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1 **Immunotargeting of antigen xCT attenuates stem-like cell behavior and metastatic progression in breast**
2 **cancer**

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12
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30 **Abstract**

31 Resistance to therapy and lack of curative treatments for metastatic breast cancer suggest that current therapies
32 may be missing the subpopulation of chemo- and radio-resistant cancer stem cells (CSC). The ultimate success
33 of any treatment may well rest on CSC eradication, but specific anti-CSC therapies are still limited. A
34 comparison of the transcriptional profiles of murine Her2+ breast tumor TUBO cells and their derived CSC-
35 enriched tumorspheres has identified xCT, the functional subunit of the cystine/glutamate antiporter system xc-,
36 as a surface protein that is upregulated specifically in tumorspheres. We validated this finding by
37 cytofluorimetric analysis and immunofluorescence in TUBO-derived tumorspheres and in a panel of mouse and
38 human triple negative breast cancer (TNBC) cell-derived tumorspheres. We further show that downregulation
39 of xCT impaired tumorsphere generation and altered CSC intracellular redox balance in vitro, suggesting that
40 xCT plays a functional role in CSC biology. DNA vaccination-based immunotargeting of xCT in mice
41 challenged with syngeneic tumorsphere-derived cells delayed established subcutaneous tumor growth and
42 strongly impaired pulmonary metastasis formation by generating anti-xCT antibodies able to alter CSC self-
43 renewal and redox balance. Finally, anti-xCT vaccination increased CSC chemosensitivity to doxorubicin in
44 vivo, indicating that xCT immunotargeting may be an effective adjuvant to chemotherapy.

45

46 **Precis**

47 Immunotargeting of breast cancer stem-like cells can sensitize them to chemotherapy, offering an effective
48 strategy to overcome drug resistance and limit metastatic progression.

49

50 Introduction

51 Despite recent advances in breast cancer management resulting in a decrease in overall mortality (1), minimal
52 residual disease and local and distant post-treatment recurrences are still major obstacles to complete remission.
53 According to the cancer stem cell (CSC) model, these residual elements are caused by a stem-like
54 subpopulation of tumor cells that are endowed with self-renewal and multi-lineage differentiation capabilities,
55 chemo- and radio-resistance and the ability to give metastases (2). The therapeutic implication of CSC model is
56 that a tumor needs to be deprived of its CSC population to be completely eradicated. Novel anticancer strategies
57 must therefore be developed to face this new challenge.

58 Active immunotherapy, i.e. vaccination, is an attractive approach to target CSC. Preclinical studies have shown
59 that CSC are immunogenic and a more effective antigen source for inducing protective anti-tumor immunity in
60 mice than unselected tumor cells (3). Several clinical trials in which CSC lysate or mRNA are used to pulse or
61 transfect autologous dendritic cells (DC), that are then injected back into the patients, are currently underway in
62 various tumor settings (4). However, this technique presents the difficulty of setting up standardized functional
63 DC production procedures (5). A promising alternative can be found in powerful and versatile DNA-based
64 vaccines which combines lower manufacturing costs and more standardized production processes while also
65 inducing strong immunological responses against tumor antigens. Nevertheless, one must identify a suitable
66 target to develop a DNA-based vaccine; as no consensus on the expression of specific vaccination targets by
67 CSC currently exists, pre-clinical screening by high-throughput technologies can be of use in uncovering
68 antigens for CSC-targeted genetic vaccines.

69 We have compared the transcription profile of the murine Her2⁺ breast cancer TUBO cell line (6) with that of
70 its CSC-enriched tumorspheres in order to identify antigens expressed by mammary CSC. Among the genes
71 upregulated in tumorspheres and associated with poor prognosis in several datasets of human mammary cancer,
72 we focused on xCT. It is the light chain of the antiporter system x_c⁻, which imports the aminoacid cystine into
73 cells in exchange with glutamate. Cystine is the rate-limiting substrate for the synthesis of the antioxidant
74 glutathione (GSH), which is known to be involved in the detoxification of reactive oxygen species (ROS) (7).

75 xCT is highly expressed by a variety of malignant tumors (7-12) and plays an important role in cancer growth,
76 progression, metastatic dissemination (13-15) and drug resistance (16). Moreover, its membrane expression is
77 stabilized via direct interaction with a CD44 variant (13), while targeting xCT has been found to deplete
78 undifferentiated CD44v-expressing cancer cells in a xenografted model of head and neck squamous cell
79 carcinoma, sensitizing the tumor to other therapies (17).

80 We herein report that xCT upregulation is a general feature of breast CSC and that plays a functional role in
81 CSC biology and intracellular redox balance. We demonstrate that anti-xCT DNA vaccination slows established
82 subcutaneous tumor growth, efficiently impairs lung metastasis formation and increases CSC chemosensitivity,
83 thus making it a novel therapeutic approach for breast cancer treatment.

84

85 **Materials and Methods**

86 *Cell and tumorsphere cultures*

87 MDA-MB-231, HCC-1806 and 4T1 cells were purchased from ATCC (LGC Standards) and cultured as in (18).
88 NIH/3T3 cells were cultured as in (19). Cells were passaged in our laboratory for fewer than six months after
89 their resuscitation. TUBO cells and tumorspheres were generated as in (18). Human cell lines were tested
90 utilizing Short Tandem Repeat (STR) profiling.

91 *FACS analysis*

92 Cells and tumorspheres were stained with AlexaFluor647-anti-Sca-1, PE-anti-CD44 and PE/Cy7-anti-CD24
93 (Biolegend), and with goat anti-xCT (Santa Cruz Biotechnology) antibodies followed by rabbit FITC-anti-goat
94 Ig (Dako), or with Aldefluor kit (StemCell Technologies), as in (18). To quantify anti-xCT antibody titers,
95 tumorsphere-derived and NIH/3T3 cells were incubated with sera of vaccinated mice and subsequently with
96 rabbit FITC-anti-mouse Ig (Dako). Cells were stained with 2',7'-dihydrochlorofluorescein diacetate (DHCF-DA,
97 Sigma-Aldrich) as in (20). All samples were analyzed on a CyAnADP Flow Cytometer, using Summit 4.3
98 software (Beckman Coulter).

99 *Fluorescent microscopy*

100 Tumor microarrays (TMA) (Biochain # T8234700-2, lot # B406087, and Biochain # T6235086-5, lot #
101 B112136) of normal or tumor human tissues were blocked in 3% H_2O_2 (Sigma-Aldrich), followed by 1%BSA,
102 and then incubated with anti-xCT or isotype-matched control antibodies (Abcam). The signal was amplified as
103 in (21) and sections were fixed in 1%formaldehyde (Sigma-Aldrich), counterstained with DAPI (Sigma-
104 Aldrich) and mounted in Mowiol® (Calbiochem). Images were acquired using a confocal microscope LSM700
105 and Zen software 7.0.0.285 (Zeiss). Slides were scanned on a slide scanner (Hamamatsu Nanozoomer 2.0RS)
106 using the Calopix software. xCT⁺ cell percentage was defined by quantifying blue (nuclei) and red (xCT)
107 surface areas and calculated as the ratio of xCT expression (i.e. xCT stained surface/xCT+nuclei surface).

108 Tumorspheres were cytopinned to glass slides, fixed in 4%formaldehyde and then incubated with rabbit anti-
109 OCT4 (Abcam), rat PE-anti-Sca-1 (Santa Cruz biotechnology), mouse APC/eFluor780-anti-Thy1.1

110 (eBioscience) or the matched isotype control antibodies. Cytospinned tumorspheres or NIH/3T3 cultured on
111 glass coverslips were stained with IgG purified from immunized mice sera and then with rabbit AlexaFluor488-
112 anti-mouse or goat Texas red-anti-rabbit secondary antibodies (Life Technologies). Images were acquired on
113 ApoTome system and AxioVision Release4.8 software (Zeiss).

114 *In vitro cytotoxicity*

115 24 hours after TUBO cells and tumorspheres seeding in 96-well plates, scalar doses of doxorubicin or
116 sulfasalazine (SASP) (Sigma-Aldrich) were added and incubated at 37°C for 72 hours. Cytotoxicity was
117 evaluated with MTT using the Cell Proliferation Kit I (Roche Diagnostics).

118 *RNA interference*

119 xCT downregulation in tumorspheres was performed using a pool of specific siRNAs, or scrambled siRNAs
120 (Invitrogen Corp.), as in (18).

121 *SASP effects on tumorsphere formation*

122 Dissociated tumorspheres were cultured with scalar doses of SASP or its diluent DMSO (Sigma-Aldrich), and
123 the total number of tumorspheres/well was counted 5 days later.

124 *Measurement of ROS and GSH*

125 ROS amount was analyzed as 2',7'-dichlorofluorescein (DCF) formation in cells incubated with 5µM DHCF-DA
126 for 20 minutes at 37°C using the Luminescence Spectrometer LS 55 (Perkin–Elmer), quantified using a DCF
127 standard curve and expressed as pmol DCF formed/min/mg protein (22). GSH content was assessed by
128 determining non-protein sulphhydryl content, as in (23), and calculated using a GSH standard curve. Results are
129 expressed as µg GSH/mg of cellular proteins.

130 *Plasmids*

131 The cDNA sequence for mouse xCT (NM_011990.2), in the pDream2.1 plasmid (GenScript), was cloned in a
132 pVAX1 (Invitrogen) plasmid (pVAX1-xCT), sequenced (BMR Genomics), and produced with EndoFree
133 Plasmid Giga kits (Qiagen Inc.).

134 *Immune sera effect on tumorsphere formation*

135 Serum IgG from vaccinated mice were purified using the Melon Gel purification kit (Thermo Scientific) and
136 incubated with tumorsphere-derived cells. After 5 days, spheres were counted and analyzed for CSC markers
137 expression and ROS production by FACS.

138 *In Vivo Treatments*

139 Wild type (Charles River Laboratories) and Ig μ -chain gene knocked out (BALB- μ IgKO) (24) BALB/c mice
140 were maintained at the Molecular Biotechnology Center, University of Torino, and treated in accordance with
141 University Ethical Committee and European guidelines under Directive 2010/63. Vaccination, performed either
142 before or after tumor challenge, consisted of two i.m. electroporation at two weeks interval, of pVAX1 or
143 pVAX1-xCT plasmids as previously described (25).

144 Primary s.c. tumors were induced by injecting 1×10^4 TUBO or 4T1 tumorsphere-derived cells. Some tumors
145 were explanted and tumorspheres generated as in (26). Lung metastases were induced either by injecting i.v.
146 5×10^4 TUBO tumorsphere-derived cells or by injecting s.c. 1×10^4 4T1 tumorsphere-derived cells. In the latter
147 case, lungs were removed when s.c. tumors reached 10 mm mean diameter. Micrometastases were counted on a
148 Nikon SMZ1000 stereomicroscope (Mager Scientific).

149 Doxorubicin treatment consisted of the i.v. administration of a total dose of 10 mg/Kg either in a single
150 injection or in two administration at a week interval.

151 *Statistical analysis*

152 Differences in latency, sphere formation, protein expression, GSH and ROS levels and metastasis number were
153 evaluated using a Student's *t*-test. Data are shown as the mean \pm SEM unless otherwise stated. Values of *P*
154 $< 0,05$ were considered significant.

155

156 **xCT is upregulated in breast CSC**

157 To identify the transcripts associated with mouse and human mammary CSC, we compared the transcription
158 profile of Her2⁺ murine TUBO cells which had been cultured as an epithelial monolayer, with the profiles of the
159 first three *in vitro* passages of their derived tumorspheres (P1, P2 and P3) using MouseWG-6 v2.0 Illumina
160 beadchips (GSE21451) (Supplementary Figure 1A). This analysis uncovered a cluster of transcripts whose
161 expression rose, as well as three clusters whose expression decreased from TUBO through P1 to P3 cells
162 (Supplementary Figure 1B).

163 We devised a ranking procedure according to the clinical outcome (CO) of tumors expressing the transcripts
164 that we found increased in tumorspheres (27), using data from six public human breast cancer datasets (see
165 Supplementary methods). One of the genes with the best CO score was xCT (7) (Slc7a11, Supplementary
166 Figure 1C), whose expression increased progressively from TUBO to P3 tumorspheres, as confirmed by FACS
167 (Figure 1A) and qPCR (Supplementary Figure 2) analyses. Interestingly, most P3-derived cells that express the
168 stem cell marker Sca-1 (26) are also xCT⁺ (Figure 1B). The immunofluorescence analysis revealed widespread
169 xCT positivity in tumorspheres that are essentially composed of CSC, as confirmed by Sca-1, OCT4 and Thy1.1
170 marker expression patterns (Figure 1C). xCT upregulation is a feature of breast CSC and is not due to
171 tumorsphere culture conditions, since it was also observed on the small CD44^{high}CD24^{low} CSC population
172 present in TUBO cells (Figure 1D). Moreover, xCT upregulation is not restricted to TUBO-derived CSC as it
173 was also observed in tumorspheres derived from mouse (4T1) and human (HCC-1806 and MDA-MB-231)
174 TNBC cell lines (Figure 1E), suggesting that xCT may be a hallmark of breast cancer CSC.

175 xCT expression in the TMA of normal and neoplastic samples was evaluated to address its distribution in
176 human cancers. xCT expression was low in normal mammary glands (Figure 1F, left panel) as it was in the
177 other normal tissues tested (Supplementary Figure 3A). By contrast, xCT was expressed at high levels in many
178 neoplastic tissues (Supplementary Figure 3B), including hyperplastic mammary lesions and invasive ductal
179 breast carcinomas (IDC) (Figure 1F, middle and right panels) displaying a pattern in which it is confined to
180 neoplastic cells. In particular, we found xCT expression in 62% of Her2⁺, 57% of estrogen/progesterone

181 receptor⁺ Her2⁻ (ER/PR⁺Her2⁻), and 35% of TNBC samples (Figure 1G), suggesting that xCT may well be a
182 commonly upregulated target in breast cancers.

183 **xCT downregulation impairs tumorsphere generation and alters intracellular redox balance**

184 A MTT test was performed on TUBO cells and tumorspheres that had either been treated or not with scalar
185 doses of xCT inhibitor SASP (28). While SASP did not decrease TUBO cell viability, except for the highest
186 dose (100 μ M; IC₅₀ 126,1 \pm 25,7 μ M; Figure 2A), tumorsphere viability was inhibited in a dose dependent
187 manner (IC₅₀ 51,6 \pm 3,5 μ M; Figure 2B), suggesting that CSC are sensitive to xCT inhibition. Similarly, xCT
188 silencing through a pool of specific siRNAs impaired tumorspheres but not TUBO cell viability (Figures 2A,
189 B). Moreover, SASP treatment and xCT silencing impaired tumorsphere generation (Figure 2C, D). FACS
190 analyses performed 24 hours after siRNA transfection showed that the reduction in xCT⁺ cells (Figure 2E, F) is
191 accompanied by a reduction in CSC, i.e. Sca1⁺ and CD44^{high}/CD24^{low} cells (Figure 2F). On the contrary, xCT
192 overexpression increases colony generating ability, as confirmed by the higher number of colonies generated in
193 soft agar by NIH/3T3 and HEK-293 cells transfected with xCT when compared to the corresponding cells
194 transfected with empty plasmids (Supplementary Figure 4). Taken together, these data suggest that xCT plays
195 an important role in CSC maintenance and sphere generation.

196 As xCT is an important determinant of redox balance (13), we evaluated GSH and ROS levels in TUBO cells
197 and tumorspheres. GSH amount was significantly greater in tumorspheres than in TUBO cells (Figure 2G),
198 whereas ROS levels were lower (Figure 2H). xCT downregulation caused a significant decrease in GSH and an
199 increase in ROS levels (Figure 2G, H) as compared to controls, suggesting that CSC have a higher ROS defense
200 capability than epithelial tumor cells.

201 **Anti-xCT vaccination induces antibodies that inhibit CSC**

202 BALB/c mice were vaccinated with either pVAX1-xCT or pVAX1 to evaluate whether xCT is a potential target
203 for cancer immunotherapy. No T-cell response was observed against the H-2K^d dominant mouse xCT peptide
204 (Supplementary Figure 5A, B). Tumorsphere-derived cells were stained with the sera of vaccinated mice to

205 evaluate their humoral response, and specific antibody binding was analyzed by FACS. pVAX1-xCT
206 vaccination induced the production of CSC-binding antibodies, which were not detectable in empty pVAX1
207 vaccinated mouse sera (Figure 3A-C). These results were confirmed by the ability of purified IgG, from
208 pVAX1-xCT vaccinated mouse sera, to stain tumorspheres (Figure 3D). These antibodies are specific for xCT,
209 since no binding was observed in NIH/3T3 cells negative for xCT expression (Figure 3E-H).

210 Of note, TUBO cells incubated with IgG purified from pVAX1-xCT vaccinated mice displayed reduced sphere-
211 generation ability (Figure 3I), a lower percentage of stem cell marker positive cells (Figure 3L), but increased
212 ROS content as compared to control IgG (Figure 3M).

213 These results suggest that anti-xCT vaccination induces antibodies targeting xCT, thus affecting self-renewal
214 and ROS production in CSC.

215 **Anti-xCT vaccination slows *in vivo* breast tumor growth**

216 TUBO-derived tumorspheres were s.c. implanted into BALB/c mice that were vaccinated when tumors reached
217 2 or 4 mm mean diameter to evaluate whether xCT immune-targeting hinders breast cancer growth (Figure 4A-
218 D). Tumors grew progressively in the pVAX1 group of 2 mm vaccinated mice (Figure 4A), while tumors
219 regressed in 23,8% of pVAX1-xCT vaccinated mice (Figure 4B). Tumor growth kinetics were slower in the
220 latter group than in the pVAX1 group, as proven by the significantly shorter time required for tumors to reach 4
221 or 6 mm mean diameter ($20.7 \pm 2,7$ and $30.7 \pm 3,6$ days in pVAX1-mxCT vaccinated mice versus 12.9 ± 2 and
222 $20.8 \pm 2,5$ days in control mice). Anti-xCT vaccination also induced tumor regression in 16% of mice that were
223 treated when their tumors measured 4 mm mean diameter (Figure 4D), while all tumors in the pVAX1 group
224 reached 10 mm mean diameter in less than 60 days (Figure 4C). The efficacy of anti-xCT vaccination was then
225 evaluated against 2 or 4 mm mean diameter tumors obtained when 4T1 tumorsphere derived-cells were injected
226 s.c. (Figure 4E-H). In 2 mm tumor vaccinated mice, tumors grew rapidly in pVAX1 group (Figure 4E), while
227 tumor growth kinetics were generally slower and the time required for the tumors to reach 4, 6, 8 or 10 mm
228 mean diameter was significantly longer in the pVAX1-xCT vaccinated group (10.4 ± 1.3 ; 15.6 ± 1.6 ; 20.4 ± 1.3 ;
229 23.4 ± 1.2 days in pVAX1-xCT vaccinated mice versus 4.9 ± 0.5 ; 10 ± 1.1 ; 14.6 ± 1.0 ; 17.4 ± 0.8 days in control

230 mice). Similarly, the 4 mm tumor vaccinated group displayed slower tumor growth in pVAX1-xCT vaccinated
231 mice (Figure 4E, H), and the time required for the tumors to reach 6, 8 or 10 mm mean diameter was
232 significantly longer (9.2 ± 0.9 ; 13.1 ± 0.9 ; 17.0 ± 0.5 days in pVAX1-xCT vaccinated mice versus 5.2 ± 0.9 ; 8.8 ± 1.0 ;
233 13.0 ± 1.6 days in control mice), indicating that xCT immunotherapy may be beneficial in various breast cancer
234 subtypes.

235 Tumor remission in vaccinated mice might be due to a reduction of CSC frequency as a consequence of the
236 treatment, as suggested by the decrease in the percentage of Aldefluor⁺ cells in regressing tumors from mice
237 vaccinated with pVAX1-xCT plasmid (Figure 4I). Moreover, the cells composing the tumor mass had a
238 significantly decreased tumorsphere forming ability (Figure 4L) when compared with cells derived from tumors
239 grown in pVAX1 vaccinated mice.

240 **Anti-xCT vaccination prevents lung metastasis formation**

241 BALB/c mice were vaccinated with pVAX1 or pVAX1-xCT plasmids and i.v. injected with TUBO-derived
242 tumorspheres to evaluate the effects of anti-xCT vaccination on lung metastasis formation. Metastasis number
243 was significantly reduced after pVAX1-xCT vaccination, as reported in Figures 5A and B. This anti-metastatic
244 effect is dependent on the specific antibodies elicited by anti-xCT vaccination, since no effect was observed
245 vaccinating BALB- μ IgKO mice i.v. injected with TUBO-derived tumorspheres (Figures 5C, D).

246 Anti-xCT vaccination was also able to reduce the number of spontaneous metastases generated from the s.c.
247 injection of 4T1-derived tumorspheres, either when vaccination was performed before tumorspheres injection
248 (Figure 5E, F) or when mice already had a 2 mm mean diameter tumor (Figure 5G, H).

249 Altogether, these findings suggest that anti-xCT vaccination interferes with CSC metastatic properties both in a
250 preventive and therapeutic setting. This anti-metastatic activity is due to CSC immunotargeting, since no effect
251 was observed in xCT-vaccinated mice injected with differentiated tumor cells (Supplementary Figure 6A, B) or
252 in mice vaccinated against Her3 (29) and injected with TUBO-derived tumorspheres (Supplementary Figure 7
253 A-F). In this model Her3 is not a CSC-specific antigen, since it is equally expressed on TUBO cells and
254 tumorspheres.

255 **Anti-xCT vaccination enhances the effect of doxorubicin**

256 In accordance with CSC resistance to chemotherapy (2), TUBO cells display a higher sensitivity to doxorubicin
257 than tumorspheres (Figure 6A, B). Since xCT is involved in maintaining the intracellular redox balance, thus
258 counteracting the effects of ROS-generating cytotoxic drugs (13), it is likely that targeting xCT could increase
259 CSC chemosensitivity. In order to explore this hypothesis *in vivo*, unvaccinated, pVAX1-xCT and pVAX1
260 vaccinated mice were i.v. injected with TUBO-derived tumorspheres and either treated with doxorubicin or not.
261 As shown in Figure 6C, pVAX1-xCT determined a decrease in the number of lung metastases compared to the
262 control and doxorubicin treated mice and the combination of vaccination and doxorubicin significantly
263 improved the activity of individual treatments.

264 Similar results were observed in mice challenged with s.c. injection of TUBO-derived tumorspheres and
265 subjected to vaccination and chemotherapy when tumors reached 2 mm mean diameter. The tumor regressed in
266 25% of mice treated with doxorubicin alone (Figure 6D) or in combination with pVAX1 plasmid (Figure 6E),
267 while the combination of doxorubicin and anti-xCT vaccination stopped tumor progression in 60% mice (Figure
268 6F).

269 All together, these data suggest that anti-xCT vaccination may well be an efficient adjuvant treatment for
270 chemotherapy both in a preventive and in a therapeutic setting.

271

272 **Discussion**

273 A key challenge in anticancer therapy is the development of treatments able to both shrink a tumor and kill
274 CSC, which are resistant to current chemo- and radio-therapies, and are considered the source of tumor
275 recurrence and metastatic spread. However, the identification of ideal CSC-associated targets is a particularly
276 tough task since CSC appear to be “moving targets” that switch between different cell states during cancer
277 progression (30). Hence, antigens that are upregulated in CSC but also present in more differentiated cancer
278 cells would appear to be outstanding candidates.

279 A possible candidate with these features is xCT, that we have identified as upregulated in breast Her2⁺ and
280 TNBC CSC. Importantly, its expression is not confined to CSC, since we were able to detect its presence on
281 cancer cells in human hyperplastic mammary glands as well as in IDC, independently from their histological
282 subtype. xCT possesses all the features of an ideal target for immunotherapy against undifferentiated and more
283 differentiated cancer cells. This speculation is strengthened by our meta-analyses, performed on human breast
284 cancer datasets, that link high xCT expression to poor prognosis.

285 Moreover, xCT expression is not limited to breast cancer. Our analyses, performed on a plethora of healthy and
286 neoplastic tissues, and data obtained elsewhere in other tumors (7,31,32) have identified xCT as a distinctive
287 cancer marker and suggested that its targeting may be of benefit in the treatment of a wide range of neoplastic
288 diseases.

289 Another important feature of xCT as immunotherapy target is its functional role, which becomes essential in
290 CSC. Indeed, the impairment of sphere-generation following the pharmacological or genetic downregulation of
291 xCT reflects the role of this protein in the regulation of CSC self-renewal, indicating that xCT is not a simple
292 bystander of the stem-like phenotype in breast cancer, but that it also plays a role in CSC biology.

293 xCT function in CSC self-renewal might be mediated by the regulation of the mechanisms that govern CSC
294 intracellular redox balance. Although further studies are needed to better define this mechanism in our model, it
295 has been shown that intracellular ROS concentration can affect CSC viability and self-renewal (33-35). CSC
296 upregulate the key regulator of the anti-oxidant response Nuclear factor erythroid 2-related factor 2 (36) and

297 many anti-oxidant enzymes, such as superoxide dismutase 2, glutathione peroxidases and heme oxygenase 1,
298 that attempt to maintain intracellular ROS at levels lower than those observed in differentiated cancer cells (37).
299 In accordance with this, a basal increase in GSH and decrease in ROS levels were observed in tumorspheres as
300 compared to TUBO cells, which can be explained by xCT upregulation in tumorspheres and proven by the fact
301 that xCT downregulation reverts this phenotype.

302 Several authors have advocated the use of xCT as a therapeutic target for pharmacological inhibition using the
303 administration of SASP, a FDA-approved, anti-inflammatory drug for the treatment of inflammatory bowel
304 disease, ulcerative colitis and Crohn's disease (38). However, SASP exerts many effects in addition to its ability
305 to interfere with xCT function, so it cannot be considered a specific xCT inhibitor (39). Although recent
306 preclinical models demonstrated that SASP impairs cancer growth and metastatic spread via xCT inhibition
307 (15,17,40), its use in cancer patients is hampered by its low specificity, short bio-availability and numerous side
308 effects (41).

309 We have chosen DNA-based antitumor vaccination as our xCT targeting option on the basis of our consolidated
310 expertise in the field and the considerations stated above. In fact, DNA vaccination is a cost-effective
311 technology able to induce a significant immune activation against tumor antigens in a specific and well tolerated
312 way, thus reducing off-target effects (42). This paper sees the first report of xCT being targeted with a DNA-
313 based vaccination strategy that efficiently slows mammary tumor growth and prevents lung metastasis
314 formation. Our data show that, despite xCT being a self-antigen, DNA vaccination is able to induce an
315 antibody-based immune response against the tumor; a lack of T-cell response against xCT may be caused by a
316 thymic depletion of high-avidity T-cell clones, as we have previously reported for the Her2 antigen in the
317 BALB-neuT model (43). Notably, the anti-tumor effects were lost in B cell deficient mice, confirming the
318 central role of vaccine-induced antibodies. The anti-tumor effects observed *in vivo* may result from the CSC
319 self-renewal and redox balance impairment induced by anti-xCT antibodies, as indicated by our *in vitro*
320 observations. This is particularly evident in the inhibition of lung metastasis formation, most likely because
321 CSC self-renewal is essential for re-initiating growth at the metastatic site (44).

322 Interestingly, we were not able to detect any vaccine side effect as xCT expression in normal tissues is almost
323 completely limited to the brain, which can barely be reached by circulating antibodies by virtue of the blood-
324 brain barrier (45). However, it has been reported that xCT may also regulate immune cell functions. In fact,
325 xCT mediates cystine uptake in macrophages and dendritic cells, which are the only source of free cysteine
326 release which, in turn, is essential for the antigen-driven activation of T lymphocytes (46). However, by
327 vaccinating mice against both xCT and Her2, we observed that xCT targeting does not impair the Her2 specific
328 T cell response (Supplementary Figure 5C, D). The safety of xCT immune targeting is further sustained by the
329 fact that its genetic ablation in mice does not alter vital biological functions (47).

330 xCT participate in cancer cells' resistance to a variety of antitumor drugs (16). Its action here is thought to be
331 linked to its role in intracellular redox balance, as many chemotherapeutic drugs exert their function, at least in
332 part, by increasing oxidative stress (48). It is worth noting that tumorspheres display significantly increased
333 resistance to doxorubicin, a drug largely used in breast cancer therapy, as compared to epithelial TUBO cells.
334 This fits with the observation that CSC are chemoresistant (2), and may reflect the increased expression of xCT
335 in tumorspheres. In this regard, it has been demonstrated that xCT inhibition promotes the sensitization of tumor
336 cells to doxorubicin (49,50). In accordance with these findings, we have observed that a combination of anti-
337 xCT vaccination and doxorubicin strongly enhanced the anti-metastatic potential of the individual treatments.
338 This observation strengthens the translatability of this immunotherapeutic approach to clinical trials, where new
339 experimental protocols are routinely tested in combination with standard treatments.

340 In conclusion, we have shown for the first time that xCT immunotargeting is effective in impairing tumor
341 growth and metastasis formation *in vivo*. We propose a mechanism by which anti-xCT vaccination exerts its
342 anti-neoplastic function across two separate, but complementary, fronts: i) a direct effect on CSC through
343 immune-mediated eradication and ii) inhibition of xCT function on CSC, leading to impairment of tumorigenic
344 and stem-like properties and their sensitizing to chemotherapy. Moreover, a possible additional anti-cancer
345 mechanism may occur, by which the anti-xCT antibodies induced by vaccination may suppress the myeloid

346 derived suppressor cells that exploit xCT for their inhibitory activity (46). However, this hypothesis is still to be
347 verified.

348 Further studies will thoroughly investigate the interactions between chemotherapy and anti-xCT vaccination and
349 all possible side effects in order to accelerate translation to the clinic, as a safe tool to combat CSC with in
350 patients is sorely needed.

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466

467 **Figure legends**

468 **Figure 1. xCT expression in breast CSC and tumors.** (A) FACS analysis of xCT expression in TUBO cells
469 and P1 to P3 tumorsphere passages over six independent experiments. (B) Representative density plots of xCT
470 and Sca-1 expression on TUBO and tumorspheres. Numbers show the percentage of cells in each quadrant. (C)
471 Representative immunofluorescence staining of xCT, Sca-1, OCT4 and Thy1.1 on tumorspheres. DAPI stains
472 the nucleus. Scale bar 20 μm . (D) Representative density plots of xCT expression in TUBO cells stained with
473 CD44 and CD24. (E) FACS analysis of xCT expression in HCC-1806, MDA-MB-231 and 4T1 cells and their
474 derived tumorspheres over three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Student's *t*-test.
475 (F) Immunofluorescence of xCT expression (red) in normal breast, hyperplastic and IDC breast carcinoma.
476 Scale bar 20 μm . (G) Percentage of xCT⁺ samples in normal mammary gland and in TNBC, Her2⁺ or
477 ER/PR⁺Her2⁻ breast cancer subtypes.

478 **Figure 2. xCT regulates CSC self-renewal and the intracellular redox balance.** (A, B) MTT assay of the
479 cytotoxic effect exerted by scalar doses of SASP or by anti-xCT siRNAs on (A) TUBO and (B) tumorspheres.
480 (C) Sphere generation ability relative to untreated cells of tumorspheres incubated with SASP. (D) Sphere
481 generation ability of tumorspheres incubated with siRNAs to xCT, scrambled siRNA or not at all shown as
482 tumorsphere number/ 10^3 plated cells. (E, F) FACS analysis of xCT and CSC marker expression in spheres 24
483 hours after transfection with siRNA to xCT or scrambled siRNA. (E) Gray histograms show xCT expression,
484 open histograms show the background of negative control IgG stained cells from one representative experiment.
485 (F) Relative expression (%) of xCT⁺, Sca-1⁺ and CD44^{high}/CD24^{low} cells in tumorsphere-derived cells
486 transfected with siRNAs to xCT (black bars) compared to cells transfected with scrambled siRNA (dashed line).
487 (G, H) GSH (G) and ROS (H) levels in TUBO cells and their derived tumorspheres after either seeding in
488 normal conditions, transfection with siRNAs to xCT or scrambled siRNA over three independent experiments.
489 * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Student's *t*-test.

490 **Figure 3. Vaccine-induced antibodies target CSC and affect their self-renewal and ROS flux.** (A-C)
491 TUBO-derived tumorsphere or (E-G) NIH/3T3 cell staining by antibodies present in the sera of BALB/c mice

492 vaccinated with pVAX1 or pVAX1-xCT, analyzed by FACS. Results are reported as (A, E) the mean
493 fluorescence intensity (MFI) from 7 mice per group, (B, F) the percentage of positive cells, and (C, G) two
494 representative dot plots. (D, H) Representative images of (D) TUBO-tumorspheres or (H) NIH/3T3 cells stained
495 with IgG purified from sera of mice vaccinated with pVAX1 or pVAX1-xCT. Scale bar 20 μ M. (I) Sphere
496 generating ability of tumorspheres incubated for 5 days with IgG purified from the sera of mice vaccinated with
497 pVAX1, pVAX1-xCT or not at all. Graph shows tumorsphere number/ 10^3 plated cells. FACS analysis of (L)
498 CSC marker expression or (M) ROS production in tumorspheres incubated for 5 days with IgG purified from
499 the sera of vaccinated mice or not at all, reported as (D) percentage of positive cells or of the (E) DCF MFI
500 from four independent experiments. * $P < 0.05$, ** $P < 0.01$, Student's *t*-test.

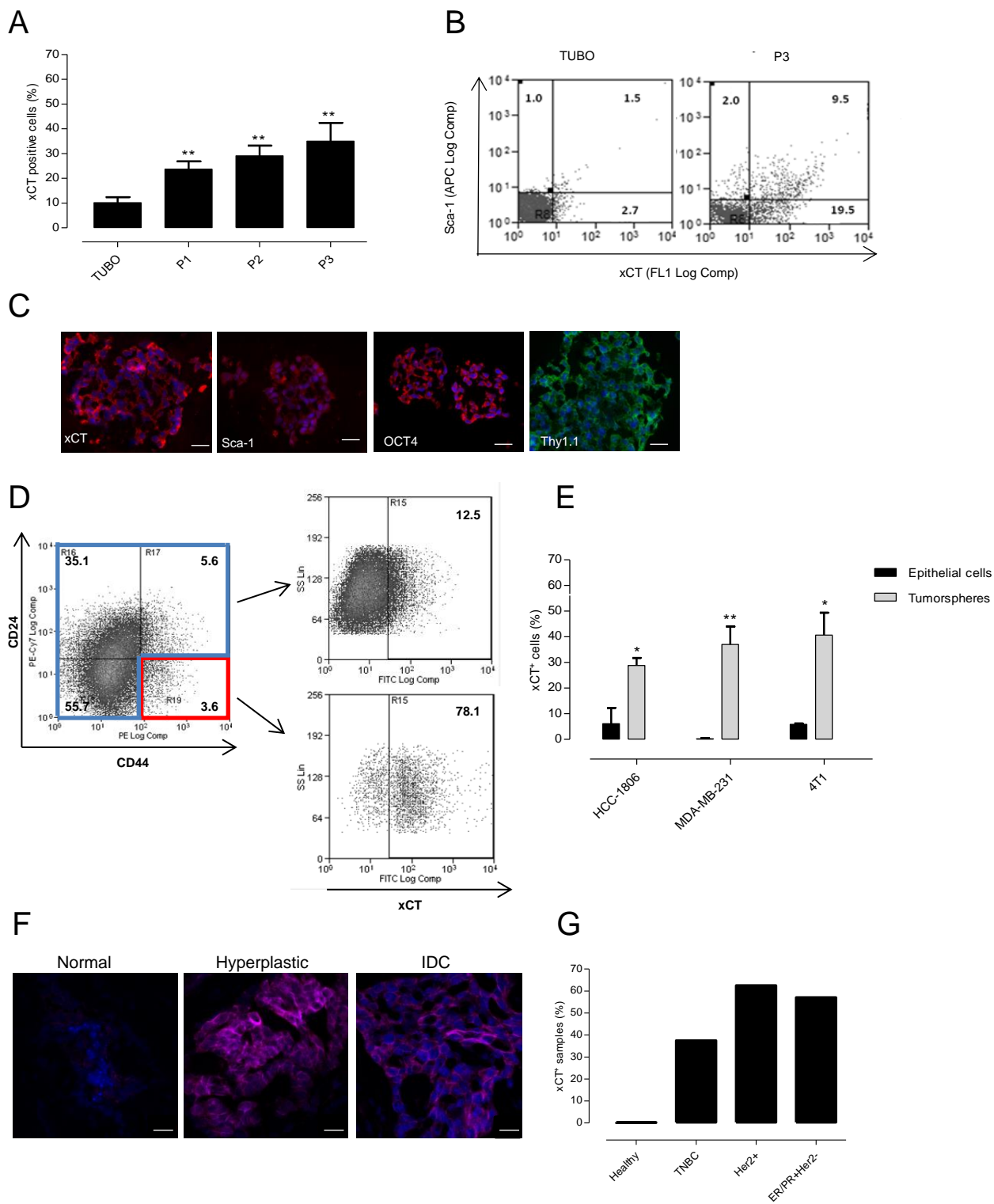
501 **Figure 4. Anti-xCT vaccination delays CSC-induced tumor growth *in vivo*.**

502 BALB/c mice were s.c. challenged with tumorspheres derived from either (A-D) TUBO or (E-H) 4T1 cells and
503 electroporated with (A, C, E, G) pVAX1 or (B, D, F, H) pVAX1-xCT plasmids when their tumor reached (A,
504 B, E, F) 2 or (C, D, G, H) 4 mm mean diameter. Each black line depicts the growth of a single tumor. Data were
505 cumulated from three independent and concordant experiments. Statistically significant differences in mean
506 time required for pVAX1-xCT group and pVAX1 group tumors to reach 4, 6, 8 or 10 mm mean diameter are
507 indicated by dashed gray lines. (I, L) Analysis of (I) the percentage of Aldefluor⁺ cells in tumors explanted from
508 vaccinated mice challenged s.c. with TUBO-derived tumorspheres and (L) number of tumorspheres generated
509 *in vitro* by cells from the same tumors. * $P < 0.05$, ** $P < 0.01$.

510 **Figure 5. Anti-xCT vaccination reduces CSC-generated lung metastasis formation. (A-B and E-H)**

511 BALB/c and (C, D) BALB- μ IgKO mice were vaccinated with either pVAX1 or pVAX1-xCT plasmids (A, C,
512 E) before tumorspheres injection or (G) when mice had 2 mm mean diameter tumor. (A, C, E, G) Number of
513 lung metastases in mice challenged (A, C) i.v. with TUBO- or (E, G) s.c. with 4T1-derived tumorspheres and
514 enumerated (A, C) 20 days later or (E,G) when the primary tumor reached 10 mm mean diameter. (B, D, F, H)
515 representative images of lung metastases after H&E staining ** $P < 0.01$, *** $P < 0.001$, Student's *t*-test.

516 **Figure 6. Anti-xCT vaccination enhances the effect of doxorubicin *in vivo*.** (A, B) MTT assay of the
517 cytotoxic effect exerted by incubation with scalar doses of doxorubicin in (A) TUBO and (B) tumorspheres. (C)
518 Number of lung metastases in mice challenged i.v. with TUBO-derived tumorspheres and either vaccinated or
519 not with pVAX1 and pVAX1-xCT plasmids alone or in combination with doxorubicin administration. (D-F)
520 Tumor growth curves of BALB/c mice s.c. injected with TUBO derived tumorspheres and treated with
521 doxorubicin in combination with (E) pVAX1 or (F) pVAX1-xCT vaccination when their tumors reached 2 mm
522 mean diameter. Treatments were repeated the week later. Each black line depicts the growth of a single tumor.
523 Student's *t*-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Student's *t*-test.



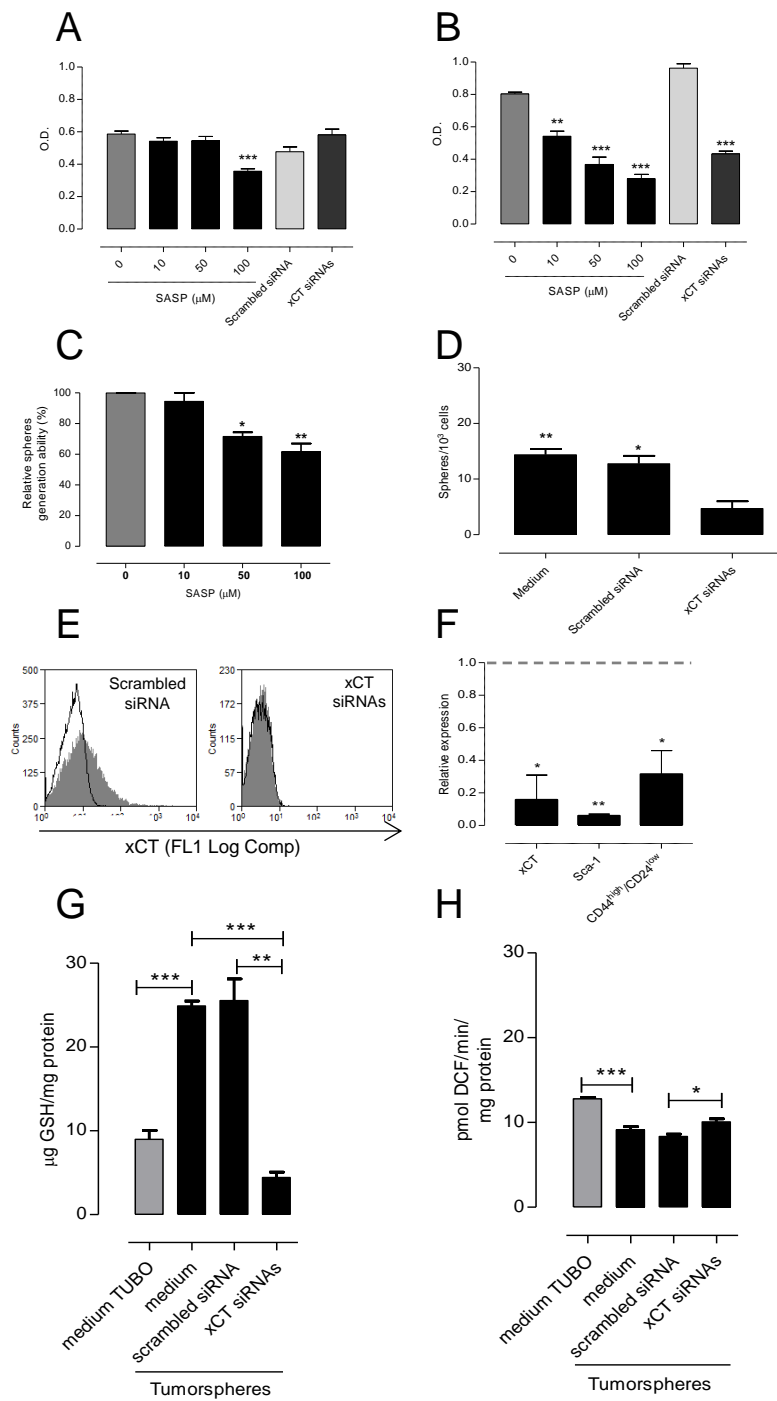
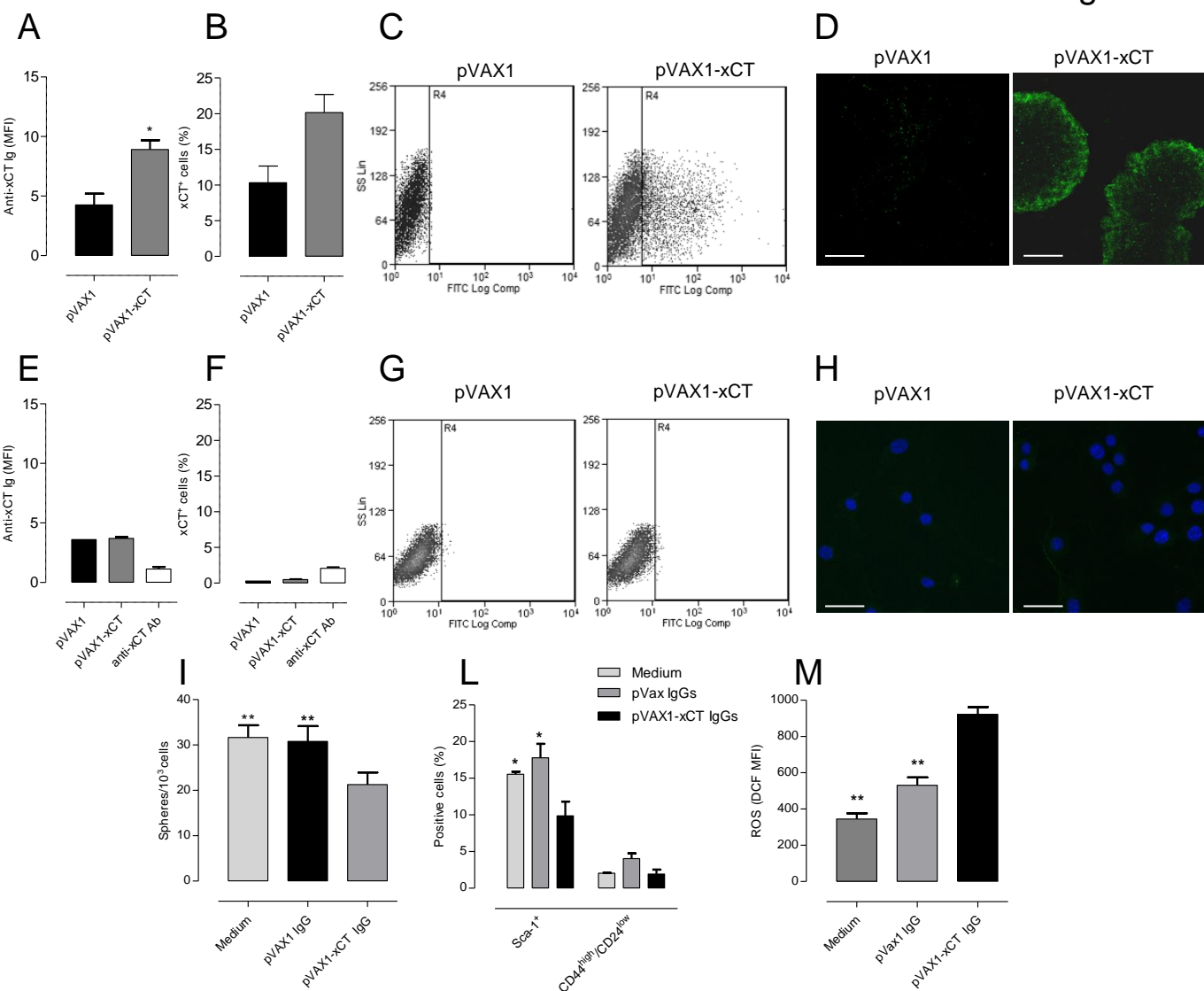
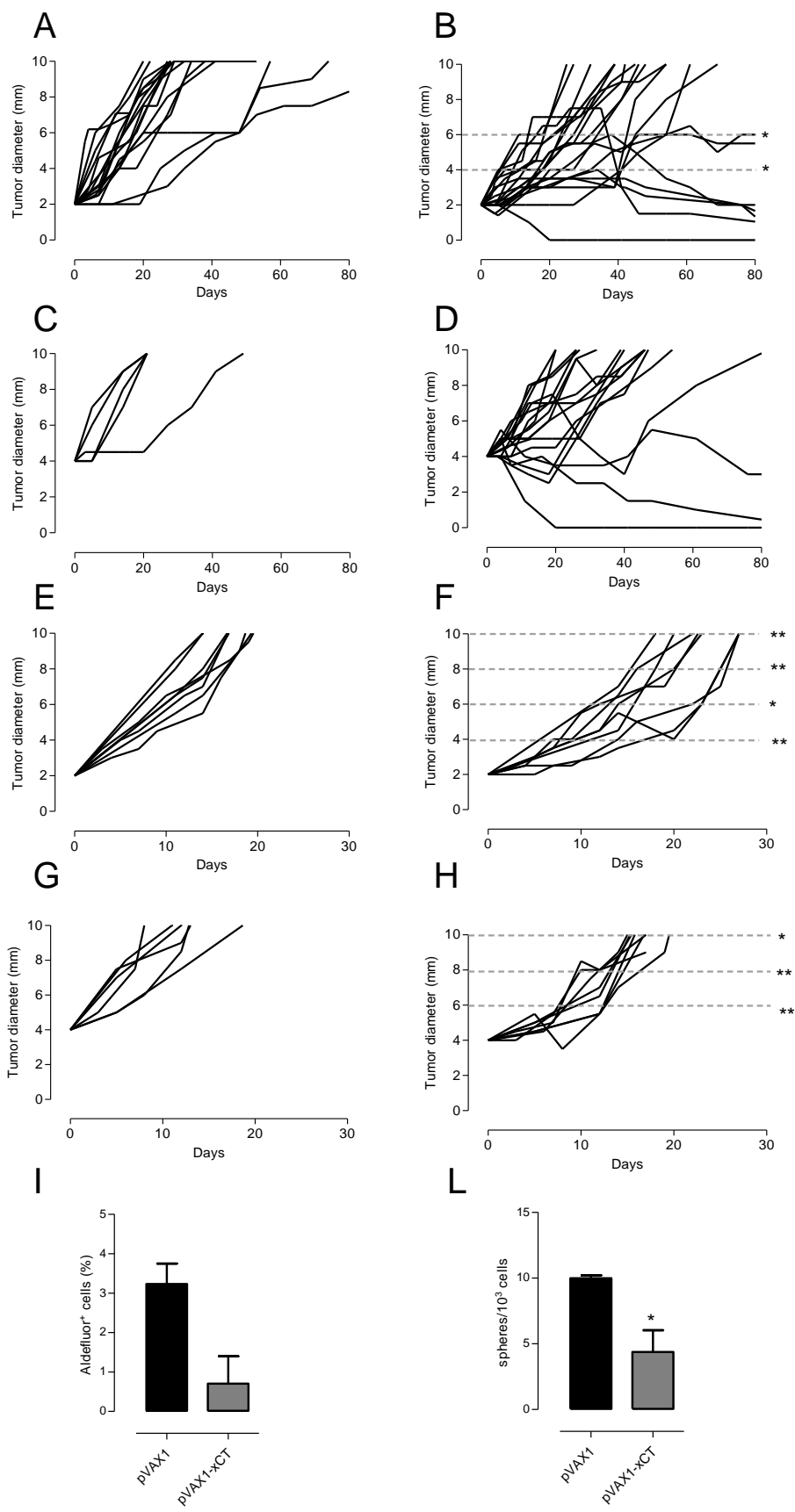


Figure 3





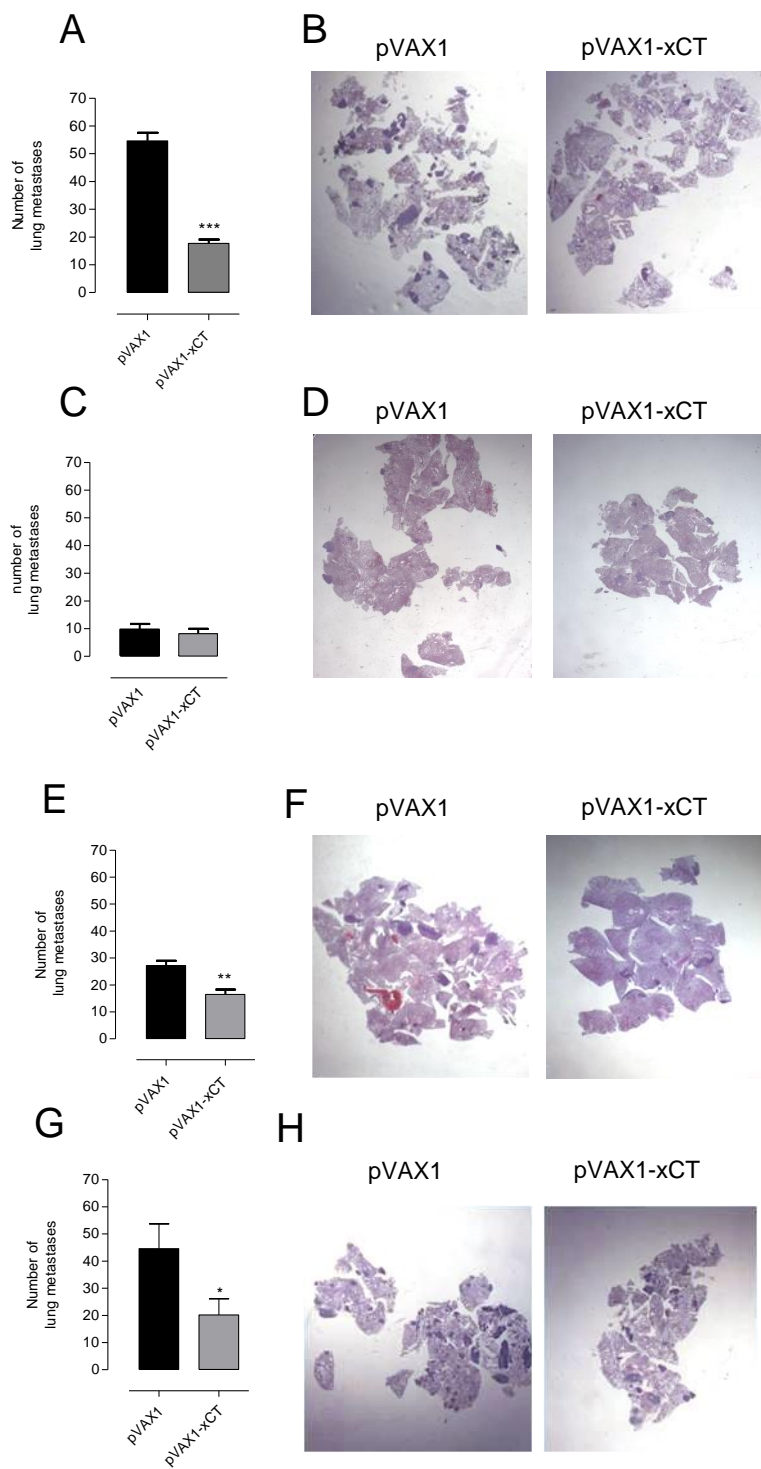


Figure 6

