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Research

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Novel intratumoral treatment with 5-Androstene-3 β , 17 α -diol inhibits tumor size and metastasis in a mice model

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20 Abstract

21 **Background:** Breast cancer treatment-failure is related to low compliance rates, high costs,
22 and long-term toxicities. Thus, it is necessary to find less toxic and cheaper treatments. The
23 epimereandrostene-3 β ,17 α -diol (α -AED) has been proven to inhibit breast tumor cell
24 proliferation *in vitro*, being an ideal candidate to treat mammary tumors. The aim of this study
25 was to evaluate the *in situ* effects of α -AED on tumor mammary growth. 4T1 tumors with 14
26 days of growth were injected with an equivalent dose to the IC₅₀ (100 μ M) and its double
27 concentration (200 μ M) of α -AED. **Methods:** The cell viability and cell proliferation of murine
28 and human cancer cell lines after the treatment of α -AED were evaluated with
29 sulforhodamine assay and bromodeoxyuridine incorporation, respectively. We also
30 evaluated the size and tumor growth of the orthotopic tumor model after the treatment with
31 α -AED. The tumor infiltration changes and cytokine determination into the tumor
32 microenvironment were determined by flow cytometry and ELISA methods, respectively.
33 The humoral response denoted by the production of IgG anti-4T1 antibodies were also
34 determined by ELISA method. **Results:** Low and high concentrations of α -AED administered
35 intratumorally reduce the size and average tumor. α -AED also increased the percentages of
36 NK, plasmatic and plasmablast cells in mice tumors injected with 100 μ M of α -AED.
37 Meanwhile, tumors injected with 200 μ M of α -AED contained an elevated proportion of
38 plasmablast and B cells. Of notice, VEGF levels in all α -AED-treated tumors were lower than
39 the control and vehicle group. The tumor *in situ* response was reflected systemically by the
40 increase of the anti-4T1 IgG in serum from α -AED-treated mice, but no other associated
41 systemic changes were detected. The reduction in tumor size for to the local injection of α -
42 AED is produced by the anti-proliferative effect of this hormone, that maybe associated to

43 the VEGF reduction and metastasis. **Conclusion:** We observed an astonishing tumor size
44 reduction and metastasis after the treatment with α -AED. The above suggest that α -AED
45 can be used in clinical studies in order to prove its efficacy as an alternative of novel breast
46 tumor treatment.

47

48 **Keywords:** Breast tumor, intratumoral treatment, alpha-androstenediol, translational
49 medicine, metastasis, tumor microenvironment.

50

51 **Background**

52 There is a broad range of accepted therapeutic approaches to treat breast cancer to this
53 day. These treatments have as an objective to reduce tumor size and risk of metastasis
54 development **(1)**. Among these therapies, the estrogen antagonist, tamoxifen, and
55 aromatase inhibitors are commonly used as adjuvant therapy to treat hormone receptor-
56 positive breast cancer despite that toxicity associated with these treatments is related to low
57 compliance rates and long-term toxicities **(2)**. Additionally, patients with triple-negative
58 breast cancer (TNBC) do not benefit from anti-estrogenic therapy **(3)**.

59

60 Aside from estrogenic antagonists, other steroid compounds such as
61 dehydroepiandrosterone (DHEA) have been proven to inhibit the proliferation and migration
62 of breast cancer cell lines *in vitro*, and to prevent the development of breast cancer after
63 mutagen administration **(4, 5)**. Another steroid that possesses a potent anti-proliferative
64 effect, is the DHEA-analogue named 5-Androstene-3 β , 17 α -diol (α -AED). This hormone is
65 demonstrated to be more effective in the inhibition of MCF-7 and MDA-MB231 breast cancer
66 cell lines **(4, 6)** . In regard to α -AED, its antiproliferative effect is independent of either the α
67 estrogen or the androgen receptors **(7)**.

68

69 The androstene α -AED is an epimer of 5-Androstene-3 β , 17 β -diol (β -AED), which has been
70 shown to upregulate immune activity **(8)**. Both steroids occur naturally, being α -AED found
71 to be secreted in the spermatic vein of human testes **(9)**. This steroid is also been found in
72 amniotic fluid and fetal-placenta circulation of normal pregnancies and low levels were
73 related to pathological pregnancies linked to diabetes, toxemia and placental insufficiency
74 **(10)**.

75

76 Despite the fact that androstenes are related to immune-regulation, the main effect of α -
77 AED is on the proliferation of tumor cells. Their effects have been widely studied on *in vitro*
78 experiments in order to evaluate changes on the immune system and the development of
79 tumor cells, which have been done independently. Thus, the resulting interaction between
80 the immune system and cancer context remains unknown. The interaction aforementioned
81 is relevant knowing that immune cells influence the microenvironment of the tumor and can
82 determine if the tumor development halts or progresses (11, 12).

83

84 In addition to immune cell proportions and phenotypes, other important drivers in the tumor
85 microenvironment are the soluble factors that play an important role in the immune tumor
86 milieu. In this sense, angiogenesis is associated with tumor nutrient supply and metastasis.
87 Besides, the angiogenesis is importantly drive through the secretion of Vascular Endothelial
88 Growth Factor (VEGF) by tumor and immune cells in response to hypoxia (13). Hence,
89 elevated levels of VEGF correlate with lymph node metastasis augmentation and worse
90 prognosis in patients with breast tumors (14).

91

92 All things considered; it is possible to directly modify the tumor microenvironment by a direct
93 administration of pharmacological agents (15). Thus, to address the local
94 microenvironmental modifications of the tumor and immune cells by α -AED, this compound
95 was administered directly into mice tumors induced by 4T1 mammary tumor cells.

96

97 **Materials and Methods**

98 **Ethic statement**

99 All the experimental procedures and animal studies were performed within the standards
100 established by the Institutional Care and Animal Use Committee (CICUAL) (permit number
101 2017-208), in accordance with Mexican regulation (NOM-062-ZOO-1999) and with the
102 Guide for the Care and Use of Laboratory Animals of the National Institute of Health (NIH)
103 of the United States of America. Animal procedures were performed at the IIB, UNAM, in
104 the Biological Models Unit (Unidad de Modelos Biológicos, UMB).

105 **Animals**

106 Female BALB/c AnN mice (MGI Cat# 5654849, RRID: MGI:5654849) of 8 to 9 weeks old,
107 were obtained from Envigo México (Facultad de Química, UNAM, México). The animals
108 were housed 4-5 per with 12 hours of alternating light at 22 °. Food (Envigo LabDiet 5015 -
109 Cat# 0001328 Purina, St. Louis, MO-) and water were delivered *ad libitum* in sterile
110 conditions.

111 **Cell culture**

112 The 4T1 murine mammary carcinoma cell line and the HCC1937, a human breast cancer
113 cell line (ATCC Cat# CRL-2539, RRID: CVCL_0125) were cultured in RMPI 1640 medium
114 (Sigma, St. Louis, MO) supplemented with 10% of fetal bovine serum (FBS) for murine cells
115 and 5% of FBS for human cell line (ByProducts, Guadalajara, Mexico), 1.0 mM sodium
116 pyruvate, 100 units/mL penicillin, and 100 mg/ml streptomycin. 4T1 cells were maintained at
117 37°C, 5% CO₂ atmosphere, and 95% humidity.

118 **Cell viability assay**

119 For the Sulforhodamine B (SRB) assay (Sigma, St. Louis MO), 4T1 and the triple-negative
120 breast cancer cell line; HCC1937, were seeded in 96-well culture plates (1x10³- 2x10³ cells
121 per well) in media supplemented with 5 or 10% heat-inactivated and charcoal treated-FBS,

100 units/mL penicillin plus 100 µg/mL streptomycin and maintained with a 5% atmosphere of CO₂ at 37°C and 95% humidity. 24 hours after seeding, cells were treated with vehicle (0.1 % v/v EtOH) or with increasing concentrations of α -AED and incubated for 72 hours. Next, cell viability was obtained using SRB assay that determines cell density by measuring protein content, performed according to (16), with modifications. In brief, cells were fixed with 100 µl of cold trichloroacetic acid (10%) for 1 hour, washed 3 times with water, and air-dried overnight. Afterward, cells were stained with 100 µl of SRB 0.057% in 1% acetic acid during 30 min, washed with 150 µl of acetic acid (1%), and air-dried for 2 hours. Next, 100 µl of 10 mM Tris Base solution (pH 10) were aggregated, to solubilize the protein-bound dye, and the plates were agitated during 30 min. Plates were read at 492 nm in a Stat Fax 4200 microplate reader (Awareness Technology). Data were normalized to run between 0-100% and 0-1 as follows: Cell viability= optical density (OD) 492 in treatment wells/OD492 in control wells x 100 and x1, respectively. Experiments were performed in triplicate on 3 different occasions. The values of the IC₅₀ were calculated with Prism 6® software (GraphPad Software Inc.).

Cell proliferation assay

To quantify 4T1 cell proliferation, we determined the Bromodeoxyuridine (BrdU) incorporation during DNA synthesis. This assay was performed in cells maintained in the same culture conditions that the ones for SRB assay and treated with vehicle, 100 µM, and 200 µM, during 72 hours. Procedures were performed according to the manufacturer's protocol (BD Apoptosis, DNA Damage and Cell Proliferation Kit, Cat 15821759, Thermo Fischer Scientific) with PerCP-Cy5.5 Mouse Anti-BrdU. Cell analysis was performed with BD FACSCalibur™ (BD Biosciences) flow cytometer and the data were analyzed with

145 FlowJo software (Treestar Inc.). Compensation was assessed in BD FACSCalibur™ and
146 FlowJo software with unstained samples.

147 **Orthotopic tumor cell induction**

148 After a second subculture at 80% of confluency, 4T1 cells were harvested and suspended
149 in sterile 0.9% NaCl solution in a concentration of 250,000 cells/ml. They were conserved in
150 ice until the inoculation into the mice. For the mammary tumor induction, mice were
151 anesthetized with Sevoflurane 5% (Abbot, Mexico), the abdominal area was cleaned with
152 EtOH 70%, and in the fat pad under the second last right nipple were injected
153 subcutaneously 4T1 cells 10^4 . Mice recovery was supervised.

154 **Tumor model and α -AED treatment**

155 To test the intratumoral effect of α -AED on the tumor growth, female mice were randomized
156 into four experimental groups: 1) 4T1 group of animals with untreated tumors (12); 2) Vh
157 group with tumors injected with 40 μ l of corn oil-vehicle (14); 3) α -AED 100 μ M group of mice
158 with intratumoral injection of 580 ng of α -AED in 40 μ l of corn oil (15), and 4) α -AED 200
159 μ M: intratumoral injection of 1160 ng of α -AED (12), 40 μ l. Tumor growth was observed for
160 28 days. Finally, the tumor weight was obtained at the moment of the euthanization at day
161 28 post-inoculation.

162 **Flow cytometry**

163 The left and right peripheral (inguinal) lymph nodes (PLNs) and the spleen were excised
164 and mechanically disaggregated through a 50 μ m nylon mesh with PBS. Tumors were
165 excised and minced with a scalpel. After the PBS wash, the cells of the lymph nodes were
166 resuspended in FACS buffer (PBS, 2% FBS, 0.02% NaN₃). Erythrocytes in splenic
167 suspension were lysed for 10 minutes with ACK buffer (150 mM NH₄Cl, 10 mM KHCO₃, 0.1

168 mM Na₂ EDTA, pH 7.3), washed with PBS and resuspended in FACS buffer. The minced
169 tumors were incubated in digestion medium (RPMI 1640, 10 U/ml DNase, Roche,
170 Mannheim, Germany; 0.5 mg/ml type IV Collagenase, Sigma, St. Louis, MO) for 20 minutes,
171 and 50 µl FBS was added to stop digestion. Mechanical disruption in a 50 µm nylon mesh
172 was performed. After the PBS wash, the cells were resuspended in FACS buffer.
173 Approximately 1x10⁶ cells were incubated (20 minutes at 4°C) with anti-CD16/CD32
174 (TruStain®, Cat# 101319, Clone 93, RRID:AB_1574973, BioLegend, San Diego, CA) and
175 washed. Then, they were stained with the following panels. For T lymphocyte:
176 AlexaFluor®488-conjugated anti-CD3ε (Cat# 100321, Clone 145-2C11, RRID:AB_389301)
177 1:100, PE-conjugated anti-CD4 (Cat# 100407, Clone GK1.5, RRID:AB_2075573) 1:300,
178 PerCP-conjugated anti-CD8 (Cat# 100732, Clone 53-6.7, RRID:AB_893423) 1:100, and
179 AlexaFluor®647-conjugated anti-Foxp3 (Cat# 320013, Clone 150D, RRID:AB_439750)
180 1:100. For macrophage and NK: AlexaFluor® 647-conjugated anti-F4/80 (Cat# 123122,
181 Clone BM8, RRID:AB_893492) and PE-conjugated anti-NKp46 (Cat# 137604, Clone
182 29A1.4, RRID:AB_2235755). To B lymphocyte: PE-conjugated anti-CD19 (Cat# 115507,
183 Clone 6D5, RRID:AB_313642), 1:200, plasmatic cells: Brilliant Violet 421™ anti-mouse
184 CD138 (Syndecan-1) (Cat# 142507, Clone 281-2, RRID: AB_2565621). Antibodies from
185 BioLegend, San Diego, CA, and the Foxp3/Transcription Factor Staining Buffer kit (Cat#
186 TNB-0607-KIT, Tonbo Biosciences, San Diego, CA) were used for intracellular Foxp3
187 staining, according to the manufacturer's protocol.

188 Cell analysis was performed with a BD FACSCalibur™ (BD Biosciences) flow cytometer.
189 The data was analyzed with FlowJo software (Treestar Inc.). Compensation was assessed
190 in BD FACSCalibur™ and FlowJo software with unstained samples, single stain controls,
191 and FMO for Foxp3⁺ (CD3⁺/CD4⁺; CD3⁺/CD8⁺).

Cytokine determination

The tumors from the mice were stored in TRIzol™ reagent (Cat# 15596026, Invitrogen) at -70°C until use. Protein isolation was performed according to the procedural guidelines for TRIzol® reagent use. Protein quantification was done with a NanoDrop 1000 spectrophotometer (Thermo Scientific). An amount of 10 µg of protein was used to determine cytokine tissue levels.

Tumor cytokines were measured with ABTS ELISA kits (PeproTech) with the following antibodies: TNF-α (Cat# 500-P64bt, RRID:AB_147984), IFN-γ (Cat# 500-P119bt, RRID:AB_148087), IL-4 (Cat# 500-P54bt, RRID:AB_147636), IL-5 (Cat# 500-P55), and IL-10 (Cat# 500-P60, RRID:AB_147978), and unconjugated antibodies were used for cytokine capture, according to the manufacturer's instructions, with modifications. Briefly, coated plates (96-well plate, MaxiSorp Nunc Cat# NNC#442404) with 50 µl (2 µg/ml) of different antibodies were incubated overnight. After 3 washes (wash buffer, PeproTech), the plates were blocked (block buffer: PeproTech) and then washed again. 50 µl of sera (1:2 dilution) or tissue protein (10 µg) was added in duplicate (in diluent solution, PeproTech), maintained at 4°C for 2 h, and washed three times. An enzyme-substrate reaction was developed with ABTS liquid substrate (PeproTech). All solutions were from ABTS ELISA buffer kit (Cat# 900-K00). The plates were read at a wavelength of 405 nm with a wavelength correction set at 650 nm at different time points in a Stat Fax 4200 microplate reader (Awareness Technology).

VEGF quantification

Polystyrene wells (96-well plate, MaxiSorp Nunc Cat# NNC#442404) were coated with 50 µl of tumor protein (10 µg) or standard curve (0.001-1 ng) with VEGF mBA-165 (Cat# sc-4571, Santa Cruz Biotechnology) in bicarbonate buffer (pH 9.6) per duplicate and incubated

216 at 4°C overnight. The plate was washed and blocked with 200 µl of PBS/BSA 1%/Tween 20
217 0.05% for 1 hour at 4°C. After washing, 50 µl of anti-VEGF/C-1 antibody (Cat# sc-7269,
218 RRID:AB_628430, Santa Cruz Biotechnology) in a 1:200 dilution was added and incubated
219 for 1 h at 4°C. After washing, 50 µl of m-IgGκ/BP-HRP (Cat# sc-516102, RRID:AB_2687626,
220 Santa Cruz Biotechnology) (1:400) was added and maintained for 2 hours at room
221 temperature. An enzyme-substrate reaction was developed with 50 µl of substrate solution
222 and stopped after 15 minutes with 50 µl 2N sulfuric acid. The plates were read at a
223 wavelength of 492 nm in a Stat Fax 4200 microplate reader (Awareness Technology).
224 Cytokine and VEGF concentrations were calculated by interpolation from a standard curve.

225 **IgG detection**

226 Coated polystyrene wells (96-well plate, MaxiSorp Nunc Cat# NNC#442404) with 50 µl of
227 mice sera/bicarbonate buffer (pH 9.6) suspension (1:1000) or with 1 µg/1ml of 4T1 crude
228 extract, overnight at 4°C. The plate was washed three times with (PBS/Tween 20 0.05%)
229 and blocked with 200 µl of 3% bovine serum albumin (BSA) and washing solution, for 30
230 minutes at room temperature. The plate was washed as mentioned. For the anti-4T1 IgG
231 determination plates were incubated with mice sera (1:200) for 2 hours at room temperature
232 and washed. In both cases were added 50 µl of peroxidase goat anti-mouse IgG (Jackson,
233 RRID:AB_2338511) at 1:10,000 dilution was added during 90 min at room temperature. An
234 enzyme-substrate reaction was developed by the addition of 50 µl of freshly prepared
235 substrate solution (0.05% o-phenylenediamine/0.01% H₂O₂/0.1 M sodium citrate/0.1 M citric
236 acid) and stopped after 10 min with 50 µl 2N sulfuric acid. The plate was read at a
237 wavelength of 492 nm in a Stat Fax 4200 microplate reader (Awareness
238 Technology). Cytokine, VEGF and antibody determination were performed after proper
239 ELISA standardization.

Statistical analysis

Data were charted as mean $\pm$ SD. To compare the differences between a non-parametrical Kruskal-Wallis test and Dunn test for multiple comparisons were used. The differences were considered significant when $p \leq 0.05$. All the analyses were calculated with Prism 6® software (GraphPad Software Inc.).

Results

α -AED treatment reduce the viability of murine mammary tumor 4T1 cells and human breast cancer cells *in vitro*

To obtain the IC₅₀ value of α -AED, we measured the viability of the 4T1 cell line after the *in vitro* exposition to increasing concentrations of α -AED (1×10^{-12} to $1 \times 10^{-3.3}$ Molar) during 72 hours (Fig. 1A). SRB assay was used to quantify the cell density through the determination of cellular protein content. The IC₅₀ value was determinate at $1 \times 10^{-4.035}$ with an interval of $1 \times 10^{-4.253}$ to $1 \times 10^{-3.817}$ Molar. Of notice, low concentrations of α -AED (1×10^{-12} to 1×10^{-8} Molar) slightly increased 4T1 cell density, meanwhile, high doses (1×10^{-6} to $1 \times 10^{-3.3}$ Molar) reduced the quantity of 4T1 cells (Fig. 1A, B). This reduction was 3.2 ($p \leq 0.01$), 2.4 ($p \leq 0.05$), and 2.8 ($p \leq 0.01$) times lower than the vehicle, with 1×10^{-4} Molar (100 μ M), $1 \times 10^{-3.7}$ Molar (200 μ M) and $1 \times 10^{-3.3}$ Molar (500 μ M) concentrations, respectively (Figure 1B).

We also assessed the effect of α -AED on the viability of the triple-negative HCC1937 breast cancer cell line in order to compare the results found in the murine cell line with the effects of α -AED in a human breast cancer cell counterpart. The data showed that after 72 hours, the treatment of α -AE significantly increase the cell density of the HCC1937 cells from concentrations between 1×10^{-12} – 1×10^{-8} Molar. However, the cell number was drastically

decreased only at 1×10^{-7} Molar, being less than 50% with respect to the control and with respect to the other concentrations tested (Figure 1C).

In order to assess the α -AED anti-proliferative effect, we measure BrdU incorporation in 4T1 proliferating cells treated with vehicle (EtOH 0.2%), α -AED 1×10^{-4} Molar (100 μ M) and twice this concentration ($1 \times 10^{-3.7}$ Molar or 200 μ M). Average BrdU positive-cells was 9-fold lower when treated with α -AED 1×10^{-4} Molar (100 μ M) and 4-fold inferior with $1 \times 10^{-3.3}$ Molar (200 μ M) ($p \leq 0.01$ and $p \leq 0.05$, respectively), compared with vehicle-treated cells, (Figure 1D and 1E).

Intratumoral injection with α -AED reduced tumor size and weight

After 28 days from 4T1 cell inoculation and 14 days after intratumoral injection, the mean weight of tumors treated with α -AED was smaller than the untreated (4T1) and the vehicle (Vh) injected tumors (Fig. 2A). As observed *in vitro* (Fig. 1), the average weight of tumors treated with α -AED 100 μ M (1×10^{-4} Molar) was smaller than the treated with twice this dose (200 μ M= $1 \times 10^{-3.3}$ Molar) (Fig. 2A). Tumor mass weight was 65% and 50% smaller than α -AED treated-groups (α -AED 100= p 0.0001, α -AED 200= $p \leq 0.05$), when compared with the Vh group.

α -AED *in situ* treatment decreased metastatic-like macroscopic lung lesions

Aside to assess the effect of α -AED on tumor growth, we detected metastatic-like lesions on the lung surface. These macroscopic lesions were much more evident in the non-treated tumors (4T1), as well as, in vehicle-treated (Vh), while in the α -AED there was a clear decrease in the lesions (Figure 3).

Diminished tumor growth is associated with minor changes in the tumor microenvironment

Androstene steroids have been linked to immune response regulation (17), therefore, we determined some cell populations of innate (macrophages and NK cells) and adaptive (T and B lymphocytes) immune response within the tumor. Mean percentages of F4/80⁺ macrophages and T cells (CD3⁺, CD4⁺, CD8⁺, CD4⁺Foxp3⁺) intratumoral cells, remains without statistical difference (Fig. 4). Meanwhile, the percentage of NK cells was higher ($p \leq 0.01$) in tumors, when treated with 100 μM (Fig. 4F).

Humoral response B cells in the tumor

Aside from NK cell proportion rise, some changes were also noticed in regard to the mean proportion of humoral-related cells inside the tumor (Fig. 5). The first of these changes was the elevated percentage ($p \leq 0.05$) of plasmatic cells (CD138⁺) in α -AED 100 μM treated group (Fig. 5A), the second was the increase of B lymphocytes (CD19⁺) proportion ($p \leq 0.05$) in α -AED 200 μM (Fig. 5C), and the third was associated to plasmablast cells (CD19⁺/CD138⁺) that had different proportions when treated with α -AED, but this population is very scarce inside 4T1 tumors (Fig. 5B).

Microenvironmental VEGF is reduced in tumors treated with α -AED.

Because of the importance of tumor cell milieu communication, additionally to immune cell proportions, we search for another way by which α -AED could be mediating the reduction of tumor size, thus we determine tumor VEGF concentration. Notably, VEGF levels in α -AED-treated tumors were lower (100 μM = $p \leq 0.01$, 200 μM = $p \leq 0.0001$) than the Vh group (Figure 6).

Tumor cytokine levels

309 Since local treatment with α -AED 200 μ M did not have the same effect on the reduction of
310 tumor size compared with 100 μ M intratumoral administration, we chose to measure some
311 representative interleukins in the tumor of Vh and α -AED 100 μ M only (Fig. 7). We
312 determined Type 1 (TNF- α , IFN- γ), Type 2 (IL-4 and IL-5), and regulatory (IL-10) cytokines.
313 No changes were observed in these interleukins within the tumor (Fig. 7)

314 **Specific humoral response against 4T1 cells is enhanced in mice treated with α -AED**

315 As part of the systemic humoral response analysis in tumor-bearing mice, we determined
316 the percentage of B (CD19⁺), plasmatic (CD138⁺) and plasmacytoid cells (CD19⁺/CD138⁺)
317 from the spleen. None of these three humoral-related cell proportions were different
318 (Supplementary Figure 1 G,H and I).

319 Additionally to this analysis, we obtained IgG and anti-4T1 IgG serum levels, of which,
320 unspecific levels of IgG (Fig. 8A), in response to α -AED treatment remain with no statistical
321 change. Nevertheless, anti-4T1-IgG from α -AED-treated mice were higher in serum from
322 mice injected with 100 μ M (p 0.001) and 200 μ M of α -AED ($p \leq 0.05$), (Fig. 8B).

323 **Systemic innate and adaptive immune response is preserved in tumor-bearing mice** 324 **treated with α -AED**

325 Because the injection of α -AED was performed inside the tumor, and we want to find out if
326 this local treatment could reach a systemic level, we analyzed the effect of this local
327 treatment in the systemic immune response in secondary lymphoid organs. In order to
328 assess whether these modifications in splenic cell proportions were different in treated and
329 untreated mice (Supplementary Figure 1). Innate and adaptive cells obtained from the
330 spleen from mice treated with α -AED, conserved their proportions when compared with Vh
331 group, with exception of CD4⁺/Foxp3⁺ T cells. The proportion of this regulatory population

was augmented in the spleen of tumor-bearing mice treated with α -AED 100 μ M (p 0.001) (Supplementary Figure 1d).

It is interesting to note that, the same pattern found in the spleen, was found in the closer lymphoid organ to the tumors, the inguinal lymphatic nodes. Innate and adaptive cells obtained from the spleen from mice treated with α -AED, conserved their proportions when compared with Vh group, with exception of CD4⁺/Foxp3⁺ T cells. The proportion of this regulatory population was augmented in the spleen of tumor-bearing mice treated with α -AED 100 μ M ($p \leq 0.001$) (Supplementary Figure 2d).

Discussion

The neurosteroid α -AED has been widely proved as a proliferation inhibitor in different cancer cell lines and its IC₅₀ values vary according to the type of the cell line. For instance, the IC₅₀ value for glioma cells of human origin ranges from 8.5 $\pm$ 1.30 μ M to 22.35 μ M; for rat glioma cells F98 and RT-2 are 13.65 $\pm$ 1.55 μ M and 18.05 $\pm$ 0.25 μ M, respectively. Lastly, the IC₅₀ value of the murine glioma cell line GL261 is 17.25 $\pm$ 1.05 (**18**). In regard to the murine 4T1 cell line, the IC₅₀ value determined in our study was 100.875 μ M (1 $\times$ 10^{-4.035} Molar). This concentration was higher than the one reported for glioma cell lines, but as mention before the IC₅₀ of α -AED varies among cell lines. In addition to the inhibitory effect of α -AED on glioma cell growth, this epimer also inhibited the proliferation of breast cancer cell lines *in vitro*. Examples of it include breast adenocarcinoma (MCF-7, MDA-MB231), and mammary ductal carcinoma (T47D and TTU-1) (**6**). Despite, the IC₅₀ value was not assessed in these cell lines, but lethal dose (LD)₅₀ was established between 8 - 15 μ M (**6**). In line with the results of Graf et al, we also observed that the triple-negative breast cancer cell line, HCC1937,

355 displayed low sensitivity to the inhibitory cell viability effect to the α -AED, showing its best
356 effect at 10 μ M. The above may be partly due to the fact that this cell line lacks one of the
357 crucial cell targets for the effect of this compound, as is the estrogen receptor. Therefore,
358 we extended the insights evoked previously by dehydroepiandrosterone in other breast
359 cancer cells that are similar to α -AED, where a reduction in cell proliferation has achieved
360 in high doses of the adrenal hormone dehydroepiandrosterone (4, 6, 19). Besides, we
361 hypothesize that these findings also open the study for the searching of different cell targets
362 with alternative techniques. In fact, our research group has recently reported that although
363 there was not reported a direct proliferative effect of different estrogenic compounds in
364 human triple-negative breast cancer cells, several crucial molecular factors involved in
365 epithelial transition or hypoxia processes, among others were modulated after their
366 exposure (20).

367 On the other hand, the proliferation and viability have been increased in cells treated with
368 lower concentration of α -AED than 10 nM. This effect has also been observed in murine
369 macrophage-like cell line RAW 264.7 (concentration lower than 6.25 nM) cultured for 48h,
370 and in human breast ductal carcinoma cells ZR75-1, as well as, MDA-MB231 breast tumor
371 cells (lower than 12.5 nM) cultured during 6 days (8).

372 In line with the reduction of 4T1 cell density treated with α -AED 100 μ M (1×10^{-4} Molar) and
373 200 μ M ($1 \times 10^{-3.7}$ Molar) concentrations ($p \leq 0.01$ and $p \leq 0.05$), (Fig. 1A, B), we assessed if
374 this effect was associated with the inhibition of the proliferation through the BrdU
375 incorporation in proliferating cells. After 72 hours of treatment with 100 μ M and 200 μ M we
376 observed a dramatic reduction in the percentage of proliferating cells BrdU⁺ ($p \leq 0.01$ and p
377 ≤ 0.05 , respectively), (Fig. 1C, D). These results suggest that the reduction in cell density

378 detected through SRB assay was associated with a decrease in the proliferation of 4T1 cells
379 treated with α -AED (7) .

380 After we assessed the size reduction of tumors treated with 100 μ M and 200 μ M of α -AED,
381 we determined the effect of intratumoral treatment on immune cell infiltration in the 4T1
382 tumor model. Accordingly to Loria 2002, immune regulation has associated with β -AED
383 exposure, which is chemically identical to α -AED, and the anti-tumoral effect has linked to
384 α -AED regimen (8, 21). But, because of the structural similarities between of androstenediol
385 α and β , and the lack of information associated with the immune tumor cell
386 microenvironment, we search for some immune components of this milieu in order to
387 determine if α -AED intratumoral treatment exert local immune changes.

388 Cancer is a complex disease that involves tumor development and cell metastasis from this
389 tumor to other distant tissues. Primary tumor growth itself rarely causes the death of its
390 carrier and is the metastatic disease the cause of the vast majority (about 90%) of the
391 cancer-related deaths (22). In this sense, we observed that lungs from 4T1 group mice
392 exhibited metastatic-like lesions on the lung surface. These macroscopic metastases
393 disappear when tumors were treated with α -AED (100 μ M and 200 μ M), (Fig. 3).

394 For instance, NK cell proportion was augmented within the tumors treated with α -AED 100
395 μ M (Fig. 4F). NK cells are known as cytotoxic cells of the innate response, and in tumors
396 are principal effectors of the cancer immunoediting by recognizing and destroying tumor
397 cells directly through the exocytosis of granules with perforin and granzyme, apoptosis
398 mediated by different death receptors (FasL, TRAIL and TNF- α) and IFN- γ secretion (12,
399 23, 24). Another way by which NK cells exert their cytotoxic effect in solid tumors is by the

antibody-dependent cell-mediated cytotoxicity activated by antibodies linked to target cells
(25).

In order with these findings in the present study, we also search for humoral-response components in the tumor milieu as we described as follows. In treated tumors, the percentages of plasmatic CD138⁺ and plasmablast cells CD19⁺/CD138⁺ were higher in tumors injected with 100 μ M of α -AED ($p \leq 0.05$ and $p 0. \leq 001$, respectively), (Fig. 5A, B). Meanwhile, the tumors injected with 200 μ M of α -AED contained an elevated proportion of plasmablast and B cells CD19⁺ ($p \leq 0.05$) (Fig. 5B, C). To our knowledge, there is no report about the effect of α -AED on the humoral-related response. Thus, these findings are the beginning of a further investigation, nevertheless, the presence of naïve or mature B cells within different types of breast tumors, is related to a positive prognostic effect in 54 cohorts studies (26). Also, the presence of B lymphocytes CD19⁺ is associated with a favorable prognostic in the overall survival of patients with tongue squamous cell carcinoma (27). In a similar case, elevated plasma cell density is linked to a longer time to relapse in triple-negative breast cancer tumors (TNBC) (28).

Consequently to this knowledge, and the increased proportions of B and plasmatic cells in the milieu of α -AED treated-tumors, we determined the systemic humoral response of these tumor-bearing mice. Neither of the population's changes observed in the tumor were detected in the spleen or PLN (Fig. 6)

Additionally to immune cells proportions, we search for complementary ways by which α -AED mediates the reduction of the tumor size, thus we determine tumor soluble factors, that included angiogenic (VEGF), Type 1 response (TNF- α , IFN- γ), Type 2 response (IL-4, IL-5) and regulatory (IL-10) soluble factors. Notably, VEGF levels in α -AED-treated tumors were lower (100 μ M= $p \leq 0.01$) than the Vh group (Fig. 7F). As previously mentioned, VEGF is related to vessel growth, nutrient

supply and tumor cell dispersion to other tissues, thus its decrease within the tumor may lead to the reduction of tumor growth and metastasis **(13)**. The diminished concentration of VEGF level in α -AED 100 μ M treated tumors may be associated with a direct production inhibition of the tumor milieu cells, or linked to other microenvironmental factors. The VEGF expression is modified by hypoxia, free radicals, pH imbalance and nutrient deficiency, that increase at the same time that the tumor grows, therefore, the inhibition of the proliferation of the 4T1 cells mediated by α -AED could reduce these pro-angiogenic factors within the treated tumors and in consequence the VEGF production **(29)**. Further studies to unveil these mechanisms are needed.

Since local treatment with α -AED 200 μ M did not have the same effect on the reduction of tumor size compared with the intratumoral administration of α -AED 100 μ M, we chose to exclude this group from this determination (Fig. 8). None of Type 1, Type 2 and regulatory response interleukins were different in α -AED-injected tumors (Fig 6 8-E).

Intratumoral drug delivery is not a common practice in breast cancer patients, being intravenous systemic chemotherapy the most common administration route. But only a small fraction of the anticancer drug reaches the tumor, therefore high doses of the medicament have to be administrated, which in turn increase the undesirable side effects of chemotherapeutic compounds that affect healthy tissues **(30)**. A leading objective in cancer research is to avoid or reduce the toxicity of the conventional cytotoxic treatment, and a promising method is the local delivery of the anticancer drug **(31)**, which may be used as a neoadjuvant therapy before tumor removal. For that reasons, different studies in diverse types of cancer has focused in different intratumorally alternatives that indeed has been proposed and applied in some cases in the clinical and palliative cancer therapy **(32, 33)**, highlighting the translational impact of this work. Thus, in this study, we injected in the tumor

the anti-proliferative neurosteroid α -AED and besides local changes, we look for its immune systemic effect in secondary lymphoid organs such as the spleen and PLN. Following the previous findings, the reduction in tumor size accompanied by changes in humoral-related response within the neoplasias treated with α -AED, suggest a role of these cells in tumor growth control. This could be explained because in tumor the microenvironment, the B lymphocytes promote T-cell responses through the production of cytokines and chemokines, they also differentiate into producing-antibodies plasmatic cells (26). Specific antibodies may be able to recognize tumor-associated antigens and act directly on target proteins or through their Fc receptor trigger the ADCC, and in turn improve antigen presentation and activation of T cells (26). Thus, to improve our understanding in humoral-related mechanisms in the 4T1 tumor mice model treated with α -AED, we determined the levels of unspecific IgG and anti-4T1 IgG in mice serum. As well as B, plasmatic and plasmablasts cells, we found no-changes in unspecific antibodies (IgG). In contrast, the 4T1-specific IgG levels were elevated in mice treated with 100 μ M and 200 μ M of α -AED (Fig. 6).

Conclusion

In summary, we have established a direct intratumorally delivery of a α -AED that affect not only the proliferation rate of human and murine cancer cells but also the immune modulation in a mouse breast cancer model. Using this strategy, we demonstrate that intratumorally delivery of α -AED might significantly enhance different immunotherapies, modulating *per se* different immune populations and inhibiting the cancer metastasis through the downregulation of VEGF secretion. Our results highlight the great potential of α -AED for mediating antitumor activity will surely needs to be prove in human studies. Therefore, our

471 results offer a significant bridge between basic and pre-clinical medicine towards
472 translational studies as an urgent demand for therapeutic options.

473

474 List of abbreviations

475 α -AED epimereandrostene-3 β ,17 α -diol

476 IFN- γ interferon-gamma

477 IgG Immunoglobulin G

478 NK natural killer cells

479 TNF- α tumor necrosis factor alpha

480 TRAIL TNF-related apoptosis-inducing ligand

481 VEGF vascular endothelial growth factor

482

483

484 **Declarations**

485 Ethics approval and consent to participate. Animal care and experiments at the Instituto de
486 Investigaciones Biomédicas are constantly evaluated and approved by the Experimental
487 Animal Care and Use Committee of the Institute (Comité de Cuidado y Uso de Animales
488 de Experimentación, CICUAL, permit number 2016-00133) adhering to the official Mexican
489 standards (NOM-062-ZOO-1999). Mexican regulations are in strict accordance with the
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514 **References**

515 1. Waks AG, Winer EP. Breast Cancer Treatment: A Review. Jama. 2019;321(3):288-
516 300. Epub 2019/01/23.

- 517 2. van Hellemond IEG, Geurts SME, Tjan-Heijnen VCG. Current Status of Extended
518 Adjuvant Endocrine Therapy in Early Stage Breast Cancer. Current treatment options in
519 oncology. 2018;19(5):26. Epub 2018/04/29.
- 520 3. Wahba HA, El-Hadaad HA. Current approaches in treatment of triple-negative breast
521 cancer. Cancer biology & medicine. 2015;12(2):106-16. Epub 2015/07/16.
- 522 4. Lopez-Marure R, Contreras PG, Dillon JS. Effects of dehydroepiandrosterone on
523 proliferation, migration, and death of breast cancer cells. European journal of pharmacology.
524 2011;660(2-3):268-74. Epub 2011/04/19.
- 525 5. McCormick DL, Rao KV, Johnson WD, Bowman-Gram TA, Steele VE, Lubet RA, et
526 al. Exceptional chemopreventive activity of low-dose dehydroepiandrosterone in the rat
527 mammary gland. Cancer research. 1996;56(8):1724-6. Epub 1996/04/15.
- 528 6. Graf MR, Jia W, Powell T, Loria RM. 5-androstene 3 β ,17 α diol induces autophagic
529 cell death of human breast cancer cells and enhances radiation cytotoxicity. journal of
530 Cancer Therapeutics and Research. 2012;1(1).
- 531 7. Huynh PN, Carter WH, Jr., Loria RM. 17 alpha androstenediol inhibition of breast
532 tumor cell proliferation in estrogen receptor-positive and -negative cell lines. Cancer
533 detection and prevention. 2000;24(5):435-44. Epub 2000/12/29.
- 534 8. Loria RM. Immune up-regulation and tumor apoptosis by androstene steroids.
535 Steroids. 2002;67(12):953-66. Epub 2002/10/26.
- 536 9. Dehennin L. Secretion by the human testis of epitestosterone, with its sulfoconjugate
537 and precursor androgen 5-androstene-3 beta,17 alpha-diol. The Journal of steroid
538 biochemistry and molecular biology. 1993;44(2):171-7. Epub 1993/02/01.

- 539 10. Mancuso S, Bellante FP, Angelini A, Marana R. Amniotic 5-androstene-3 beta, 17
540 alpha-diol in high risk pregnancy. *Journal of steroid biochemistry*. 1980;12:95-6. Epub
541 1980/01/01.
- 542 11. Emens LA, Silverstein SC, Khleif S, Marincola FM, Galon J. Toward integrative
543 cancer immunotherapy: targeting the tumor microenvironment. *Journal of translational*
544 *medicine*. 2012;10:70. Epub 2012/04/12.
- 545 12. Zitvogel L, Tesniere A, Kroemer G. Cancer despite immunosurveillance:
546 immunoselection and immunosubversion. *Nature reviews Immunology*. 2006;6(10):715-27.
547 Epub 2006/09/16.
- 548 13. Bohn KA, Adkins CE, Nounou MI, Lockman PR. Inhibition of VEGF and Angiopoietin-
549 2 to Reduce Brain Metastases of Breast Cancer Burden. *Frontiers in pharmacology*.
550 2017;8:193. Epub 2017/04/27.
- 551 14. Konecny GE, Meng YG, Untch M, Wang HJ, Bauerfeind I, Epstein M, et al.
552 Association between HER-2/neu and vascular endothelial growth factor expression predicts
553 clinical outcome in primary breast cancer patients. *Clinical cancer research : an official*
554 *journal of the American Association for Cancer Research*. 2004;10(5):1706-16. Epub
555 2004/03/12.
- 556 15. Kim DY, Kwon DY, Kwon JS, Park JH, Park SH, Oh HJ, et al. Synergistic anti-tumor
557 activity through combinational intratumoral injection of an in-situ injectable drug depot.
558 *Biomaterials*. 2016;85:232-45. Epub 2016/02/14.
- 559 16. Vichai V, Kirtikara K. Sulforhodamine B colorimetric assay for cytotoxicity screening.
560 *Nature protocols*. 2006;1(3):1112-6. Epub 2007/04/05.
- 561 17. Urban NH, Chamberlin B, Ramage S, Roberts Z, Loria RM, Beckman MJ. Effects of
562 alpha/beta-androstenediol immune regulating hormones on bone remodeling and apoptosis

563 in osteoblasts. The Journal of steroid biochemistry and molecular biology. 2008;110(3-
564 5):223-9. Epub 2008/06/03.

565 18. Loria RM, Graf MR. 17alpha-androstenediol-mediated oncopromotion of tumor cells by
566 different mechanisms is determined by the target tumor. Annals of the New York Academy
567 of Sciences. 2012;1262:127-33. Epub 2012/07/25.

568 19. Gayosso V, Montano LF, Lopez-Marure R. DHEA-induced antiproliferative effect in
569 MCF-7 cells is androgen- and estrogen receptor-independent. Cancer J. 2006;12(2):160-5.
570 Epub 2006/04/25.

571 20. Segovia-Mendoza M, Gómez de León CT, Garcia-Becerra R, Ambrosio J, Nava-
572 Castro KE, Morales-Montor J. The chemical environmental pollutants BPA and BPS induce
573 alterations of the proteomic profile of different phenotypes of human breast cancer cells: A
574 proposed interactome. Environmental research. 2020;191:109960.

575 21. Graf MR, Jia W, Lewbart ML, Loria RM. The anti-tumor effects of androstene steroids
576 exhibit a strict structure-activity relationship dependent upon the orientation of the hydroxyl
577 group on carbon-17. Chemical biology & drug design. 2009;74(6):625-9. Epub 2009/10/15.

578 22. Lambert AW, Pattabiraman DR, Weinberg RA. Emerging Biological Principles of
579 Metastasis. Cell. 2017;168(4):670-91. Epub 2017/02/12.

580 23. Gross E, Sunwoo JB, Bui JD. Cancer immunosurveillance and immunoediting by
581 natural killer cells. Cancer J. 2013;19(6):483-9. Epub 2013/11/26.

582 24. Smyth MJ, Cretney E, Kelly JM, Westwood JA, Street SE, Yagita H, et al. Activation
583 of NK cell cytotoxicity. Molecular immunology. 2005;42(4):501-10. Epub 2004/12/21.

584 25. Lo Nigro C, Macagno M, Sangiolo D, Bertolaccini L, Aglietta M, Merlano MC. NK-
585 mediated antibody-dependent cell-mediated cytotoxicity in solid tumors: biological evidence
586 and clinical perspectives. Annals of translational medicine. 2019;7(5):105. Epub 2019/04/26.

- 587 26. Wouters MCA, Nelson BH. Prognostic Significance of Tumor-Infiltrating B Cells and
588 Plasma Cells in Human Cancer. *Clinical cancer research : an official journal of the American*
589 *Association for Cancer Research*. 2018;24(24):6125-35. Epub 2018/07/28.
- 590 27. Lao XM, Liang YJ, Su YX, Zhang SE, Zhou XI, Liao GQ. Distribution and significance
591 of interstitial fibrosis and stroma-infiltrating B cells in tongue squamous cell carcinoma.
592 *Oncology letters*. 2016;11(3):2027-34. Epub 2016/03/22.
- 593 28. Yeong J, Lim JCT, Lee B, Li H, Chia N, Ong CCH, et al. High Densities of Tumor-
594 Associated Plasma Cells Predict Improved Prognosis in Triple Negative Breast Cancer.
595 *Frontiers in immunology*. 2018;9:1209. Epub 2018/06/15.
- 596 29. Wang F, Xu P, Xie KC, Chen XF, Li CY, Huang Q. Effects of tumor microenviromental
597 factors on VEGF expression. *Biomedical reports*. 2013;1(4):539-44. Epub 2014/03/22.
- 598 30. Weinberg BD, Blanco E, Gao J. Polymer implants for intratumoral drug delivery and
599 cancer therapy. *Journal of pharmaceutical sciences*. 2008;97(5):1681-702. Epub
600 2007/09/12.
- 601 31. Wolinsky JB, Colson YL, Grinstaff MW. Local drug delivery strategies for cancer
602 treatment: gels, nanoparticles, polymeric films, rods, and wafers. *Journal of controlled*
603 *release : official journal of the Controlled Release Society*. 2012;159(1):14-26. Epub
604 2011/12/14.
- 605 32. Goldberg EP, Hadba AR, Almond BA, Marotta JS. Intratumoral cancer chemotherapy
606 and immunotherapy: opportunities for nonsystemic preoperative drug delivery. *The Journal*
607 *of pharmacy and pharmacology*. 2002;54(2):159-80. Epub 2002/02/19.
- 608 33. Chu XY, Huang W, Wang YL, Meng LW, Chen LQ, Jin MJ, et al. Improving antitumor
609 outcomes for palliative intratumoral injection therapy through lecithin- chitosan nanoparticles

loading paclitaxel- cholesterol complex. International journal of nanomedicine. 2019;14:689-705. Epub 2019/02/19.

Figure Legends

Figure 1. Effect of α -AED in 4T1 and HCC1937 cells. The 4T1 cell line was exposed to vehicle (EtOH 0.2%) and α -AED 1×10^{-12} to $1 \times 10^{-3.3}$ Molar for 72 hours. Cell density was measured through SRB assay. A. Cell viability adjusted as a percentage to obtain half-maximal inhibitory concentration (IC_{50}). B. Cell density adjusted to the vehicle (Vh). Graphs represent the mean $\pm$ SD of the data from three experiments in triplicate. C. Cell density of HCC1937 triple-negative breast cancer cells was evaluated after 72 hours of exposure of vehicle (EtOH) or 1×10^{-12} to 1×10^{-7} Molar of α -AED. Each bar represents the mean $\pm$ standard deviation of three different experiments realized by triplicate (*, $p \leq 0.05$). D. BrdU incorporation was determined in proliferative cells: offset histogram (left) and separated histograms of untreated 4T1 cells (M, Medium), treated with vehicle (Vh), α -AED 100 μ M and 200 μ M (1×10^{-4} -and $1 \times 10^{-3.3}$ Molar). D. Percentage of proliferating cells. Graph represent the mean $\pm$ SD of the data from four experiments (Medium, $n = 6$; Vh, $n = 6$; AED 100 μ M, $n = 4$; AED 200 μ M, $n = 4$). Statistical significance was calculated using Kruskal-Wallis test (*, $p \leq 0.05$; **, $p \leq 0.01$).

Figure 2. Effect of α -AED in 4T1 tumor growth in BALB/c mice. After 14 days of growth tumors were injected with vehicle -corn oil 40 μ l- (Vh), α -AED 100 μ M (1×10^{-4} Molar) or 200

633 μM ($1 \times 10^{-3.3}$ Molar), and excised after 28 days of tumor development. A. Tumor weight from
634 intact tumors (4T1), Vh, $\alpha\text{-AED}$ 100 μM and $\alpha\text{-AED}$ 200 μM groups. (B) Representative
635 images of tumors from all groups. Millimetric grid as a background; black bar = 0.5 cm.
636 Graphs represent the mean $\pm$ SD of the data from three experiments. Statistical significance
637 was calculated using Kruskal-Wallis test (*, $p \leq 0.05$; ****, $p \leq 0.0001$).

638

639 **Figure 3. Effect of $\alpha\text{-AED}$ in 4T1 tumor growth in BALB/c mice.** After 14 days of growth
640 tumors were injected with the vehicle -corn oil 40 μl - (Vh), $\alpha\text{-AED}$ 100 μM (1×10^{-4} Molar) and
641 200 μM ($1 \times 10^{-3.3}$ Molar), and excised after 28 days of tumor development. A. Tumor weight
642 from intact tumors (4T1), Vh, $\alpha\text{-AED}$ 100 μM and $\alpha\text{-AED}$ 200 μM groups. (B) Representative
643 images of tumors from all groups. Millimetric grid as a background; black bar = 0.5 cm.
644 Graphs represent the mean $\pm$ SD of the data from three experiments. Statistical significance
645 was calculated using Kruskal-Wallis test (*, $p \leq 0.05$; ****, $p \leq 0.0001$).

646

647 **Figure 4. Effect of intratumoral $\alpha\text{-AED}$ treatment on immune cell infiltration in the 4T1**
648 **tumor model.** Innate and adaptive immune cells in the tumor microenvironment.
649 Determination of innate and adaptive immune subpopulations by flow cytometry within the
650 tumor. A. T CD3⁺ lymphocytes B. CD8⁺ T cytotoxic. C helper CD4⁺ T lymphocytes. D.
651 CD4⁺/Foxp3⁺ lymphocytes. E. F4/80⁺ macrophages. F. NKp46⁺ cells. Graphs represent the
652 mean $\pm$ SD of the data from three independent experiments. Representative dot plots of the
653 cytometric analysis of the corresponding population tumor cells (below). Gate from 10,000

cells was collected. Statistical significance was calculated using a Kruskal-Wallis test (**p ≤ 0.01).

Figure 5. Effect of α -AED local injection on the humoral-related cell infiltration in the 4T1 tumor model. Humoral-related cell subpopulations by flow cytometry of the tumor. A. Plasmatic cells (CD138⁺). B. Plasmablasts (CD138⁺/CD19⁺). C. B cells (CD19⁺). D. Representative dot plots of the cytometric analysis of the corresponding population tumor cells. Graphs represent the mean±SD of the data from three independent experiments. Gate from 10,000 cells was collected. Statistical significance was calculated using a Kruskal-Wallis test (*, p ≤ 0.05; ***, p ≤ 0.001).

Figure 6. Analysis of the VEGF expression in the tumor microenvironment. Graph show data from tumor proteins obtained from two independent experiments. Bars represent the mean±SD of cytokine levels (pg/10 µg of tumor protein). Statistical significance was calculated using a Kruskal-Wallis test (**p ≤ 0.01; ****p ≤ 0.0001).

Figure 7. Expression of soluble factors in the tumor microenvironment. Analysis of type 1 (IFN- γ and TNF- α), type 2 (IL-4, IL-5), and regulatory (IL-10) cytokines. Graphs indicate data of tumor proteins obtained from two independent experiments. Bars represent the mean±SD of cytokine levels (pg/10 µg of tumor protein).

Figure 8. Systemic humoral response to intratumoral α -AED treatment in BALB/c mice. A. Serum IgG levels B. IgG anti-4T1 levels, in sera from intact (Int), tumor-bearing mice with no treatment (4T1), and sera from mice injected into the tumor with vehicle (corn

oil 40 μ l), α -AED 100 μ M or α -200 μ M. Graphs represent the mean $\pm$ SD of data from two experiments. Statistical significance was calculated using Kruskal-Wallis test (*, $p \leq 0.05$; ** $P \leq .01$; *** $P \leq .001$; **** $P \leq .0001$).

Supplementary Figure 1. Effect of intratumoral α -AED treatment in the immune cell infiltration of secondary lymphoid organ, in the 4T1 tumor model. Determination of innate and adaptive immune subpopulations by flow cytometry in the spleen. a. T lymphocytes CD3⁺.. b. CD8⁺ T cytotoxic lymphocytes. c. CD4⁺ T helper lymphocytes . d. CD4⁺/Foxp3⁺ T lymphocytes. e. NKp46⁺ cells. f. F4/80⁺ macrophages. g. CD19⁺ B lymphocytes. h. Plasmatic cells (CD138⁺). i. Plasmablasts (CD138⁺/CD19⁺). Graphs represent the mean $\pm$ SD of the data from three independent experiments. Gate from 10,000 cells was collected. Statistical significance was calculated using a Kruskal-Wallis test (*, $p \leq 0.05$; ***, $p \leq 0.001$).

Supplementary Figure 2. Effect of intratumoral α -AED treatment in the immune cell infiltration of secondary lymphoid organs, in the 4T1 tumor model. Determination of innate and adaptive immune subpopulations by flow cytometry in lymph nodes. T lymphocytes CD3⁺.. b. CD8⁺ T cytotoxic lymphocytes. c. CD4⁺ T helper lymphocytes. d. CD4⁺/Foxp3⁺ T lymphocytes. e. NKp46⁺ cells. f. F4/80⁺ macrophages. g. CD19⁺ B lymphocytes. h. Plasmatic cells (CD138⁺). i. Plasmablasts (CD138⁺/CD19⁺). Graphs represent the mean $\pm$ SD of the data from three independent experiments. Gate from 10,000 cells was collected. Statistical significance was calculated using a Kruskal-Wallis test (*, $p \leq 0.05$).

Figures

Figure 1

See the Supplemental Files section for the complete figure caption.

A

Tumor weight

B

Figure 2

See the Supplemental Files section for the complete figure caption.

Figure 3

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Figure 4

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Figure 5

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Figure 6

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VEGF

Figure 7

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Figure 8

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Supplementary Files

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