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Bisphenol A disruption promotes mammary tumor microenvironment via phenotypic cell polarization and inflammatory response

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Abstract

Inflammation in the established tumor microenvironment (TME) is often associated with a poor prognosis of breast cancer. Bisphenol A (BPA) is an endocrine-disrupting chemical that acts as inflammatory promoter and tumoral facilitator in mammary tissue. Previous studies demonstrated the onset of mammary carcinogenesis at aging when BPA exposure occurred in windows of development/susceptibility. We aim to investigate the inflammatory repercussions of BPA in TME in mammary gland (MG) during neoplastic development in aging. Female Mongolian gerbils were exposed to low (50 µg/kg) or high BPA (5000 µg/kg) doses during pregnancy and lactation. They were euthanized at 18 months of age (aging) and the MG were collected for inflammatory markers and histopathological analysis. Contrarily to control MG, BPA induced carcinogenic development mediated by COX-2 and p-STAT3 expression. BPA was also able to promote macrophage and mast cell (MC) polarization in tumoral phenotype, evidenced by pathways for recruitment and activation of these inflammatory cells and tissue invasiveness triggered by tumor necrosis factor-alpha and transforming growth factor-beta 1 (TGF-β1). Increase of tumor-associated macrophages, M1 (CD68 + iNOS+) and M2 (CD163+) expressing pro-tumoral mediators and metalloproteases was observed; this aspect greatly contributed to stromal remodeling and invasion of neoplastic cells. In addition, the MC population drastically increased in BPA-exposed MG. Tryptase-positive MCs increased in disrupted MG and expressed TGF-β1, contributing to EMT process during carcinogenesis mediated by BPA. BPA exposure interfered in inflammatory response by releasing and enhancing the expression of mediators that contribute to tumor growth and recruitment of inflammatory cells that promote a malignant profile.

KEYWORDS

bisphenol A, mast cells, TGF-β1, TNF-α, tumor-associated macrophages

1 | INTRODUCTION

Tumor microenvironment (TME) is an important target studied in cancer development and therapeutic approaches. TME promotes the neoplastic cross-talk required for tumor progression (Bussard et al., 2016). One of the main elements for the TME promotion is the establishment of inflammatory process, by production of cytokines and inflammatory-tumor mediators (Polyak & Kalluri, 2010). Breast cancer is one type of adenocarcinoma that presents a great imbalance between inflammation and malignancy (Liubomirski et al., 2019). These phenomena are associated to cancer progression through inflammatory mediators that stimulate the micrometastasis and recruitment of tumor-associated stromal cells (Fouad et al., 2014).

Exposure to environmental chemicals and pollutants increase the risk of inflammation and breast cancer (Koual et al., 2020). Several compounds act as disruptors of stromal-epithelial interaction, impairing tissue homeostasis and developing neoplasms (Koual et al., 2020). Bisphenol A (BPA) is considered a multitarget disruptor of the female reproductive system (Pivonello et al., 2020) and is among the compounds recognized as tumor promoters in the mammary gland (MG). In addition to being considered a promoter of breast cancer (Nair et al., 2020), BPA has been linked as an promoter of inflammatory process (Murata & Kang, 2018). This is due to the fact that exposure to BPA upregulates the expression of inflammatory cytokines recognized as attractant chemokines for inflammatory cells (Kanaya et al., 2019).

We recently demonstrated the carcinogenic potential of BPA in MG of elderly gerbil females after previous exposure during pregnancy and lactation (Ruiz, Colleta, Leonel, et al., 2021). Safe (50 µg/kg/daily) and acute (5000 µg/kg/daily) dosages promoted tumoral onset in MG in a long-term, considering the exposure during pregnancy and lactation (Ruiz, Colleta, Leonel, et al., 2021; Ruiz, Colleta, Zuccari et al., 2021). In these phases an intense remodeling occurs in epithelial and stromal compartments (Terry et al., 2019) and exposure to BPA triggers a late tumor development onset at aging (Ruiz, Colleta, Leonel, et al., 2021). Also, recent studies have shown that BPA acts as an inflammatory modulator in MG in adult females exposed *in utero* (Leonel et al., 2021).

Since the TME at aging predicts a worse prognosis (Fane & Weeraratna, 2020) and BPA is one of the most environmental-spread endocrine disruptors (Vandenberg et al., 2007), the crosstalk between neoplastic development and BPA-exposed TME are matter of concern and deserve to be studied. Here, we investigate the expression of inflammatory cytokines in epithelial and stromal compartments and describe the phenotype of recruited inflammatory cells, mast cells (MC) and macrophages, in the TME during BPA-induced carcinogenesis of aged gerbil females exposed in gestational/lactational window.

2 | MATERIALS AND METHODS

2.1 | Experimental design and animals

Female Mongolian gerbils (*Meriones unguiculatus*) were allocated with fertile males in reproductive age (4 months old) for allowing copulation. On the 8th gestational day, the pregnant females were sorted into four experimental groups ($n = 8$): control (subjected to water gavage/daily); vehicle (subjected to 0.1 ml corn oil gavage/daily); ↓BPA (subjected to 50 µg/kg of BPA/daily in corn oil by gavage/daily—reference dose with carcinogenic effects; see Ruiz, Colleta, Leonel, et al. (2021); Ruiz, Colleta, Zuccari et al. (2021) and Kwon (2022); BPA, Merck Milllipore, 239658); and ↑BPA (subjected to 5000 µg/kg of BPA/daily—high dose). Gavage was performed for 39 days—18 days of pregnancy and 21 days of lactation. After, females were kept with no other treatment until 18 months of age when they were anesthetized and euthanized. Euthanasia was performed in the estrous phase of cycle, confirmed by vaginal smear. Left MG was collected and fixed in paraformaldehyde 4%, processed in histological routine and serially sectioned (4.5 µm) for immunohistochemistry (IHC) analysis. Right MG was frozen at −80°C for Western blot analysis analysis.

The animals were kept in Animal Breeding Center of the Institute of Biosciences, Humanities and Exact Sciences (IBILCE; UNESP). They were allocated in polysulfone isolators with balanced food and water *ad libitum*. Ethical approval and international protocols for animal handling were registered in Local Ethical Committee (Protocol Number 217/2019).

2.2 | IHC and histochemical analysis

Histological sections were destined to IHC technique for inflammatory cells and markers detection. The sections were deparaffinized and hydrated. Antigen retrieval was performed with 10 mM citrate buffer (95°C; pH 6.0) or Tris + EDTA (90°C; pH 9.0) for 45 min. H_2O_2 5% diluted in methanol was used for peroxidase blockage. Blocking of nonspecific proteins was performed in 10% skimmed milk. After, sections were incubated overnight with primary antibodies for p-STAT3 (1:100, rabbit monoclonal, D3A7, #9145; Cell Signaling), COX-2 (1:75; rabbit monoclonal, D5H5, #12282; Cell Signaling), tumor necrosis factor-alpha (TNF-α) (1:75, rabbit monoclonal, D2D4, #11948; Cell Signaling), F4/80 (1:100, rabbit monoclonal, IgG, D2S9R, #70076; Cell Signaling), and IL-6 (1:100, mouse monoclonal, E-4, sc-28343, Santa Cruz Biotechnology). These steps were interspersed with buffer washes in PBS or TBS. Slides were incubated with secondary antibody and after with polymer (Novolink™, Leica Biosystems Newcastle Ltd.). Positive staining detection of IHC reaction was performed with 3–30 diaminobenzidine tetrahydrochloride (DAB) (Novolink™; Leica Biosystems) and counterstaining with hematoxylin. Also, Toluidine blue (pH 4.0) staining was performed in histological sections for total MCs detection. Positive and negative controls for specific IHC of the present study were provided in Supplementary file. Other specificity antibodies assay for Mongolian gerbil were demonstrated in Ruiz, Colleta, Leonel, et al. (2021); Ruiz, Colleta, Zuccari et al. (2021).

2.3 | Double immunofluorescence assay

Double immunofluorescence assay was performed to determine co-localization or specific cell markers. The steps were similar to IHC techniques, except for the peroxidase blockage that was not carried. Incubation of the following primary antibodies was performed with a (1:50) dilution: CD68 (goat polyclonal, M-20, Santa Cruz Biotechnology), CD163 (rabbit polyclonal, M-96, Santa Cruz Biotechnology; or mouse monoclonal, ED2, sc-58965, Santa Cruz Biotechnology), NOS2/iNOS (mouse monoclonal, C-11, sc-7271, Santa Cruz Biotechnology), MC tryptase (mouse monoclonal, MAB1222, Sigma-Aldrich; or, rabbit polyclonal, ab196772, Abcam), MC chymase (mouse monoclonal, ab2377, Abcam), COX-2 (rabbit monoclonal, D5H5, #12282; Cell Signaling), MMP2 (mouse monoclonal, sc-13595, Santa Cruz Biotechnology), MMP9 (mouse monoclonal, sc-21733, Santa Cruz Biotechnology), transforming growth factor-beta 1 (TGF-β1) (rabbit polyclonal, sc-146), TNF-α (rabbit monoclonal, D2D4, #11948; Cell Signaling), and IL-6 (mouse monoclonal, E-4, sc-28343, Santa Cruz Biotechnology). After, the incubation with specific fluorochrome conjugated secondary antibodies was performed, following the host of primary antibodies: anti-mouse fluorescein isothicyanate (FITC) (sc-2010, Santa Cruz Biotechnology); anti-rabbit FITC (sc-2359, Santa Cruz Biotechnology); anti-mouse Rhodamine (sc-2092, Santa Cruz Biotechnology); anti-goat Texas Red (sc-2783, Santa Cruz Biotechnology) and/or anti-rabbit Texas Red (sc-2780, Santa Cruz Biotechnology). -4',6-diamidino-2-phenyl-indole (DAPI) (Fluoroshield™ with DAPI, F6057, histology mounting medium, Merck) was used for nuclear fluorescence and mounting. Negative controls were performed by placing 1% bovine serum albumine in the primary antibody step, and subsequently, all conjugation and assembly steps were performed.

2.4 | Western blot analysis

MG was stored in −80°C for Western blot analysis analysis. Protein extraction was performed with an extraction buffer (Tris-EDTA −50 mmol/L Tris, 5 mmol/L EDTA, 250 mmol/L NaCl; NP-40 1%, SDS 0.1%; NP-40 1%; and SDS 0.1%) containing protease inhibitor (Protease Inhibitor Cocktail, 1:100, P8340, Sigma Aldrich) in smashed samples, which were then centrifuged (20 min; 14,000 rpm; 4°C). Protein concentration was quantified by absorbance using BCA Protein Assay Kit (Pierce BCA Protein Assay Kit, 23,227, Thermo Fischer Scientific) in microplate reader SPECTROstar Omega (BMG Labtech). Electrophoresis in SDS page gel (15 µg of protein per sample; 105 V; 90 min) and electrophoretic transfer (90 min) to a nitrocellulose membrane (Amersham Protram, 10,600,003, GE Healthcare) were performed. TNF-α expression amount was evaluated by Western blot analysis assay. Blots were blocked with skimmed milk 3% in TBS + Tween (0.1%). After, they were incubated with primary antibody anti-TNF-α (17 kDa, rabbit monoclonal, D2D4, #11948; Cell Signaling) and anti-GAPDH (36 kDa, rabbit monoclonal, 14C10, #2118, Cell Signaling, used as endogenous positive control) overnight. Secondary antibody incubation was performed with horseradish peroxidase conjugated (anti-rabbit IgG, HRP-linked Antibody, #7074). All steps were interposed with tris buffered saline plus tween washes. Antibody detection was made with Chemiluminescent substrates—Luminol Enhancer and Peroxide solution reagents (Westar Antares, Cyanagen, XLS142,0250) and revealed in ChemiDoc Image System (BioRad). Image J software (version 1.52a) was used for densitometry analysis and protein quantification. Normalization was made by comparing the expression of proteins after incubation with TNF-α and the corresponding expression of GAPDH, in the same membrane. Western blot analysis analysis were employed to IHC analysis that did not present different significance of positive cells among groups.

2.5 | Quantification and statistical analysis

For the quantitative analyses, the number of positive cells per whole mammary tissue area (cell/mm2) was considered. For IHC (pSTAT3, TNF-α, $CD163^+$, COX-2, $F4/80^+$), analyzes were performed in QuPath software (version 0.1.2, an open-source pathology software platform). Specific cell detection was evaluated by double immunofluorescence (CD68 plus iNOS, COX2, MMP9, and TGFβ1; $Tryptase^+Chymase^+$ or $Tryptase^+Chymase^-$ plus TGFβ1 and MMP2). For these analyses, eight random fields were captured (x100) per sample on the AX10 Fluorescence Microscope (Zeiss) coupled with AxioVision (Zeiss) software. The number of positive cells was counted after merge fluorescence channels—total positive cells/analyzed area (mm^2); or percentage of double-positive cells by total number of the cell type.

Statistical analyses were performed in GraphPad Prism 6.00 for Windows (GraphPad Software). Kolmogorov–Smirnov test was used to check data normality distribution. One-way analysis of variance followed by Tukey's test was applied in parametric data (total and cytoplasmic p-STAT3; COX2; F4/80; CD68 plus iNOS, COX2, MMP9, and TGFβ1; CD163; IL6; Tryptase/chymase; and plus, TGFβ1 and MMP2). Kruskal–Wallis's test followed by Dunn's test was applied in non-parametric data (TNF-α, nuclear p-STAT3, CD68/TNF-α). The $p < .05$ was considered as significant for comparative analysis.

3 | RESULTS

3.1 | Expression of inflammatory markers in mammary tissue disrupted by BPA

Inflammatory mediators in mammary tissue were evaluated by IHC in aged females of four experimental groups. Expression of p-STAT3 (phosphorylated signal transducer and activator of transcription 3) showed higher rates in MG exposed to BPA at both dosages, than in control groups (Figure 1a). P-STAT3 expression enhanced in vehicle group compared to control, in which positive cells presented nuclear staining (Figure 1b). In both BPA-exposed groups, contrarily,

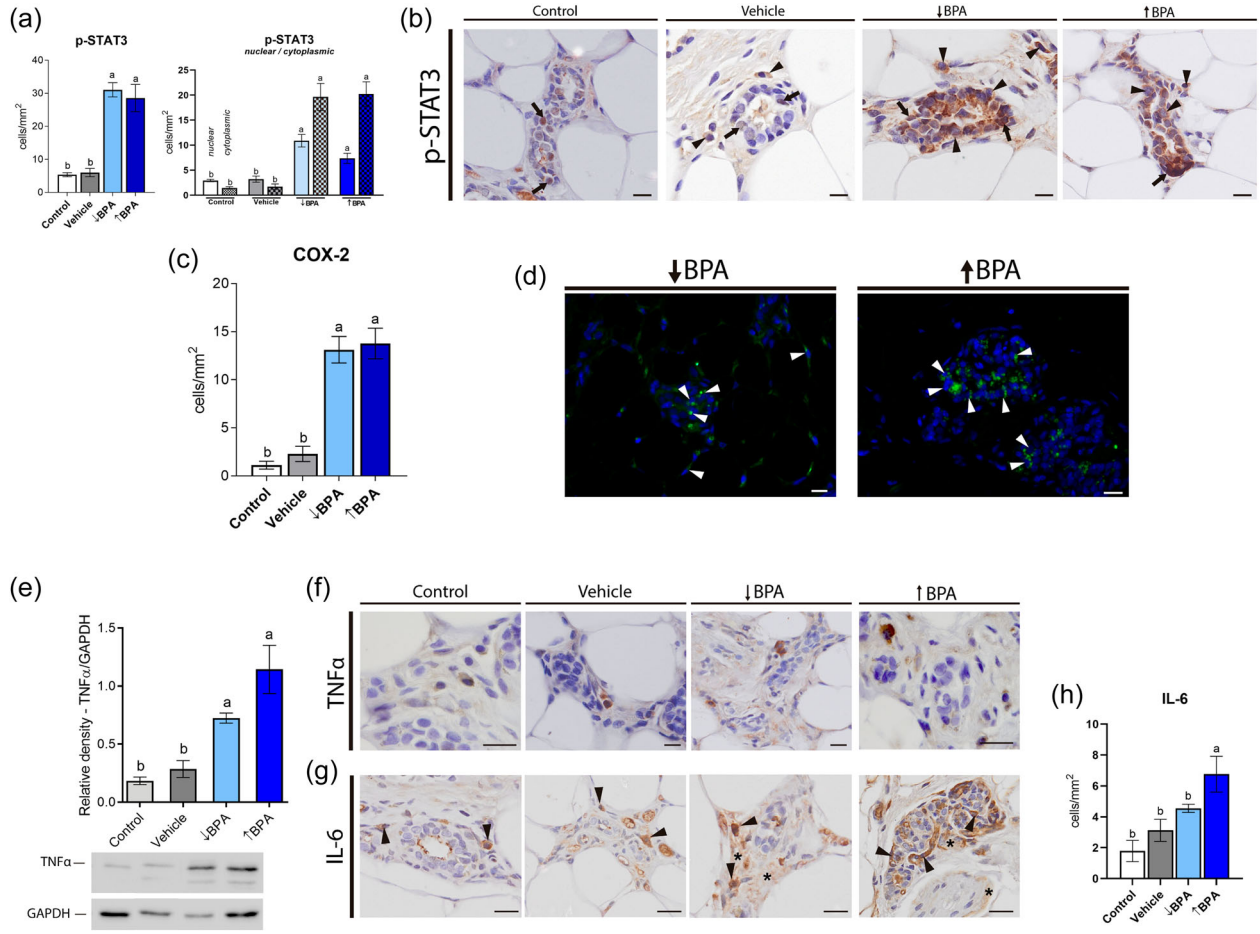

FIGURE 1 Inflammatory markers in the MG exposed to BPA. (a, b) p-STAT3. (a) p-STAT3-positive cells: total cells (*first*) and nuclear/cytoplasmic expression (*second*) graphs. (b) Representative figures of p-STAT3, *arrowheads* indicate cytoplasmic staining and *arrows*, the nuclear staining. (c, d) COX-2. (c) COX-2 positive cells. (d) Representative illustration of carcinoma cells in immunofluorescence expressing cytoplasmic COX-2 (*arrowheads*) in BPA groups. (e, f) TNF-α. (e) Western blot analysis analysis of TNF-α. (f) Immunohistochemistry for TNF-α. (g, h) IL-6. Cytoplasmic staining of stromal cells (arrowheads) and diffuse staining (asterisks) was demonstrated in immunohistochemistry and immunofluorescence assay. Graphs: Mean ± SEM is expressed in graphical figures; different letters (a, b, c) indicate the significant difference ($p < .05$) among experimental groups. Quantification was performed in IHC staining of all proteins. Scale Bars: (b) 20 μm; (d) 30 μm; (f) 20 μm; (g) 30 μm. IHC, immunohistochemistry; TNF-α, tumor necrosis factor-alpha.

p-STAT3 was observed in cytoplasm of stromal and epithelial cells (Figure 1b). Although, expression rates of nuclear and cytoplasmatic P-STAT3 in BPA groups were increased compared to control and vehicle groups (Figure 1a).

Control and vehicle groups presented the lowest values of COX-2 (cyclooxygenase-2) expression (Figure 1c), observed scarcely in stromal cells. Compared to control groups, expression of COX-2 increased almost 4-folds in BPA exposed animals. In BPA groups, COX-2 was expressed in the cytoplasm of normal epithelium, carcinoma cells, and surrounding stroma (Figure 1d). In IHC analysis, TNF-α-positive cells did not present statistical differences among groups (Figure 1e,f). We investigate the amount of TNF-α by Western blot analysis assay. This analysis detected an increase of this protein expression (concentration) in BPA groups (Figure 1e). IL-6 (interleukin 6) showed less cytoplasmic positivity of stromal cells in control and vehicle groups, whereas in ↑BPA group IL-6-positive cells were more increased (Figure 1g,h). Also, diffuse staining in stroma of BPA groups for IL-6 was confirmed by immunofluorescence (Figure 1g).

3.2 | Macrophages in TME promoted by BPA in aged females

Total macrophages in mammary tissue were evaluated by F4/80 IHC (Figure 2a,b). BPA groups presented high rates of macrophages in comparison to control and vehicle groups, which also differed between them. Clusters of macrophages were observed near to disrupted tissue; intraepithelial macrophages were also observed in BPA groups (Figure 2b). Furthermore, M1 ($CD68^{+}iNOS^{+}$) and M2 ($CD163^{+}$) macrophages were quantified and compared among groups (Figure 2c). BPA groups presented an increase in M1 macrophages

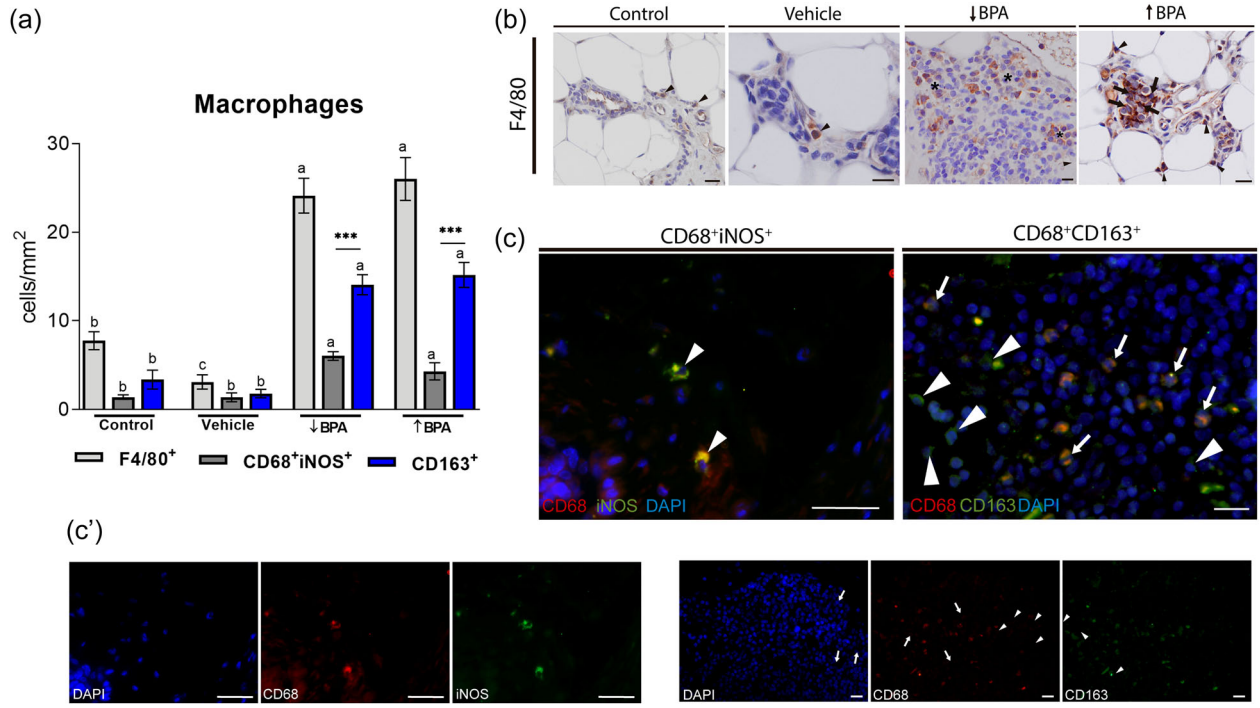

FIGURE 2 Macrophages in MG of aged females exposed to BPA during pregnancy/lactation window. (a) Macrophage population in mammary tissues. Total macrophages (F4/80$^+$), M2 macrophages (CD163$^+$) and M1 macrophages (CD68$^+$iNOS$^+$) expression in mammary tissue. Mean ± SEM is expressed in graphical figures; different letters (a, b, c) indicate the significant difference ($p < .05$) in the incidence of each cell type among experimental groups; significant difference ($p < .01$) between M1 and M2 incidence within ↓BPA and ↑BPA groups (*asterisks*) is shown. (b) Stromal F4/80$^+$ macrophages (*arrowheads*) and, carcinoma (*asterisks*) and intraepithelial (*arrows*) macrophages. (c–c') Tumor-associated macrophages (TAMs). M1 macrophages (CD68$^+$iNOS$^+$) and M2 macrophages (CD163$^+$). CD68$^+$CD163$^+$ (*arrows*) and CD68$^-$CD163$^+$ (*arrowheads*) in carcinoma features. (c') Representative channels of image (c). Cell incidence quantification was performed by IF staining. Scale Bars: (b) 20 μm; (c–c') 40 μm. BPA, Bisphenol A; MG, mammary gland; TNF-α, tumor necrosis factor-alpha.

compared to control and vehicle groups (Figure 2a). Also, M2 macrophages were twofold increased in ↓BPA and threefold increased in ↑BPA compared to M1 macrophages in the same group. Macrophages CD68$^+$ expressed TNF-α and COX-2 in peritumoral tissue and nondisrupted structures of BPA groups (Figure 3a). The percentages of CD68 + TNF-α+ and CD68 + COX2+ macrophages were enhanced in BPA groups (Figure 3a'). In addition, CD68 + /TGF-β1+ coexpression was increased compared to the control and vehicle groups (Figure 3b–b'). CD163$^+$ macrophages presented TGF-β1 positivity in BPA groups (Figure 3b), which was enhanced in comparison to the control groups (Figure 3b'). MMP9 was increased in c-localization with CD68$^+$ and CD163$^+$ macrophages (Figure 3c).

3.3 | MCs in TME promoted by BPA in aged females

Total MC were evaluated by toluidine blue staining in mammary tissue (Figure 4a,b). MCs were increased in BPA groups, mainly in ↑BPA. Analysis of tryptase$^-$/chymase$^+$ and tryptase$^+$/chymase$^+$ were performed to detect different mature MC populations (Figure 4a,c). Tryptase$^+$ MC (MC_T—tryptase$^+$/chymase$^-$) were enhanced in BPA groups, presenting higher rates in ↓BPA. Also, chymase$^+$ MC (MC_{CT}—tryptase$^+$/chymase$^+$) presented no statistical differences among control, vehicle and ↓BPA groups, whereas ↑BPA presented high rate of MC_{CT}. MC_T expressed TFG-β1 and MMP2 in the stromal compartment of BPA groups (Figure 4d), which were both increased (Figure 4d'). Also, MC_{CT} presented positivity for TGF-β1 (Figure 4d), incrased in BPA groups (Figure 4d'), and also expressed MMP2, which did not present significative difference among experimental groups (Figure 4d').

4 | DISCUSSION

We demonstrate in the present study the patterns of expression of inflammatory markers and cells in the TME of BPA-induced carcinogenesis. Both BPA dosages were able to promote an inflammatory stroma. This is a warning due to possible outcomes related to MG development and woman health risk, since the worldwide production of BPA is supposed to increase in next years (Rogers, 2021). Epithelial and stromal inflammatory responses indicate cancer prognosis of MG tissue and a possible target for therapeutics (Baumgarten & Frasor, 2012). Nonetheless, inflammation is a remarkable feature disturbed by endocrine disruptors, which unbalance the communication between compartments (Murata &

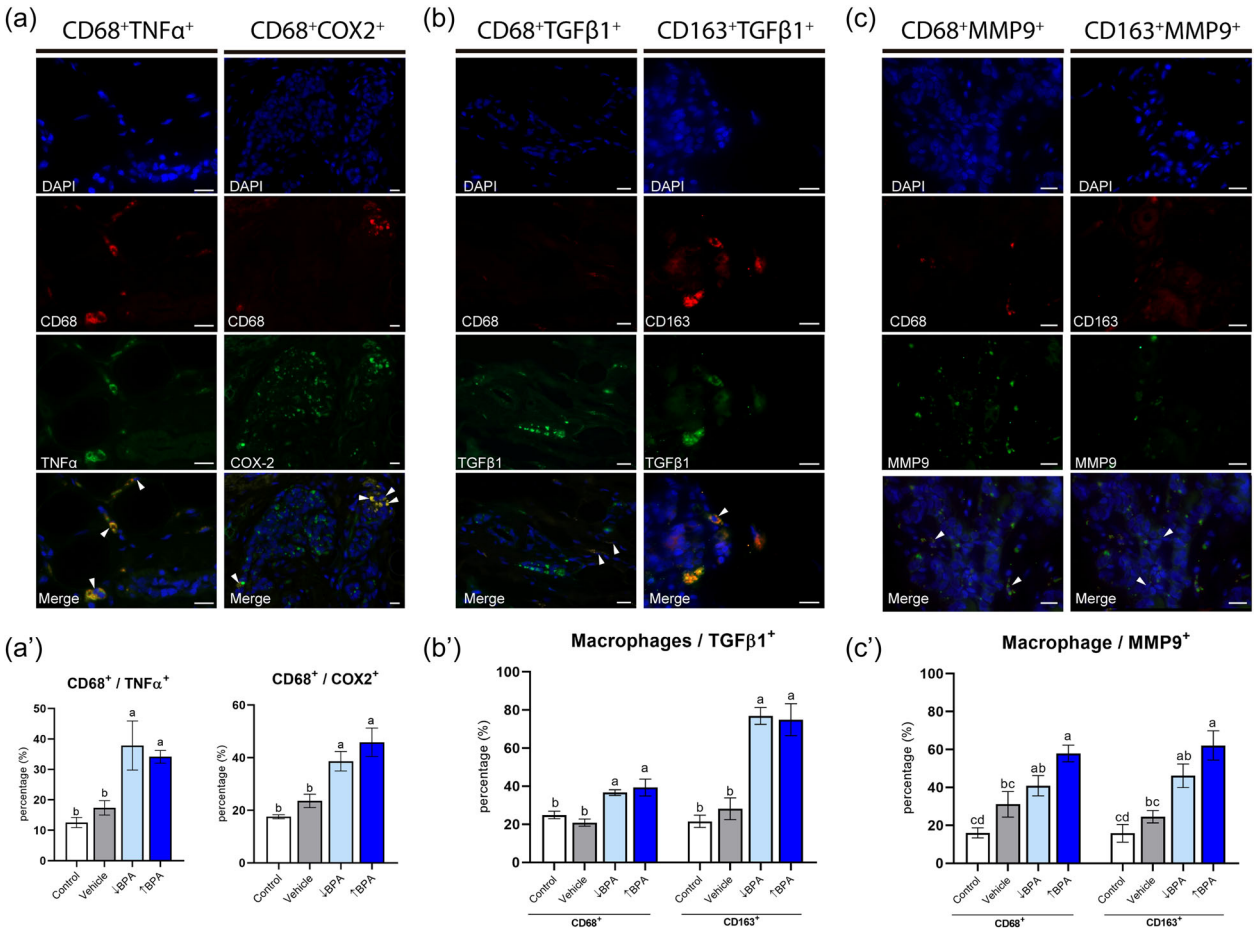

FIGURE 3 Expression of $CD68^+$ and $CD163^+$ macrophages of cytokines and inflammatory markers. (a–a') $CD68^+$ cells (*arrowheads*) expressing TNF-α and COX-2. (b–b') TGF-β1 (*arrowheads*) expressed by $CD68^+$ and $CD163^+$ cells, as well as (c–c') MMP9 (arrowheads). Graphs: Mean ± SEM is expressed in graphical figures; different letters (a, b, c) indicate the significant difference ($p < .05$) among experimental groups. Cell incidence and double expression quantifications were performed by IF staining. Groups: (a, b) ↓ BPA; (c) ↑ BPA. Scale Bars: (a–c) 20 μm. BPA, Bisphenol A; TNF-α, tumor necrosis factor-alpha.

Kang, 2018). We demonstrated that increased expression of COX-2 and p-STAT3 in mammary cells after BPA exposure, in addition to TGF-β1, are presented as pathways to establish neoplastic process and reactive surrounding stroma.

Tumor-associated macrophages are mainly activated and recruited via TGF-β1 signaling (Gratchev, 2017). In addition to the expression of $CD68^+$, M1 macrophages are also positive for iNOS and TNF-α (Mantovani et al., 2002). The presence of M1 phenotype is associated to an increasing tissue inflammation in the TME (Murray et al., 2014). Since BPA increases the expression of these proteins in epithelial cells and macrophages, the increase in M1 is probably linked to this proinflammatory cascade. We demonstrated that M1 macrophages expressed TGF-β1, a remarkable signaling molecule in BPA tumorigenesis in MG highlighted in recent studies (Ruiz, Colleta, Leonel, et al., 2021; Shen et al., 2018; Wang et al., 2019). TGF-β1 is amplified by COX-2 expression (Gan et al., 2016; Hugo et al., 2015) in addition to p-STAT3 (Clarkson et al., 2006), both high expressed in epithelial compartment in the present study. Thus, disruption of BPA in the MG of aged females could be linked to a communication between inflammatory markers expressed in the epithelium and stromal cells.

Also, the marker CD163 was used in the present work as a marker for M2 macrophages (M. G. Kim et al., 2020). Even though an anti-inflammatory function is signaled for this macrophage phenotype (He et al., 2014), studies suggest a stimulatory effect on tumor progression (M. G. Kim et al., 2020; Najafi et al., 2019). The proportion of M2 is almost twofold that of M1 in the MG of aged females exposed to BPA. Thus, M2 macrophage polarization was favored during BPA endocrine disruption. Differences in the number of these macrophage subtypes indicate favoring or balancing the TME in the differentiation (polarization) of this cell type (H. Kim et al., 2019; Mantovani et al., 2002). In a tumorigenic environment, M2 macrophages orchestrated by epithelial-derived cancer cells influence extracellular matrix (ECM) remodeling, mainly by MMP9 (Sousa et al., 2015). Also, M2 enhance is related to a poor prognosis due to its association to an invasive environment (Jeong et al., 2019). BPA-promoted polarization by estrogenic pathways (Teixeira et al., 2016)

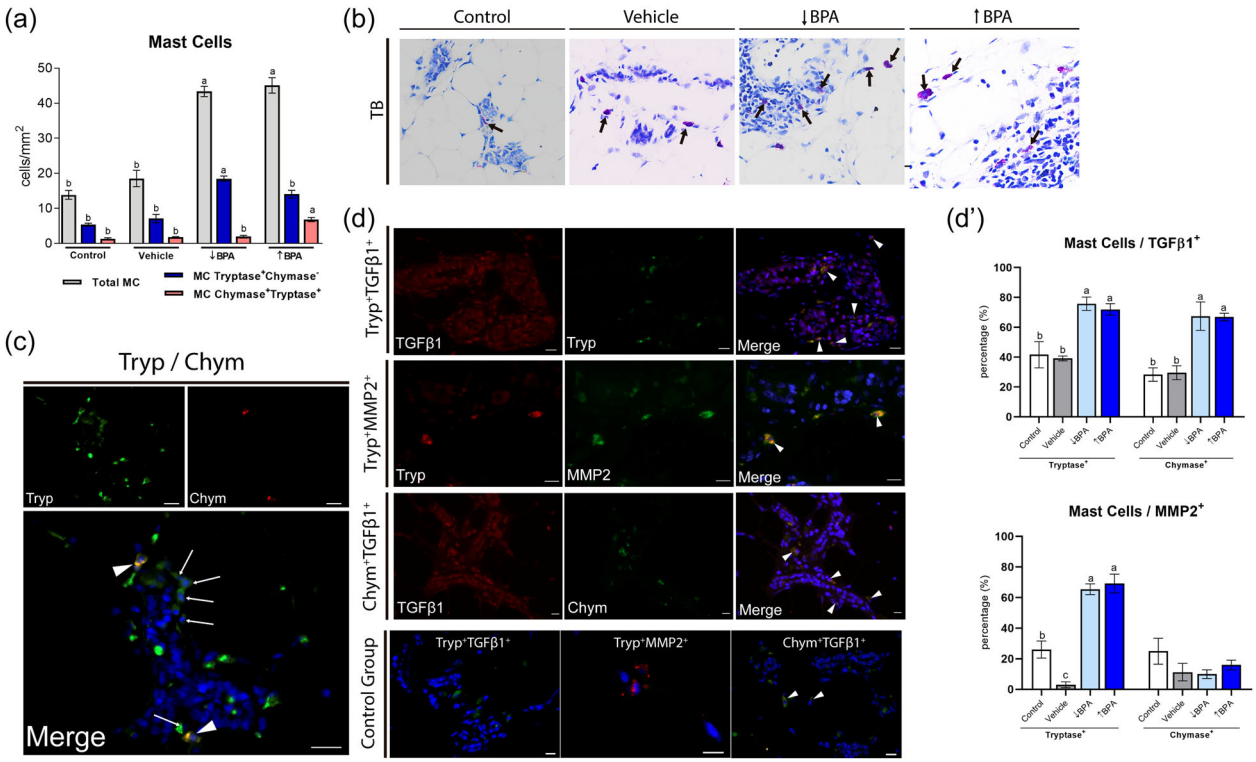

FIGURE 4 MC in MG of aged females exposed to BPA during pregnancy/lactation window. (a) MC population in mammary tissues. Total MC by toluidine blue (TB), Tryptase$^+$Chymase$^-$ cells and Tryptase$^+$Chymase$^+$ cells were quantified. (b) Toluidine blue (pH 4) presented a metachromatic reaction (purple) with MC cytoplasm/granules (*arrows*). (c) MC population. MCT (Tryptase$^+$Chymase$^-$, *arrows*) and MCCT (Tryptase$^+$Chymase$^+$, *arrowhead*). (d) Tryptase$^+$ cells expressing TGF-β1 in neoplasia and MMP2 in stroma of BPA exposed MG. Chymase$^+$ cells also express TGF-β1 (arrowhead). Control group was also shown. (D') Percentage of MC subtypes expression of TGF-β1 and MMP2. Graphs: Mean ± SEM is expressed in graphical figures; different letters (a, b, c) indicate the significant difference ($p < .05$) among experimental groups. MCT and MCCT were quantified by double immunohistochemistry for CD68 and CD163. The expression of TGF-β1 and MMP2 was quantified by double immunohistochemistry with CD68 or CD163, and the data were presented in percentage of the total of each subtype of MC that expressed the proteins. Cell incidence quantification and protein colocalization were performed by IF staining. Groups: (c), (d—Tryp$^+$TGF-β1$^+$) $^{\downarrow}$BPA; (d—Tryp$^+$MMP2$^+$; Chym$^+$TGF$^-$β1$^+$) $^{\uparrow}$BPA. Scale Bars: 20 μm. BPA, Bisphenol A; MG, mammary gland; TNF-α, tumor necrosis factor-alpha.

that were favored in the MG of aged females, as previously shown (Ruiz, Colleta, Zuccari et al., 2021).

MC are responsible for important events in tumor signaling and ECM remodeling during carcinogenesis (Komi & Redegeld, 2020). We demonstrated that exposure to BPA during pregnancy and lactation increases MC recruitment to mammary tissue in the onset of neoplastic development in aging. MC_T was the increased phenotype in the stroma of both BPA exposure doses, whereas MC_{CT} was more recruited in MG exposed to high dose BPA, probably as a long-term response to a high exposure. MC_T initiates differentiation and proliferation of fibroblasts and stromal remodeling by releasing degradative proteases, such as MMPs, in the TME (Mangia et al., 2011). MMP9 is expressed by macrophages (Zollo et al., 2014) and MC (Xu et al., 2017) in response to active estrogenic pathways (Inman et al., 2015). Also, MC_T is involved with collagen remodeling in the ECM (Westbury et al., 2014). Expression of MMPs was observed in differentiated MC, such as MC_T and MC_{CT}, after endocrine disruption by BPA. Both MMP-2 and MMP-9 expressed in active MC phenotypes hardly contribute to neoplastic-associated tissue remodeling (Di Cara et al., 2018).

In addition, MC_T is related to increasing of angiogenesis and micrometastasis in the early stages of carcinogenesis (Ranieri et al., 2009). Mainly in breast carcinoma, an increase of MC_T (Kankkunen et al., 1997) is associated with invasion and migration (Liu et al., 2011). In this condition, MC_T are found peri and intratumor and are also associated with the communication process between disrupted/cancerous cells and stromal compartments (Khan et al., 2013). This crosstalk between disrupted epithelial cells and MC_T could be facilitated by chemotactic action of TGF-β1 (Ramírez-Valadez et al., 2017). Since it was enhanced in coexpression of MC markers in MG from aged females exposed to BPA, MC communicates with breast cancer cells via TGF-β1, promoting their proliferation and stromal migration (Zollo et al., 2012). As we described previously (Ruiz, Colleta, Leonel, et al., 2021), TGF-β1 is a potential candidate for mediating the EMT process in BPA disrupted tissues. Also, EMT influences this process through inflammatory cytokines that enable tumor cell communication, and prepares invasion fields for cancerous cells (Aponte-López et al., 2018).

BPA induced an increase in mature MC, contrarily to what was observed in control and vehicle groups, which had low number of total MC. This demonstrates the recruiting and maturation effect of this cell type in MG exposed to BPA, as well as an unexplored molecular mechanism of cell-mediated TME (Leoh et al., 2015). Also, the increase in TNF-α and TGF-β1 by BPA in other cell types may be associated to the increase of mature MC in these groups and the development of a TME (Fleming et al., 2012). In addition to TNF-α, IL-6 mediates inflammation via MC, promoting macrophage activation (Dahdah et al., 2014) and MC own activation (Elieh Ali Komi et al., 2020). Thus, BPA could modulate MC self-activation and co-activation of inflammatory cells during MG carcinogenesis in aged females (Figure 5).

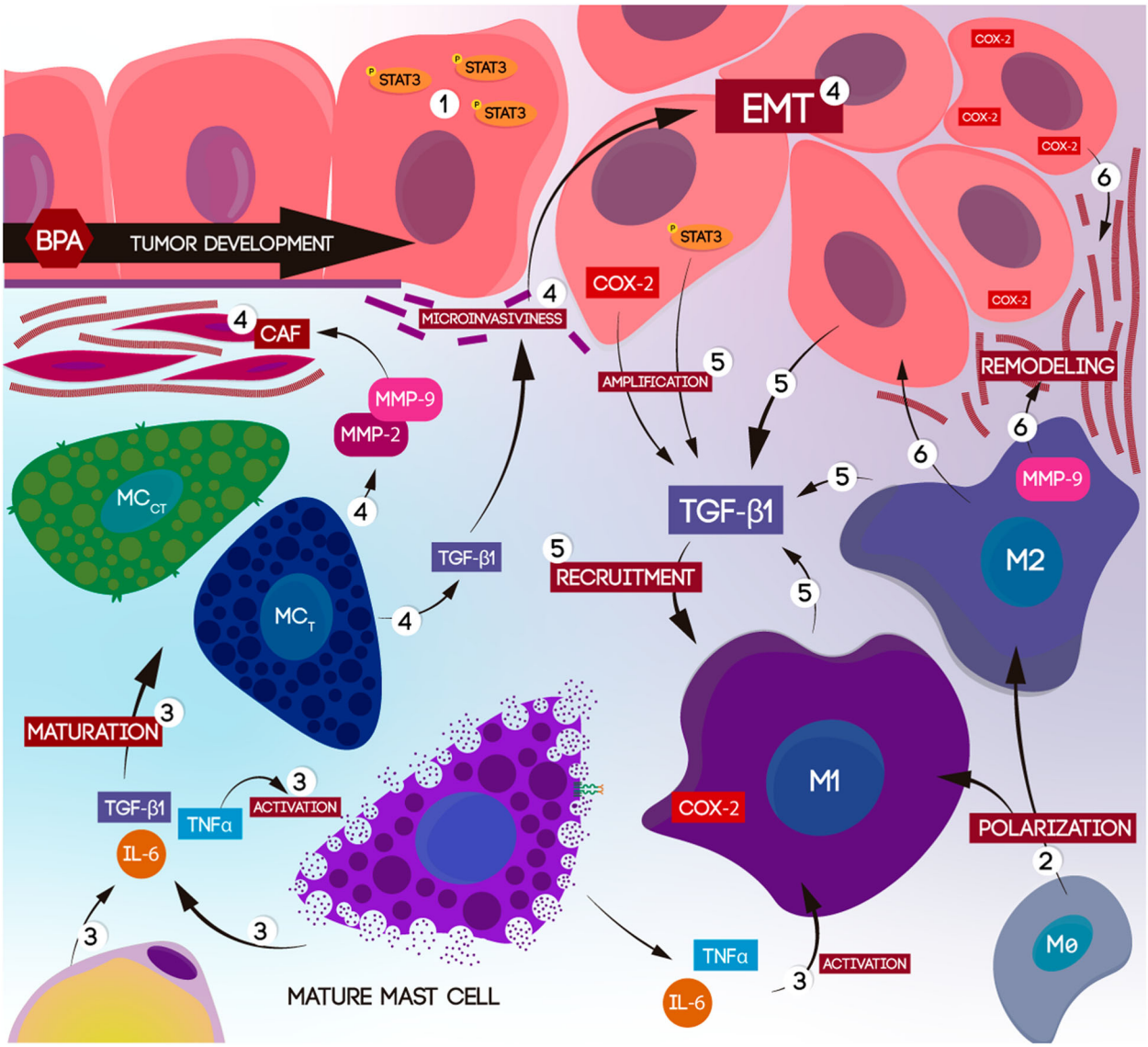

FIGURE 5 Tumor microenvironment in MG from aged females exposed to BPA. (1) p-STAT3: cytoplasmic expression in epithelial normal and neoplastic cells in disrupted tissue. (2) Macrophage polarization: BPA interfered in the polarization of resident and recruited macrophages. Induction of M2 protumoral phenotype and M1 proinflammatory phenotype was stimulated by BPA. (3) Activation and maturation of M1 macrophages and MC: IL-6 and TNF-α expressed and released by mature MC act as activation-key chemokines for M1 and MC. With TGF-β1, these chemokines act in maturation of MC_T ($Tryptase^+Chymase^-$) and MC_{CT} ($Tryptase^+Chymase^+$). (4) Role of MC_T: expression of MMP2 and MMP9 are associated with remodeling of stromal compartment. These proteases alter fibrillar elements of ECM and promote the activation of fibroblasts, influencing the recruitment of cancer-associated fibroblasts (CAF). Also, MCT participates in microinvasive profile and epithelial-mesenchymal transition of neoplastic cells through TGF-β1 pathway. (5) Role of M1 macrophages: this tumor-associated macrophage phenotype acts expressing COX-2 and TGF-β1. COX-2 and p-STAT3 expressed by carcinoma cells amplified TGF-β1 signaling pathway to recruit inflammatory cells, particularly macrophages. TGF-β1 expressed by carcinoma cells, M1 and M2 also enhances the TGF-β1 synthesis and activity in these cells. (6) Role of M2: M2 macrophages express MMP9 that exerts a remodeling activity in ECM collagen. COX-2 expressed by carcinoma cells also contributes to matrix remodeling, promoting a metastatic environment. ECM, extracellular matrix; MC, mast cells; TNF-α. tumor necrosis factor-alpha; TNF-β1, tumor necrosis factor-beta 1.

5 | CONCLUSION

In summary, BPA modulates the progress of the TME in MG of aged females during the onset of neoplasia. Mammary cells exposed to BPA express COX-2 and p-STAT3 that contribute to EMT process and inflammation. M1 and M2 macrophages phenotypes promote the TME establishment. MC enhances inflammatory-chemokine signaling and communication for EMT progress of BPA-disrupted epithelial cells. Thus, BPA can induce an TME in MG of aged females by inflammatory responses and signaling, which also contributes to tumor growth and invasiveness.

AUTHOR CONTRIBUTIONS

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DATA AVAILABILITY STATEMENT

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

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