

A chaperone protein-enriched tumor cell lysate vaccine generates protective humoral immunity in a mouse breast cancer model

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Abstract

We have documented previously that a multiple chaperone protein vaccine termed chaperone-rich cell lysate (CRCL) promotes tumor-specific T-cell responses leading to cancer regression in several mouse tumor models. We report here that CRCL vaccine generated from a mouse breast cancer (TUBO, HER2/*neu* positive) is also capable of eliciting humoral immunity. Administration of TUBO CRCL triggered anti-HER2/*neu* antibody production and delayed the progression of established tumors. This antitumor activity can be transferred through the serum isolated from TUBO CRCL-immunized animals and involved both B cells and CD4⁺ T lymphocytes. Further evaluation of the mechanisms underlying TUBO CRCL-mediated humoral immunity highlighted the role of antibody-dependent cell-mediated cytotoxicity. These results suggest that tumor-derived CRCL vaccine has a wider applicability as a cancer vaccine because it can target both T-cell- and B-cell-specific responses and may represent a promising approach for the immunotherapy of cancer. [Mol Cancer Ther 2008;7(3):721–9]

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Note: G. Li and S. Andreansky contributed equally to this work.

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Introduction

Tumors express specific patterns of mutated or overexpressed antigens that may serve as potential targets for activated effector immune cells (1, 2). Therefore, vaccines that deliver multiple tumor-derived antigens are being increasingly investigated, as they may stimulate polyvalent T-cell and B-cell responses against the tumor cells from which the vaccine has been generated. We have developed one such autologous tumor-derived vaccine termed chaperone-rich cell lysate (CRCL) and have shown that CRCL vaccine generates anticancer protection through T-cell-mediated immunity in several tumor models (3, 4). We have now extended our work by investigating whether tumor-derived CRCL can also promote tumor-specific responses through adaptive humoral immunity. Because CRCL is a complex of several chaperone proteins (3), it has the possibility to deliver both MHC class I and II epitopes and generate tumor-specific helper T cells, CTLs, and antibodies that may target tumor cells.

The CRCL vaccine was derived from HER2/*neu*-positive tumor (TUBO) and was evaluated as a therapeutic vaccine in an established tumor setting. HER2/*neu* overexpression occurs in 20% to 30% of breast cancer cases and is a marker for poor prognosis (5). HER2/*neu* is a self-protein, but preexisting host immunity to HER2/*neu* antigen has been shown in breast cancer patients (6). Active immunization strategies that generate optimal antibody and T-cell-specific responses are being used in treating HER2/*neu* cancers (7). The induction of a humoral response is particularly important due to the overexpression of HER2/*neu* protein on the surface of the cancer cells. Thus it is not surprising that Herceptin, a humanized monoclonal antibody, has been approved for clinical use (8).

We report here that the administration of CRCL derived from TUBO tumors delayed growth of preestablished tumors. Analysis of the B-cell-specific response showed the presence of anti-HER2/*neu* antibody in the serum of TUBO CRCL vaccinated animals. We have investigated the mechanisms of antibody-mediated tumor destruction in response to TUBO CRCL vaccine and have shown that multiple immune effectors such as CD4⁺ T lymphocytes and B cells are required. We have thus documented that CRCL, in addition to its potential to stimulate efficient antitumor T-cell responses, is also capable of generating potent humoral responses that can lead to delay in growth of preestablished tumors.

Materials and Methods

Mice

Female wild-type (WT) or severe combined immunodeficient BALB/c (H2^d) mice (6–8 weeks old) were obtained

from the National Cancer Institute. B-cell-deficient BALB/c (H2^d) mice (9) were purchased from Taconic Farms. Animal experiments were conducted according to the guidelines of the University of Arizona Institutional Animal Care and Use Committee.

Cell Lines

TUBO is a cloned cell line derived from BALB/c mice transgenic for the transforming rat HER2/*neu* oncogene (BALB-NeuT; ref. 10). Dr. Guido Forni (University of Turin) and Dr. Carla de Giovanni (University of Bologna) generously provided the TUBO cells. Rat p185 *neu* is a xenogeneic protein in normal mice (11) and does not induce anti-rat p185 *neu* antibodies or any detectable CTL when implanted in WT BALB/c mice (10). TUBO cells were cultured at 37°C, 5% CO₂ in DMEM (Life Technologies) supplemented with 25 mmol/L HEPES, pyridoxine-HCl (Life Technologies) with 20% heat-inactivated fetal bovine serum (Gemini). 12B1 is a murine leukemia cell line (kindly provided by Dr. W. Chen, Cleveland Clinic) in BALB/c background (4) and was used as an irrelevant antigen control. The cells were cultured in RPMI 1640 (Life Technologies) supplemented with 10% fetal bovine serum.

CRCL Preparation

CRCL was prepared from tumors that were implanted s.c. in BALB/c mice as described previously (3, 4). In brief, tumor tissues were homogenized in detergent-containing buffers, and the high-speed supernatants underwent forward solution isoelectric focusing in a Rotofor device (Bio-Rad) at 15 W constant power. Fractions were harvested and analyzed for chaperone protein content. Fractions of interests were pooled and prepared as vaccines by dialysis, detergent removal, and centrifugal concentration. The level of endotoxin in CRCL was lower than that in media control (<0.01 EU/μg) as determined by Limulus amebocyte lysate assay kit (Cambrex Bio Science).

In vivo Tumor Growth Experiments

WT or B-cell-deficient BALB/c mice were injected with 1×10^5 viable TUBO cells s.c. in the right groin. The mice were vaccinated six times on the opposite groin with 20 μg tumor-derived CRCL on days 0, 2, 4, 7, 10, and 14 after palpable tumor development (tumor diameter, 2–3 mm). Mice injected with saline and 12B1 CRCL (BCR-ABL positive) served as control groups. Tumor volume was calculated every week and mice that reached 4,000 mm³ in tumor volume were euthanized. The data were analyzed with GraphPad Prism software (GraphPad Software) and the log-rank statistics were used to test differences between groups (12).

Measurement of HER2/*neu*-Specific Antibodies by ELISA

HER2/*neu*-specific ELISA was done with sera collected from tumor-bearing vaccinated mice. Mice were bled from the lateral tail vein before tumor implantation (day 0), during vaccination (after third vaccination, day 16), and 1 week (day 30) and 3 weeks (day 44) after the last immunization and stored at -80°C. They were analyzed for antibodies to extracellular domain of human HER2/*neu* (ECD^{HER2}) by ELISA using 96-well microtiter plates coated

with 1 μg/mL ECD^{HER2} protein (13–15). Soluble human ECD^{HER2} was purified from BHK/erbB2 cell culture supernatants (generously provided by Dr. James D. Marks, University of California-San Francisco) using affinity chromatography with anti-HER2/*neu* IgG3 immobilized on CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech). The ECD^{HER2}-coated plates were blocked with bovine serum albumin (BSA; Sigma) and incubated overnight at 4°C with serum dilutions. Alkaline phosphatase-labeled rat anti-mouse IgG1 and IgG2a (Zymed) or alkaline phosphatase-labeled goat anti-mouse IgG3 (Southern Biotechnology Associates) were used to detect bound IgG and absorbance values were read at 410 nm after color development for 2 h with *p*-nitrophenyl phosphate disodium (Sigma).

Flow Cytometry

Sera from mice immunized with PBS or TUBO CRCL were collected a week after the administration of six doses. A total of 2×10^5 TUBO cells were stained by an indirect immunofluorescence method. Tumor cells were incubated with naive or immune sera at 1:80 serum dilution followed by fluorescein (Alexa Fluor 488)-conjugated goat anti-mouse IgG (Molecular Probes). The cells were evaluated using a FACScan (Becton Dickinson) and the data were analyzed with Cell Quest Software (Becton Dickinson).

Transfer of Immune Sera

Severe combined immunodeficient BALB/c mice were randomized and distributed into six mice per group. Sera from mice vaccinated with PBS, 12B1 CRCL, or TUBO CRCL were pooled 1 week after the last dose of vaccine and stored at -80°C. Mice received i.v. injection of 200 μL pooled sera on day -1 and were then challenged with 10^5 tumor cells a day later (13). A group of mice that did not receive any serum were used as controls for the transfer groups.

Internalization of Tumor Antigens from the Surface of Cells

Down-regulation of tumor antigen molecules, including HER2/*neu* molecules, from the cell surface of TUBO cells were assessed by confocal microscopy after incubation with sera collected a week after administering six doses of vaccine. TUBO cells were incubated with sera of various dilutions and permeabilized to detect cytoplasmic expression of tumor antigens. Alexa Fluor 488-conjugated IgG (Molecular Probes) was used to assess the expression of membrane-bound and cytoplasmic tumor antigens. Internalization of fluorescence was measured on a confocal microscope (LFM310; Zeiss; 488 nm argon laser). Green fluorescence was detected after excitation at 488 nm and images were recorded as TIF files and processed (LSM Image Examiner; Zeiss) to subtract background and enhance lower and middle intensity fluorescence (10).

Antibody-Dependent Cell-Mediated Cytotoxicity Assay

Antibodies contained in the sera of TUBO CRCL vaccinated mice were evaluated for tumor cell lyses by antibody-dependent cell-mediated cytotoxicity (ADCC) assay. Tumor cells were plated at 1×10^4 per well in 96-multiwell plates and allowed to attach overnight at

37°C. Cultures were incubated for 2 h on ice with sera from experimental and control mice at different dilutions. After washing, spleen cells from naive BALB/c mice were added at 50:1 effector-to-target ratio and plates were incubated at 37°C overnight. Nonadherent cells were carefully washed out and 20 μ L WST-1 solution (Roche Diagnostics) was added to each well. The absorbance at 450 nm was read after 4-h incubation.

Depletion of Natural Killer Cells and T Cells followed by Tumor Challenge

Natural killer cells were depleted with anti-asialo GM1 (25 μ L/mouse, 1/8 diluted with PBS; Wako Chemical). Rat anti-mouse CD4⁺ (GK1.5) and rat anti-mouse CD8⁺ (2.43.7) hybridomas (American Type Culture Collection) were sent to Taconic Farms for the production of mouse ascites. Mice were depleted of CD4⁺ or CD8⁺ cells by i.p. injection with 50 μ L GK1.5 or 2.43.7 in PBS. Immune cells were depleted on -3 and -1 days before s.c. injection with 10⁶ TUBO cells. This was followed by two more depletions 7 and 21 days after tumor implantation to maintain the depletion of specific cell types. Mice were vaccinated six times after palpable tumor development as described previously.

Statistical Analysis

All statistical analyses were done using the Student's *t* test to evaluate significance between groups, except that the log-rank statistics was used to test differences between groups. For all cases, results were regarded significant if *P* values were <0.05.

Results

CRCL Vaccination of Mice Bearing Established TUBO Tumors Leads to Tumor Growth Delay

CRCL was generated from TUBO tumors and Western blot analysis established the presence of heat shock

proteins 110, 90, 70, and 60 and glucose-regulated protein 94, confirming our previous reports (3, 4, 12, 16, 17). To evaluate the efficacy of CRCL vaccine in this mouse breast cancer model, groups of 12 BALB/c mice were injected with 10⁵ TUBO cells s.c. and were monitored for tumor growth. Mice were randomized and only animals with palpable tumors were vaccinated. The results indicate that vaccination with TUBO CRCL significantly delayed tumor growth when compared with nonimmunized mice or mice immunized with irrelevant 12B1 CRCL (Fig. 1). By day 44, nonimmunized and 12B1 CRCL-immunized mice exhibit an average tumor volume of 4,351 \pm 248 and 4,122 \pm 338 mm³ respectively, whereas the tumor volume of mice immunized with TUBO CRCL was 2,635 \pm 305 mm³ (*P* < 0.01). This delay in tumor volumes confirmed the antitumor efficacy of TUBO CRCL against preestablished murine tumors.

Vaccination with TUBO CRCL Elicits HER2/*neu* Antibody Production

It has been shown that antibody plays a critical role in HER2/*neu*-positive tumor immunity (18–22). Because TUBO cells overexpress rat p185 *neu*, we predicted that CRCL derived from TUBO tumors might chaperone HER2/*neu* antigen. We therefore measured anti-HER2/*neu* antibody titers in tumor-bearing mice vaccinated with TUBO CRCL. Sera from PBS and 12B1 CRCL-immunized animals were used as controls for the specificity of the antigen. A purified extracellular domain from human HER2/*neu* was used to test HER2/*neu*-specific isotypes (14, 15). It has been shown previously that antibodies against rat p185 *neu* can cross-react to the ECD^{HER2} of human origin (13, 14), because rat *neu* protein is 89% homologous to human HER2/*neu* protein (23). Increase in total IgG1 response to ECD^{HER2} was evident in the

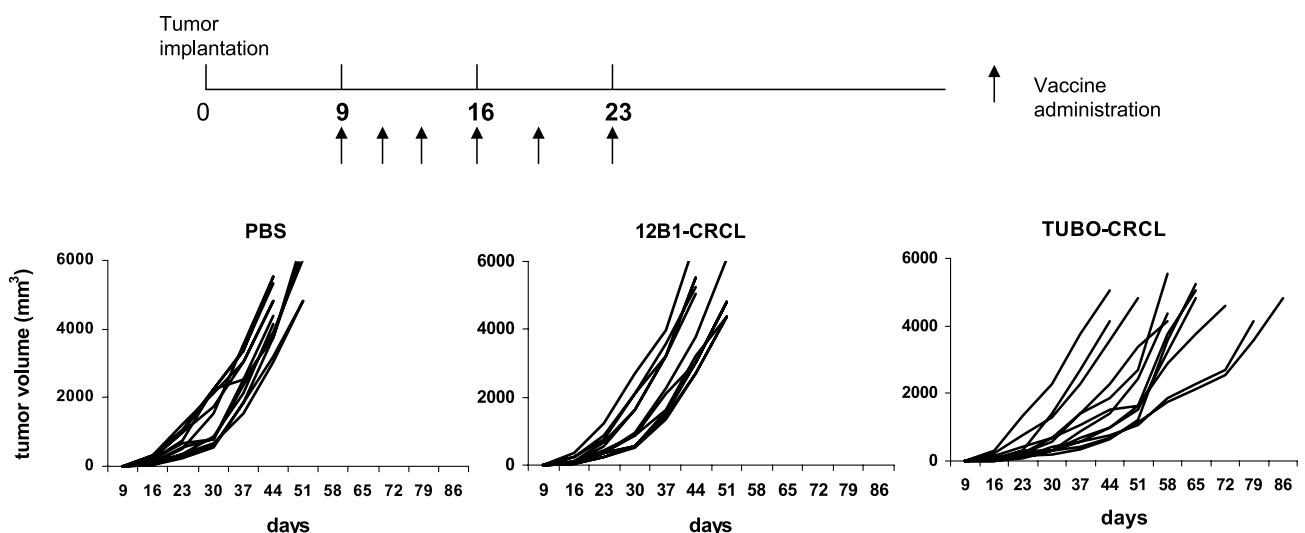


Figure 1. TUBO CRCL delays growth of established tumors. Female BALB/c mice (*n* = 12) were injected s.c. with 1 \times 10⁵ TUBO cells. After the tumors were palpable on day 9 (2–3 in diameter), mice were vaccinated s.c. on the opposite groin with PBS, 12B1 CRCL, or TUBO CRCL (20 μ g/mouse/injection) at the indicated days. CRCL vaccine was administered on the days indicated (arrows). Individual tumor size was measured every week starting from day 9. Mice with tumors exceeding 4,000 mm³ in volume were euthanized and considered dead. Representative of three independent experiments.

HER2/*neu*-positive CRCL-immunized group (Fig. 2) when compared with sera from preimmune mice (day 0; $P < 0.01$). These absorbance values were six times higher a week after the last dose (day 30) and this difference was sustained for at least an additional 2 weeks (day 44). In comparison, analysis of saline and 12B1 CRCL-immunized sera did not show the same specificity ($P > 0.05$). Analysis of IgG2a and IgG3 levels showed substantial increase compared with the controls ($P < 0.05$) but lower in contrast to IgG1 production.

CRCL-Mediated Tumor Growth Delay Depends on B Cells

The importance of B cells in delaying the onset of mammary carcinoma cells has been documented in HER2/*neu* mouse models (15, 24). To examine the role of B cells in

tumor growth delay, we used J_H knockout mice lacking B cells and antibodies ($Ig^{-/-}$) and compared them with tumor growth in WT BALB/c mice. The mice were injected s.c. on day 0 with TUBO cells and mice with measurable tumors were vaccinated with tumor-derived CRCL. In $Ig^{-/-}$ tumor-bearing mice, immunization with TUBO CRCL provided no significant protection compared with saline controls. By day 32, they had an average tumor volume of $3,032 \pm 373$ and $2,439 \pm 212 \text{ mm}^3$, respectively ($P > 0.05$; Fig. 3A and B). Tumor growth kinetics in $Ig^{-/-}$ mice immunized with TUBO CRCL was significantly faster compared with TUBO CRCL-immunized WT BALB/c mice ($P < 0.05$), indicating that B-cell response generated by TUBO CRCL vaccination played a role against the growth of TUBO tumors.

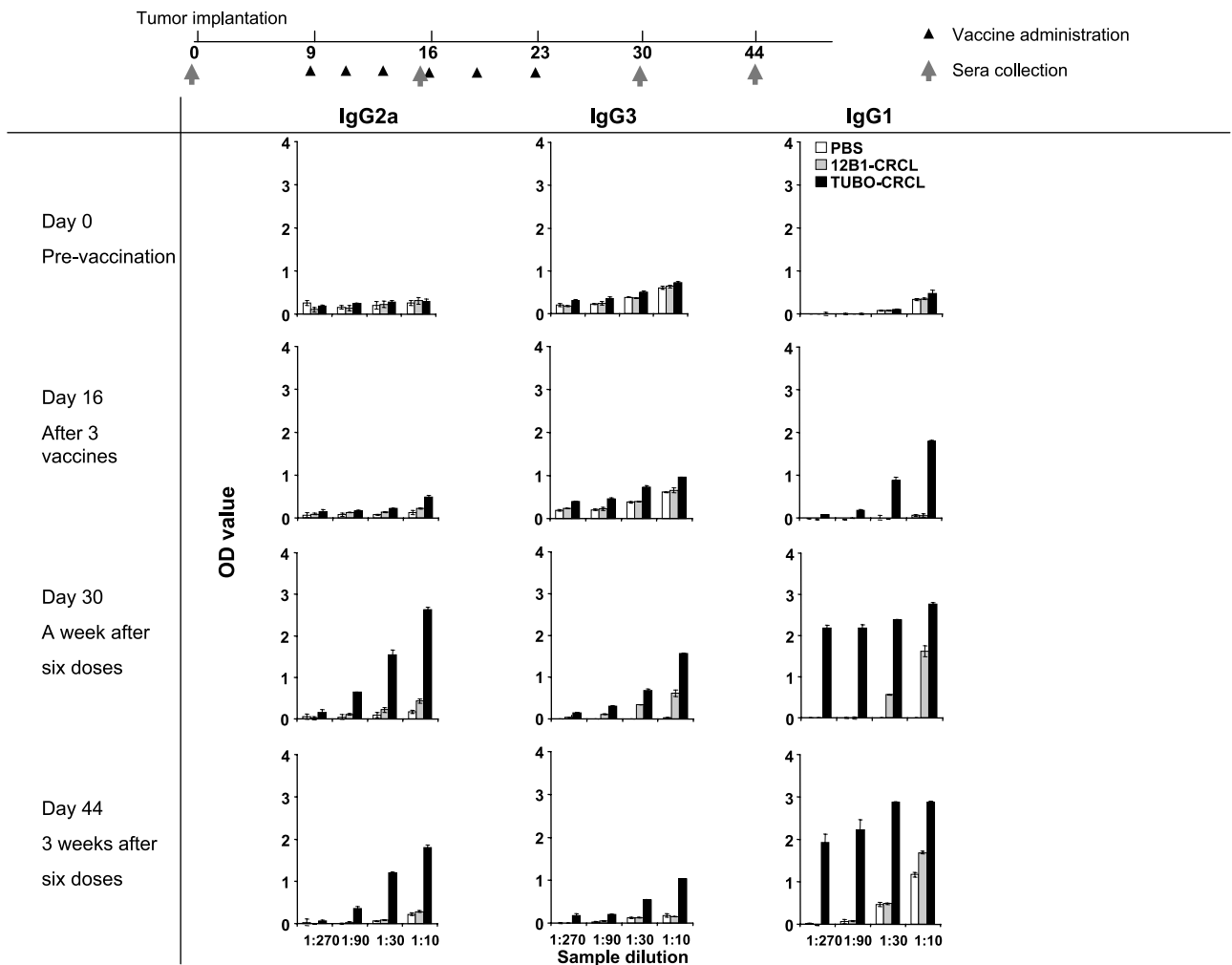


Figure 2. TUBO CRCL vaccination induces anti-HER2/*neu* antibody production in tumor-bearing mice. Groups of mice ($n = 8$) were injected with TUBO cells and vaccinated as described in Fig. 1. Mice were bled before tumor implantation (day 0), during vaccination (after third vaccination, day 16), and 1 wk (day 30) and 3 wk (day 44) after the last immunization. Sera from each group were pooled and examined for anti-HER2/*neu* IgG1, IgG2a, and IgG3 isotypes by ELISA. Plates were blocked with 3% BSA and dilutions of sera in PBS containing 1% BSA were added to the wells. After an overnight incubation, bound IgG was detected by incubating for 1 h at 37°C with alkaline phosphatase-labeled rat anti-mouse IgG1 or IgG2a and alkaline phosphatase-labeled goat anti-mouse IgG3. The wells were developed with *p*-nitrophenyl phosphate disodium dissolved in diethanolamine buffer and were read at 410 nm after 2 h. Average intensity at $A_{410 \text{ nm}}$ of duplicate wells at 1:270, 1:90, 1:30, or 1:10 dilutions. Sera from mice injected with PBS and 12B1 CRCL were used as controls. Representative of two independent experiments done in duplicate.

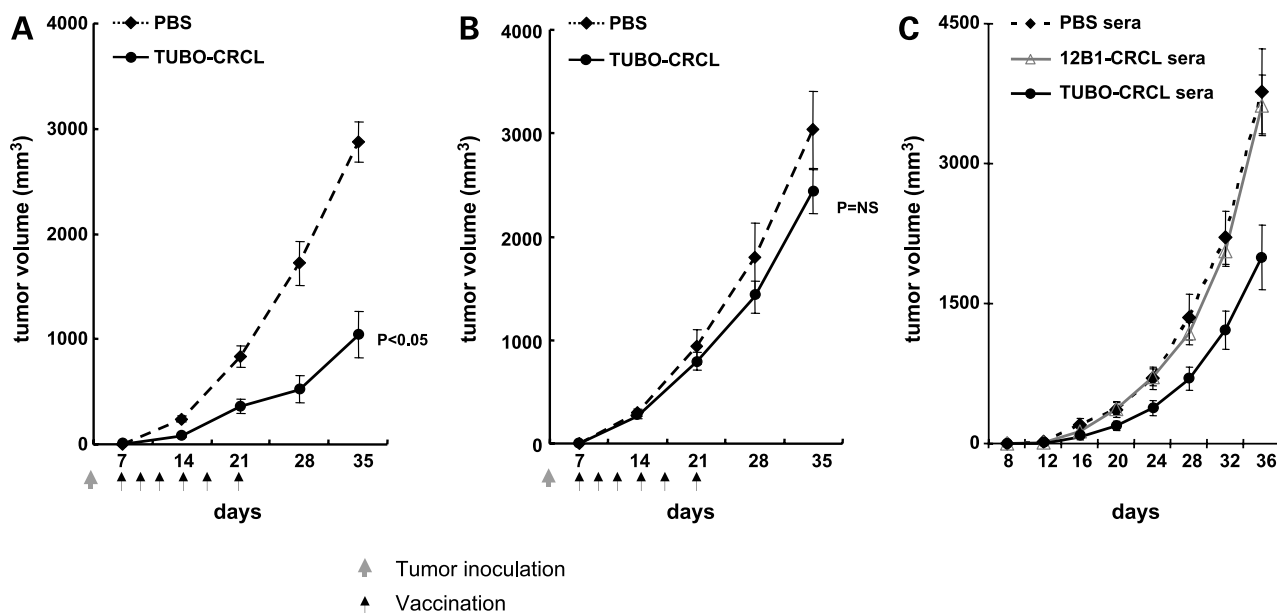


Figure 3. Therapeutic efficacy of TUBO CRCL is abrogated in B-cell-deficient mice and can be transferred by sera from TUBO CRCL-vaccinated mice. WT (A) and B-cell-deficient BALB/c (B) mice ($n = 8$) were injected s.c. on day 0 with 1×10^5 TUBO cells. After the tumor became palpable (2–3 mm in diameter), the mice were immunized with saline or TUBO CRCL (20 $\mu\text{g}/\text{mouse}$). Tumor volume was measured weekly. No significant statistical difference was observed between untreated and CRCL-immunized mice in the B-cell-deficient groups ($P > 0.05$) compared with the groups in WT controls ($P < 0.05$). Representative of two independent experiments. C, naive BALB/c mice were vaccinated with PBS, 12B1 CRCL, or TUBO CRCL. Sera were collected 1 wk after the last vaccination by tail vein bleed. On day -1, severe combined immunodeficient mice ($n = 8$) were injected i.v. with 200 μL pooled sera and 1×10^5 TUBO cells were injected in the right groin on day 0. Tumor volume was measured twice a week. The differences between the groups were statistically significant from day 24 ($P < 0.05$). Representative of two independent experiments.

Antitumor Activity Can Be Transferred with Immune Sera

Next, we addressed whether antitumor activity can be transferred through immune sera. CB.17 severe combined immunodeficient mice were injected i.v. with 100 μL pooled sera from TUBO CRCL-immunized mice. Following transfer of sera, mice were challenged the following day with TUBO cells. As shown in Fig. 3C, no apparent antitumor activity was observed with sera from control mice receiving PBS or irrelevant 12B1 CRCL ($P > 0.05$). Conversely, mice receiving sera from TUBO CRCL-immunized animals showed a significant tumor growth delay ($P < 0.05$) from day 24 onwards (380 ± 78 versus 702 ± 112 and 710 ± 92 mm³). This provides additional evidence that TUBO CRCL-associated antibodies have an effect on delaying tumor growth.

Serum from TUBO CRCL-Vaccinated Mice Facilitates ADCC

Various mechanisms have been proposed to account for the antitumor activity of antibodies. We have shown substantial IgG1, IgG2a, and IgG3 production against ECD^{HER2} from mice immunized with TUBO CRCL. These isotypes are known to mediate ADCC analogous to the antitumor activity of trastuzumab (Herceptin) *in vivo* (25). Therefore, to understand whether TUBO CRCL-induced antibodies can cause tumor cell lyses, we did ADCC assays using mouse splenocytes as effectors. Figure 4 shows that at 1:5 dilution of the sera ~30% cytotoxicity was detected

from TUBO CRCL-vaccinated mice when compared with the 5% killing with control sera ($P < 0.05$). This effect was specific as we were able to abrogate lysis of tumor cells with increasing dilutions of the sera obtained by TUBO CRCL immunization.

Sera from TUBO CRCL-Immunized Mice Contain IgG That Binds to Cell Surface and Are Subsequently Internalized

It has been reported that antibody binding to the HER2/*neu* antigen can cross-link HER2/*neu* molecules on the cell surface and trigger endocytosis (18). Reduced HER2/*neu* at the cell surface allows less HER2/*neu* heterodimer formation, potentially resulting in reduction in growth factor-induced signaling and proliferation. Based on the results depicted in Fig. 2, we reasoned that the antibodies contained in the sera of TUBO CRCL-immunized mice may bind to and dampen the expression of tumor antigens at the tumor cell surface. Flow cytometry analysis indicated a positive staining only in the cells incubated with anti-TUBO CRCL serum (Fig. 5A), where the average mean fluorescence intensity of experimental sera was 121.3 ± 21.9 when compared with 31.7 ± 5.2 of control sera. This indicates that the sera of TUBO CRCL-immunized mice contain antibodies (IgG) that can specifically bind to TUBO cells, some of which may presumably be anti-HER2/*neu* antibodies.

Next, we analyzed by confocal microscopy whether binding of these antibodies can induce the internalization

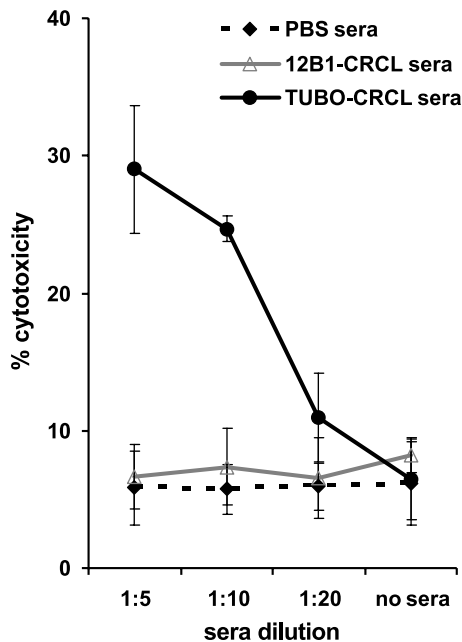


Figure 4. ADCC with sera from mice vaccinated with TUBO CRCL. TUBO cells were plated (1×10^4 per well) and allowed to attach overnight. Cultures were incubated for 2 h at 4°C with sera from the indicated group of mice at different dilutions. These sera were collected 1 wk after the last vaccine dose. After washing, BALB/c spleen cells from naive mice were added at 50:1 effector-to-target ratio and plates were incubated at 37°C overnight. Points, mean of triplicate sera sample from mice bled after six vaccinations with 12B1 or TUBO CRCL; bars, SE. Representative of three independent experiments.

of the IgG/anti-IgG-fluorochrome complexes from the tumor cell surface. TUBO cells were incubated with 1:10 dilution of immune sera for 3 h at 4°C and 37°C , respectively. Figure 5B shows that at 4°C sera from mice immunized with TUBO CRCL bound to the TUBO cell membrane as shown by the green fluorescence stain on the surface of the tumor cells. When the cells were exposed to 37°C , the bound complexes were then internalized and confined to the cytoplasm of the tumor cells as shown by the presence of large cytoplasmic dots. No such effect was observed with TUBO cells incubated with sera from PBS or 12B1 CRCL-vaccinated mice, showing that this response was TUBO CRCL specific. Although it is possible that anti-HER2/*neu* antibodies present in the sera may contribute significantly to this process, we cannot exclude that TUBO cells may also express antigens analogous to HER2/*neu* that are also susceptible to internalization by the binding of antibody to the surface of the cells.

CD4⁺ T Cells but Not CD8⁺ T Cells Are Required for the Generation of Antitumor Effects in Vaccinated Mice

We have shown previously in 12B1 BCR-ABL⁺ leukemia model that the protective effect of CRCL vaccination was completely abrogated in the absence of CD8⁺ and CD4⁺ T cells (12). Therefore, to assess the contribution of specific T cells in the TUBO model, we depleted either CD8⁺ or

CD4⁺ T cells before and after CRCL vaccine administration. CD8⁺ T did not play a role in antitumor effect, as both the TUBO CRCL-vaccinated groups (one with intact T cells and the other with depleted CD8⁺ T cells) revealed no significant difference in tumor growth delay (Fig. 6A). By day 49, the average tumor volumes of the undepleted group ($2,139 \pm 178 \text{ mm}^3$) and CD8-depleted group ($2,061 \pm 330 \text{ mm}^3$) were similar ($P > 0.05$). In contrast, protective immunity could not be elicited without CD4⁺ T cells as all mice in depleted group ($3,329 \pm 429 \text{ mm}^3$) grew tumors significantly faster ($P < 0.05$) than the immunized mice with intact CD4⁺ ($2,139 \pm 178 \text{ mm}^3$; Fig. 6B).

Discussion

Polyvalent autologous cancer vaccines circumvent issues associated with peptide-specific immunization by including a multitude of tumor-specific antigens in one vaccine preparation. Such vaccines may trigger the activation of effector polyclonal immune responses, which may overcome the emergence of escape tumor cell variants. We have developed one such vaccine (CRCL) and have now reported that, in addition to tumor-specific T-cell responses (3, 4, 16), CRCL can also provide protective immunity by the induction of humoral immunity. Several reports have indicated that single chaperone protein vaccines can induce antigen-specific antibodies (19, 26); however, it is not known whether humoral responses directly account for the antitumor effect.

Herein, we document that CRCL vaccine isolated from mammary carcinoma delays growth of preestablished tumors and that tumor-specific immunity involves antibody production, B cells, and CD4⁺ T lymphocytes. Additionally, transfer of serum to severe combined immunodeficient mice implied that protection against TUBO is mediated by humoral immunity. These findings are consistent with other reports highlighting the importance of B cells in tumor growth control following immunization with antibody-cytokine fusion proteins (13, 15) or DNA plasmids encoding both extracellular domain and intracellular domain of HER2/*neu* (10, 20).

In the TUBO model, the role of the humoral response seems to be particularly prominent because of the dual role of rat p185 *neu*, which is both a target tumor antigen and a membrane-exposed receptor regulating cell growth (21). The extracellular domain of HER2/*neu* contains several B-cell epitopes and it has been shown that active immunization with a combination of these epitopes is effective in preventing mammary tumors (22, 27). Because CRCL was isolated from TUBO cells (expressing rat p185 *neu*), we evaluated the presence of anti-HER2/*neu* antibodies in the serum of TUBO CRCL-immunized animals. Anti-HER2/*neu* IgG1, IgG2a, and IgG3 isotypes analyzed in tumor-bearing vaccinated mice increased 1 week after the first dose of vaccine and persisted for at least 3 weeks after the last dose, suggesting that a long-term immunity can be elicited. Subclass of the antibody response has distinct biological function and can affect antigen specificity, as

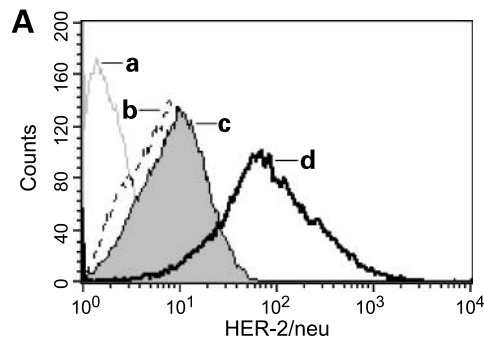
Fc γ R-mediated effector responses such as ADCC is primarily mediated by IgG1 and IgG3 isotypes (28). The latter have also been reported to be associated with T helper 1 immune response (29), whereas IgG1 production is usually linked to T helper 2 response (13). Thus, generation of wider isotype specificity in response to CRCL immunization suggests that polyclonal expansion of antigen-reactive B cells may be possible in this model.

Several B-cell epitopes reside in the extracellular domain of the HER2/*neu* antigen (27). Although further confirmation is required, HER2/*neu* antigen chaperoned within the CRCL preparation may deliver several of these immunogenic B-cell epitopes. Similar to peptide vaccines, endogenous and persisting antibody titers generated by CRCL would be more valuable compared with repeated administration of trastuzumab (30). Indeed, passive administration of antibodies may be cleared from the circulation, which may lessen their therapeutic potential (31). Further-

more, compared with vaccines that target single or limited antigens, antibodies generated by TUBO CRCL immunization may target other unidentified antigens that are naturally expressed *in vivo* on the surface of TUBO cells.

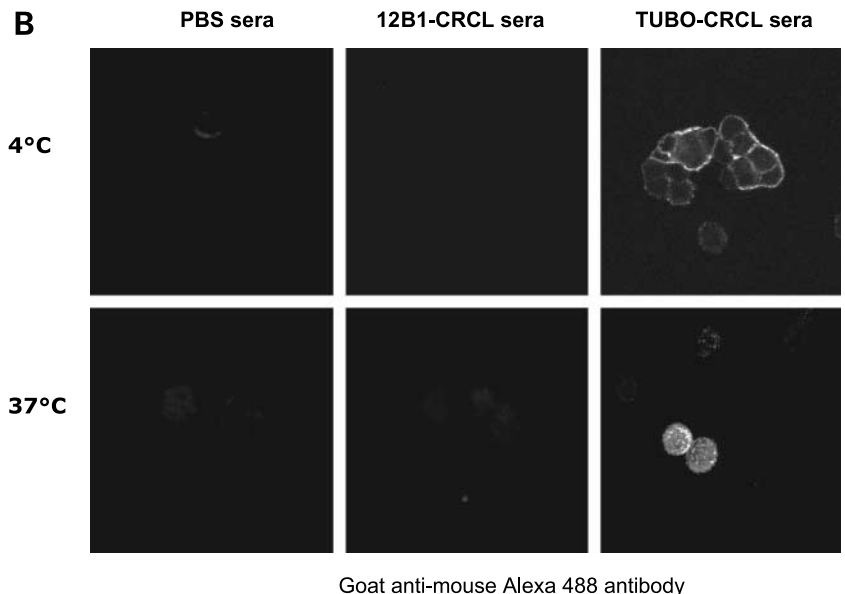
Depletion of natural killer cells before tumor implantation and during vaccine administration had no consequence in tumor delay (data not shown), suggesting that CRCL-induced antibodies may interact with Fc receptors on other effectors such as macrophages and neutrophils (32). A recent report highlights interaction of trastuzumab with specific Fc receptor subtypes and that Fc γ R is a dominant component of the activity of trastuzumab and similar monoclonal antibodies (25). Therefore, the role of these effectors in eliciting ADCC in the response to CRCL vaccination remains to be investigated.

CD4⁺ T cells are important in antitumor immunity as they can produce cytokines supporting an evolving B-cell immunity. In the TUBO model, tumor delay was achieved



- a. TUBO cells only
- b. Cells + Alexa Fluor 488
- c. Cells + Sera from control mice + Alexa Fluor 488
- d. Cells + Sera from TUBO-CRCL immunized mice + Alexa Fluor 488

Figure 5. Serum from TUBO CRCL-vaccinated mice contains IgG that can bind to TUBO cells and are subsequently internalized. **A**, flow cytometric analysis of the binding of IgG contained in the sera of TUBO CRCL-immunized mice. A total of 2×10^5 TUBO cells were stained in a standard indirect immunofluorescence procedure with 50 μ L of a 1:80 dilution in PBS-BSA of naive or immune sera followed by a secondary antibody, fluorescein-conjugated anti-mouse IgG (Alexa Fluor 488). *a*, TUBO cells only; *b*, cells stained with secondary antibody; *c*, cells stained with sera from control mice and secondary antibody; *d*, cells stained with sera from TUBO CRCL-vaccinated mice and secondary antibody. **B**, internalization of the IgG contained in the sera of TUBO CRCL-vaccinated mice at 37°C. A total of 2×10^5 TUBO cells were resuspended in medium and incubated with 50 μ L of a 1:10 dilution in PBS-BSA from PBS or 12B1 CRCL or TUBO CRCL-immunized mice for 3 h at 4°C or at 37°C. For detection of cytoplasmic tumor antigens, TUBO cells were incubated with 1 mL PBS-4% paraformaldehyde at 4°C for 20 min. The cells were washed twice with cold PBS-BSA and incubated with 1 mL PBS-0.3% Triton X-100 for 30 min at room temperature and washed again. Confocal microscopy was used to assess the expression of membrane-bound and cytoplasmic tumor antigens. Representative of two separate experiments.



Goat anti-mouse Alexa 488 antibody

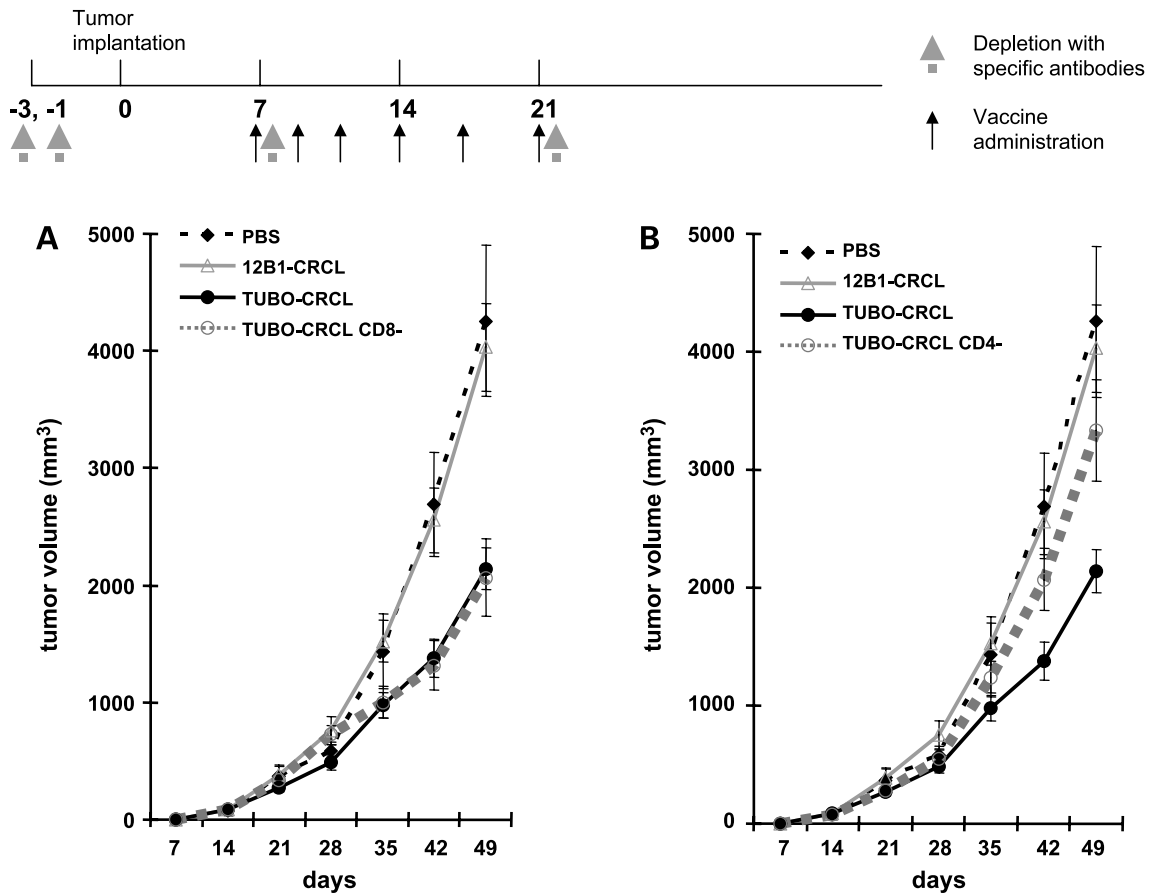


Figure 6. Effects of TUBO CRCL on tumor growth in CD8⁺ (A) or CD4⁺ (B) T-cell-depleted mice. BALB/c mice ($n = 8$) were depleted with anti-CD8⁺ (2.43) or CD4⁺ (GK1.5) antibodies as indicated. In brief, mice were depleted twice before tumor implantation (days -3 and -1) and were depleted twice during vaccine administration (days 7 and 21). On day 0, 1×10^5 TUBO cells were administered. After the tumor became palpable (day 7), animals were vaccinated six times as indicated and monitored for tumor growth every week. Representative of two independent experiments.

in the absence of CD8⁺ T cells, but CD4⁺ T cells were required. Other investigators have also established that CTLs are difficult to detect in this tumor model and that tumor-reactive CD4⁺ T lymphocytes are crucial for the induction of antitumor immunity (10, 33, 34).

Our data also indicate that B cells play a major role in CRCL-induced protection in mice. It is known that, in addition to the production of antibodies, B cells also execute multiple functions in the immune response (35, 36). These cells may indeed act as antigen-presenting cells and present antigens to CD4⁺ T cells and modulate their function by promoting up-regulation of costimulatory signals required for their expansion and differentiation (36).

One significant aspect of this work lies in the observation that tumor-derived CRCL delays tumor growth by the induction of a humoral immunity in an established tumor model. The work presented here is part of an ongoing effort to better understand the immune responses elicited by the tumor-derived multichaperone protein vaccine and its potential for further clinical application. The above data

also advance our knowledge on the utility of CRCL vaccine and provide a foundation that could lead to improved therapy for cancers responding to antibodies, such as HER2/*neu*-expressing tumors.

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