER1 selective ligand G1, which we analyzed in parallel with 4HT, TAM and E2. While the highest G1 level provided similar effects on Vegfα expression, strong differences were detected on IL1b and Arg1, as the expression of these genes was strongly inhibited by 4HT and TAM, but significantly induced by G1 and E2 (Figure 2). Such differences were not due to ligand-induced alterations in receptor expression, as ERα and GPER1 mRNAs did not vary in response to any treatment used (Supplementary Figure 2A). Altogether, these and previous results show that high concentrations of 4HT and TAM elicit macrophage responses that are ERα and GPER1-independent and distinct from those induced by the physiological ligand, E2.

Role of NRF2 and PI3K/Akt in the immunoregulatory response of macrophages to 4HT and TAM

It is known that xenobiotics activate NRF2 and regulate immune gene transcription, including a reduction of IL1β mRNA. 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 AREs-containing promoter (Rizzi et al., 2018), to readily acquire evidence for this hypothesis. Notably, 4HT increased the luciferase activity in peritoneal macrophages obtained from ARE-luc2 mice (Figure 5A), 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 (Figure 5B). 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 (Figure 3C). Moreover, a strong increase in Hmox-1 protein levels was observed following TAM and 4HT treatments, providing a biological evidence for NRF2 mediated effects of these drugs. Positive effects were also observed for other NRF2 target genes, namely NADP dehydrogenase quinone 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 (Figure 5E). The effects on VEGFα 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 Figure 5F). On the other hand, PI3K inhibition did not modify E2 action on VEGFα 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.

Role of TAM in immune functions 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 could alter cell uptake ability, a key step in microbe elimination by macrophages. 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. TAM induced a faster accumulation of nanoparticles and a higher number of cells involved in the phagocytosis process (Figure 6). As expected, similar results are obtained when cells are treated with 4HT.

Next, we evaluated whether TAM regulates the immune responses of macrophages induced by microbial signals, such as LPS. This bacterial endotoxin increased the mRNA levels coding inflammatory proteins, such as TNFα, IL6 and IL1β, with a more potent effect observed shortly after stimulation (Figure 7A). Notably, TAM increased both the short and long-term LPS effects on TNFα mRNA. Interestingly, the long-term treatment with TAM alone induced a 5-fold increase in this cytokine mRNA. On the other hand, the induction of IL1β by LPS is significantly reduced by TAM at all time points analyzed, with a reduced expression of this cytokine when TAM was assayed alone. On the other hand, induction of IL6 by LPS is affected by TAM only shortly after treatment. To substantiate the biological relevance of these results, we analyzed the amount of these inflammatory cytokines that is secreted by macrophages in response to the above-mentioned signals. As expected, TNFα and IL6 proteins are modulated by TAM and LPS in accordance with the effects on the mRNA (Figure 7B). Strikingly, IL1β levels are 8-fold higher when TAM is combined with LPS at both time points analyzed, in contrast to what observed for IL1β mRNA. Superimposable immunomodulatory effects and increased IL1β protein levels were obtained with 4HT (see Supplementary Figure 3). After its biosynthesis, IL1β is secreted by macrophages through the activity of the NLRP3 inflammasome complex and the transformation of pro-caspase1 into the active caspase-1 enzyme, allowing the active form of IL1β to be produced and secreted by cells. Gene expression analyses did not show any effect of TAM on NLRP3 inflammasome components (Nlrp3, Pycard and Caspase-1, see Supplementary Figure 4). Interestingly, the results of Figure 7C showed the appearance of active caspase-1 in macrophages treated with TAM, as well as 4HT, at all the time points tested. These results help explaining the increase in IL1β protein levels following TAM+LPS treatment, despite the reduction in mRNA levels, triggered by a dual mechanism of TAM-induced caspase-1 activation and LPS-increased cytokine expression. This inflammatory burst induced by TAM+LPS treatment did not result in cell toxicity, while TAM was able to attenuate the proliferative effects of LPS on macrophages (See Supplementary Figure 5). Since the PI3K/Akt pathway has been reported to regulate inflammasome activation, we asked whether its induction by TAM may also be involved in caspase-1 activation. However, the presence of LY294,002 did not modify the effect of TAM and 4HT (see Figure 7C), suggesting that PI3K/Akt activation is not required for TAM and 4HT-induced caspase-1 activation.

Taken together, these results demonstrate that pharmacological concentrations of TAM are able to alter the immune functions of macrophages and their phenotypic activation induced by inflammatory conditions, by promoting a TAM-specific macrophage phenotype that may counteract pathogen infections.

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 (Lee et al., 2000; Bowie et al., 2004; Jiang et al., 2013; Liu et al., 2015; Bekele et al., 2016). 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 IL1b protein secretion and altering the endotoxin-induced mRNA levels encoding inflammatory mediators, such as TNFα, IL1β and IL6. 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 (Villa et al., 2015; Pepe et al., 2018). 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 induce antioxidant, phagocytic and inflammatory responses that are similar to those here described for TAM in macrophages, such as the inhibition of the LPS-induced expression of IL1β and IL6 and increase in LPS-positive effects on TNFα (Harvey et al., 2011; Kobayashi et al., 2016; Wang et al., 2017b; Bewley et al., 2018; Mornata et al., 2020). Activation of NRF2 by TAM has been previously described in epithelial cells (Feng et al., 2017). 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 (Lee et al., 2000; Bowie et al., 2004; Liu et al., 2015; Bekele et al., 2016).

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 from damaging macrophages themselves, although these processes are highly activated in macrophages and are essential for killing pathogens and activating inflammation. These protective systems may also be involved in the observed macrophage-specific effects of TAM, uncoupling the oxidative and inflammatory responses induced by this drug 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 (Kisanga et al., 2004); higher dosages are used for off-target indications, supposedly reaching micromolar drug concentrations in patients serum (Kisanga et al., 2004). Our data show that these pharmacological doses of TAM triggers immunomodulatory effects, which may also be potentiated by high E2 concentrations (>1-100nM) that are reached in the peritoneal fluid following ovulation in the breast adipose tissue (Koninckx et al., 1998; Lønning et al., 2011b, 2011a). This leads us to hypothesize that TAM immune activity may contribute to its clinical outcome. Indeed, TAM antitumor efficacy 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 cancer and TAM use in oncology is associated with modifications in immune cell composition (Bekele et al., 2016; Larsson et al., 2019). Thus, existing evidence support a possible contribution of inflammatory cells in TAM efficacy in breast cancer. From the data shown here, we speculate that the TAM-induced potentiation of the inflammatory response and increase in cellular uptake induce a more efficient disposal of apoptotic cancer cells. Moreover, long-term TAM therapy is associated with the acquisition of drug resistance, that eventually leads to disease relapse and the appearance of side effects, such as retinopathy. Interestingly, TAM resistance has been associated with non-cell autonomous processes that may involve NRF2, as this transcription factor is implicated in

chemotherapeutics and TAM resistance in epithelial cells (Kim et al., 2008; Bekele et al., 2016; Sanghvi et al., 2019). On the other hand, 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 (Wang et al., 2017a). Thus, the role of inflammatory cells in mediating both the therapeutic as well as adverse effects of TAM 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 (Behjati and Frank, 2009; Vaglio et al., 2011; Dellê et al., 2012). One of its major exploitation pertains a broad range of human infections (Vargas-Villavicencio et al., 2007; Nicolao et al., 2014; Sik Jang et al., 2015; Montoya and Krysan, 2018; Weinstock et al., 2019). We here suggest that TAM may potentiate the macrophage response to infective agents and improve microbicidal activity by activating phagocytosis and modulating macrophage phenotypic activation, particularly against pathogens persisting within macrophages. Indeed, TAM has been shown to increase intracellular killing of*Mycobacterium tuberculosis* in macrophages (Sik Jang et al., 2015) 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.

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.

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