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# **Contribution of humoral immune responses to the antitumor effects mediated by anthracyclines.**

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**Running title:** Contribution of B cell responses after chemotherapy

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Authors declare no conflict of interest.

## Abstract

Immunogenic cell death (ICD) induced by cytotoxic compounds contributes to the success of selected chemotherapies by eliciting a protective anticancer immune response, which is mediated by CD4<sup>+</sup> and CD8<sup>+</sup> T cells producing interferon- $\gamma$  (IFN $\gamma$ ). In many instances, cancer progression is associated with high titers of tumor-specific antibodies, which become detectable in the serum but whose functional relevance is elusive. Here we explored the role of humoral immune responses in the anticancer efficacy of anthracyclines. Chemotherapy reduced the number of tumor-infiltrating B cells and failed to promote humoral responses against immunodominant tumor antigens. While anthracycline-based anticancer chemotherapies failed in T cell-deficient mice, they successfully reduced the growth of cancers developing in mice lacking B lymphocytes (due to the injection of a B cell-depleting anti-CD20 antibody), immunoglobulins or immunoglobulin receptors (FcR) due to genetic manipulations. These results suggest that the humoral arm of antitumor immunity is dispensable for the immune-dependent therapeutic effect of anthracyclines against mouse sarcoma. In addition, we show here that the titers of IgA and IgG antibodies directed against an autoantigen appearing at the cell surface of tumor cells post-chemotherapy (calreticulin) did not significantly increase in patients treated with anthracyclines and that anti-calreticulin antibodies had no prognostic or predictive significance. Collectively, our data indicate that humoral anticancer immune responses differ from cellular responses in thus far that they do not contribute to the success of anthracycline-mediated anticancer therapies in human breast cancers and mouse sarcomas.

**KEYWORDS:** antibodies, immunogenic cell death, chemotherapy, humoral immunity, Fc $\gamma$ R, cancer.

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**ABBREVIATIONS:**

ADCC: Antibody-dependent cell cytotoxicity

ATP: Adenosine triphosphate

CRT: Calreticulin

CTL: Cytotoxic T Lymphocyte

DC: Dendritic cell

ER: Endoplasmic Reticulum

FcR: Fc Receptor

HMGB1: High mobility Group Box-1

ICD: Immunogenic Cell Death

Mincle: Macrophage-inducible C type lectin

Myd88: Myeloid differentiation primary response gene

SNP: Single nucleotide polymorphism

TLR: Toll-like receptor

## Introduction

Cancer often results from a chronic inflammatory process in which multiple hematopoietic and/or immune components participate.<sup>1, 2</sup> The question arises as to which immune cell subset directly or indirectly promote malignancy, and whether these chronic infiltrates are generic across multiple solid tumor types or instead reflect tissue-selective, or oncogenic pathway-selective, profiles. If the contribution of leukocyte populations to oncogenesis or tumor progression was generic, therapeutics aimed at quelling the protumorigenic activities of such subsets could be proposed. Hence, it is of utmost importance to investigate how the interplay between inflammatory cells is orchestrated and modulated during anticancer therapies.

Dendritic cells (DCs) are bone marrow-derived cells that are present in all tissues<sup>3</sup> where they scan the environment and take up foreign antigens (including tumor antigens) before migrating to lymphoid organs to trigger adaptive immune responses.<sup>3</sup> Upon recognition of a foreign antigen, T and B lymphocytes undergo clonal expansion and mount antigen-specific “cognate” responses. Once the adaptive immune response has ensued, the source of danger or damage is eliminated, inflammation declines and tissue homeostasis is restored. In tumors, these well-orchestrated series of events fail to resolve and lead to chronic inflammation. Activated leukocytes (mostly represented by myeloid cells) supply direct and indirect mitogenic growth mediators and proteolytic enzymes that stimulate expansion and dissemination of both neoplastic and stromal cells<sup>4</sup>. Moreover, the chronic presence of paracrine and juxtacrine mitogenic and tissue-remodeling molecules fuels programs that impair anti-tumor cytotoxic T lymphocyte (CTL) or NK/NKT cell-mediated killing of “damaged” (cancer) cells (reviewed in<sup>5</sup>).

In addition to myeloid cells, lymphocytes play an important role in chronic inflammation. B cells display variable pro- and anti-tumor bioactivities deriving from their functional plasticity and phenotypic heterogeneity. As the sole producers of immunoglobulins (Ig), B cells are critical initiators and modulators of humoral immunity, tailoring specific responses to infections. In addition, B lymphocyte-derived paracrine factors are causative and/or

potentiating mechanisms of disease, as shown in autoimmunity.<sup>6</sup> Moreover, there is accumulating evidence that B cell function is relevant to carcinogenesis. Cancer patients often develop specific antibody responses against tumor antigens,<sup>6</sup> which are mostly correlated with poor survival.<sup>7</sup> Progression of human papillomavirus type 16 E6/E7-driven squamous carcinogenesis in transgenic mice is significantly diminished in the absence of B cells.<sup>8,9</sup> In this preclinical tumor model, promotion of the early steps of carcinogenesis is achieved through deposition of IgG autoantibody in neoplastic parenchyma and immune complex/FcγR ligation of mast cells and macrophages, eventually fostering pro-angiogenic and immunosuppressive gene expression programs.<sup>8,9</sup> Similarly, skin chemical carcinogenesis by two-stage application of the DNA damaging agent DMBA and the tumor promoter TPA occurs more rapidly in B cell -proficient mice than in B cell-deficient mice.<sup>10</sup> As a possibility, B cells may modulate myeloid cell phenotypes through the secretion of IL-10 and TNFα, thereby accelerating tumor progression. Finally, B cell-derived lymphotoxin β is responsible for promoting castration resistance in mice with transgene-induced prostate cancer, likely by stimulating IKKα and STAT3 activity in malignant cells.<sup>11</sup> Whereas these studies revealed protumorigenic potential for B cells, others reported no specific functional significance for B cells during mammary carcinogenesis.<sup>12</sup>

The efficacy of anticancer therapies is determined by tumor cell-intrinsic as well as by cell-extrinsic, in part immune factors. Thus, therapeutic settings that induce tumor antigen-specific Th1/Tc1 responses (i.e. immune responses that involve specific T cell subsets producing IFNγ) are particularly efficient in eliminating residual tumor cells. The way cancer cells die when exposed to selected chemotherapeutics (such as anthracyclines and oxaliplatin) die, ultimately dictates the immunogenicity of cell death. “Immunogenic cell death”(ICD) can be defined as a process in which cell death is preceded by a preapoptotic endoplasmic reticulum stress response and autophagic program, facilitating the recruitment, activation and antigen-presenting function of DCs.<sup>13</sup> Indeed, ICD is accompanied by exposure or secretion of cell death-associated molecular patterns such as calreticulin (for the engulfment of dead bodies by DC), HMGB1 (for the TLR4/Myd88-dependent processing of the phagocytic cargo by DC) and ATP (for DC recruitment and the Nlrp3-dependent IL-1β release culminating in Tc1 polarization).<sup>13</sup> Accordingly, after

exposure to neoadjuvant chemotherapy, approximately one third of stage 2/3 breast cancers appeared highly infiltrated by cytotoxic T cells with a concomitant reduction of B lymphocytes and Th2 cells.<sup>14</sup> Moreover, the relative proportions of macrophages and CD8<sup>+</sup> T cells predict pathological responses to neoadjuvant anthracycline-based therapy and overall survival in locally advanced breast cancer.<sup>15</sup>

Since conventional chemotherapies can elicit tumor-specific T cell immunity, which then contribute to durably and negatively inflecting the tumor growth curve, we addressed the question whether anticancer therapies would also stimulate B cell- or FcR-dependent immune responses. Using a variety of complementary experimental settings, we came to the conclusion that humoral immune responses are not specifically induced by chemotherapy and that they do not significantly contribute to tumor control.

## Results

**Intratumoral accumulation of antigen-specific T cells but depletion of B cells after chemotherapy.** To evaluate possible anticancer immune responses mediated by B cells, we determined the absolute numbers of CD19<sup>+</sup>B220<sup>+</sup> B lymphocytes in the CD45<sup>+</sup> leukocytic fraction from tumors established subcutaneously for 18 days and harvested at day 8 post-chemotherapy with anthracyclines, following previously established experimental settings.<sup>16, 17</sup> MCA205-OVA, a methylcholanthrene-induced sarcoma cell line genetically modified to express the candidate tumor antigen OVA, was injected under the skin of histocompatible C57Bl/6 mice, and the developing cancers were treated by intratumoral injections of anthracyclines (doxorubicin). CD45.2 OVA (SIINFEKL)-specific H2-K<sup>b</sup>-restricted TCR transgenic CD8<sup>+</sup> T cells (CTL) that were adoptively transferred into CD45.1 congenic mice on the first day post-chemotherapy proliferated and accumulated in tumor beds (Fig. 1A-B). This expansion of OVA-specific T cells reflects a general increase in the frequency of Th1 and Tc1 cells post-chemotherapy.<sup>16, 17</sup> In sharp contrast, doxorubicin depleted intratumoral B cells by more than 70% (Fig. 1C). Moreover, a single systemic injection of cyclophosphamide reduced the number of splenic B cells, while it increased the frequency of CTL in the spleen (Suppl. Fig.1A-C). Thus conventional chemotherapeutic regimens that stimulate local or systemic T cell responses, can reduce the number of B cells.

**Contribution of cytotoxic T cells but not of B lymphocytes to the therapeutic effect of anthracyclines.** To further assess the role of B cells, we compared the antitumor effects of doxorubicin on MCA205 cancers established in C57Bl6 WT *versus*  $\mu$ MT mice, which lack immunoglobulins and B lymphocytes.<sup>18</sup> In  $\mu$ MT mice, anthracyclines successfully reduced tumor growth, and this effect was undistinguishable from that observed in WT control mice (Fig. 2A). Next, we took advantage of an anti-CD20 antibody,<sup>19</sup> which was applied at different time points pre/post-tumor inoculation and pre/post-doxorubicin (Fig. 3A) to deplete CD19<sup>+</sup>CD20<sup>+</sup> B lymphocytes (Fig. 3B, not shown). B cell depletion did not affect the natural expansion of MCA205 cells (which lack CD20 expression, not shown) in vivo and failed to compromise the efficacy of chemotherapy against this MCA205 sarcomas (Fig. 3C-E). In contrast, depletion of CTLs using anti-CD8 $\alpha$  antibodies completely abrogated the

antitumor effects of anthracyclines (Fig. 4A-B), confirming their contribution to the efficacy of chemotherapy.<sup>13</sup>

Hence, genetic deficiency or antibody-induced depletion of B lymphocytes did not interfere with the therapeutic activity of anthracyclines.

**No contribution of immunoglobulins and Fc receptors to the efficacy of anticancer chemotherapy.** To investigate to which extent immunoglobulins (Ig) might modulate the therapeutic efficacy of chemotherapy, we analyzed the titers of Ig directed against the immunodominant tumor antigen OVA in our model system. OVA-specific IgG/M/A antibody titers (monitored at day 15 and 21 post-therapy) were not boosted by chemotherapy (not shown and Fig. 2B). It is known that leukocyte Fc $\gamma$ R is necessary for squamous carcinogenesis,<sup>8</sup> as well as for short and long-term effects (involving memory T cell responses) in lymphoma bearing mice treated with antibody-dependent cell cytotoxicity (ADCC)-mediating Ab.<sup>20</sup> Therefore, we assessed the putative contribution of Fc $\gamma$ R to the efficacy of chemotherapy against MCA205-OVA sarcoma. Fc $\gamma$ Rs are broadly expressed on immune cells and encompass both activating, e.g., Fc $\gamma$ RI, III, and IV (including the Fc $\gamma$ R subunit), and inhibitory, e.g., Fc $\gamma$ RIIB, subtype complexes.<sup>21</sup> MCA205-OVA was inoculated into mice genetically manipulated to lack specific Fc $\gamma$ R subtypes, namely Fc $\gamma$ RIII,<sup>22</sup> Fc $\gamma$ RI,<sup>23</sup> Fc $\gamma$ RIV,<sup>24</sup> as well as into « Fc $\gamma$ RIV-only » mice (which lack Fc $\gamma$ RI/IIB/III/Fc $\epsilon$ RI/II genes)<sup>25</sup> or « Fc $\gamma$ RIIB only » mice (which lack the Fc $\gamma$ RI/IIIA/IV genes).<sup>26</sup> Thus anthracyclines similarly reduced tumor growth in these Fc $\gamma$ R-deficient mice (Fig. 5A-F) as it did in WT C57Bl6 controls (see above, Fig. 2A). Of note, Fc $\gamma$ R does not bind immunoglobulins but is a subunit that mediates signal transduction by other receptors relevant to myeloid cell function including the macrophage-inducible C-type lectin (Mincle),<sup>27</sup> which plays a cardinal role in antimycobacterial responses.<sup>28</sup> In accord with the observation that Fc $\gamma$ RIII did not affect anthracycline responses (Fig. 5), the efficacy of anthracyclines was not compromised in Mincle knockout mice either (Suppl. Fig. 2).

Altogether, humoral immune responses directed against immunodominant tumor antigens as well as FcR are dispensable for the chemotherapeutic activity of anthracyclines.

**Humoral immune responses against ICD-related auto-antigens in patients.** The chaperone calreticulin (CRT) is sequestered in the endoplasmic reticulum (ER) in unstressed cells but relocates to the plasma membrane upon induction of ER stress by anthracyclines<sup>13</sup> or hypericin-based photodynamic therapy.<sup>29</sup> Its immunohistochemical detection in paraffin-embedded primary breast cancer tissues has been correlated with serum levels of anti-CRT antibodies.<sup>30</sup> We analyzed the titers of IgA and IgG antibodies in 75 healthy controls and 63 breast cancer patients (Table 1, Fig. 6A) at diagnosis, during and after systemic chemotherapy (Fig. 6B). Both anti-CRT IgA and IgG serum levels were slightly higher in cancer patients than in control individuals (Fig. 6A). However, chemotherapy failed to significantly increase the humoral immune response against CRT (Fig. 6B). Paired serum specimens were available in 4 cases, revealing no specific increase post-therapy (Suppl. Fig. 3). The seroconversion (defined as antibody titers reaching levels above the detection threshold of the ELISA test<sup>30</sup>) of patients either at diagnosis or after neoadjuvant chemotherapy failed to correlate with pathological complete responses (Fig. 6C) or metastases-free survival (Fig. 6D). We conclude that in human breast cancer, humoral immune responses directed against CRT lack clinical significance.

## Discussion

Our findings strongly suggest that humoral immunity directed against immunodominant tumor antigens or self-epitopes related to ER stress-associated chaperones (CRT) may not contribute to the chemotherapy-mediated control of tumor growth. Using three distinct model systems (B cell depletion by anti-CD20 antibodies, constitutive Ig deficiency in  $\mu$ MT mice, lack of activatory or inhibitory Fc $\gamma$ R resulting from homologous recombination), we demonstrated that B cells or Ig-specific receptors are dispensable for anthracycline-mediated antitumor effects, contrasting with the essential role of T-lymphocyte-mediated immune responses observable in the same settings. Indeed, in these OVA-engineered or wild type mouse sarcoma models, anthracyclines did not readily mobilize the humoral arm of immunity.

These data do not rule out the possibility that, in other circumstances of dosing or scheduling or other therapeutic regimen, anti-tumor B cell responses be triggered. Humoral immune responses have been described in the context of various immunotherapy strategies. Ipilimumab, a fully human mAb directed against cytotoxic T-lymphocyte antigen-4 that improves overall survival in metastatic melanoma patients, slightly increased antibody levels against tumor antigens (NY-ESO-1, MART-1, SSX2, p53, MAGE-A4) and boosted humoral immune responses against tetanus, pneumococcal and influenza vaccines.<sup>31, 32</sup> However, NY-ESO-1 seropositivity was only predictive of clinical benefit in cases of the concomitant elicitation of NY-ESO1-specific CD8<sup>+</sup> T cell responses.<sup>32</sup> Repeated vaccination with NY-ESO-1 protein in adjuvants (Montanide plus CpG ODN) induced an integrated Th1 CD4<sup>+</sup> T cell and antibody response against NY-ESO-1 that was eventually completed by CD8<sup>+</sup> T cell immunity. Vaccine-induced antibodies facilitated cross-presentation of NY-ESO-1 epitopes by dendritic cells to CD8<sup>+</sup> T cells.<sup>33</sup> Similarly, therapy-induced antibodies directed against anti-MHC class I-like chain related protein A molecules (MICA) enhanced cross-presentation to T cells and immune-mediated antitumor effects.<sup>34</sup> Antibody responses developed towards neovessels following immunization were instrumental to create ischemic tumor necrosis.<sup>35</sup> Hence, GM-CSF-engineered tumor vaccines combined with anti-CTLA4 engendered a coordinated cellular and humoral immunity associated with clinically significant tumor regressions in advanced solid cancers.<sup>36</sup> In such regressing

tumor burdens, T cell infiltrates were juxtaposed to antibody-producing B cells in long term responders. Humoral reactivity was reported against proangiogenic cytokines such as angiopoietin-1 and -2 blocking endothelial cell tube formation and/or macrophage inhibitory factor attenuating macrophage Tie-2 and MMP9 expression <sup>35</sup>. Likewise, antibodies against protein disulfide isomerases (PDI, ERp5, <sup>37</sup>) or an accessory unit of the vacuolar H<sup>+</sup>-ATPase complex <sup>38</sup> appeared of clinical significance. These data are in line with other observations stating that the presence of tertiary lymphoid organogenesis (TLO), high endothelial venules (HEV) or germinal centers (GC) residing around tumor nests are associated with good prognosis in breast carcinoma, non-small cell lung cancer and melanoma. <sup>39-42</sup> Although some particular chemotherapy regimens elicit a therapy-relevant T cell immunity, they fail to ignite protective B cell responses, at least in mouse sarcomas and human breast cancers, which however have been investigated at relatively early time points. Further in-depth investigation should address antitumor serum reactivities in clinical cases where TLO or HEV or GC are observed post-chemotherapy.

We found that anti-CD20 antibodies did not affect the efficacy of anthracyclines. As a caveat, it should be noted that a subset of regulatory CD5<sup>+</sup> CD1d<sup>high</sup> IL-10 producing B cells (B1 cells) fails to be depleted by anti-CD20 antibodies. <sup>43</sup> Interestingly, secretion of autoantibodies is a function exclusive to the B1 subset and the majority of human cancer patients mount specific autoantibody responses against their tumors. <sup>44</sup> Moreover, the population of CD20-resistant B cells has been reported to enhance A20 lymphoma growth in an IL-10-dependent manner. <sup>19</sup> Future studies must evaluate in which particular settings B1 cells may modulate the growth of human tumors.

FcγR (CD64 and CD16) have been shown to have a positive role in the treatment of mouse melanomas with specific antibodies. <sup>45</sup> These preclinical findings are in accordance with human data showing that single nucleotide polymorphisms (SNP) affecting the protein structure of FcγRIIIA/CD16A determine the in vivo efficacy of monoclonal ADCC-mediating antibodies (such as Rituximab, Trastuzumab and Erbitux). <sup>46</sup> To our knowledge, the clinical significance of such SNPs has not been reported for anthracyclines or oxaliplatin, supporting the findings reported in this paper.

Antibodies against calreticulin (CRT), a Ca<sup>+</sup>-binding ER residing chaperone, represent a diagnostic tool for various infectious, autoimmune and cancer-associated disorders. <sup>47-50</sup> Interestingly, anti-CRT IgA titers are more useful than anti-CRT IgG levels for detecting mammary, colorectal, hepatocellular and pancreatic carcinomas, alcoholic hepatitis, refractory coeliac disease, or primary biliary cirrhosis. <sup>30, 51-53</sup> High concentrations of anti-CRT IgA antibodies were more frequently detected in lymph node-positive breast cancers than IgG antibodies but failed to correlate with CRT membrane expression <sup>30</sup>. The biological significance of anti-CRT antibodies is unclear. However, experimental immunization against CRT could induce liver focal necrosis and hepatitis. <sup>54</sup> *Trypanosoma cruzi* CRT is highly immunogenic and exhibits cross-reactivity with isogenic cardiomyocytes, thus causing a local autoimmune reaction relevant to Chagas disease. <sup>55</sup>

The observation that anti-CRT IgA or IgG serum levels did not significantly increase after anthracycline-based chemotherapy of breast cancer patients may reflect the inability of many tumors to translocate CRT to the cell surface <sup>56, 57</sup>, defects in other hallmarks of ICD such as absent expression of HMGB1 (Yamazaki T *et al.* same issue), or deficient autophagy <sup>58</sup> or the general irrelevance of humoral responses to clinical outcome. Future studies must correlate the therapy-induced raise in anti-CRT IgA antibodies or other immunological alterations, including changing frequencies in tumor antigen-specific T lymphocytes, with tumor cell-intrinsic, ICD-relevant parameters.

## Material and Methods

**Mouse strains.** All mice were bred and maintained according to both the FELASA and the Animal Experimental Ethics Committee Guidelines (Val de Marne, France). WT SPF C57Bl/6J mice were obtained from Harlan. OT-1 C57Bl/6J mice and Ly5.1 congenic mice were obtained from Jackson Lab. C57Bl/6J FcγRI<sup>-/-</sup> mice<sup>23</sup>, FcγRI/IIIA/IV ('FcγRIIB-only')<sup>26</sup>, FcγRIII<sup>-/-</sup> mice,<sup>22</sup> FcγRIV<sup>-/-</sup> mice,<sup>24</sup> and FcγRI/II B/III FcεRI/II (5KO, also known as "FcγRIV-only") mice<sup>25</sup> have been reported previously and bred at the Institut Pasteur animal facility. C57Bl/6J μM KO mice were obtained from Centre d'Élevage d'Orléans (Dr Ryffel) and bred at the Institut Gustave Roussy animal facility.

**Reagents and antibodies.** Doxorubicin was purchased from Sigma Aldrich. Anti-mouse antibodies for CD3ε (145-2C11), CD4 (GK1.5), CD8α (53-6.7), CD16/32 (2.4G2), CD19 (1D3), CD45.2 (104), were obtained from BioLegend or eBioscience. LIVE/DEAD fixable yellow stain fluorescence for viability staining was purchased from Invitrogen/Molecular Probes. Flow cytometry analyses have been performed on a Cyan (Beckman Coulter) flow cytometer with FloJo (Tree Star) software or on a FACSCanto II and Diva software (BD Bioscience).

**Transplantable tumor cell lines.** MCA205 OVA fibrosarcoma cells (syngenic from C57Bl/6 mice) were cultured at 37 °C under 5% CO<sub>2</sub> in RPMI 1640 containing 10% FCS, 2 mM L-glutamine, 100 IU/ml penicillin/streptomycin, 1 mM sodium pyruvate and MEM non-essential amino acids (Invitrogen). OVA expressing tumor cells have been selected by antibiotic selection (Hygromycin (50μg/ml, Invitrogen))

**Chemotherapy treatment of established tumors in mice.** Tumors were subcutaneously inoculated in the right flank by injecting 1x10<sup>6</sup> of MCA205-OVA cells. When tumor size reached 30-45 mm<sup>2</sup>, mice were treated with 2μM doxorubicin, injected intratumorally in 50 μl of PBS, or PBS alone as control. Tumor growths were monitored every 3-4 days using a caliper. In some experiments, MCA205 OVA tumor cell line has been subcutaneously inoculated to CD45.1 C57Bl/6 mice. Seven days later, mice have been treated or not by doxorubicin (i.t). One day post chemotherapy, 1x10<sup>6</sup> CD45.2 congenic naive OT-I T cells have been injected i.v. day 8 post-chemotherapy. After mouse sacrifice and tumor harvesting and enzymatic dissociation (see below), the amounts of tumor infiltrating OT-I or B cells have been assessed by flow cytometry.

**Tumor infiltrating lymphocytes analyses.** At indicated time points, tumors were harvested, cut into small pieces and digested in Liberase TM 25μg/ml(Roche) and DNase I 150 U/ml (Calbiochem) for 30 min at 37°C. Single-cell suspension was obtained by crushing the digested tissue with a syringe plunger and filtering through a 100 μm cell strainer. After washing, cells have been used for subsequent immunostaining.

***In vivo* cell depletion.** At indicated time point, MCA 205 OVA tumor bearing mice received a single 250 µg of anti-CD20 (MB20-11) or IgG2a control i.v, as previously described <sup>59</sup>. B cell depletion was checked in spleen and tumors from antibody administrated mice. CD8+ T cells have been depleted by using anti-CD8α Ab (53-6.72, Bioxcell) or Rat IgG2a control (2A3, Bioxcell). The depletions have been started 1 day prior chemotherapy treatment and maintained during the whole experiment. Mice received 100 µg of Ab in 100µl of PBS, i.p, every 3 days. T cell depletion was checked in spleen and tumor from antibody administrated mice.

**Mouse Anti-OVA Ig dosing.** MCA 205 OVA-bearing mice have been treated or not with doxorubicin at day 7 post-tumor inoculation. Two to 3 weeks later, sera have been collected and the presence of OVA specific IgG, IgA and IgM has been determined by using mouse anti-ovalbumin Igs (total G+A+M) ELISA kit (alpha diagnostic), according to manufacturer's instructions.

**Patients' cohort.** Breast cancer patients who has signed an informed consent for investigations of research purposes were selected from a database of 591 patients who received preoperative anthracyclines-based chemotherapy regimen (plus or minus taxanes and herceptin for HER2 BC) at the Institut Gustave Roussy between 1987 and 2003 (Table 1). Pathologic complete response (pCR) were defined as the absence of any invasive cancer or isolated tumor cells only in the breast and lymph nodes after completion of chemotherapy. Serum was drawn for CA15.3 dosages and remaining material kept frozen for parallel investigations including anti-CRT IgG/A concentration assessments. Therefore, serum withdrawn at diagnosis prior to chemotherapy, during the 8 cycles of therapy and/or after completion of neoadjuvant treatment at follow up consultations was kept at -20°C until ELISA was performed.

**Human Anti-CRT IgG dosing.** Human anti-CRT IgG and IgA sera concentrations have been evaluated as previously described. <sup>60</sup> Antibody response against CRT has been assessed in a cohort of 63 breast cancer patients and 75 healthy volunteers. The cut-off values for Ig were as follows: detection threshold of 30 UI/ml for IgA and 100 for IgG).

**Statistical analyses.** Results are expressed as mean ± SEM. All experiments were repeated at least twice, yielding similar results. Normal distributions were compared by unpaired, two-tailed Student's t tests. Statistical analyses were performed by means of the software Prism 5 (GraphPad, San Diego, CA, USA) and *p* values < 0.05 were considered as statistically significant.

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## Figure Legends

### **Figure 1. Ag specific T cells but not B cell accumulation in the tumor bed post chemotherapy.**

(A-B). *T cell accumulation in tumors post-anthracyclines.* Day 7 established MCA205 OVA implanted in CD45.1 C57Bl/6 mice were treated with doxorubicin (i.t). One day post chemotherapy,  $1 \times 10^6$  CD45.2 congenic naive OT-I T cells were injected i.v. and their frequency among CD8/CD45.2 cells (A) and absolute number (B) were assessed in flow cytometry in TILs. N= 5 mice/group. A representative dot plot is shown (A) and the data from one representative experiment out of 3 are depicted (B). (C) *B cell depletion post-Doxo.* Absolute intratumoral B cell numbers (defined as CD19<sup>+</sup> B220<sup>+</sup> live CD45<sup>+</sup> cells) were assessed 8 days post-chemotherapy in TILs using flow cytometry. N= 5 mice/group. A representative graph out of two independent experiments is shown. Student t test: \*\* p < 0.01

### **Figure 2. Chemotherapy efficacy is not impaired in $\mu$ MT<sup>-/-</sup> mice.**

A. *Ig and B cell depleted mice.* Tumor growth curves of MCA205 OVA were monitored in WT or  $\mu$ MT KO mice, treated or not with doxorubicin at day 7 post-tumor inoculation. Tumor growth has been assessed every 4 days using a caliper. N= 5 mice /group. The graph is representative of one experiment out of 2 independent ones. B. *Ig levels in serum post-anthracyclines.* MCA 205 OVA-bearing mice have been treated or not by doxorubicin at day 7 post-tumor inoculation. Three weeks later, sera have been collected and the concentrations of OVA-specific IgG, IgA and IgM have been determined in ELISA. N= 10 mice/group. Student t test: \*\*\* p < 0.001, ns: not significant.

### **Figure 3. Anti-CD20 Ab-mediated B cell depletion does not impair chemotherapy efficacy.**

(A). *Experimental setting.* B cells have been depleted using anti-CD20 antibody either before tumor inoculation (D-14), before chemotherapy (D-2) or 6 days after chemotherapy treatment (D+6). Mice received doxorubicin (i.t) at day 0 and the tumor growth was monitored every 3-4 days. B cell depletion was analyzed by flow cytometry in the spleen 1 week after starting anti-CD20 injections. (B) Dot plots show the depletion in the « D-14 » group as a representative example. (C-E) Tumor growth curves of control or doxorubicin-treated MCA205 bearing mice treated with anti-CD20 or IgG control Ab. N= 5-7 mice /group. Student t test or Anova:\*\*\* p<0.001, ns: not significant.

### **Figure 4. CD8<sup>+</sup> T cells are indispensable for chemotherapy efficacy.**

(A). *Experimental setting.* CD8<sup>+</sup> T cells have been depleted from day -1 before chemotherapy throughout the experiment using 100 $\mu$ g of anti-CD8 antibodies (53.6.72) or rat IgG2a control Ab (2A3) every 3 days. (B). Tumor growth curves of control or doxorubicin-treated MCA205- bearing mice treated with anti-CD8 or IgG controls are depicted in a typical experiment out of three. Student t test:\*\*\* p<0.001, ns: not significant.

**Figure 5. Chemotherapy efficacy does not rely on antibody response.**

MCA205 –OVA was established in various C57BL/6 mouse backgrounds (WT (A) or FcR deficient as indicated i.e “Fc $\gamma$ RIV only” (Fc $\gamma$ RI/IIB/III Fc $\epsilon$ RI/II KO) (B) FcR $\gamma$  KO (Fc $\gamma$ RI/III/IV KO) (C), Fc $\gamma$ RI KO (D) FcRIII KO (E) and FcRIV KO (F)) and treated or not with doxorubicin at day 7 post-tumor inoculation. Tumor growth kinetics is indicated in one representative experiment out of two for each group of mice. N= 5-7 mice/group. Student t test: \*\*\*p<0.001, ns= not significant

**Figure 6. No clinical significance of anti-CRT antibodies in neoadjuvant chemotherapy-treated advanced breast cancers.** A. Comparisons of anti-CRT IgA and IgG serum levels in sex and age matched-75 healthy volunteers and 63 advanced BC patients. B. Kinetics of anti-CRT IgA and IgG serum levels in advanced BC patients defined in Table 1. T0: diagnosis, T1: Under chemotherapy, T2: Follow up 1 and T3: Follow up 2. C-D. Frequency of pathological complete responses (pCR (C)) and metastasis free survival (D) in patients bearing advanced BC treated with neoadjuvant anthracycline-based chemotherapy. Probability to develop a pCR or to remain free of metastases at 3 years and 6 months of follow up are represented according to patients’ seropositivity for anti-CRT IgA or IgG (threshold of 60 and 90 arbitrary units respectively). Diagnosis n=26; After chemotherapy n=36. Student t test: \*p<0.05, ns= not significant.

**Table1: Characteristics of breast cancer patients**

Patients	N=63	%
<b>Age at diagnosis</b>		
Mean	48	
Range	10	
<b>Sex</b>		
Female	63	100%
<b>Histology</b>		
Ductal invasive	63	100%
<b>Grade</b>		
1	3	5%
2	23	37%
3	35	56%
ND	2	3%
<b>ER status</b>		
Positive	29	46%
Negative	34	54%
<b>HER2-status</b>		
Positive	4	6%
Negative	59	94%
<b>Chemotherapy</b>		
Neoadjuvant	63	100%
Adjuvant	16	25%
<b>Neoadjuvant therapy</b>		
FEC + TXT	50	79%
TXT + FEC	4	6%
Others (with Epirubicine)	9	14%
<b>Adjuvant therapy</b>		
Hormonotherapy	28	44%
RX	41	65%
RX/FUN	12	19%
RX/TXT	4	6%
Others	6	10%

Abbreviations : FEC = 5-fluorouracil / epirubicin / cyclophosphamide ; TXT = taxotere ; FUN = 5-fluorouracil / navelbine