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HER-2 therapeutic vaccine is not hampered by concurrent HER-2 monoclonal antibody

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ES2B-C001 represents a new generation of vaccines based on virus-like particles (VLP) that display the full extracellular domain of HER-2 and has now entered clinical development. ES2B-C001 elicited strong anti-HER-2 antibody responses that cured human HER-2 transgenic tumors and metastases in mice. Early vaccine trials may include patients who are still receiving anti-HER-2 monoclonal antibody (MAb) therapy, a clinical scenario that has not typically been addressed in preclinical studies. To evaluate whether the concurrent administration of 4D5 anti-HER-2 MAb affects the immunogenicity or the therapeutic efficacy of ES2B-C001, we administered concurrent treatments to tumor-free or to human HER-2 transgenic mammary carcinoma-bearing mice. In tumor-free mice, 4D5 treatment did not hamper either ES2B-C001 activity, which induced a strong anti-HER-2 IgG production, or T cell responses. In tumor-bearing mice, the therapeutic efficacy of ES2B-C001 was not compromised by 4D5, resulting in 13/20 long-term tumor-free mice with ES2B-C001 alone, versus 15/20 with the combined treatment. ES2B-C001 elicited robust HER-2-specific antibody responses, reaching concentrations in the milligram/mL range and persisting for >6 months post-treatments, regardless of prior 4D5 administration. These findings indicate that co-administration of an anti-HER-2 MAb does not impair the efficacy of ES2B-C001, supporting the feasibility of vaccinating patients undergoing trastuzumab therapy.

The HER-2 oncogene drives the growth of more than 20% of breast cancers, and it is involved in variable proportions of other tumor histotypes[1–6]. HER-2 is also an antigen, which can be recognized by the immune system of the host, thus leading to a decades-long quest for therapeutic vaccines endowed with clinical efficacy[7–9].

Recent advances in vaccine technologies, especially based on virus-like particles (VLP)[10–12], fostered a new generation of HER-2 vaccines, some of which have recently entered the clinical arena[1,8,13–19].

In a proof-of-concept study, a prototypic HER-2-VLP vaccine demonstrated a robust therapeutic response against a human HER-2 transgenic mammary carcinoma[20]. This vaccine was subsequently re-engineered by ExpreS^{2}ion Biotechnologies (Hørsholm, Denmark) for human administration (referred to as ES2B-C001). The ES2B-C001 VLP vaccine is based on the SpyTag-SpyCatcher conjugation system, which exploits two highly reactive protein SpyTag/SpyCatcher binding partners that spontaneously form covalent isopeptide bonds. The Catcher sequence is linked through a BiP (Binding immunoglobulin Protein) secretion signal at the N-terminus of the extracellular domain sequence of HER-2 (amino acids 23-652), and a purification tag is added at the C-terminus (C-tag). The final Catcher-HER-2 sequence was codon optimized in S2 insect cells, subcloned, selected, and purified by ExpreS^{2}ion Biotechnologies. Separately, the Tag sequence was linked through a linker sequence (GSGTAGGGSGS) at the N-terminus of the Acinetobacter phage AP205 coat protein, from which the VLP structure is derived. The sequence was inserted in a plasmid vector, expressed in *E. coli* cells, and purified by ExpreS^{2}ion Biotechnologies, obtaining the Tag peptides expressed on the surface of VLP[19]. The purified Catcher-HER-2 and Tag-VLP were subsequently mixed in a 2:1 molar ratio, allowing spontaneous covalent coupling between the HER-2 ECD (extracellular domain) and the VLP through the SpyTag–SpyCatcher system. This process yielded a stable, conjugated HER-2-VLP characterized by a directional, high-density, "virus-like" presentation of the HER-2 antigen on the VLP surface (Fig. 1a)[19,20]. In our previous study, ES2B-C001 administered

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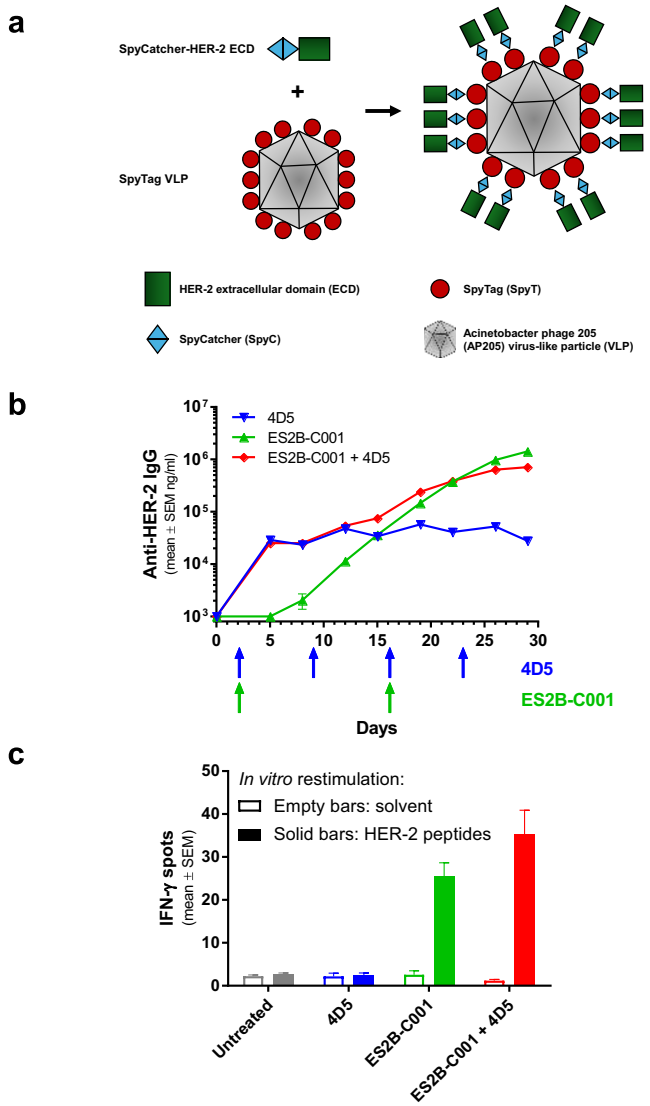

Fig. 1 | Anti-HER-2 immune responses of five healthy FVB mice receiving ES2B-C001 vaccinations and/or 4D5 MAb treatments. a Schematic design and assembly of the ES2B-C001 vaccine based on the SpyTag-SpyCatcher conjugation system. The extracellular domain of HER-2 (green) is linked at the N-terminus to the SpyCatcher (SpyC, blue) protein. Spy-tagged VLP subunits (red) and SpyCatcher-HER-2 fusion proteins are mixed, obtaining the HER-2-VLP. **b** Kinetics of serum anti-HER-2 antibodies, as measured by ELISA against a human HER-2 extracellular domain. Each point represents the mean and SEM ($n = 5$). The arrows under the x-axis indicate the day on which mice received 4D5 treatments (blue arrows) and/or ES2B-C001 vaccinations (green arrows). **c** IFN-γ release by splenocytes, re-stimulated in vitro with a mixture of human HER-2 peptides, as measured by ELISpot (*see* Materials and Methods). Each bar represents the mean and SEM ($n = 5$).

with Montanide ISA 51 adjuvant resulted in the inhibition of tumor growth and metastatic dissemination in FVB mice challenged with a human HER-2 positive cell line. In particular, ES2B-C001 elicited a strong and persistent polyclonal anti-HER-2 antibody response, comprising all IgG isotypes, and a moderate HER-2-specific T cell response. Mice remained tumor-free for more than one year after cell challenge[19].

At variance with the preclinical conditions in which HER-2 vaccines are routinely tested, early clinical trials might also enroll patients who are still being treated with HER-2 monoclonal antibodies (MAb), like trastuzumab[21–23]. Since the full HER-2 ECD is exposed on the ES2B-C001 vaccine, comprising the epitope in subdomain IV recognized by trastuzumab (Fig. 1a)[24], this could entail potential interactions between the two agents. Such interactions might range from synergistic, since they both target the same antigen, to antagonistic, because anti-HER-2 monoclonal antibodies might intercept or neutralize the vaccine, reducing its ability to elicit host immune responses[21,22,25–28].

We present here a preclinical study of anti-HER-2 vaccine–antibody interactions, showing that the two agents can be safely administered together without any loss of therapeutic activity.

Results

Anti-HER-2 vaccine + antibody in tumor-free mice

To analyze the interactions between the HER-2 VLP vaccine ES2B-C001, which is currently being tested in a first-in-human clinical study (NCT06746688), and the 4D5 monoclonal antibody (MAb), the mouse hybridoma from which trastuzumab was derived by humanization[29–32], we administered both agents to healthy, i.e., tumor-free, FVB female mice.

The half-life of monoclonal antibodies in mice was reported to be one week[33]; our previous experience with ES2B-C001 and similar HER-2 VLP vaccines was based on two-week intervals between administrations[19,20]. Therefore, we scheduled weekly administrations for the 4D5 MAb and bi-weekly vaccinations with ES2B-C001. To maximize the potential interference between the two agents, 4D5 was administered i.p. in the morning on the same days on which mice received i.m. vaccinations in the afternoon (*see* arrows in Fig. 1b).

Figure 1b shows the HER-2-specific IgG levels detected by ELISA against the human HER-2 extracellular domain protein. In mice treated with 4D5, serum anti-HER-2 IgG levels showed minor fluctuations around a plateau attained after the first MAb administration. Vaccinated mice had increasing serum anti-HER-2 IgG, in the order of magnitude of hundreds of micrograms/mL, after the second vaccination. The kinetics of anti-HER-2 IgG in mice receiving the combined treatment overlapped that of the MAb at the early time points, and that of vaccinated mice at later time points, in practice corresponding to the sum of single treatments.

To evaluate specific T cell responses, spleen cells were re-stimulated in vitro with a mixture of human HER-2 peptides and tested in a gamma-interferon (IFN-γ) ELISpot assay. Figure 1c shows that the combined treatment elicited an IFN-γ response similar to that of the vaccine alone.

Altogether, the results suggest that the combined treatment did not hamper the T and B cell immunogenicity of the ES2B-C001 vaccine. However, two issues deserve further investigation. Firstly, this set of experiments was conducted in tumor-free mice, but the presence of neoplastic masses can heavily influence all immune responses[34]; secondly, previous results with ES2B-C001 showed the induction of long-lasting immune memory, with high antibody titers persisting almost indefinitely[19], hence the long-term effects of MAb administration need to be analyzed.

Therapy of HER-2$^+$ mammary carcinomas

It was previously shown that optimal scheduling of either anti-HER-2 vaccines or 4D5 MAb can completely inhibit the growth in mice of mammary carcinoma cells expressing human HER-2[19,20,35,36]. Therefore, to allow for the evaluation of either antagonistic or additive/synergistic effects of the combination, we delayed the start of treatments to 26 days after tumor cell challenge, to permit a more advanced tumor growth. Under these conditions, we expected a broader individual variability in the control of tumor growth, hence we performed two independent tumor therapy studies.

Figure 2 shows the tumor-free survival curves of individual experiments (Fig. 2a, b), and the combined survival curves (Fig. 2c); individual tumor growth curves are shown in Fig. 3. In the first experiment (Fig. 2a), the combination resulted in a higher proportion of long-term tumor-free mice than individual treatments. In the second experiment (Fig. 2b), all therapeutic treatments yielded similar proportions of tumor-free mice. Altogether, the combined vaccine + MAb treatment was not significantly different from vaccine alone (Fig. 2c). Before stopping 4D5 administration, about 90% and 50% of mice were tumor-free in the first and second experiment, respectively. Considering the two experiments together, most mice developed tumors after 4D5 interruption, ending with only 30% of

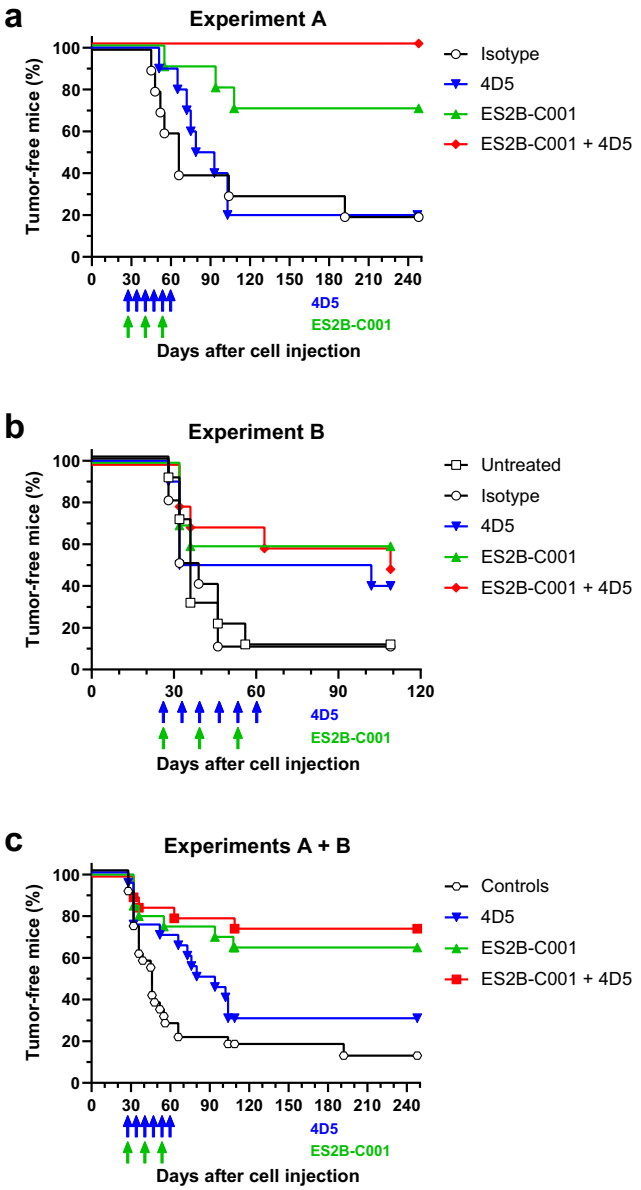

Fig. 2 | Tumor-free survival curves. Tumor-free survival curves of mice challenged in the mammary fat pad with human HER-2 transgenic mouse mammary carcinoma cells ($n = 10$ mice per group). Two independent experiments, designated as A and B, are shown in the upper (**a**) and middle (**b**) panels, respectively; the lower panel (**c**) shows the pooled survival curves of both experiments, and the curve labeled as "Controls" includes all Isotype and Untreated controls. The arrows under the *x*-axis indicate the day on which mice received 4D5 (or isotype control) treatments (blue arrows) and/or ES2B-C001 vaccinations (green arrows). The pooled survival curve of mice receiving both ES2B-C001 and 4D5 was not significantly different (by the log-rank test) from that of mice receiving ES2B-C001 alone.

mice being tumor-free versus 65% and 75% of mice treated with vaccine alone or combination treatment, respectively (Figs. 2c and 3).

In a non-inferiority perspective, it can be concluded that the simultaneous administration of an anti-HER-2 monoclonal antibody did not hamper the antitumor therapeutic activity of the ES2B-C001 vaccine.

Anti-HER-2 antibody response

To evaluate the kinetics of anti-HER-2 antibodies, blood samples were taken before each treatment (tumor cell challenge and MAb/vaccine administration) and at later time points. The results are presented separately for tumor-negative and tumor-positive mice (Fig. 4a, b), since tumor growth is known to affect the immune response of the host. Large tumors are more immunosuppressive than small tumors both locally and systemically, thereby affecting the ability of the immune system to elicit both natural and immunotherapy-induced immune responses[34].

It should be noted that, for antibodies directed against membrane-associated antigens, the clearance may be influenced by the target binding, followed by possible internalization of the antibody-antigen complexes and subsequent degradation. Furthermore, a tumor mass expressing high HER-2 levels can act as a "sink" for circulating anti-HER-2 antibodies[37,38]. Indeed, this was clearly evident in mice treated with 4D5 MAb. An inverse correlation was observed between tumor volume and circulating antibody levels measured six days after the fifth administration of 4D5. Mice bearing sizeable tumors exhibited lower levels of circulating anti-HER-2 MAbs, as reported in Supplementary Fig. 1. Notably, all mice with tumors had a two-log reduction in circulating anti-HER-2 antibodies in comparison to tumor-free mice. The reduction in anti-HER-2 antibodies was also evident in all vaccinated mice (Fig. 4a, b). However, the huge endogenous antibody titers elicited by the ES2B-C001 vaccine, which reached plateau levels in the milligrams/mL range, only resulted in a quantitative reduction, whereas exogenous 4D5 completely disappeared from the serum of mice bearing advanced tumors. HER-2 expression, as evaluated by flow cytometry, in progressive tumors was similar in control and vaccinated mice (Supplementary Fig. 2).

The direct comparison of mice receiving ES2B-C001 vaccine alone, or in combination with 4D5, showed an initial additive effect of exogenous MAb, similar to that previously observed in tumor-free mice (see Fig. 1b). In the long run, when antibodies elicited by ES2B-C001 greatly overcame circulating 4D5, the anti-HER-2 IgG levels became similar in both groups. It is interesting to note that, months after the end of treatments, all surviving vaccinated mice had overlapping titers, thus suggesting that MAb administration had no effect on long-term immune memory.

Discussion

The results presented here provide a clear and definitive answer to the question of whether anti-HER-2 MAb administration interferes with the therapeutic efficacy of HER-2 targeted vaccination. Our data demonstrates that there is no detrimental effect.

The immunogenicity of the ES2B-C001 vaccine, as measured by the kinetics and magnitude of anti-HER-2 antibody responses, remained unaffected by the concurrent administration of 4D5, the murine analog of trastuzumab. Notably, several months after treatment cessation, all surviving mice maintained high levels of circulating anti-HER-2 antibodies, regardless of whether they received the vaccine alone or in combination with the MAb. Similarly, vaccine-induced T cell-specific responses, measured by IFN-γ ELISpot, were not impaired by MAb co-administration. A further anti-tumor immune mechanism that we plan to investigate in future studies is the antibody-dependent cell-mediated cytotoxicity (ADCC), in which natural killer (NK) cells and other leukocytes with Fc receptors can kill target tumor cells expressing the antigen recognized by antibodies after the binding with their Fc region[39,40]. In fact, both 4D5 and trastuzumab were reported to perform ADCC[29,31,41].

Our findings showed that the therapeutic efficacy of ES2B-C001 in controlling tumor growth was not compromised by the simultaneous administration of the anti-HER-2 MAb. These preclinical results suggest no contraindications to the clinical evaluation of ES2B-C001 in patients undergoing treatment with therapeutic anti-HER-2 MAbs such as trastuzumab.

Patients with advanced HER-2-positive breast cancer are now being treated with antibody drug conjugates (ADC), such as trastuzumab emtansine or trastuzumab deruxtecan[42–45], which offer distinctive therapeutic benefits and toxicity profiles compared to conventional MAbs[46,47]. We are currently planning to extend our investigations to assess the potential interactions between ES2B-C001 and ADCs. A further therapeutic option consists of the combination of trastuzumab and pertuzumab, another anti-HER-2 MAb. While trastuzumab binds to subdomain IV of the HER-2 ECD, pertuzumab binds to subdomain II. Recent studies have

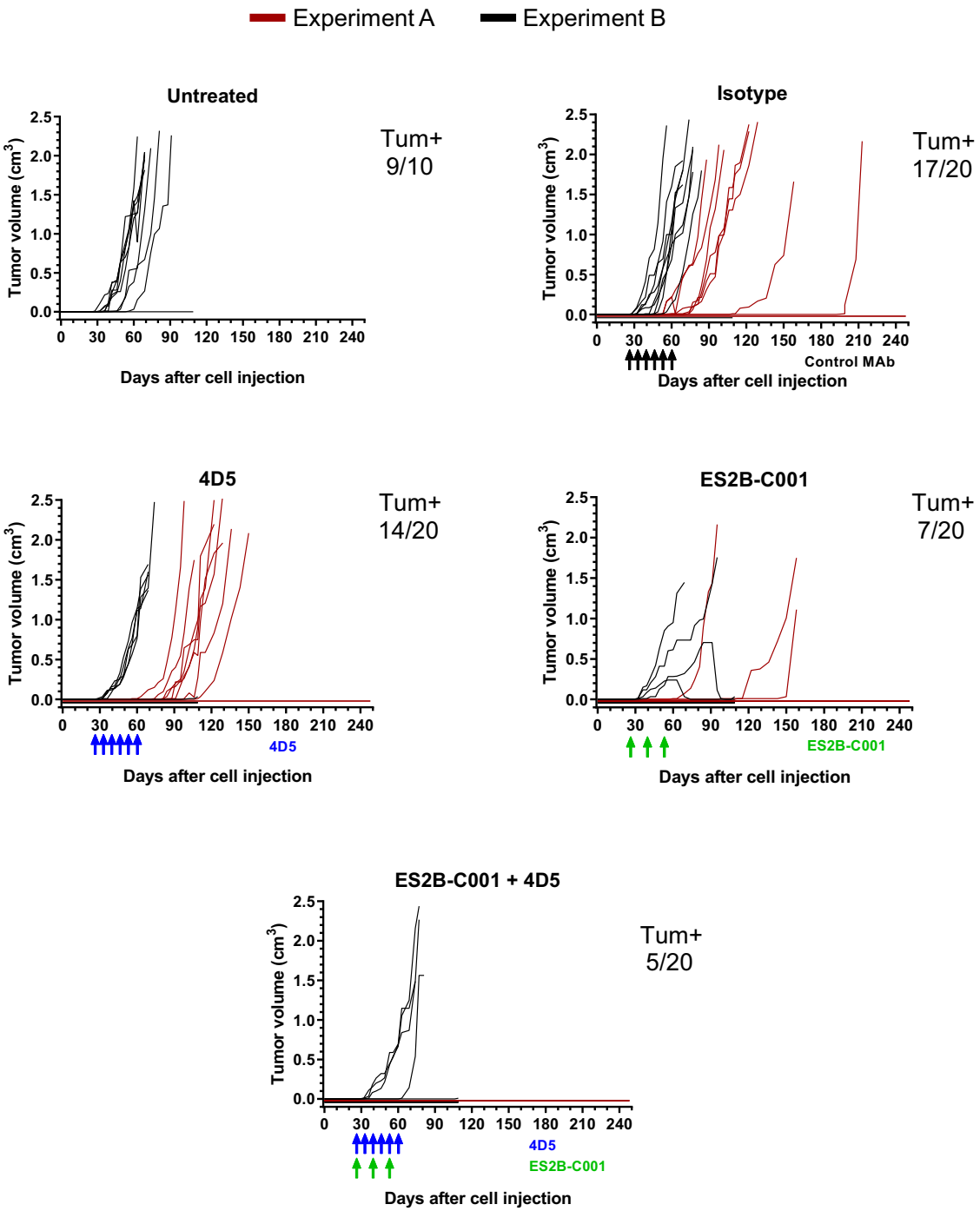

Fig. 3 | Tumor growth curves. Each line represents one mouse; brown lines: experiment A, black lines: experiment B. The arrows under the *x*-axis indicate the day on which mice received treatments. Tumors were measured with calipers, and volumes were calculated as described in the Materials and Methods. The number of mice with progressive tumors (tum +) is shown in the upper right corner of each graph.

evidenced that this regimen was able to improve progression-free survival and overall survival[9,48]. Since this regimen is based on the simultaneous administration of two MAbs directed against two different HER-2 epitopes, the interaction of ES2B-C001 with this treatment may also be evaluated.

An additional point of interest is the potential for enhanced therapeutic benefit through sequential or combined administration of MAbs and HER-2 vaccination. From an immunological perspective, this strategy could provide immediate passive immunity via the MAb, bridging the time required for the vaccine to elicit a robust and durable active immune response (see Fig. 4). Preliminary data suggests possible additive effects (see Fig. 2a); however, overall outcomes (*see* Fig. 2c) showed only a marginal, statistically non-significant (*see* Results), advantage for the combination therapy. Further studies are warranted to explore this possibility, including optimization of dosing regimens, treatment schedules, and alternative experimental models, such as lung metastasis, which has previously proven to be a relevant target for evaluating the therapeutic potential of ES2B-C001[19].

Methods

Cells

The D16-BO-QD cell line (referred to as QD) was established in our laboratory from a transgenic HER-2-positive mammary carcinoma of a

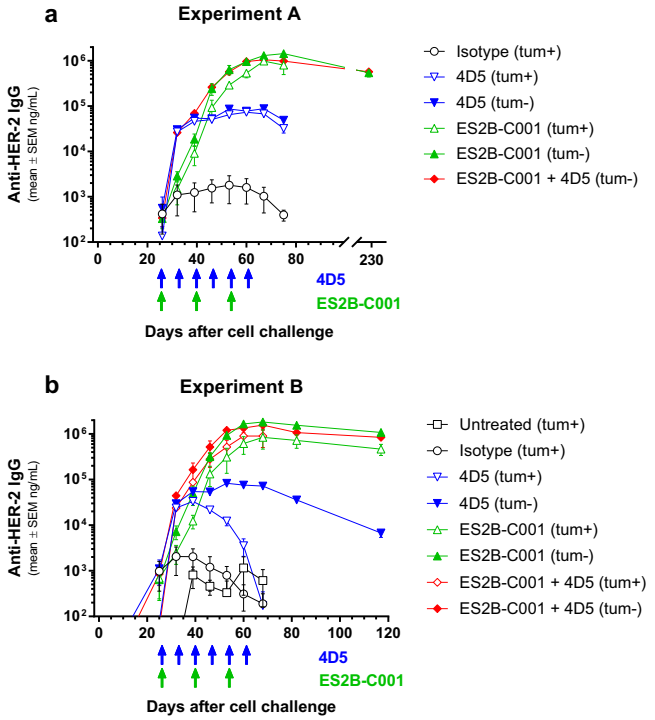

Fig. 4 | Kinetics of serum anti-HER-2 antibodies, as measured by ELISA against a human HER-2 extracellular domain. For each treatment, the values of mice with progressive tumors (tum+) are plotted separately from those of tumor-free mice (tum-); the latter include mice with transient tumor growth followed by regression. The arrows under the *x*-axis indicate the day on which mice received 4D5 (or isotype control) treatments (blue arrows) and/or ES2B-C001 vaccinations (green arrows). The number of mice in each group was as follows. Experiment A, Isotype (tum+): 6, 4D5 (tum+): 8, 4D5 (tum-): 2; ES2B-C001 (tum+): 3, ES2B-C001 (tum-): 7, ES2B-C001 + 4D5 (tum-): 10. Experiment B, Untreated (tum+): 4, Isotype (tum+): 4, 4D5 (tum+): 5, 4D5 (tum-): 5; ES2B-C001 (tum+): 4, ES2B-C001 (tum-): 6, ES2B-C001 + 4D5 (tum+): 5, ES2B-C001 + 4D5 (tum-): 5. Each point represents the mean and SEM. ELISA titers were compared by Tukey's test. **a** $p < 0.05$ Isotype (tum+) vs ES2B-C001 (tum+) and $p < 0.001$ Isotype (tum+) vs ES2B-C001 (tum-) and ES2B-C001 + 4D5 (tum-) at day 67 and 75. Titers of ES2B-C001 + 4D5 (tum-) were not statistically different from ES2B-C001 (tum+) and ES2B-C001 (tum-) at days 67 and 75. **b** $p < 0.01$ Untreated (tum+)/Isotype (tum+) vs ES2B-C001 (tum-) and $p < 0.05$ Untreated (tum+)/Isotype (tum+) vs ES2B-C001 + 4D5 (tum-) at day 68. Titers of ES2B-C001 + 4D5 (tum-) were not statistically different from ES2B-C001 (tum-) at day 68. Titers of ES2B-C001 + 4D5 (tum+) were not statistically different from ES2B-C001 (tum+) at day 68.

Delta16 female mouse[49,50]. QD cells were cultured in MammoCult medium (STEMCELL Technologies, Vancouver, Canada) supplemented with 1% fetal bovine serum (FBS), 100 U/mL penicillin, and 10 µg/mL streptomycin (Thermo Fisher Scientific, Monza, Italy) as previously reported[19]. Cells were cultured at 37 °C in a humidified 5% CO_2 atmosphere and were split once or twice a week according to cell density, using 0.05% trypsin-EDTA (Thermo Fisher Scientific).

Vaccine formulation

Design, expression, and purification of the ES2B-C001 vaccine were performed by ExpreS^{2}ion Biotechnologies (Hørsholm, Denmark) as previously reported[19]. ES2B-C001 VLP vaccine is based on the SpyTag-SpyCatcher conjugation system, in which SpyTag virus-like particles (VLP) of Acinetobacter phage 205 (AP205) are conjugated with SpyCatcher-HER-2 ECD, as previously described[19,20]. The ES2B-C001 vaccine was formulated with Montanide ISA 51 (Seppic, Courbevoie, France), referred to as Montanide, in a 50:50 volume.

Mice, tumor cells challenge, vaccination, and bleedings

Six-eight-week-old FVB female mice were purchased from Envigo RMS (S. Pietro al Natisone, Udine, Italy) or Charles River Laboratories (Calco, Lecco, Italy).

The vaccine was administered intramuscularly (i.m.) in the left hind leg at a dose of 10 µg per mouse per administration, as reported previously[18–20].

The mouse anti-human HER-2 IgG2a monoclonal antibody 4D5, kindly provided by Genentech (South San Francisco, CA, USA), was administered intraperitoneally (i.p.) at 100 µg per mouse. Control groups received an IgG2a isotype control antibody i.p. (BioXcell, Lebanon, NH, USA).

We used 4D5 instead of trastuzumab to maintain species consistency within our murine model. Furthermore, using humanized antibodies in immunocompetent mice could lead to the formation of anti-drug antibodies (ADAs)[51].

To investigate the potential interactions between the two treatments, groups of five healthy, tumor-free mice received two bi-weekly vaccine administrations and/or four weekly MAb administrations (see Fig. 1b arrows). For ELISpot assays (*see* below), performed 12–25 days after the last 4D5 treatment, mice received one additional treatment; spleens were collected seven days later.

For tumor therapy, groups of ten mice were challenged with 10^6 QD cells in the mammary fat pad (i.m.f.p.) of the fourth mammary area. Treatments started 26 days after cell challenge; mice received three bi-weekly vaccine administrations and six weekly MAb administrations (see arrows in Figs. 2, 3, and 4). All mice were bled before each treatment, two weeks after the last vaccination, and periodically thereafter, to monitor antibody titers; sera were stored at minus 80 °C. All mice were monitored daily and weighed twice weekly. Tumors were measured with calipers twice a week; tumor volume was calculated as $\pi/6*(\sqrt{ab})^3$ where a = maximal tumor diameter and b = maximal tumor diameter perpendicular to a. All the procedures described above did not require animal anesthesia. Mice were sacrificed if they showed any signs of distress or tumor volume reached 2.5 cm^3. Mice were euthanized by CO_2 inhalation and subsequent cervical dislocation.

All in vivo experiments were performed according to Italian and European laws and were authorized by the Italian Ministry of Health (letter 396-2023-PR).

Enzyme-Linked Immunosorbent Assay (ELISA)

Serum anti-HER-2 IgGs were measured in a customized ELISA. Human HER-2 extracellular domain comprising amino acids 22–652 (Gene ID: NP_004439), codon optimized for *Drosophila melanogaster* S2 insect cells and produced in ExpreS^{2}ion (ExpreS^{2}ion Biotechnologies) as described[19], with a C-terminal Strep-tag II for affinity purification, was used as coating antigen.

Immunoplate Nunc Maxisorp 96-well microplates (Merck, Darmstadt, Germany) were coated overnight with the human HER-2 ECD at 1 µg/mL in carbonate-bicarbonate buffer (Merck). A standard curve with 4D5 (0.04 to 30 ng/mL) was run in parallel with experimental sera. A horseradish peroxidase-labeled goat anti-mouse Ig antibody (Thermo Fisher Scientific) diluted 1:30'000 was used for detection.

Enzyme-Linked ImmunoSpot (ELISpot)

Splenocytes of five mice per group were isolated from spleens by passage through 40 µm Falcon Cell Strainers (Sial, Rome, Italy). Red blood cells were removed using Red Blood Cell Lysis Solution (Miltenyi Biotec, Bergisch Gladbach, Germany). The ELISpot Mouse IFN-γ kit (R&D Systems, Minneapolis, MN, USA) was used according to the manufacturer's protocol. Splenocytes were seeded in triplicate in the 96-well plate (0.2×10^6 splenocytes per well) and exposed to either a HER-2 peptide pool (PepMix, JPT Peptide Technologies GmbH, Berlin, Germany) dissolved in dimethyl sulfoxide (DMSO, Merck), or to DMSO alone (referred to as solvent). PepMix consisted of a mixture of peptides of 15 amino acids in length, spanning the complete sequence of the extracellular domain of HER-2

protein, for a total of 161 peptides with an 11 amino acid overlap between adjacent peptides. This pool of HER-2 peptides contained all linear epitopes resulting from ES2B-C001 vaccination, ensuring an effective in vitro re-stimulation of T cells. Forty-eight hours later, plates were washed and consecutively treated with the anti-IFN-γ antibody, streptavidin alkaline phosphatase, and the BCIP-NBT substrate, according to the manufacturer's protocol. After the last washing with deionized water, plates were dried overnight, and spots were counted under a dissection microscope.

Statistical analysis

All statistical analyses were performed using GraphPad Prism (GraphPad Software, Boston, Massachusetts, USA, www.graphpad.com pad.com).

The Kaplan–Meier tumor-free survival curve, as a method for estimating the survival function from time datapoints, was applied to events of progression: events occurred when a specific tumor volume (≥ 0.01 cm^3) was reached. Comparison of the Kaplan–Meier curves was carried out with the Log-Rank (Mantel-Cox) test, to detect differences between groups when the risk of an event is not equally distributed. Antibody titers were compared using one-way ANOVA followed by Tukey's test for pairwise comparisons.

Ethics approval

The animal study protocol was approved by the Institutional Review Board of the University of Bologna and authorized by the Italian Ministry of Health (letter 396-2023-PR).

Data availability

The data that support the findings of this study are available at https://doi.org/10.5281/zenodo.18434422.

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