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The immune activity of selective estrogen receptor modulators is gene and macrophage subtype-specific yet converges on *Il1b* downregulation

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ABSTRACT

Raloxifene belongs to the family of Selective Estrogen Receptor Modulators (SERMs), which are drugs widely prescribed for Estrogen Receptor alpha (ERα)-related pathologies. Recently, SERMs are being tested in repurposing strategies for ERα-independent clinical indications, including a wide range of microbial infections. Macrophages are central in the fight against pathogen invasion. Despite estrogens have been shown to regulate macrophage phenotype, SERMs activity in these cells is still poorly defined. We investigated the activity of Raloxifene in comparison with another widely used SERM, Tamoxifen, on immune gene expression in macrophages obtained from mouse and human tissues, including mouse peritoneal macrophages, bone marrow-derived macrophages, microglia or human blood-derived macrophages, assaying for the involvement of the ERα, PI3K and NRF2 pathways also under inflammatory conditions. Our data demonstrate that Raloxifene acts by a dual mechanism, which entails ERα antagonism and off-target mediators. Moreover, micromolar concentrations of Raloxifene increase the expression of immune metabolic genes, such as *Vegfa* and *Hmox1*, through PI3K and NRF2 activation selectively in peritoneal macrophages. Conversely, *Il1b* mRNA down-regulation by SERMs is consistently observed in all macrophage subtypes and unrelated to the PI3K/NRF2 system. Importantly, the production of the inflammatory cytokine TNFα induced by the bacterial endotoxin, LPS, is potentiated by SERMs and paralleled by the cell subtype-specific increase in IL1β secretion. This work extends our knowledge on the biological and molecular mechanisms of SERMs immune activity and indicate macrophages as a pharmacological target for the exploitation of the antimicrobial potential of these drugs.

1. Introduction

Selective Estrogen Receptors Modulators (SERMs), such as Raloxifene (RAL) and Tamoxifen (TAM), are well-known regulators of the Estrogen Receptors-α (ERα) and ERβ [3]. Upon binding to endogenous estrogen hormones, ERs undergo conformational changes that allow receptor binding to specific DNA sequences within gene promoters and interaction with nuclear co-regulators, leading to the transcriptional control of ER-target genes. Importantly, SERMs binding to ERs triggers tissue-specific agonist or antagonist effects depending on the availability of ER co-regulators [43]. For instance, RAL displays estrogen-like activities in osteoclasts and antagonist effects in epithelial cells of the endometrium. Based on this mechanistic hypothesis, RAL is prescribed to postmenopausal women for the therapy of osteoporosis without the

Abbreviations: Akt, protein kinase B; *Hmox1*/HMOX1, heme oxigenase-1; LPS, lipopolysaccharide; *Nrf2*/NRF2, Nuclear factor erythroid 2-related factor 2; PI3K, phosphoinositide 3-kinase, *Tnfa*/TNFα, tumor necrosis factors-α; *Il1b*/IL1β, interleukin 1 β; *Vegfa*, vascular endothelial growth factor-α.

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risk of endometrial neoplasia [66].

More recently, compelling evidence also showed ERα-independent biological responses activated by high SERMs concentrations. Although still poorly defined, such "off-target" effects have been reconciled with the activation of a list of intracellular mediators, including transcription factors, such as NRF2, and enzymes linked with cholesterol and lipid metabolism [58]. It is important to recall that, in clinical settings, micromolar SERMs levels are indeed reached in patient blood and tissues when these drugs are used in chronic therapies of ERα-positive breast cancer as well as in acute administration in ERα-unrelated conditions, such as ERα-negative tumors, fibrosis and infections [34,50,52,60].

Drug repurposing strategies are pharmaceutical initiatives that make use of drugs that are already available on the market to treat diseases that differ from the medical indications for which the drugs had been originally approved [56]. Such strategies are particularly helpful against infections, in order to meet the urgent need for novel therapeutics that overcome multidrug resistance and limit the expansion of emerging pathogens, while avoiding the expensive and long-lasting processes of research and development of novel compounds [24]. SERMs are currently being assayed as a novel strategy to fight against microbial species, with encouraging results [48]. Although the wide spectrum of anti-infective activity hint to a general, host-mediated mechanism of action of SERMs that involves immune cells, beyond pathogen-specific targets, yet the mechanism underlying drug anti-infective activity is still not defined.

Macrophages are immune cells that play a primary role in the innate protection against infections. Under the influence of physio-pathological signals, macrophages acquire distinct immune phenotypes that allow these cells to activate an inflammatory response and eliminate invading pathogens. Viral and bacterial signals induce a classic proinflammatory phenotype, named M1, characterized by the production of antimicrobial and immune molecules, such as the inflammatory cytokine IL1β. On the other hand, macrophages may activate an alternative, M2 phenotype characterized by the release of anti-inflammatory signals and growth factors, that dampen inflammation and promote tissue repair [39]. These two phenotypes simplistically represent the extremes of a spectrum of intermediate functions acquired by macrophages under the combined influence of endogenous molecules and xenobiotics occurring in vivo.

Preclinical data demonstrated that TAM is able to poise macrophages towards an inflammatory phenotype through ERα-independent mechanisms that involve the NRF2 signaling pathway as well as alteration in lipid and calcium homeostasis at the endo-lysosomal membranes [55,58]. Also, RAL activity on cell membrane lipids has been shown to limit the infections driven by enveloped viruses, including SARS-CoV-2 infections. RAL immune activity may thus have a key impact against COVID-19 [23]. These preliminary observations suggest that macrophages are indeed target cells for SERMs anti-infective activity and may thus express molecular targets endowed with high pharmacological potential and translational value. However, the molecular and biological details of SERMs activity have been scarcely investigated in these cells [58].

The present study was aimed at further increasing our understanding of SERMs activity in macrophages obtained from different tissues. We found that RAL induces ERα-unrelated regulation of immune gene expression similarly to TAM, with gene-specific variations in the potency of these two drugs that depends on the tissue of origin of the cells analyzed. Importantly, both SERMs induce a significant down-regulation of *Il1b* mRNA in different subtypes of macrophages, hinting to a conserved mechanism of SERMs immune activity, which also modifies cell responsiveness to inflammatory stimuli.

2. Methods

2.1. Materials

RAL (#R1402), TAM (#T5648), E2 (#E8875), tBHQ (#112941) and LPS (from Escherichia coli O111:B4; #L4130) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). RAL, TAM and E2 were dissolved in EtOH (#20821.321, VWR, Radnor, Pennsylvania, USA) to a stock concentration of 10^{-2} M. tBHQ was dissolved in DMSO/H_2O 1:5 v/v to a stock concentration of 50 mM. LPS was dissolved in sterile H_2O to a stock concentration of 1 mg/mL. Ly294,002 (#278–038) was obtained from Alexis Biochemicals (Axxora LLC, San Diego, USA) and dissolved in DMSO to a stock concentration of 50 mM. 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 1. 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.

2.2. 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 [33]. The experiments were designed based on the 3 R 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/6 J mice were supplied by Charles River Laboratories. ERαKO female mice were obtained from P. Chambon, IGBMC, Strasbourg, France [16]. Only female mice were used in the present study and sacrificed at 4 months of age through carbon dioxide inhalation.

2.3. Primary cell cultures and cell lines

Peritoneal macrophages. Mouse peritoneal macrophages (mPM) were recovered as previously described [53]. Briefly, 5 mL of pre-chilled 0.9% NaCl were injected in the peritoneal cavity using a 21 G needle, recovered and centrifuged at 1500 rpm for 8 min; cells were incubated with ACK solution (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM EDTA; pH 7.3) for 5 min at 4 °C and seeded at the concentration of 1×10^6 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 min, cells were intensively washed with PBS and incubated in RPMI w/o phenol red with 10% dextran coated charcoal-FBS (DCC).

Bone-marrow derived macrophages. Primary cultures of mouse bone-marrow-derived macrophages (mBMDM) were prepared according to previous work [42,53]. Briefly, the bone marrow from tibia and femur was flushed with DMEM + GlutaMAX (Life Technology-Invitrogen) using a 21-gauge needle. Cells were centrifuged at 1200 rpm for 5 min at 10 °C, seeded in flask cell culture T75 in DMEM + GlutaMAX supplemented with 10% endotoxin-free FBS, 1% penicillin/streptomycin and 1% Na pyruvate and incubated o/n. On the next day, the supernatant was collected, seeded at the concentration of 3×10^6 cells/dish and grown for 6 days in DMEM + GlutaMAX containing 20% endotoxin-free FBS, 30% L929-cell conditioned media, 1% penicillin and streptomycin, and 1% Na pyruvate. After 6 days BMDMs were harvested with Accutase (Merck-Millipore) and plated in 12-well plate at the concentration of 4.5×10^5 cells/well. After 45 min, cells were intensively washed with PBS

and incubated in RPMI w/o phenol red with 10% DCC.

Microglia. Primary cultures of mouse microglia (mMG) were prepared from 2-dayold newborn mice as previously described [63]. After meninges removal, brains were mechanically dissociated and digested in a solution of 2.5% trypsin (Sigma-Aldrich) and 1% DNAse (Sigma-Aldrich), filtered through a 100-μm cell strainer, and seeded at the confluence of 5×10^6 in a 75-cm^2 flask in minimum essential Eagle's medium (MEM) supplemented with 10% FBS, 0.6% glucose, 1% penicillin and streptomycin, and 1% L-glutammine (MEM + 10% FBS). Glial cells were grown at 37 °C under a humidified 5% CO2 and 95% air atmosphere, and medium was replaced every 3 days. After 14 days, microglia were obtained by shaking the confluent monolayer of mixed glial cells at 260 rpm for 1.30 h and seeded in 24-well plates at the confluence of 3×10^5 cells/well. The medium was changed with RPMI w/o phenol red supplemented with 10% DCC 45 min after microglia plating in order to remove contaminating cells.

BV2 cell line. BV2 mouse microglial cell line (from Elisabetta Blasi, University of Perugia) was cultured in DMEM + GlutaMAX, supplemented with 10% endotoxin-free FBS, 1% penicillin/streptomycin and 1% Na pyruvate. The cells were maintained at 37 °C in a 5% CO2 incubator. BV2 cells were seeded at 5×10^5 per well in a 12-well plate. After 5 h, cells were washed and incubated in RPMI w/o phenol red with 10% DCC.

Human monocyte-derived macrophages (hMDM). Peripheral blood mononuclear cells were isolated from buffy coats of male healthy donors of 25–60 years old and monocytes obtained by two-step density gradient centrifugations followed by incubation of purified cells in RPMI without serum, for 20 min at RT. Human macrophages were differentiated from monocytes by 7 days of culture with 1:1000 recombinant human (rh) M-CSF in RPMI supplemented with 10% endotoxin-free FBS, 1% penicillin/streptomycin and 1% Na pyruvate. After 7 days, the medium was changed with RPMI w/o phenol red supplemented with 10% DCC.

2.4. RNA preparation and real time PCR

Total RNA was purified using ReliaPrep RNA Cell Miniprep System (Promega, Milan, Italy), 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 s 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 using the *36b4 (Rplp0)* transcript as reference gene.

2.5. 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 1400 rpm for 30 min. 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 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

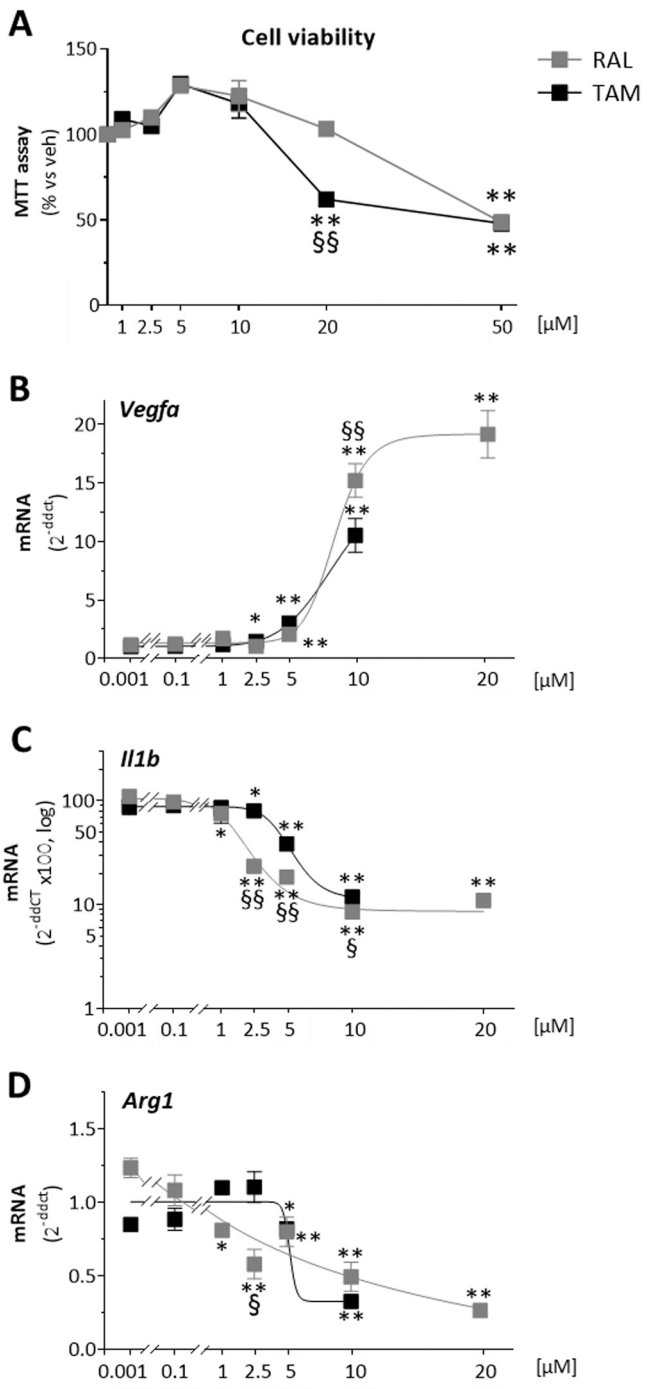

Fig. 1. Dose-dependent effects of RAL and TAM on cell viability and immune gene expression. Peritoneal macrophages were treated with vehicle (veh, not shown) or increasing concentrations of Raloxifene (RAL, grey squares) and Tamoxifen (TAM, black squares), as specified. **A)** Cell viability was assessed by the MTT assay after 24 h treatment. Data are presented as percentage of values with respect to those obtained with vehicle-treated cells. **B-D)** Cells were analyzed following 3 h treatment to assess **B)** *Vegfa*, **C)** *Il1b* and **D)** *Arg1* mRNA levels by real time PCR. Fold-changes were calculated using the 2^{-ddCt} method respect to the mean value of vehicle-treated cells (veh=1). In C), values are represented with a logarithmic scale (veh=100). Each experimental point represent mean values ± SEM (n = 5). Sigmoidal curves are generated by GraphPad Prism 8.0 (non-linear regression analysis, standard curve to interpolate: Sigmoidal, 4PL, X is concentration). One-way ANOVA followed by Tukey post hoc test, *$p < 0.05$, * *$p < 0.01$ versus veh; §$p < 0.05$, §§$p < 0.01$ RAL vs TAM.

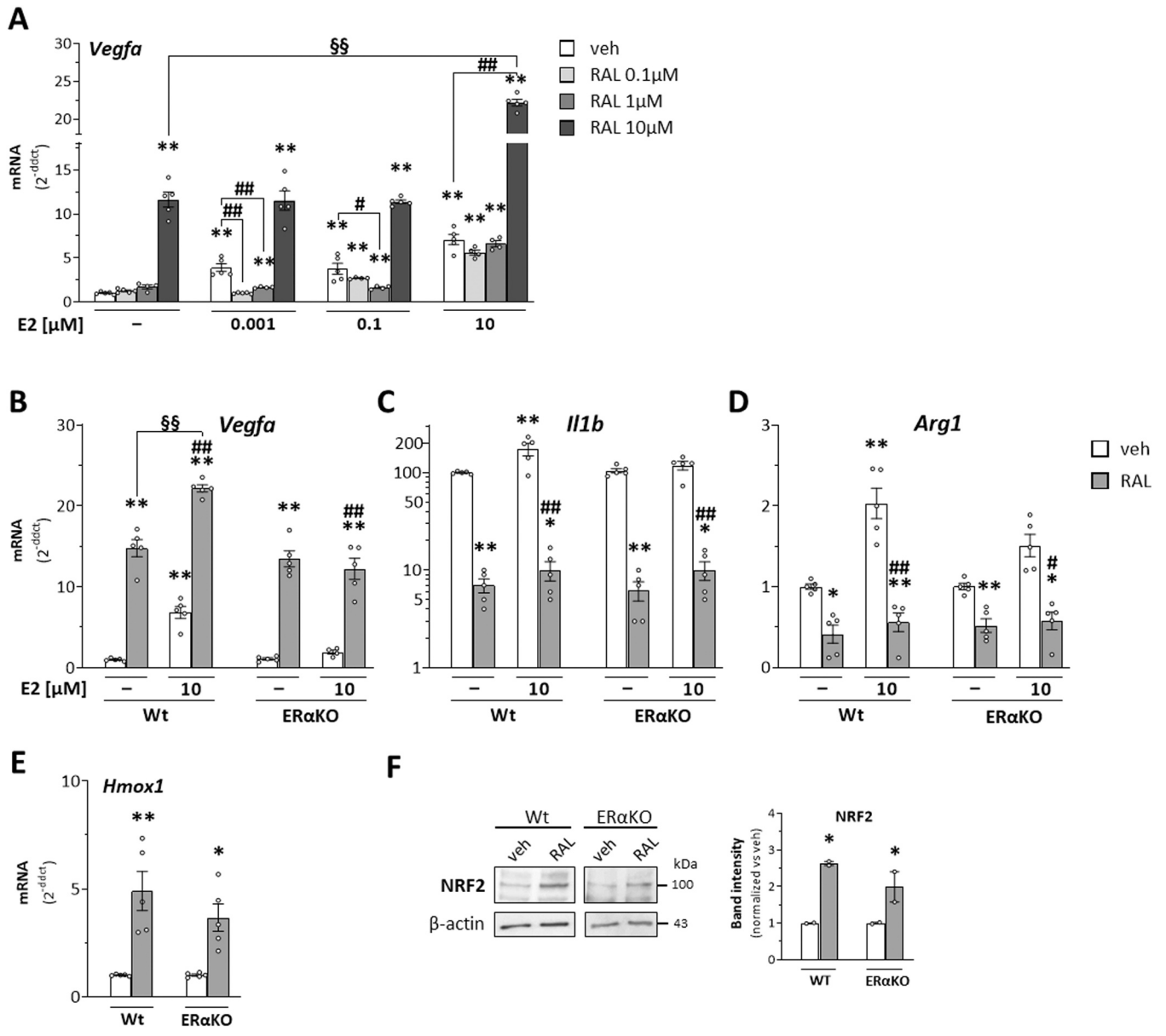

Fig. 2. Role of ERα in the immunomodulatory activity of RAL. Peritoneal macrophages from wild type (Wt) or ERαKO mice were used to analyze RAL mechanism of action. **A)** Wt mPM were treated for 3 h with increasing concentrations of 17b-Estradiol (E2) or RAL, alone or in combination, as specified, and *Vegfa* mRNA analyzed by real time PCR. **B-D)** Wt and ERαKO macrophages were treated with 10 μM RAL or 10 μM E2, as specified, and analyzed for **B)** *Vegfa,* **C)** *Il1b or* **D)** *Arg1* mRNA levels. **E)** Expression of the NRF2-target gene *Hmox1* was analyzed in Wt and ERαKO mPM after 3 h treatment 10 μM RAL. **F)** Wt and ERαKO cells were treated with vehicle (veh) or 10 μM RAL and analyzed for NRF2 and β-actin protein levels after 30' treatment; a representative Western blot is shown. In the right panel, the quantification of band signals was calculated from two independent experiments, normalized by the corresponding β-actin signal and reported as fold-induction versus veh (veh=1). (A-E) Fold changes were calculated using the 2^{-ddCt} method respect to the mean value of veh (=1). In C), values are represented with a logarithmic scale (veh=100). Bars represent mean values ± SEM (A-E: n = 5; F: n = 2). One-way ANOVA followed by Tukey post hoc test, *p < 0.05, * *p < 0.01 vs veh; $^{\#}$p < 0.05, $^{\#\#}$p < 0.01 vs E2 alone; §§p < 0.01 vs RAL alone.

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) using Odissey Fc Imaging system (LiCorBiosciences). For semiquantitative analyses, the densities of the protein bands were measured by Image Lab™ Software (Bio-Rad).

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

2.6. Enzyme-Linked Immunosorbent Assay (ELISA)

Cell supernatants were centrifuged at 450g for 5 mins and stored at − 20 °C until usage. Cytokine concentrations were determined using ELISA Duoset kits for mouse TNFα and IL1β according to the manufacturer's protocol (#DY410 and #DY401, 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.

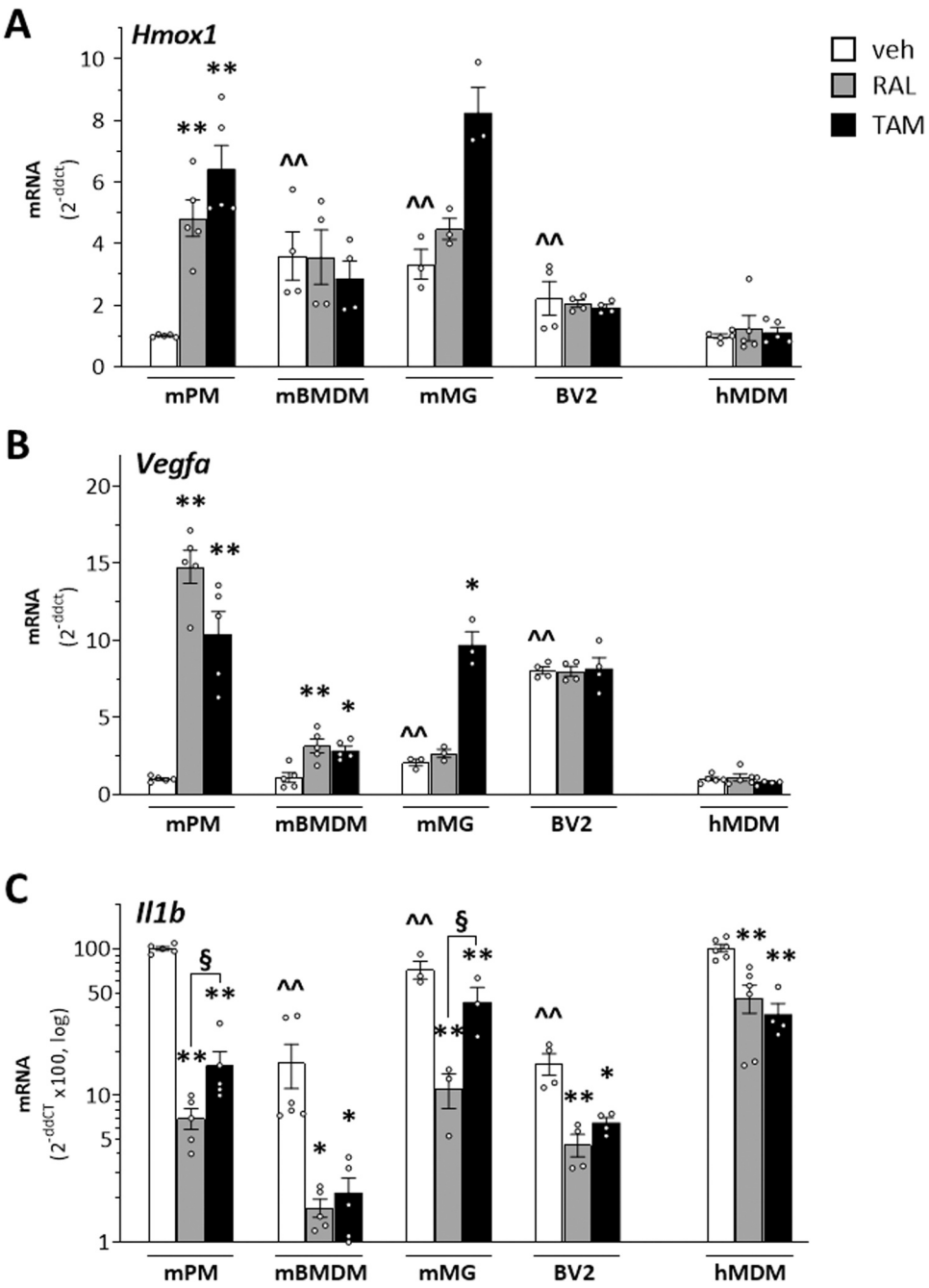

Fig. 3. Immunomodulatory effects of SERMs in macrophages obtained from different tissues. A-C) Murine peritoneal macrophages (mPM), bone marrow-derived macrophages (mBMDM), microglia (mMG), mouse BV2 microglia cell line and human monocyte-derived macrophages (hMDM) were treated with 10 μM RAL and 10 μM TAM for 3 h and analyzed for **A)** *Hmox1,* **B)** *Vegfa,* **C)** *Il1b* mRNA levels. (A-C) Fold changes for each gene were calculated using the 2^{-ddCt} method respect to the mean value of the mPM or hMDM vehicle (mPM/hMDM veh=1). In C), values are represented with a logarithmic scale (mPM/hMDM veh=100). Bars represent mean values ± SEM (n = 3–5). One-way ANOVA followed by Tukey post hoc test, *p < 0.05, * *p < 0.01 vs own veh; §p < 0.05 RAL vs TAM; ^^p < 0.1 vs mPM veh.

2.7. Cell viability assay

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 h. Then, an equal volume of the extraction buffer (20% w/v of SDS dissolved in a solution of DMF/H_20 1:1 v/v) was added to each well and incubated at 37 °C for 20 h. 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).

2.8. Statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology [14]. Experimental group size was predetermined based on prior experience in evaluating for statistical significance. One-way ANOVA or Two-way ANOVA followed by Tukey 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 = 3, where n represents the number of biological replicates derived from independent experiments. Differences are considered significant at the values of *p<0.05 and **p<0.01.

3. Results

3.1. Immunomodulatory effects of RAL in macrophages

In order to assess RAL immune activity and compare its potency with that of Tamoxifen (TAM), we used primary cultures of mouse peritoneal

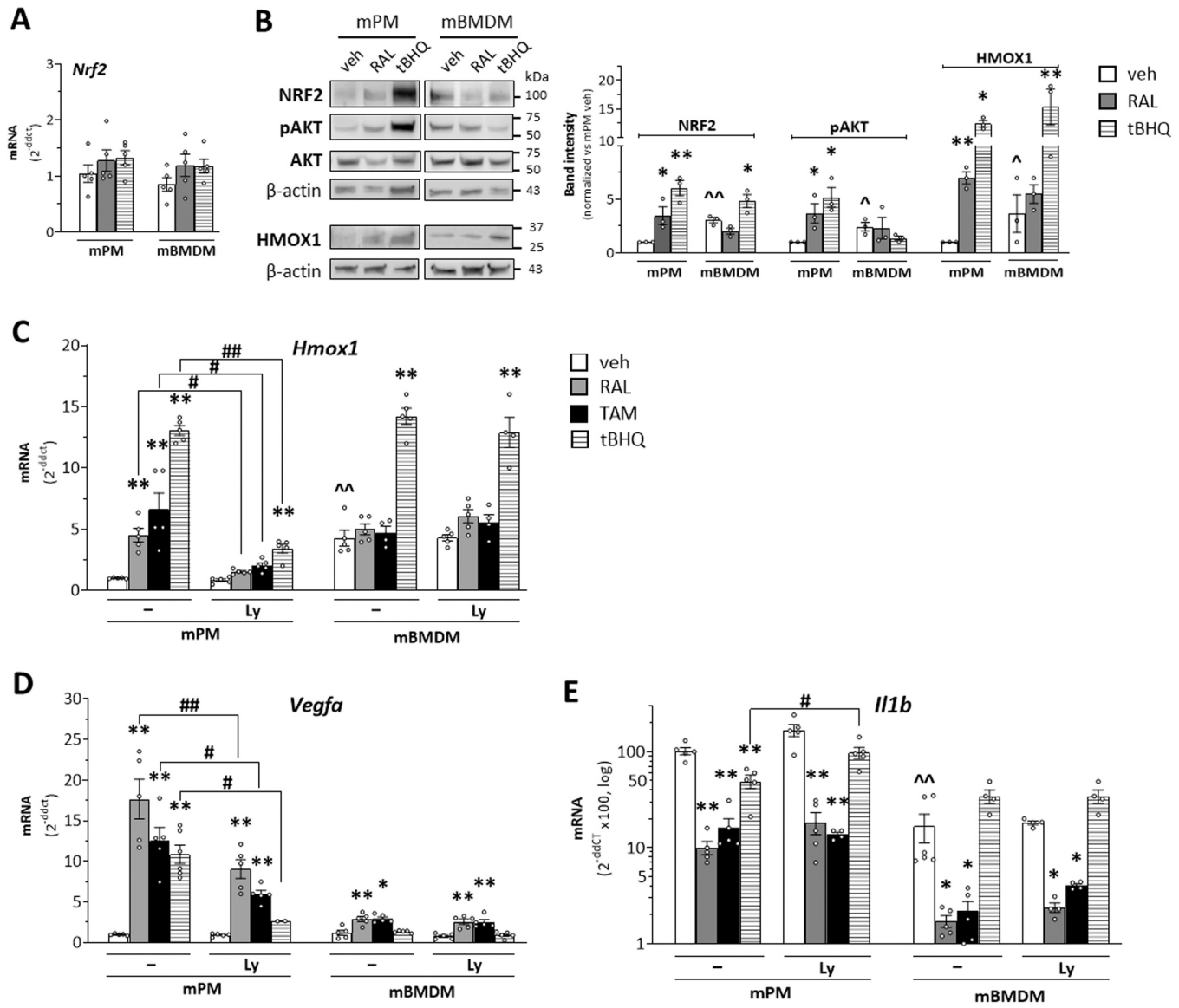

Fig. 4. Role of PI3K/Akt in the immunomodulatory activity of SERMs. Peritoneal macrophages (mPM) and bone marrow-derived macrophages (mBMDM) were treated with vehicle (veh, open bars), 10 μM Raloxifene (RAL, grey bars), Tamoxifen (TAM, black bars) or 100 μM tBHQ (dashed bars) and analyzed for **A)** *Nrf2* mRNA levels by real time PCR after 3 h treatment, or **B)** for NRF2, HMOX1, pAKT and AKT protein expression after 30' (NRF2, pAKT and AKT) or 16 h (HMOX1) treatments; representative Western blots are shown, with bands at the expected molecular weight (NRF2, 100–110 kDa; HMOX1, 28 kDa; pAKT and AKT, 60 kDa; β-actin, 43 kDa). Quantification of band signals is reported in the right panel, in which data represent mean values of 3 independent experiments, obtained from normalization by the corresponding β-actin signal and reported as fold-induction versus mPM veh (=1). **C-E)** 10 μM LY294,002 (Ly) was added for 30' prior to SERMs or tBHQ and analyzed after 3 h for **C)** *Hmox1*, **D)** *Vegfa* and **E)** *Il1b* mRNA by real time PCR. Fold-changes for each gene were calculated using the 2^{-ddCt} method respect to the mean value of the mPM veh (=1). In E), values are represented with a logarithmic scale (mPM veh=100). Bars represent mean values ± SEM (n = 3–5). One-way ANOVA followed by Tukey post hoc test, *$p < 0.05$, * *$p < 0.01$ vs veh; #$p < 0.05$, ##$p < 0.01$ combination with LY vs treatment alone; ^$p < 0.05$, ^^$p < 0.01$ vs mPM veh.

macrophages (mPM) and treated them with increasing concentrations of these two drugs. We first assessed cell viability and observed no alterations using up to 20 μM RAL, while a significant cell toxicity was registered with a similar concentration of TAM (Fig. 1A). We thus used RAL and TAM at the highest concentrations of 20 and 10 μM, respectively, to evaluate the expression of genes related to immune cell activation, namely *Vegfa*, *Il1b* and Arginase 1 (*Arg1*). Fig. 1B shows that *Vegfa* mRNA levels were increased by RAL already at 2.5 μM concentration and reached maximal levels at 20 μM, with higher efficacy and potency as compared with TAM. Moreover, both SERMs induced a down-regulatory effect on *Il1b* mRNA, which was first observed with 1 μM RAL and maximal at 10 μM RAL and TAM (Fig. 1C). A similar pharmacological profile was also observed for another immune genes, Arginase 1 (*Arg1*) (Fig. 1D). These data show that high drug levels are deprived with toxic effects, which are known to induce cell death in other non-immune cell types [6,8] and that both RAL and TAM are immunologically active in macrophages, with an overall higher potency of RAL.

3.2. ERα-dependent and independent activities of RAL

Since estrogens, such as 17β-estradiol (E2), are able to regulate the macrophage immunophenotype through the interaction with ERα [53], we evaluated the ability of RAL to compete with and modify E2 immune responses. Macrophages were treated with combinations of fixed RAL and increasing concentrations of E2, up to 10 μM. These estrogen

concentrations, that are supraphysiological in women's blood and may represent a limitation of this study, can be reached under physiological conditions, such as in the peritoneal fluid following ovulation [36], were thus chosen to warrant competition with those used for RAL. As shown in Fig. 2A, when RAL was combined with E2 in at least a 10:1 ratio (namely, 0.001 μM E2 + 0.1–1 μM RAL, and 0.1 μM E2 + 1 μM RAL) this drug was able to reduce the E2-dependent induction of *Vegfa* expression. This inhibitory effect was lost with equimolar 0.1 μM concentrations. Interestingly, the 10 μM drugs combination resulted in an additive effect, leading to a 22-fold induction of *Vegf*a mRNA levels. These effects could not be ascribed to changes in ERs expression (Supplementary Figure 1). Thus, these results demonstrate that low RAL concentrations induce ER antagonist effects, at concentrations that are consistent with a higher ER binding affinity of E2 as compared with RAL [12]. On the other hand, micromolar concentrations of RAL induce opposite responses that may add on estrogenic effects, suggesting the engagement of distinct, ER-independent molecular mechanisms by RAL, at least on the genes under study.

These results prompted us to assess the role of ERα, the only ER isoform expressed by macrophages and involved in TAM immune activity in these cells [54,55]. As expected from the above, RAL was still effective on *Vegfa* expression in macrophages isolated from ERαKO mice, while the effects of E2 alone or in combination with RAL were absent in ERα-null cells (Fig. 2B). To further substantiate these results, we extended our analysis to *Il1b*, on which RAL and E2, exert opposite effects, negative and positive, respectively. Data reported in Fig. 2C show that the inhibitory activity of RAL is maintained in Wt cells despite the presence of equimolar E2 levels, that promoted a weak increase when assayed alone; again, RAL, and not E2, was still active in ERα-null cells. Superimposable results were observed with another immune gene, *Arg1* (Fig. 2D). These data show that, while necessary for E2 immune actions, ERα is dispensable for the immune responses induced by high RAL levels, which do not engage partial ER agonist effects, at least in our experimental conditions. We then asked if the ERα-independent activity of RAL in macrophages involved NRF2, a transcription factor linked with immune metabolic and TAM-induced responses [19,55]. Fig. 2E shows that RAL increased the expression of the endogenous NRF2-target gene *Hmox1*, with a 4-fold increase in the mRNA levels, and that RAL effects were maintained in ERα-null macrophages. Western blot analysis demonstrated increased NRF2 protein levels following RAL treatment in both Wt and ERαKO macrophages, strongly supporting an increased activation of NRF2 in the presence of RAL (Fig. 2F) and similar effects were also observed for RAL on other NRF2 target genes (Supplementary Figure 1). In fact, whereas NRF2 is normally directed to proteasomal degradation [4], increased NRF2 protein levels are suggestive of increased stability and thus availability for its transcriptional activity. Altogether, our results demonstrate a dual pharmacological activity of RAL in macrophages, in that low concentrations are able to antagonize the immune effects of estrogens, while higher levels trigger ERα-unrelated immunomodulatory effects that involve NRF2 activation and may add on E2 action in a gene-specific manner.

3.3. SERMs activity in macrophages from distinct tissues

Since tissue-specific signals are able to influence the reactivity of resident immune cells, we asked whether macrophages obtained from different organs were similarly responsive to RAL and TAM. We thus compared mPM, bone marrow-derived macrophages (mBMDM), primary cultures of mouse microglia (mMG) and the microglial cell line (BV2), as well as monocyte-derived macrophages of human origin (hMDM). The basal expression of the NRF2-target gene *Hmox1* showed slightly different levels and, more importantly, could be modified by RAL or TAM treatments only in mPM, with a positive effect of TAM in mMG that did not reach statistical significance (Fig. 3A). Similar effects were observed for *Vegf*a expression, with a positive effect of SERMs in mPM and a weak although significant increase in mBMDM: again, mMG was responsive to TAM with a significant induction of *Vegf*a mRNA (Fig. 3B). Conversely, RAL and TAM were similarly potent in reducing *Il1b* mRNA levels in all the macrophage subtypes analyzed, including hMDM (Fig. 3C). In fact, despite low basal levels of *Il1β* in some cells, such as mBMDM and BV2, both SERMS induced a further 10-fold decrease in this cytokine mRNA in both mouse and human cells. Notably, a higher potency of RAL compared to TAM was generally observed and reached statistical significance in mPM, as described above, and mMG, where TAM shows again a different trend in activity as compared with other cell subtypes. The low expression level of *Arg1* in these cell types did not allow the quantification of its basal transcription and possible negative regulation (data not shown). Taken together, these data demonstrate that SERMs are able to regulate the expression of immune genes in a macrophage subtype-specific manner, with the exception of *Il1b*, whose expression is down-regulated as a conserved immune response to SERMs in all macrophage subsets of different origin.

3.4. Role of the PI3K/Akt pathway in the immunomodulatory activity of SERMs

In order to understand the molecular mechanisms underlying the macrophage subtype-specific activity of SERMs, we further investigated the involvement of NRF2 and focused on mPM and mBMDM as paradigm of gene- and cell-specific activity of SERMs. We first evaluated NRF2 expression levels and observed that mRNA levels were similar under basal conditions and not modified by SERMs treatments in both cell types (Fig. 4A). On the contrary, NRF2 protein levels were higher in mBMDM under basal conditions and were increased by RAL only in mPM (Fig. 4B). This prompted us to assess the classic NRF2 activator tert-Butylhydroquinone (tBHQ), which is known to increase NRF2 stability and transcriptional activity. As expected, tBHQ did not modify NRF2 mRNA levels (Fig. 4A) while it increased NRF2 protein stability in both cell types, although with a lower potency in mBMDM (Fig. 4B), further suggesting a different NRF2 activation state in this macrophage subtype. To shed more light on this point, we evaluated the involvement of the PI3K/AKT pathway, considering it has been described to induce NRF2 stability and activation [32,38] and to mediate TAM responses on NRF2 target genes [55]. We used the PI3K inhibitor LY294,002 added shortly before RAL or TAM and analyzed *Hmox-1* expression. As shown in Fig. 4C, the PI3K inhibitor mitigated the activity of SERMs on *Hmox1* mRNA in mPM but not in mBMDM. Also, the higher basal expression of *Hmox1* and the potency of tBHQ did not change in mBMDM treated with LY294,002. As expected, Western blot analysis showed that HMOX1 protein levels were higher in untreated mBMDMs, changed after RAL treatment only in mPM and were induced by tBHQ in both cellular systems (Fig. 4B). These data suggest that NRF2 stability is linked with PI3K activation specifically in mPM. To corroborate this evidence, we analyzed the levels of phosphorylated AKT (pAKT), which corresponds to the active form of this PI3K effector. Fig. 4B shows that untreated mBMDM contained higher pAKT levels as compared with mPM, while the total amount of AKT protein is similar. Moreover, pAKT was increased by RAL and tBHQ treatments only in mPM, showing that SERMs inefficacy in mBMDM is paralleled by the higher NRF2 stability (Fig. 4B) and activity (Fig. 4C) in these cells, at least on the genes analyzed here. Altogether, these data suggest that the PI3K and NRF2 are differentially involved in macrophage reactivity and SERMs responsiveness.

We then extended our analyses to *Vegfa* and *Il1b* genes. Consistent with effects on *Hmox1*, SERMs and tBHQ induction of *Vegfa* was significantly decreased by LY294,002 only in mPM, while their weak activity in mBMDM was not modified (Fig. 4D), further sustaining the hypothesis that a different activation state of PI3K/NRF2 in BMDM precludes SERMs activity, at least on the genes analyzed here. Interestingly, the inhibitory effect of SERMs on *Il1b* expression was insensitive to PI3K inhibition in both mPM and mBMDM (see Fig. 4E), indicating that the negative regulation of *Il1b* mRNA exerted by SERMs

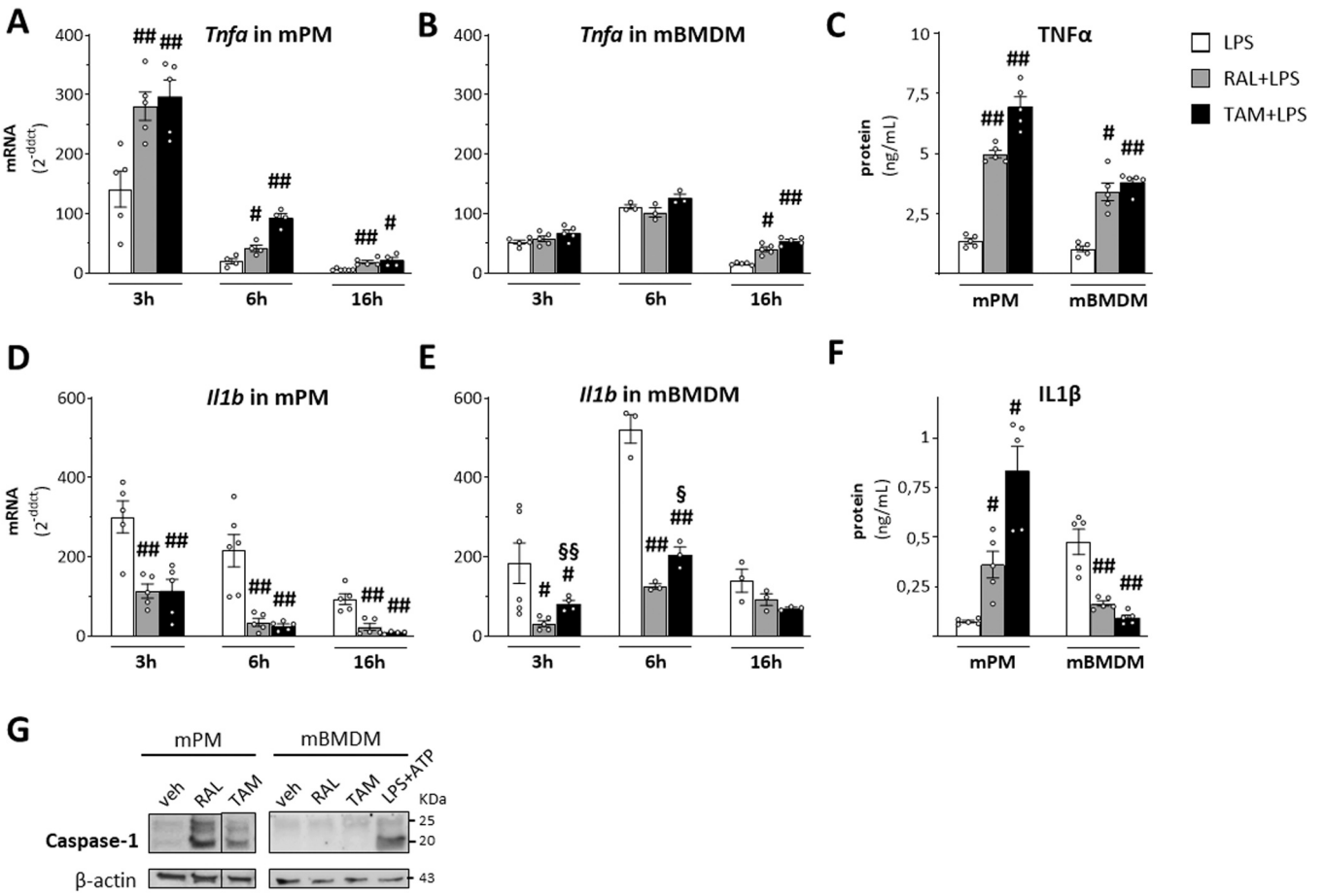

Fig. 5. Immunomodulatory effects of RAL on the LPS-induced inflammatory response. A-F) Peritoneal macrophages (mPM) and bone marrow-derived macrophages (mBMDM) were treated with 10 μM RAL or 10 μM TAM 1 h before the addition of 100 ng/mL LPS and analyzed for TNFα and IL1β expression. **A, B, D, E)** mRNA levels were evaluated by real time PCR after 3, 6 and 16 h treatment. **C, F)** protein levels were determined in cell supernatants by ELISA assay after 24 h treatment. **G)** Cells were treated with veh, 10 μM RAL, 10 μM TAM o LPS 100 ng/mL+ATP 1 mM, as control, for 6 h and cell lysates analyzed for caspase-1 and β-actin protein levels by Western blotting. Images are representative results from 3 independent experiments. A-B, D-E) Fold changes were calculated using the 2^{-ddCt} method respect to the mean value of veh (=1). Bars represent mean values ± SEM (n = 3–5). A-B, D-E) Two-way ANOVA or C-F) One-way ANOVA followed by Tukey post hoc test; statistics vs veh not specified; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ RAL+LPS vs TAM+LPS.

is independent from the PI3K pathway in different macrophage subtypes. As expected, the downregulation of *Il1b* mRNA induced by tBHQ in mPM was significantly reversed by PI3K inhibition.

Altogether, these results demonstrate that the PI3K/NRF2 pathways mediate SERMs activity on the expression of selected genes in mPM, while the lack of response in mBMDM may stem from a basal hyperactivation of these signaling pathways. Exception to this rule is *Il1b* expression, whose downregulatory mechanism by SERMs is independent on the NRF2/PI3K pathways and conserved in different macrophages.

3.5. RAL activity on the macrophage response to LPS

We next asked whether SERMs immune activity could alter the ability of mPM and mBMDM to respond to immune stimulation. We performed a time-course experiment in which macrophages were treated with the bacterial endotoxin LPS, in the absence or presence of RAL or TAM, and analyzed for the expression of two key inflammatory cytokines, TNFα and IL1β. As expected, the LPS treatment induced an increase in TNFα mRNA levels, with a stronger and faster response in mPM than in BMDM (Fig. 5A and B). Notably, this induction was significantly potentiated by SERMs, which resulted in higher fold-induction in mPM shortly after drug administration and in mBMDM at a later, 6 h treatment (Fig. 5A and B). The biological significance of these results was confirmed by evaluating TNFα protein levels. Similar amounts were detected following LPS treatment in both cell types, despite the cell type-specific kinetics of induction. Higher TNFα levels were induced by LPS when it was assayed in the presence of SERMs (Fig. 5C). Analyses of *Il1b* mRNA in cells treated with LPS showed similar results as with TNFα, with different fold-induction in mPM or mBMDM depending on the time point of LPS exposure analyzed (Fig. 5D and E). In both cell types, the effects of LPS were significantly reduced by RAL and TAM, with similar drug efficacy in mPM and stronger effects of RAL in mBMDM (Fig. 5D and E). We then assessed IL1β protein levels and observed that the effects of LPS alone correlated with those observed for IL1β mRNA, as the higher fold-induction of *Il1*β mRNA corresponded to higher protein levels in mBMDM as compared with mPM (Fig. 5F). SERMs+LPS treatment resulted in an expected reduction in protein content in mBMDM, while a potentiation was found in mPM, where SERMs induced 4–8-fold higher levels of IL1β protein as compared with LPS alone. It is known that the intracellular conversion of the precursor of IL1β into mature IL1b is mediated by caspase-1, an enzyme that is activated through the transformation of pro-caspase-1 into caspase-1 induced by secondary inflammatory signals, such as ATP [28]. We thus asked if the increased IL1β protein levels induced by RAL in mPM under inflammatory conditions, despite reduced mRNA expression, could be mediated by caspase-1 activation, similarly to what we previously described for TAM [55]. Indeed, Fig. 5 G shows the presence of the active form of caspase-1 in RAL-treated mPM, while RAL

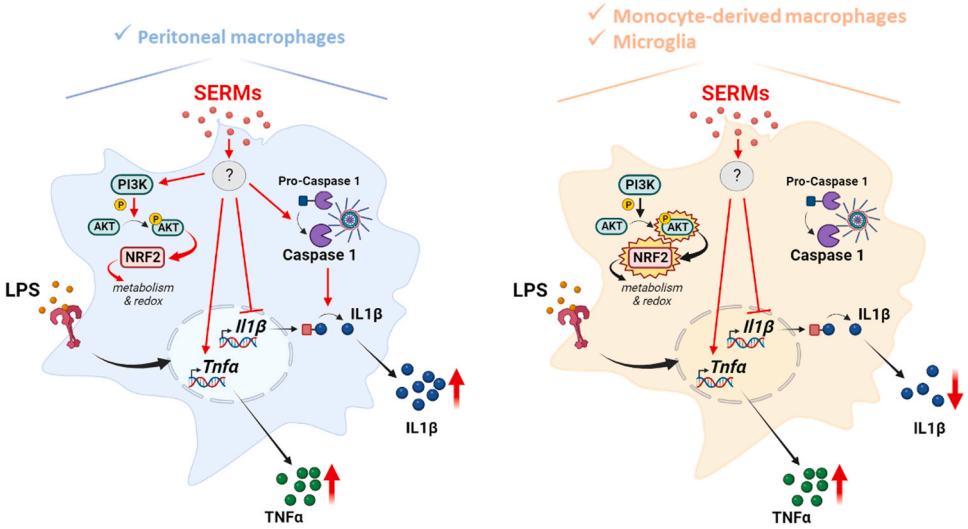

Fig. 6. Graphical abstract. SERMs act as immunoregulatory agents in macrophages, leading to the potentiation of the inflammatory response through the increase in TNFα production, as well as the cell-specific up or down-regulation of IL1β expression and the activation of the PI3K/Akt/NRF2 and caspase-1 pathways that depend on the tissue of origin of macrophages (made by BioRender.com).

and TAM were inactive in mBMDM, although these cells maintain the ability to activate caspase-1 when ATP is added together with LPS (Fig. 5G). Altogether, these data show that SERMs immune activity has distinct effects on the inflammatory response of different macrophage subtypes, with a general potentiation of TNFα production and inhibitory effects on *Il1b* mRNA, that can be counteracted by a cell type-specific increase in IL1β protein production associated with caspase-1 activation.

4. Discussion

SERMs are high-affinity ligands of ERs as well as ER-independent molecules at micromolar concentrations [58]. Therapeutic prescriptions of SERMs warrant drug blood levels that indeed reach the micromolar range and that are associated with ER-unrelated responses. The clinical use of SERMS has extended to infections with promising results against a wide spectrum of microbial species, suggesting the engagement of a host-mediated protective system by SERMs still poorly defined [2,23,29,48].

We believe that our results add more knowledge to this field. We show that the dual mechanism of action of RAL also takes place in macrophages to regulate the immune metabolic phenotype of these cells. In particular, we describe the increased production of TNFα, a potent proinflammatory cytokine involved in the fight against infections, by macrophages treated with SERMs and LPS. A more complex regulation was observed for IL1β as a novel mechanism of SERMs activity: a conserved down-regulation of *Il1β* mRNA was induced by SERMs in different macrophages, which could be compensated by the concomitant increase in IL1β protein maturation in selected macrophage subtypes, leading to a potentiated cytokine release under inflammatory conditions. The molecular mechanism and biological role of the differential regulation of TNFα and IL1β are still unclear. It is known that even small changes in the local abundance of these cytokines elicit specific responses in different cell types which impact tissue homeostasis in a context-dependent manner [31,65]. From this we can infer that specific mechanisms are needed to differentially regulate TNFα and IL1β expression and finely tune tissue homeostasis and immunity, fostering future studies to better clarify SERMs activity on these cytokines.

We here also show that SERMs can induce ERα-dependent and independent responses in macrophages. This implies that the ER antagonism, induced by lower concentrations of SERMs that hinder estrogen action, redirects macrophages towards a proinflammatory phenotype, since estrogens behave as anti-inflammatory triggers [53,64]. On the other hand, micromolar SERMs levels twist the cell immune-metabolic state and potentiate their antimicrobial potential. This dual mechanism of controlling immunity that results in a pro-inflammatory activity may underlie SERMs therapeutic efficacy when they are used as anti-infective agents [21,41,51] and ERα ligands for standard indications, such as breast cancer [1,9,47]. It is important to underline that the overall good safety profile of SERMs is associated with an increased risk for adverse reactions which, however, do not seem to involve off-target mechanisms [49,50,52]. In fact, the increased risk of thromboembolic events reported with long-term SERMs prescriptions is similar with that observed with estrogen replacement therapies, while vasomotor symptoms that may occur along with short-term, weeks-lasting treatments with SERMs are typical of anti-estrogenic molecules, pointing to ERα as the mediator of SERMs adverse reactions induced by both the agonist and antagonist effects of SERMs, respectively [25,37,51]. Altogether, our data support the hypothesis that the wide antimicrobial efficacy of SERMs is mediated by both on and off-target-mediated responses of innate immune cells that sustain host protection against infections with an overall favourable safety profile.

An intriguing question relates with the identity of the macrophage off-target mediator. We here show that SERMs engage the PI3K and NRF2 pathways and regulate specific genes in selected macrophage populations. These add to the list of ERα-unrelated SERMs effectors previously identified in this and other cell types, which includes transcription factors and signal transduction complexes [5,11,20,46,58]. It is still unclear, however, whether these mediators are directly activated by SERMs or if they are secondary effectors of upstream targets. RAL and TAM have a different chemical structure, the first containing a benzothiophene ring and the latter belonging to the triphenylethylene family. Yet, they are comparable with cationic amphiphilic drugs (CADs) which, thanks to this bimodal chemical structure, accumulate at endosomal membranes and regulate enzymatic complexes that control local lipid metabolism [10,18]. Intriguingly, previous evidence supports the existence of antiestrogen binding sites (AEBS) unrelated with ERs and located within intracellular membranes, where they regulate cholesterol and lipid metabolism [15,44,61]. However, it is still undefined whether CADs-related mechanisms and AEBS are activated by SERMs in macrophages.

It has been demonstrated that the tissue microenvironment is a major controller of the phenotype and reactivity of tissue-resident macrophages [26]. Indeed, we here demonstrate a different response to SERMs on the expression of selected genes, *Hmox1* and *Vegfa*, in macrophages

obtained from the peritoneum as compared with those differentiated from bone marrow precursors, paralleled by specific PI3K/NRF2 activation states. These two macrophage subtypes have been reported to differ in polarization signatures, phagocytosis and migration abilities [7,13,30]. This opens the possibility that SERMs might be better controllers of inflammation in selected tissues. On the other hand, the inhibition of *Il1b* by SERMs commonly occurs in different types of murine and human macrophages. A complex network of transcriptional regulators and mRNA processing systems, such as non-coding RNAs and mRNA destabilizing proteins, concur to finely tune *Il1b* expression and inflammation in innate immune cells [17,27,35,40]. Future studies are therefore needed to identify the effectors of *Il1b* down-regulation by SERMs. Altogether, we believe that, by showing the engagement of macrophages and modification of cell polarization and reactivity, our data provide an explanation to the clinical benefit of SERMs against infections [22,41,51]. Indeed, current evidence also extends the efficacy of SERMs to intracellular pathogens that survive within macrophages, including SARS-CoV-2 [45,57,59,62]. The identification of the molecular mediator of SERMs immune activity will allow a better understanding of the therapeutic activity of these agents and the development of novel drugs endowed with ERα or off-target specificity.

5. Conclusions

In summary, our study demonstrates that RAL and TAM modulate the macrophage immune activity through ERα-independent mechanisms that are specific to the tissue origin of macrophages yet converge on *Il1b* down-regulation (Fig. 6). This work extends our knowledge on the biological effects and molecular mechanisms of SERMs and indicate the immune system as a pharmacological target for these drugs, with relevant implications for SERMs therapeutic indications.

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CRediT authorship contribution statement

Chiara Sfogliarini, Giovanna Pepe, Marcello Allegretti, Candida Cesta, Massimo Locati and Elisabetta Vegeto designed the experiments; Chiara Sfogliarini and Giovanna Pepe performed the experiments and analyzed the results; Chiara Sfogliarini and Elisabetta Vegeto wrote the manuscript, with contribution from Marcello Allegretti, Candida Cesta and Massimo Locati; Massimo Locati and Elisabetta Vegeto supervised the project. All authors read and edited the final manuscript.

Declaration of Competing Interest

The authors declare that are no conflicts of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.115008.

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