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1       **Fighting breast cancer stem cells through the immune-targeting of the xCT cystine-**  
2 **glutamate antiporter**

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33 23  
34 24       **Abstract**

35 25       Tumor relapse and metastatic spreading act as major hindrances to achieve complete cure of breast  
36 26       cancer. Evidence suggests that cancer stem cells (CSC) would function as a reservoir for the local  
37 27       and distant recurrence of the disease, due to their resistance to radio- and chemotherapy and their  
38 28       ability to regenerate the tumor. Therefore, the identification of appropriate molecular targets  
39 29       expressed by CSC may be critical in the development of more effective therapies.

40 30       Our studies focused on the identification of mammary CSC antigens and on the development of  
41 31       CSC-targeting vaccines. We compared the transcriptional profile of CSC-enriched tumorspheres  
42 32       from an Her2+ breast cancer cell line with that of the more differentiated parental cells. Among the  
43 33       molecules strongly upregulated in tumorspheres we selected the transmembrane amino-acid  
44 34       antiporter xCT.

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1 In this review, we summarize the results we obtained with different xCT-targeting vaccines. We  
2 show that, despite xCT being a self-antigen, vaccination was able to induce a humoral immune  
3 response that delayed primary tumor growth and strongly impaired pulmonary metastasis formation  
4 in mice challenged with tumorsphere-derived cells. Moreover, immunotargeting of xCT was able to  
5 increase CSC chemosensitivity to doxorubicin, suggesting that it may act as an adjuvant to  
6 chemotherapy.  
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8 In conclusion, our approach based on the comparison of the transcriptome of tumorspheres and  
9 parental cells allowed us to identify a novel CSC-related target and to develop preclinical therapeutic  
10 approaches able to impact on CSC biology and therefore hampering tumor growth and dissemination.  
11

### 12 **Keywords**

13 Cancer stem cell, vaccine, tumorsphere, xCT, breast cancer, NIBIT 2017  
14

### 15 **Précis**

16 Anti-CSC vaccination through targeting of the xCT transporter elicits protective immunity against  
17 mammary tumor challenge and metastasis formation in a preclinical setting.  
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### 19 **Abbreviations**

20 ADCC antibody-dependent cell cytotoxicity  
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22 ALDH aldehyde dehydrogenase  
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24 BoHV-4 bovine herpesvirus-4  
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26 CSC cancer stem cell  
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28 ECD extracellular domain  
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30 GSH glutathione  
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32 ROS reactive oxygen species  
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34 SASP sulfasalazine  
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36 VLP Virus-Like Particles  
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### 38 **Manuscript text**

#### 39 *Introduction*

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1 Chemotherapy and radiotherapy have represented the treatments of choice for cancer patients in  
2 the past decades, often affording reduction in the tumor burden. However, a minority of cells, named  
3 cancer stem cells (CSC), has been shown to be more resistant to such therapies compared to more  
4 differentiated cancer cells of the same tumor [1], thus contributing to treatment failure and to local  
5 and distant recurrence. CSC possess the capacity to self-renew and to generate the heterogeneous  
6 lineages of cancer cells that compose the tumor, in a process that mimics normal tissue development  
7 [2]. They can be experimentally defined by their exclusive ability to recapitulate the generation of a  
8 continuously growing tumor even when injected at low cell density [2], and are thus operatively  
9 referred to as “tumor-initiating cells”, while more differentiated cells lack this ability.

10 From a clinical perspective, the CSC concept has significant implications. As mentioned above,  
11 conventional anticancer approaches are predominantly directed against the bulk population of the  
12 tumor but are not effective against CSC, because of mechanisms such as increased drug efflux and  
13 detoxification, thus allowing this self-renewing cell population to cause the perpetuation of the tumor  
14 [3]. Therefore, approaches targeting CSC could increase the long-term efficacy of currently available  
15 treatments. The potential of immunotherapy against CSC has recently become an intriguing field of  
16 research. The idea is that immune effectors not only may succeed where chemotherapy fails, but can  
17 also block the molecules involved in the chemoresistance mechanisms. Moreover, some evidence  
18 suggests that CSC are a superior target for immunotherapy compared to non-CSC. In the preclinical  
19 setting, loading DC with CSC lysates induced protective antitumor immunity in immunocompetent  
20 mice, while bulk tumor-loaded DC were unable to afford the same results [4]. This can be attributed to  
21 the fact that CSC and more differentiated cancer cells express different antigens because of their  
22 different gene expression profiles. Therefore, immunologic approaches directed against whole tumors  
23 are largely biased toward differentiated cancer cells, which represent the major proportion of the  
24 tumor mass and express tolerated differentiation antigens [4]. This might mask immunologic  
25 responses to tumor-perpetuating CSC, which represent only a minor percentage of tumor cells. CSC  
26 thus appear to be a better source of antigens compared to non-CSC, and their ablation requires a  
27 specific targeting.

28 These encouraging preclinical results led to the translation of CSC-loaded DC vaccines into the  
29 clinic, showing that they were safe, able to induce an immune response in all treated patients and to  
30 improve progression-free survival compared to unvaccinated controls [5]. However, in none of the  
31 reported anti-CSC vaccination approaches specific CSC-antigens were identified, as DC were loaded  
32 with whole protein or mRNA derived from tumor cells. This type of treatment is patient-specific and  
33 at the current state requires high manufacturing costs, thus hampering its large-scale usage [3]. Other  
34 limitations of DC-based personalized vaccines, including the difficulty in setting up standardized  
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1 procedures, ensuring the proper maturation status of the DC and the precise selection of appropriate  
2 DC subsets required to elicit the desired response, may eventually impair the efficacy of the treatment  
3 [6].  
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5 On the contrary, a large-scale clinical benefit could be achieved by combining a vaccine  
6 formulation that is cost-effective and highly reproducible, and a target antigen that is shared among  
7 a large number of patients. In this context, we coined the term “oncoantigens” to indicate tumor-  
8 associated molecules that have a causal role in the promotion of carcinogenesis and cannot be easily  
9 down-modulated or negatively selected by cancer cells under the pressure of a specific immune attack  
10 [7]. When expressed on the cell membrane they can be the target of both cell-mediated and antibody-  
11 mediated immune responses. Therefore, greater efficacy for immunotherapy could be achieved by  
12 targeting transmembrane oncoantigens expressed by CSC by virtue of their role in the progression,  
13 metastasis, resistance to therapy, and recurrence of tumors [8].  
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#### 15 *Identification of CSC oncoantigens*

16 An ideal antigen should be derived from a non- dispensable protein, in this case a protein required  
17 for CSC survival, self-renewal and tumor-initiating ability. However, no consensus exists on the  
18 expression of specific oncoantigens by CSC.  
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20 To fill this gap, we developed a pipeline to identify CSC oncoantigens. Initially, high-throughput  
21 transcription analysis is used to highlight the gene signatures that distinguish CSC from more  
22 differentiated cancer cells. Of the upregulated transcripts in CSC, which are more likely to have a  
23 functional role in their biology, only those that have an orthologue in humans, low expression in  
24 normal human tissues, high expression in human cancers and association with poor prognosis are  
25 selected as “putative” oncoantigens [9]. These candidate oncoantigens are eventually validated by  
26 immunizing tumor-bearing mice with vaccines targeting them.  
27

28 Besides the sorting based on CSC markers expression such as CD44, CD24 [10], Sca-1 [11] and  
29 aldehyde dehydrogenase (ALDH) activity [12], a tool often employed for propagating CSC  
30 populations is represented by the generation of “tumorspheres”, spherical anchorage-independent cell  
31 clones growing in serum-free medium supplemented with factors that favor stem cell growth [13].  
32 Given the exclusive ability of CSC to survive and grow under these conditions, tumorsphere  
33 generation has gained popularity as an *in vitro* surrogate assay substituting the more time and money  
34 consuming tumor-initiation assay, and has been developed for a wide range of solid tumors, including  
35 breast cancer [14].  
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37 To identify oncoantigens expressed by mammary CSC, we performed a comparative  
38 transcriptomic analysis between cells cultured as monolayer and cells cultured as tumorspheres. For  
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1 this purpose, we used the TUBO cell line, established from a Her2<sup>+</sup> mammary tumor arisen in a  
2 BALB/c female mouse transgenic for the rat Her2/neu oncogene (BALB-neuT) [15]. Serial passages  
3 of TUBO tumorspheres displayed increasing clonogenic potential, as well as a higher tumor-initiating  
4 ability, compared to TUBO cells grown as monolayer. Furthermore, tumorspheres showed stronger  
5 positivity for several CSC markers including Sca-1, Nanog, Oct-4 and ALDH activity [9]. All these  
6 observations strongly suggest that tumorspheres derived from TUBO cells are enriched in CSC.  
7 Focusing on genes strongly upregulated in tumorspheres and coding for proteins expressed on cell  
8 surface [9, 16], we selected xCT.

### 10 *xCT oncoantigen and its role in breast CSC*

11 xCT is a multipass transmembrane protein of ~500 amino-acids encoded by the gene *Solute*  
12 *Carrier Family 7, Member 11* (SLC7A11). It consists of 12 transmembrane, 7 intracellular and 6  
13 extracellular domains, with both the N- and C- termini located inside the cell (Figure 1). xCT is the  
14 light subunit of the heterodimeric amino-acid transport system x<sub>c</sub><sup>-</sup> and is coupled through a disulfide  
15 bond to the heavy subunit 4F2hc, also termed CD98 [17]. While CD98 is responsible for the  
16 trafficking of the heterodimer to the plasma membrane and acts as a subunit for several other amino  
17 acid transporters, xCT is responsible for substrate specificity and transport. System x<sub>c</sub><sup>-</sup> is an obligate,  
18 Na<sup>+</sup>-independent antiporter that exports intracellular glutamate in exchange for extracellular cystine  
19 in a gradient-dependent manner and 1:1 ratio [17]. Once inside the cell, cystine is reduced to cysteine,  
20 the rate-limiting precursor in the biosynthesis of glutathione (GSH). GSH plays a critical role in  
21 cellular defenses against oxidative stress, as it can reduce diverse reactive oxygen species (ROS).  
22 Albeit ROS are essential for biological functions, as they regulate many signaling pathways, an  
23 imbalance of ROS content within the cell can lead to harmful effects, since they can mediate an  
24 oxidative modification of biological molecules, including DNA, proteins and lipids, impairing their  
25 functions. This may result in transient cellular alterations, up to irreversible oxidative damage and  
26 cell death [18]. By providing precursors for GSH synthesis, system x<sub>c</sub><sup>-</sup> mediates free radical  
27 scavenging and detoxification, thus playing a pivotal role in intracellular redox balance regulation.

28 In our work, we have demonstrated that xCT is expressed at high levels in many cancerous human  
29 tissues compared to healthy samples from different organs [19]. Concerning mammary tissues, we  
30 observed that xCT expression is low in normal mammary gland, while it significantly increases in  
31 hyperplastic mammary tissue and invasive ductal breast carcinoma of different histological subtypes  
32 (Her2<sup>+</sup>, estrogen receptor/progesterone receptor<sup>+</sup>Her2<sup>-</sup>, triple negative). Here, xCT expression is  
33 confined to tumor cells, suggesting that xCT upregulation in the mammary tissue only occurs upon

1 oncogenic transformation. Moreover, high xCT levels in breast cancer negatively impact on patients'  
2 overall survival [20].

3 In TUBO cells, the highest percentage of xCT<sup>+</sup> cells is found in the CD44<sup>+</sup>/CD24<sup>-</sup> CSC fraction  
4 [19]. Furthermore, xCT expression increases progressively from cells growing as monolayer to cells  
5 derived from subsequent generations of tumorspheres, where the majority of Sca-1<sup>+</sup> cells are also  
6 positive for xCT. xCT upregulation in tumorsphere-derived cells was observed not only in the TUBO  
7 cell line but also in other mouse and human mammary cancer cell lines, indicating that xCT  
8 expression increases in breast CSC [19]. Notably, inhibition of **system x<sub>c</sub><sup>-</sup>** leads to decreased  
9 tumorsphere-forming ability in both mouse and human mammary cancer cell lines [21].

10 Given its detoxifying role, system x<sub>c</sub><sup>-</sup> dysfunction in cancer models has been linked mainly to the  
11 induction of ROS-dependent cell death [22]. By virtue of their increased xCT expression, TUBO  
12 tumorspheres display significantly higher GSH and lower ROS content compared to cells grown as  
13 monolayer [19]. However, xCT silencing in tumorspheres brings their GSH and ROS content back to  
14 the levels observed in parental cells, leading to impaired tumorsphere-generation ability and  
15 decreased CSC marker expression [19]. xCT thus appears to play a central role in the maintenance of  
16 the CSC-state rather than in maintaining tumor cell biology under differentiating conditions, since  
17 xCT silencing does not affect proliferation of TUBO cells growing as monolayer but only of those  
18 growing as tumorspheres [19].

19 System x<sub>c</sub><sup>-</sup> activity can be pharmacologically inhibited by the non-substrate inhibitor sulfasalazine  
20 (SASP). SASP is an FDA- and EMA-approved drug commonly used to treat chronic inflammatory  
21 diseases such as rheumatoid arthritis; it is also a potent inhibitor of NF-κB activation. However, it is  
22 insoluble in aqueous solutions and not optimized for the fortuitous interaction with xCT [23].  
23 Moreover, significant side effects associated to the use of SASP occur in 25% of treated patients, and  
24 interruption of a clinical trial due to adverse events on glioma patients has been reported [24]. In  
25 general, no specific inhibitor of system x<sub>c</sub><sup>-</sup> has been discovered yet, since all the studied compounds  
26 have shown off-target effects [17].

27 In this light, anti-xCT vaccination would provide a specific approach able to potently target xCT  
28 while at the same time avoiding undesired off-target effects.

### 29 *Development of anti-xCT vaccines*

30 Based on the central role played by xCT in mammary CSC biology, we developed multiple  
31 vaccination strategies to target xCT and impair mammary cancer progression. DNA-based antitumor  
32 vaccination was chosen as our first xCT targeting option in light of our consolidated expertise in the  
33 field and of the advantages of DNA vaccines. DNA vaccines were introduced in the early 1990s and  
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1 consisted in the subcutaneous or intramuscular administration of plasmids coding for viral or nonviral  
2 antigens [25]. Plasmids are taken up by resident antigen presenting cells (APC), monocytes and  
3 myocytes, which then express the antigen and present it to T lymphocytes, potentially inducing long-  
4 term cellular immunity [26]. When the expressed antigen is soluble or membrane-bound, a humoral  
5 response against it can be eventually mounted by activation of B lymphocytes [7]. Many anti-cancer  
6 DNA vaccines are currently tested in preventive or therapeutic protocols in the pre-clinical setting  
7 [8]. Up to now, no DNA vaccine has been approved by FDA or EMA for use in human cancer patients,  
8 but a few have been licensed for dogs and several clinical trials are ongoing in human patients, some  
9 of them with promising results [27]. DNA vaccination offers many advantages as compared to other  
10 immunotherapies, since DNA vaccines are relatively simple and inexpensive to design and produce  
11 on large scale, and are well tolerated and safe. Indeed, it has been demonstrated in preclinical models  
12 and by many clinical trials that the risk for plasmid genomic integration is very low, and no evidence  
13 of anti-DNA immune response following vaccination have been reported so far, allowing for multiple  
14 administration [8]. In the light of these observations, we generated an xCT-targeting DNA vaccine  
15 (pVAX1-mxCT) by cloning mouse xCT open reading frame (NM\_011990.2) in the FDA-approved  
16 pVAX1 plasmid, under the transcriptional control of a CMV promoter. APC have a pivotal role in  
17 immunity induction by DNA vaccines by presenting vaccine-derived peptides on MHC I and II  
18 molecules following either direct transfection of the resident APC at the injection site or DC  
19 engulfment of apoptotic transfected cells and presentation of the produced antigens, inducing CD8<sup>+</sup>  
20 and CD4<sup>+</sup> T cell activation [7]. Furthermore, plasmid DNA backbone itself acts as a pathogen-  
21 associated molecular pattern binding to TLR9 and other cytosolic double-stranded DNA sensors,  
22 eventually contributing to the intrinsic immunogenicity of DNA vaccines [28]. Thus, in theory, DNA  
23 vaccines coding the full sequence of a transmembrane protein should elicit both cellular and humoral  
24 responses against the target antigen. In practice, many hurdles need to be faced to obtain a strong  
25 immune response against a self non-mutated antigen such as xCT. Central tolerance against xCT is  
26 indeed likely to occur, thus depleting highly reactive T cells. However, as observed for another self-  
27 antigen subjected to central tolerance (rat Her2 in BALB-neuT mice), thymic depletion occurs mainly  
28 on self-reactive CD8<sup>+</sup> T cells [29]. Indeed, the protective immune response elicited in BALB-neuT  
29 mice following administration of the rat Her2 DNA vaccine rests on activation of CD4<sup>+</sup> T cells and  
30 the subsequent stimulation of anti-Her2 antibody production [29]. We can speculate that a similar  
31 mechanism takes place after xCT vaccination.

32 A potential strategy to break T cell tolerance and induce higher humoral and cellular responses is  
33 the use of recombinant viral vector-based vaccines. Indeed, strong immune responses can be induced  
34 by using viruses engineered to express exogenous antigens into host cells. Besides allowing antigen  
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1 presentation to T lymphocytes, virus-based vaccines show the advantage of creating an inflammatory  
2 environment produced by the virus intrinsic immunogenicity. In order to be a potentially translatable  
3 vector, a virus should be safe, easy to manipulate, and able to induce the transgene expression in host  
4 cells long enough to induce a potent immune reaction [30].

5 These properties are displayed by bovine herpesvirus 4 (BoHV-4), a member of the  
6 gammaherpesvirinae subfamily, genus Rhadinovirus, that has been isolated from healthy cattle and  
7 successfully used to express exogenous antigens in various cell types. An apathogenic derivative of  
8 BoHV-4, able to deliver genes to mammalian cells but endowed with a reduced replication ability in  
9 order to decrease the cytopathic effect associated to viral replication, was successfully used to  
10 vaccinate mice, rats, goats, rabbits and sheeps without any signs of pathogenicity or oncogenicity  
11 [31]. Moreover, BoHV-4 vaccination overcomes one of the major concerns of viral vectors, i.e.  
12 induction of an anti-viral immune response that gives rise to neutralizing antibodies, precluding the  
13 possibility of multiple administrations. Therefore, we cloned the full length mouse xCT open reading  
14 frame under the control of the CMV promoter in the BoHV-4 vector (BoHV-4-mxCT, Conti et al.,  
15 unpublished). When systemically administered in mice, BoHV-4 gives a persistent infection in  
16 different organs, in particular within the monocyte/macrophage lineage [32], thus generating both  
17 humoral and cellular immune responses [33]. Indeed, we have previously demonstrated that a BoHV-  
18 4-based vector delivering a chimeric rat-human Her2 protein was able to induce a higher humoral  
19 response than the corresponding DNA vaccine in BALB-neuT mice, indicating that it is superior to  
20 plasmid DNA vaccination in the activation of B and CD4<sup>+</sup> T lymphocytes [33]. Moreover, this BoHV-  
21 4 based vaccine induced a 3-fold higher specific cytotoxic response than the DNA vaccine in BALB/c  
22 mice, suggesting that it also possesses a higher ability to activate CD8<sup>+</sup> T cells [33]. We therefore  
23 expected that BoHV-4-mxCT could induce high titers of anti-xCT antibodies in BALB/c mice, and  
24 hypothesized that it could also be able to induce a T cytotoxic response.

25 Another promising strategy to break T cell tolerance with little side effects is represented by  
26 vaccination with Virus-Like Particles (VLP), artificial nanoparticles composed by self-assembled  
27 repetitive structure of viral capsid proteins that lack genetic material. VLP are safe, unable to replicate  
28 or to be pathogenic and they are produced in large-scale systems with minimal costs [34]. VLP have  
29 different applications since they are excellent delivery systems for various drugs and imaging probes  
30 [35]. They are also good candidates as vaccines able to display antigens conjugated to their surface.  
31 Moreover, their small size allows them to pass through the lymphatic vessels and reach the lymph  
32 nodes where they can induce DC antigen presentation through both MHC I and MHC II [34]. These  
33 processes activate cytotoxic CD8<sup>+</sup> T cells and either T helper 1 or 2 CD4<sup>+</sup> cells [36]. The humoral  
34 response is stimulated by T helper cells or directly by the VLP, since their repetitive structure and



1 their antigen display system improve B cell receptor binding, inducing a potent B-cell activation [37].  
2 VLP also stimulate innate immunity through the activation of Pattern Recognition Receptors  
3 mediated by their residual viral proteins [34].

4 Many VLP-based vaccines targeting non-self antigens have been approved by FDA and are  
5 commercially available for the prevention of infectious diseases, including: Engerix-B<sup>®</sup> and Sci-B-  
6 Vac<sup>™</sup> against the hepatitis B virus, Gardasil<sup>®</sup>, Cervarix<sup>®</sup> and Gardasil9<sup>®</sup> against the human papilloma  
7 virus, and Mosquirix<sup>®</sup> against malaria [38]. Many studies also focused on generation of VLP charged  
8 with tumor-associated antigen (TAA) in several preclinical models of cancer [36]. For melanoma  
9 patients, phase II clinical trial on a VLP-based vaccine has now finished with promising results [36].  
10 By virtue of the encouraging results obtained with these studies, which showed that VLP can break  
11 the immune tolerance in cancer settings, we generated two VLP vaccines based on the RNA  
12 bacteriophage MS2 vector, displaying the third and sixth extracellular domains (ECD3 or ECD6) of  
13 human xCT, respectively named AX09-0M3 and AX09-0M6 [21]. Since mouse and human ECD3  
14 and ECD6 display 73% and 100% identity, respectively, we expected to induce a strong antibody  
15 response directed to xCT extracellular loops.

#### 16 *Anti-xCT vaccination impairs mammary tumor growth and lung metastatization*

17  
18 To test the efficacy of the different anti-xCT vaccine formulations in hampering mammary cancer  
19 progression, we challenged BALB/c female mice with syngeneic TUBO tumorsphere-derived cells  
20 [19, 21] implanted subcutaneously. When implanted subcutaneously, TUBO cells give rise to Her2<sup>+</sup>  
21 tumors with a 100% penetrance and a short latency, reproducing pivotal interactions between tumor  
22 cells and the surrounding microenvironment [39]. They thus represent an interesting preclinical model  
23 to evaluate the efficacy of different immunotherapeutic approaches against tumor growth, both in a  
24 preventive and curative settings. Mice were then vaccinated with the different vaccine formulations,  
25 twice at two week intervals. In particular, DNA plasmid vaccination was performed by plasmid  
26 intramuscular injection followed by electroporation, which consists in the application of short electric  
27 pulses able to enhance DNA transfection and recruitment of immune cells to the injection site [8].  
28 BoHV-4 vaccines were administered intraperitoneally, while VLP formulations were injected  
29 intramuscularly (Figure 2).

30 When subcutaneous tumors were already established (Figure 2a), xCT immunotargeting slowed  
31 tumor growth kinetics, eventually leading to regression in some mice, suggesting that xCT  
32 vaccination may hinder primary tumor growth. Since CSC are involved in the metastatic process, and  
33 it has been demonstrated that system x<sub>c</sub><sup>-</sup> inhibition impairs metastasis formation [40], we tested the  
34 ability of anti-xCT vaccination to prevent lung colonization by TUBO-derived tumorspheres injected  
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1 intravenously into anti-xCT vaccinated syngeneic BALB/c mice (Figure 2b). All our anti-xCT  
2 vaccines proven to be able to decrease lung colonization [19, 21] (Conti et al. unpublished).

3 A less artificial metastatic model is represented by the 4T1-cells that, after subcutaneous injection  
4 in syngeneic mice, grow locally and then spontaneously disseminate to distant organs, including the  
5 lungs [41]. Anti-xCT vaccination delivered to mice bearing palpable 4T1 tumorsphere-derived  
6 tumors (Figure 2c) reduced spontaneous lung metastatization [19, 21] (Conti et al. unpublished),  
7 suggesting that xCT targeting may both impair CSC invasive capacity and affect their growth in the  
8 metastatic site.

### 9 *Vaccine-induced anti-xCT immune response*

10 The characterization of the immune responses elicited by the different vaccine formulations  
11 revealed that the mechanism of action could be mostly attributed to the generation of a polyclonal  
12 anti-xCT response, as confirmed by their inability to prevent breast cancer metastasis in BALB/c  
13 mice knockout for the  $\mu$  Ig chain, which do not produce antibodies [19]. We observed an increase of  
14 activation and IFN- $\gamma$  production in CD4<sup>+</sup> T cells from vaccinated mice following re-stimulation of  
15 splenocytes with xCT<sup>+</sup> 4T1 cells (Conti et al., unpublished). The induction of a T helper response is  
16 then accompanied by the generation of a polyclonal antibody response against xCT. In particular, we  
17 compared the ability of the different vaccines to induce antibodies able to bind full length mouse or  
18 human xCT proteins, or their extracellular loops ECD3 and ECD6, in ELISA. The results presented  
19 in Figure 3a show that while the empty vector controls BoHV-4-A29, pVAX1 and MS2 did not induce  
20 antibodies able to bind any of the xCT proteins or peptides tested (Figure 3a-e), BoHV-4-mxCT,  
21 pVAX-mxCT and AX09-0M6 vaccines induced the production of antibodies able to recognize the  
22 recombinant mouse xCT (Figure 3a). BoHV-4-mxCT and pVAX-mxCT-induced antibodies also  
23 recognized human xCT recombinant protein, which was also bound by antibodies induced by AX09-  
24 0M3 (Figure 3b). Of note, all the vaccines induced antibodies able to bind xCT extracellular domains,  
25 as demonstrated by their ability to bind to xCT ECD6 and to both mouse and human ECD3, with the  
26 highest titers induced by the corresponding VLPs (Figure 3c-e). Moreover, either sera or purified IgG  
27 from mice vaccinated with the different formulations are able to recognize xCT-expressing cells [19,  
28 21] and (Conti et al., unpublished).

29 Regarding cellular immune response, no detectable activation of CTL against xCT was triggered.  
30 In fact, upon DNA vaccination, we did not observe any specific CD8<sup>+</sup> T-cell response (assessed in  
31 an IFN- $\gamma$  ELISPOT assay) following re-stimulation of splenocytes from anti-xCT vaccinated mice  
32 with the BALB/c MHC class I H-2<sup>d</sup> dominant mouse xCT peptide [19]. A lack of T cell response  
33 against xCT may be caused by a thymic depletion of high-avidity autoreactive T-cell clones, as we  
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1 have previously reported for the Her2 antigen in the BALB-neuT model [29]. This hypothesis is  
2 strengthened by the fact that in mice vaccinated with a plasmid coding for the human xCT we  
3 observed a strong IFN- $\gamma$  production following splenocytes re-stimulation with the human  
4 immunodominant xCT peptide (Conti et al., unpublished). Similarly, following vaccination with the  
5 xCT full sequence (Conti et al., unpublished) or with AX09-0M6 [21], we could not observe any  
6 significant increase in the cytotoxic activity of splenocytes against 4T1 cells, indicating that also the  
7 response to sub-dominant peptides was absent. Regarding AX09-0M6 vaccination, this could be due  
8 to the fact that the xCT ECD6 sequence is an octamer, unsuitable to be effectively shown on MHC  
9 class I molecules. Anyway, this lack of a detectable CTL response should not be considered a  
10 limitation in the efficacy of CSC-targeting vaccines, since CSC down regulate MHC class I  
11 expression as an immune evasion mechanism [42]. Thus, CD8<sup>+</sup> T lymphocytes are expected to play  
12 only a marginal role in immunotherapies directed to CSC oncoantigens.

13 Cancer metastasis is a complex and inefficient process that requires disseminated cancer cells to  
14 find the proper microenvironment able to support metastatic growth, and both innate and adaptive  
15 immune cells in the target organ play a key role in this process [43]. Therefore, the anti-metastatic  
16 effect elicited by anti-xCT vaccines could be due, at least in part, to changes in the number and  
17 activities of the immune cells constituting the lung metastatic niche. Indeed, anti-xCT VLP-based  
18 vaccines induced a significant increase in the percentage of NK cells and a trend of increase in the  
19 percentage of T cells in the metastatic lungs [21]. Regarding the myeloid compartment, vaccinated  
20 mice displayed a trend of increase in the percentage of macrophages and a trend of decrease in the  
21 percentage of granulocytic MDSC/neutrophils compared to controls [21]. It has been demonstrated  
22 that breast cancer cells induce neutrophil recruitment in the lung pre-metastatic niche, which  
23 contribute to the growth of lung metastases [44]. Since xCT is expressed on activated neutrophils and  
24 participate to their immunosuppressive activity [45], the possibility that the decrease of neutrophil  
25 population in the lung could be induced by xCT immunotargeting deserves further investigation.

### 26 *Biological activity of anti-xCT antibodies induced by vaccination*

27 The anti-xCT antibodies induced by vaccination exert a therapeutic activity through two different  
28 mechanisms, as they i) activate the innate immune response against CSC through the induction of  
29 antibody-dependent cell cytotoxicity (ADCC), and ii) directly impair CSC self-renewal by inhibiting  
30 system x<sub>c</sub>- activity. Indeed, IgG from vaccinated mice increase the lysis of 4T1 cells by unstimulated  
31 splenocytes, likely NK cells, *in vitro* [21]. Furthermore, antibodies from vaccinated mice decrease  
32 sphere-generation ability of xCT-expressing cancer cells. The resulting spheres are smaller and  
33 contain a reduced fraction of CSC markers-positive cells compared to tumorsphere-derived cells  
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1 incubated with control mice antibodies [19, 21] (Conti et al., unpublished). Moreover, by inhibiting  
2 system  $x_c^-$  function, anti-xCT antibodies alter CSC redox balance, with a consequent increase in  
3 intracellular ROS levels [19, 21]. Elevated ROS levels negatively affect CSC survival, since they  
4 enhance sensitivity to chemotherapy and radiotherapy, inhibit  $\beta$ -catenin and consequently down-  
5 regulate stem cell genes, and promote ferroptosis, a ROS and iron-dependent cell death [46]. Indeed,  
6 sera from vaccinated mice increase the expression of Glutathione-Specific Gamma-  
7 Glutamylcyclotransferase 1 (Chac1; Conti et al., unpublished), an enzyme that degrades GSH and has  
8 been linked to ferroptosis and cell death induced by cystine starvation in breast cancer [47].

9 All these observation support the notion that xCT is not a simple fortuitous feature of the stem-  
10 like status, but it plays a functional role in CSC biology and cancer progression, decreasing the  
11 probability that xCT immunotargeting may lead to cancer escape via antigen-loss mechanisms.

#### 12 *Anti-xCT vaccination does not elicit side effects in vaccinated mice*

13 Targeting self-antigens can raise safety concerns about affecting normal cells and inducing  
14 autoimmune disease. xCT physiological expression is mainly restricted to a few normal cell types,  
15 mostly astrocytes and microglia in the central nervous system [48, 49]. xCT immunotargeting did not  
16 induce any evident adverse effects, and no inflammatory cell infiltrates or any other abnormalities  
17 were detected in the brains of vaccinated mice, maybe due to the protective effect exerted by the  
18 blood-brain barrier. Furthermore, no detectable behavioral changes were observed in vaccinated mice  
19 (Conti et al., unpublished) and [21].

20 However, system  $x_c^-$  can also regulate immune cell functions, since it mediates cystine uptake in  
21 macrophages and DC, which in turn release cysteine that is essential for T lymphocyte activation  
22 [45]. Nonetheless, the combination of xCT and Her2 immunotherapies did not impair the Her2-  
23 specific T-cell response [19], and no reduction in splenic DC, neutrophils, and macrophages was  
24 induced by vaccination [21]. The safety of xCT immunotargeting is further supported by the absence  
25 of alterations in the organs of xCT knockout mice [50]. Also from a functional point of view, xCT<sup>-/-</sup>  
26 mice show no dramatic phenotypic alterations even in the brain, where xCT is constitutively  
27 expressed, indicating that loss of xCT is largely compensated by other mechanisms *in vivo*. However,  
28 a role for **system  $x_c^-$**  emerges under induced pathological conditions. As exhaustively reviewed in  
29 [17], although xCT shows a rather restricted expression pattern under normal conditions *in vivo*, it is  
30 induced in disease states where oxidative stress and inflammation are present. However, xCT  
31 upregulation may represent a double-edged sword depending on the site of the disease. In the CNS,  
32 xCT upregulation may exacerbate the neuropathological disorders by inducing glutamate-dependent  
33 excitotoxicity in neurons. This could explain why xCT deficiency in xCT<sup>-/-</sup> mice protects against  
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1 toxin-induced Parkinson disease-like neurodegeneration and reduces susceptibility against limbic  
2 seizures [51, 52]. Moreover, system  $x_c^-$  regulates microglial reactivity in a mouse model of  
3 amyotrophic lateral sclerosis. Here, genetic deletion of xCT slows the progression of the symptoms  
4 and increases the number of surviving motor neurons at the end stage of the disease, eventually  
5 improving survival of mice following the disease onset [53]. Similarly, lack of xCT attenuates  
6 multiple sclerosis symptoms caused by acute autoimmune inflammatory demyelination [54].  
7 Conversely, deletion of xCT worsens ischemic kidney injury [55], increases lung damage and  
8 mortality following Paraquat administration [56], and aggravates acetaminophen-induced hepatic  
9 damage and mortality [57] compared to wild type mice. This supports a protective role for system  $x_c^-$   
10 against oxidative stress in tissues where excitotoxicity is not involved [17]. Therefore, possible side  
11 effects of an anti-xCT vaccination could emerge when other pathological states occur. Further  
12 investigations in this regards are surely needed.

#### 13 14 *Conclusions and future perspectives*

15 Considering the high potential of CSC to give rise to recurrences and metastasis, the identification  
16 of CSC-associated antigens, such as xCT, offers a reliable chance to design a CSC-directed  
17 immunotherapy aimed at preventing tumor evolution. We believe that our observations are  
18 particularly valuable for the clinical development of anti-CSC immunotherapies, because anti-xCT  
19 vaccination generated a robust humoral response with no toxicity.

20 Although VLP vaccination induced higher antibody titers as detected by ELISA (Figure 3), the  
21 efficacy of our three vaccines in impairing tumor growth and metastases *in vivo* was similar, and none  
22 of them was able to completely eradicate the disease [19, 21] (Conti et. al, unpublished). This  
23 observation suggests that xCT immunotargeting, independently on the vector used, could be used as  
24 an adjuvant therapy in breast cancer patients that develop resistance to standard therapies. xCT  
25 immunotargeting may be combined with different conventional or innovative treatments able to either  
26 stimulate immune responses, or to target differentiated cancer cells or CSC. In particular, xCT  
27 targeting could be successfully coupled to chemotherapy. In fact, we observed that tumorspheres  
28 display significantly increased resistance to doxorubicin, a drug largely used in breast cancer therapy,  
29 as compared to cells grown in differentiated conditions [19]. This is not surprising, since system  $x_c^-$   
30 contributes to resistance of cancer cells to many anti-tumor drugs [58], as many chemotherapeutics,  
31 including doxorubicin, exert their function, at least in part, by increasing intracellular ROS levels  
32 [59]. Furthermore, cancer cells upregulate xCT as a mechanism of self-defense in response to many  
33 chemotherapeutic drugs [58]. Therefore, vaccine-mediated inhibition of system  $x_c^-$  in combination,  
34 or sequentially, with chemotherapy may elicit clinical benefit by sensitizing bulk tumors to

1 chemotherapeutic agents as well as by targeting CSC. In support of this hypothesis, we have  
2 demonstrated that combination of anti-xCT DNA vaccination and doxorubicin strongly enhanced the  
3 anti-metastatic and anti-tumor potential of the individual treatments [19].

4 Furthermore, since PD-L1 is expressed on both CSC and myeloid cells in breast cancer, and we  
5 observed an increase in PD-1 expression on infiltrating lymphocytes upon anti-xCT vaccination  
6 (Conti et al., unpublished), combination with anti-PD1 or PD-L1 monoclonal antibodies may improve  
7 therapeutic efficacy. Many clinical trials are currently testing the administration of anti-PD1 or anti-  
8 PD-L1 antibodies in breast cancer patients, and preliminary evidence show clinical activity in a  
9 proportion of triple negative breast cancer patients treated with the anti-PD1 Pembrolizumab  
10 (Keytruda; Merck; NCT01848834) or the anti-PD-L1 atezolizumab (Tecentriq; Roche).

11 Despite our observations strongly suggest that anti-xCT vaccination is a feasible approach to  
12 induce anti-tumor protection, the evaluation of the real potential of xCT immunotargeting needs  
13 further study in appropriate preclinical models. Although transplantable tumor models have been  
14 valuable for the understanding of meaningful molecular targets and the consequences of their  
15 immunotargeting, TUBO- and 4T1-derived tumors do not represent the heterogeneity distinctive of  
16 human tumors and, because of their rapid growth rate in syngeneic mice, the long-lasting reciprocal  
17 exchange between cancer and immune cells, fundamental for tumor shaping, is lost [43]. In this view,  
18 BALB-neuT mice represent a better candidate [43]. Thanks to the slow spontaneous progression,  
19 cancer cells in BALB-neuT tumors engage long-lasting relationships with the surrounding  
20 microenvironment, including immune cells. The continuous interplay between the various cell  
21 populations in the tumor allows the generation of niches for CSC and therefore the sustaining of the  
22 tumor evolution [43]. The employment of currently available xCT null mouse models [60] crossed  
23 with BALB-neuT mice would help in dissecting the role of xCT in the CSC niche, as well as in the  
24 different steps of mammary tumor progression, from early to late stages.

25 Undoubtedly, translation of anti-xCT immunotherapy from the preclinical to the clinical setting  
26 would require a clear indication that xCT targeting would not lead to harmful side effects. As we have  
27 previously discussed, xCT expression *in vivo* is limited to few tissue types and anti-xCT vaccination  
28 did not lead to any functional or morphological alterations in vaccinated mice. Useful clues also come  
29 from studies performed on xCT-null mice. A review of the existing literature shows that xCT is  
30 dispensable *in vivo* under physiological condition, as compensatory mechanisms likely counteract the  
31 lack of this transporter [17]. On the contrary, the effects of xCT absence emerge under pathological  
32 conditions, thus requiring further investigations on the effect of anti-xCT vaccination in this context.

33 In conclusion, we showed that anti-CSC vaccination is feasible and effective in providing  
34 protection against tumor. xCT appears to be an optimal oncoantigen, since it can be targeted by  
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1 antibodies that mediate ADCC and inhibit its biological activity, which is required for CSC self-  
2 renewal, chemoresistance and tumor progression. Despite definitive proof for the safety of the vaccine  
3 needs to be provided before translation into the clinic, we are on the right path towards breaking CSC  
4 invulnerability and developing more effective anti-cancer therapies.  
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### 9 **Author contributions**

10 Roberto Ruiu, Valeria Rolih, Elisabetta Bolli, and Laura Conti produced the results discussed in  
11 this review. Roberto Ruiu, Federica Cavallo and Laura Conti provided major contribution in writing  
12 and discussing the manuscript. Federica Pericle provided the VLP technology, Gaetano Donofrio the  
13 BoHV-4 technology. Elisabetta Bolli and Valeria Rolih wrote and discussed the sections involving  
14 VLP and performed the original ELISA assay reported in this review. Giuseppina Barutello and  
15 Federica Riccardo wrote and discussed the sections involving the BALB-neuT model and the  
16 translatability of the vaccine. Roberto Ruiu produced the figures. Elena Quaglino, Federica Cavallo,  
17 Federica Pericle, Irene Fiore Merighi and Laura Conti critically revised the manuscript. All authors  
18 read and approved the final version of the manuscript.  
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### 40 **Compliance with ethical standards**

#### 41 *Conflict of interest*

42 The authors declare that no potential conflicts of interest exist  
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#### 47 *Ethical approval and ethical standards*

48 All the *in vivo* experiments were approved by the Italian Ministry of Health, authorization numbers  
49 174/2015-PR, 1042/2016-PR and 500/2017-PR.  
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#### 56 *Animal source*

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1 Mice used for the vaccination experiments reported in this paper were purchased from Charles  
2 River Laboratories or bred at the Molecular Biotechnology Center, University of Turin, where all  
3 mice were maintained and treated in accordance with the University Ethical Committee and European  
4 Union guidelines under Directive 2010/63.

## 9 **Figure legends**

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13 **8** *Figure 1. Topological model of the  $x_c$ - transporter.* xCT is composed by 12 transmembrane  
14 domains spanning the cellular membrane (red cylinders), 7 intracellular and 6 extracellular domains  
15 (ECD1-6, all represented as red lines) and is associated to CD98, the heavy chain of the  $x_c$ - transporter  
16 spanning the cellular membrane (blue cylinder). A scheme of the  $x_c$ - transporter function is also  
17 reported, with extracellular cystine (CySS) being imported in exchange for intracellular glutamate  
18 (Glu) and reduced to cysteine, the rate-limiting precursor for the synthesis of GSH, which then  
19 scavenges intracellular ROS.

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27 **16** *Figure 2. Schematic representation of the different vaccination protocols.* **a)** Therapeutic anti-  
28 tumor setting. Female BALB/c mice are challenged with a subcutaneous injection of TUBO  
29 tumorsphere-derived cells. When tumors become palpable, mice are vaccinated twice at two-week  
30 interval and tumor growth is monitored for the following days. **b)** Prophylactic anti-metastasis setting.  
31 Female BALB/c mice are vaccinated twice at two-week interval. One week after the second  
32 vaccination, mice are challenged with an intravenous injection of TUBO tumorsphere-derived cells.  
33 Few weeks after the challenge, mice are euthanized and lungs analyzed for the presence of metastasis.  
34  
35 **c)** Therapeutic anti-tumor and anti-metastasis setting. Female BALB/c mice are challenged with a  
36 subcutaneous injection of 4T1 tumorsphere-derived cells. When tumors become palpable, mice are  
37 vaccinated twice at two-week interval and the tumor growth is monitored for the following days. Few  
38 weeks after challenge, mice are euthanized and lungs analyzed for the presence of metastasis.

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47 **27** *Figure 3. Anti-xCT vaccination induces a specific humoral response.* Sera were collected from  
48 BALB/c mice vaccinated twice at two-week interval with either BoHV-4-mxCT, pVAX1-mxCT,  
49 AX09-0M6 or AX09-0M3 or control vectors (pVAX1, BoHV-4-A29 or MS2) were collected two  
50 weeks after the second vaccination, and tested by ELISA on wells coated with: **a)** full-length mouse  
51 xCT protein, **b)** full-length human xCT protein, or peptides corresponding to **c)** mouse/human xCT  
52 ECD6, **d)** mouse xCT ECD3 and **e)** human xCT ECD3. ELISA was performed from pools (each  
53 composed by 5 mice) of sera from 6 independent experiments. Graphs show mean  $\pm$  SEM of the  
54 OD<sub>450</sub> of each pool after the subtraction of the OD<sub>450</sub> of the corresponding pool from untreated mice.  
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1 Sera from mice vaccinated with the empty vectors did not bind to either full length xCT or its ECD,  
2 while those from BoHV-4-mxCT, pVAX-mxCT, AX09-0M6 and AX09-0M3 vaccinated mice  
3 recognized both recombinant xCT proteins and their ECD, although with variable efficiency.  
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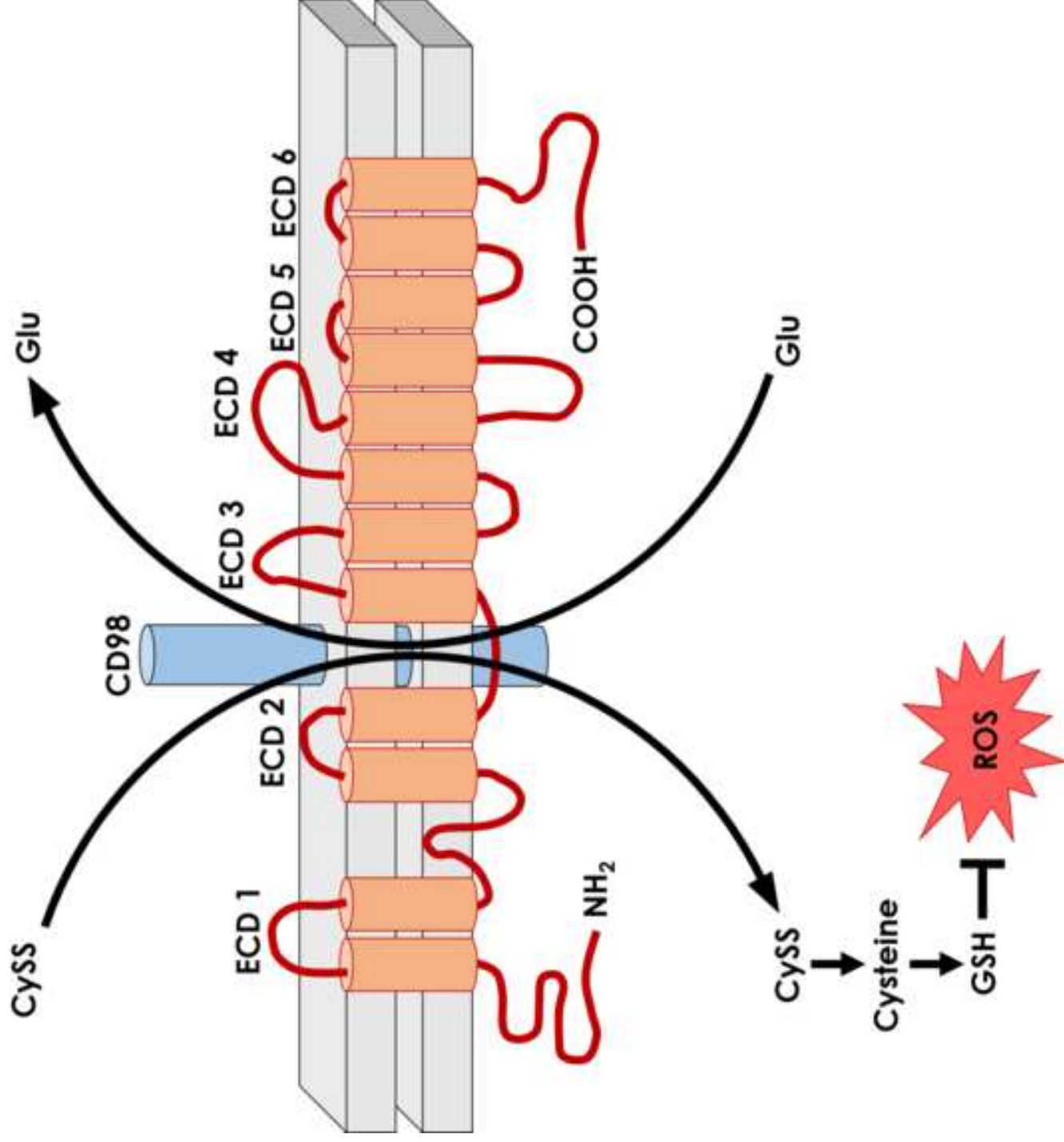
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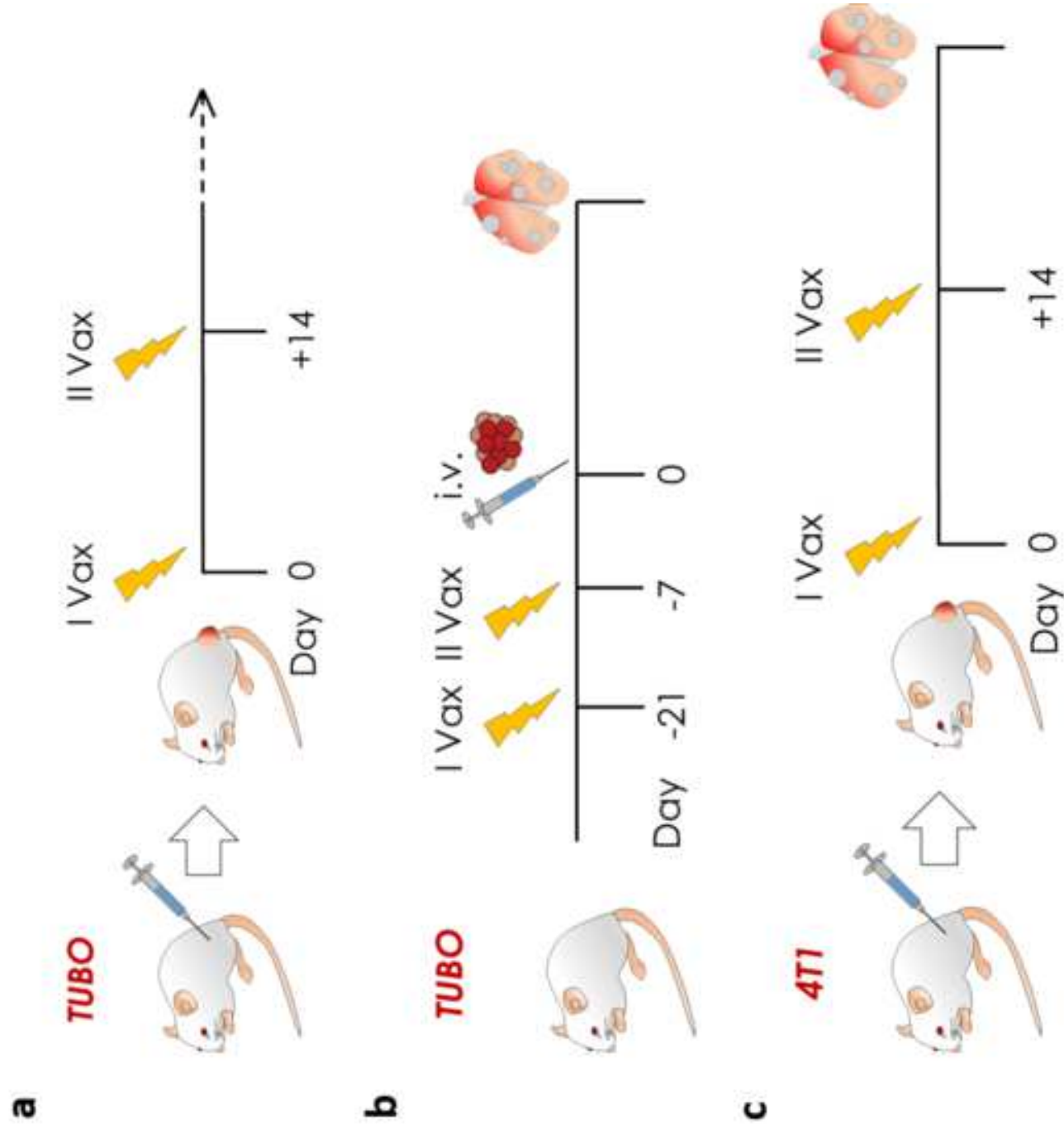
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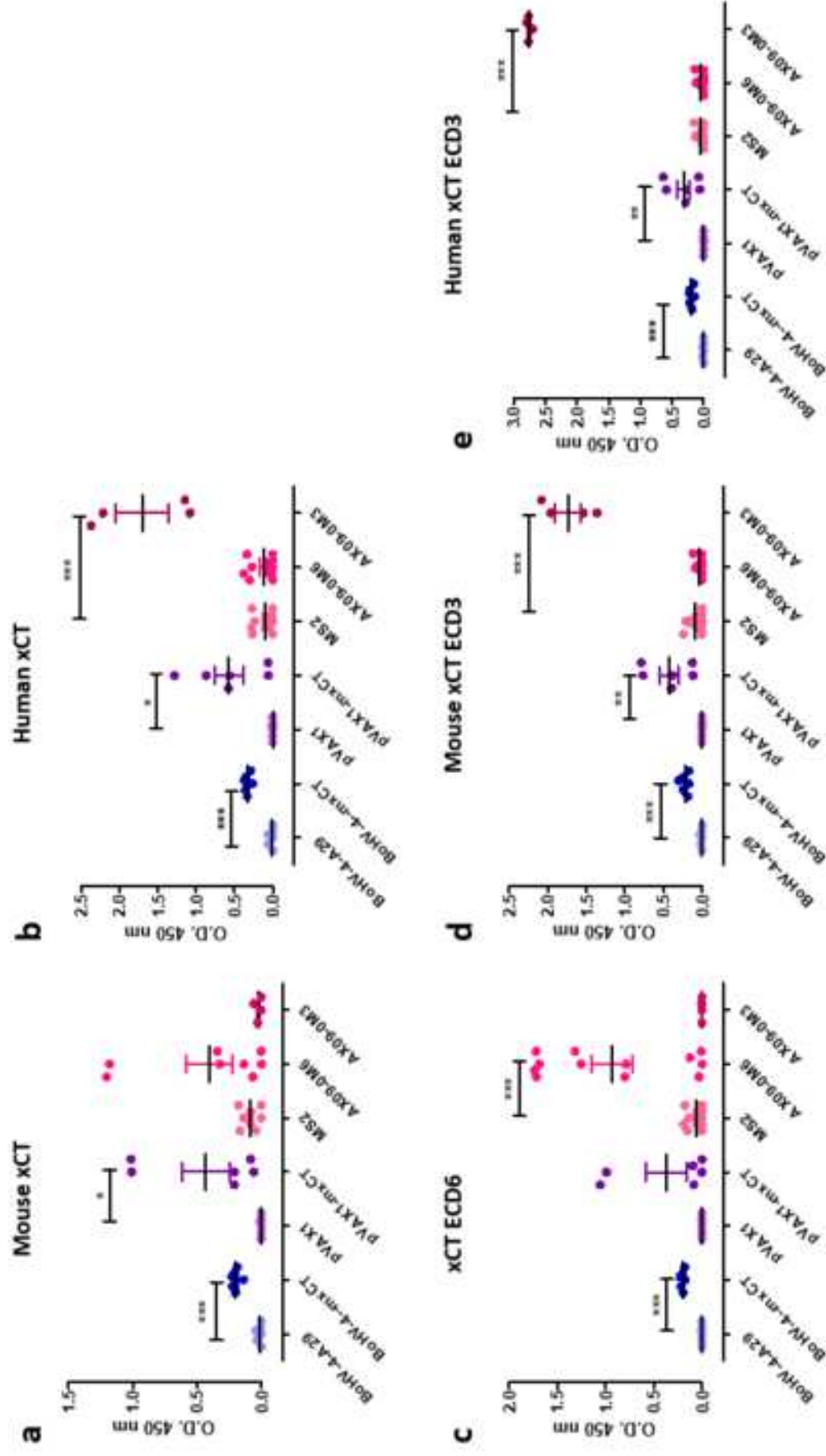
Figure 1

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This paper is a Focussed Research Review based on a presentation given at the *Fifteenth Meeting of the Network Italiano per la Bioterapia dei Tumori (NIBIT) on Cancer Bio-Immunotherapy*, held in Siena, Italy, 5<sup>th</sup>- 7<sup>th</sup> October 2017. It is part of a series of Focussed Research Reviews and meeting report in *Cancer Immunology, Immunotherapy*.