

Chaperone-rich cell lysate embedded with BCR-ABL peptide demonstrates enhanced anti-tumor activity against a murine BCR-ABL positive leukemia

Kerri L. Kislin,^{*,‡} Marilyn T. Marron,[‡] Gang Li,[‡] Michael W. Graner,^{†,1,2} and Emmanuel Katsanis^{‡,1}

^{*}Cancer Biology Interdisciplinary Program, University of Arizona, Tucson, Arizona, USA; [†]Duke University Medical Center, Durham, North Carolina, USA; and [‡]Steele Children's Research Center, University of Arizona, Tucson Arizona, USA

ABSTRACT Chaperone proteins are effective antitumor vaccines when purified from a tumor source, some of which are in clinical trials. Such vaccines culminate in tumor-specific T cell responses, implicating the role of adaptive immunity. We have developed a rapid and efficient procedure utilizing an isoelectric focusing technique to obtain vaccines from tumor or normal tissues called chaperone-rich cell lysate (CRCL). Tumor-associated peptides, the currency of T cell-mediated anticancer immunity, are believed to be purveyed by chaperone vaccines. Our purpose was to demonstrate our ability to manipulate the peptide antigen repertoire of CRCL vaccines as a novel anticancer strategy. Our methods allow us to prepare "designer" CRCL, utilizing the immunostimulation activity and the carrying capacity of CRCL to quantitatively acquire and deliver exogenous antigenic peptides (e.g., derived from the oncogenic BCR/ABL protein in chronic myelogenous leukemia). Using fluorescence-based and antigen-presentation assays, we determined that significant quantities of exogenously added peptide could accumulate in "designer" CRCL and could stimulate T cell activation. Further, we concluded that peptide-embedded CRCL, devoid of other antigens, could generate potent immunity against pre-established murine leukemia. Designer CRCL allows for the development of personalized vaccines against cancers expressing known antigens, by embedding antigens into CRCL derived from normal tissue.—Kislin, K. L., Marron, M. T., Li, G., Graner, M. W., Katsanis, E. Chaperone-rich cell lysate embedded with BCR-ABL peptide demonstrates enhanced anti-tumor activity against a murine BCR-ABL positive leukemia *FASEB J.* 21, 2173–2184 (2007)

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CHAPERONE PROTEINS ARE UBIQUITOUS in all living things, with both constitutively expressed and stress-inducible members. Molecular chaperones include the heat-shock protein (HSP) and glucose-regulated protein (GRP) families, as well as other members, and are

generally considered to belong to superfamilies based on their sequence homology and molecular weight, *i.e.*, HSP90, HSP70, *etc.* (1, 2). The chaperone roles of these proteins largely involve prevention of inappropriate protein-protein interactions (particularly during nascent polypeptide translation), and maintenance of thermodynamically stable structures and larger protein complexes. When cellular stress imparts non-native protein conformations, chaperone proteins will recognize, bind, and stabilize the potentially denaturing proteins (3), often in the form of chaperone/cochaperone/client complexes. Chaperones are also responsible for unfolding and refolding proteins during intraorganellar transport, and for labeling senescent proteins for degradation. A controversial topic in chaperone biology is whether chaperone proteins also have the ability to bind and shuttle peptides intracellularly (3–9).

From a cancer perspective, chaperone proteins purified from a tumor source and utilized in a vaccine setting have the ability to mediate specific antitumor immunity (10–13). Antigenic peptides associated with chaperones (5, 6, 14) appear to be responsible for the immunogenicity of the preparations rather than the chaperones themselves, although debate remains over the true nature of the peptide/chaperone complex (9, 15, 16). By removing bound peptides, the specific immunogenic capabilities of chaperone preparations are abrogated (6, 14, 17), while others have shown that the chaperones themselves possess potent innate immune stimulus (16, 18–20). Current data suggest that dendritic cells (DCs) take up chaperone-peptide complexes through specific receptors, such as CD91 (10–12), the scavenger receptors (21, 22), CD40 (23), LOX-1 (24), and the Toll-like receptors (25). DCs represent the peptides on MHC molecules, although the mechanisms are not fully defined (26). Thus,

¹ Co-senior authors.

² Correspondence: Duke University Medical Center, Pathology/Preston Robert Tisch Brain Tumor Center at Duke, Box 3156, Medical Sciences Research Bldg. 173A, Durham, NC 27710, USA. E-mail: michael.graner@duke.edu
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exogenous chaperones may be a means of directing peptides into DC antigen presentation pathways, while also providing activation or “danger” signals to the DCs. This culminates in the activation and stimulation of antigen-specific T-cells.

Through the use of free solution-isoelectric focusing (FS-IEF), we can enrich multiple immunogenic chaperone protein complexes from tumor- or normal tissue-derived lysates (27). We refer to these chaperone-rich cell lysates as “CRCL”. Given the capabilities of chaperones to effectively interact with exogenous free peptide (9, 15), CRCL may present an effective and novel method of transport for exogenous tumor-associated or tumor-specific peptides. This would allow for the generation of “designer” vaccines that may have off-the-shelf potential for cancer immunotherapy.

We have previously demonstrated that CRCL preparations from normal tissue have potent adjuvant effects when combined with apoptotic tumor cell material as an antileukemia vaccine (28). We have also shown that murine leukemia CRCL contains known peptide antigens such as the BCR/ABL fusion peptide (26). With emphasis on identifiable specificity in immune monitoring (29, 30), as well as an increasing database of known and putative tumor peptide antigens, we hypothesized that we could add tumor peptides exogenously to CRCL in a way that could enhance the antitumor immunity in a T cell-specific fashion. In this report, we show that an exogenous BCR/ABL peptide derived from the chronic myelogenous leukemia p210 fusion oncoprotein (GFKQSSKAL) and ovalbumin OVA_{257–264} peptide (SIINFEKL) can indeed incorporate into leukemia-derived and liver-derived CRCL. This is a relatively high-yield process in terms of amount of peptide taken up by CRCL. On pulsing the peptide-laden CRCL onto DCs, these peptides are then taken up and presented by DCs with high efficiency to stimulate T-cells in *in vitro* assays. Biochemical assays indicate that HSC70 is a major peptide binding protein in liver CRCL vaccines. Our findings also show that peptide-embedded CRCL administered *in vivo* has potent antitumor effects. Thus, these results indicate that CRCL could be the carrier of choice for exogenous tumor-related antigenic peptides. The novel concept of creating a peptide-“designer” CRCL will offer the ability to personalize enhanced vaccines for those afflicted with cancers containing known antigens, such as the BCR/ABL fusion peptide, or to generate vaccine with known antigens where autologous tumor is not available. The use of a chaperone matrix (CRCL) derived from nontumor tissue that can have antigens of choice functionally embedded in it may be a means of producing high-specificity “designer” vaccines as an off-the-shelf product.

MATERIALS AND METHODS

12B1 BCR/ABL leukemia cell line

12B1 is an aggressive murine leukemia line (BALB/c strain) expressing human BCR/ABL protein and has been described

previously (26, 28, 31, 32). This is a very aggressive, nonimmunogenic leukemia resembling blast phase of chronic myelogenous leukemia, despite the presence of the xenogenic p210 BCR/ABL protein. Inoculation with as few as 100 cells intravenously or 1000 cells subcutaneously results in uniform lethality within 25 d due to disseminated disease.

Cell culture/tumor generation/murine bone marrow-derived dendritic cell generation

All tissue/cell culture reagents were purchased from Invitrogen (Gaithersburg, MD, USA). 12B1 cells were cultured as described previously, and tumor generation was also as described (26, 31). Dendritic cells (DC) were harvested and cultured from syngeneic mouse bone marrow as described previously in detail (13). Cells were cultured at 37°C (6% CO₂) in RPMI supplemented with 10% heat-inactivated fetal calf serum and supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin sulfate, 0.05 mM MEM nonessential amino acids, and 1 mM sodium pyruvate (RPMI Complete media). All tissue/cell culture reagents were purchased from Invitrogen. 12B1 cells were cultured as described previously, and tumor generation was also as described (26, 31). Dendritic cells (DC) were harvested and cultured from syngeneic mouse bone marrow as described previously in detail (13). Cells were cultured at 37°C (6% CO₂) in RPMI supplemented with 10% heat-inactivated fetal calf serum and supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin sulfate, and 0.05 mM MEM nonessential amino acids, and 1 mM sodium pyruvate.

The B3Z cell line is a murine (H2K^b) T cell hybridoma that has specific reactivity against the ovalbumin peptide OVA_{257–264} in the context of MHC I by the T cell receptor (TCR). It has been engineered to express beta-galactosidase on triggering of the TCR with peptide ligand as a surrogate for interleukin-2 (IL-2) expression (33). It was cultured at 37°C (10% CO₂) in DMEM supplemented with 10% heat-inactivated fetal calf serum and supplemented with 2mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin sulfate, 0.05 mM MEM nonessential amino acids, and 1 mM sodium pyruvate.

Peptides

Ovalbumin (OVA_{257–264}) peptide–SIINFEKL (single letter amino acid designation) and BCR/ABL fusion junction peptide–GFKQSSKAL (GFK), both from Anaspec, Inc. (San Jose, CA, USA), were reconstituted in water. The latter was also synthesized as a fluorescein isothiocyanate (FITC) conjugate at the N terminus by standard Fmoc chemistry.

Chaperone protein enrichment and purification

12B1 tumors were used in the making of tumor-derived chaperone-rich cell lysate (CRCL). Normal livers were harvested from BALB/c and C57BL/6 mice for the preparation of (normal tissue-derived) liver CRCL. Free solution-isoelectric focusing (FS-IEF) enrichment of tumor and normal tissue-derived CRCL was performed as described previously (32). Briefly, tumor or liver tissues were homogenized in detergent-containing buffers, and high-speed supernatants were obtained. The dialyzed supernatants (which were also referred to as “cell lysates” for experiments listed below) were mixed with detergents and conjugate acid-base pairs (for pH gradient establishment), and the solution was made to 6 M urea. The mixture was subjected to FS-IEF in a Rotofor device (Bio-Rad Laboratories, Hercules, CA, USA) at 15W constant

power, and fractions were harvested and analyzed for chaperone content. Fractions of interest were pooled and prepared as vaccines by dialysis, detergent removal, and centrifugal concentration. Vaccines were tested for endotoxin with the Limulus Amebocyte Lysate assay (BioWhittaker, Walkersville, MD, USA) and were found to contain less than 0.01 EU/ μg protein (13).

For FS-IEF peptide incorporation studies, 5 mg SIINFEKL peptide was added to the liver lysate/high-speed supernatant isofocusing mixture prior to the loading of the Rotofor device and application of power. Following isofocusing, each of the 20 fractions was handled individually for dialysis, chaperone protein characterization, vaccine preparation, and immunological analysis for peptide content (see below).

Preparation of vaccines

Following the dialysis steps described above and previously (32), proteins were prepared for use as vaccines by concentration in Centricon-10 devices (Millipore, Bedford, MA, USA) and passage over Extracti-gel D columns (Pierce Endogen, Rockford, IL, USA) to remove residual detergents. Protein concentrations were determined using BCA assays with bovine serum albumin as standard, and proteins were diluted to 50 $\mu\text{g}/\text{ml}$ in PBS for pulsing onto dendritic cells.

Peptide incorporation into chaperone-rich cell lysate by simple mixing

Peptides were added to CRCL vaccine at a 1:1 microgram ratio and incubated at room temperature for 40 min. The peptide/CRCL solution was centrifuged at 9.5×10^3 rpm via a 10 kDa cut-off membrane filter (Vivaspin 500, ISCBioexpress, Kaysville, UT, USA). The retentate was then washed three times with PBS by centrifugation through the filter and then collected for use.

Native agarose gel electrophoresis and sample extractions

CRCL (60 μg) containing FITC-peptide was loaded onto a 1% agarose gel (Amresco, Solon, OH, USA) run under native conditions at 100V for 4 h at 4°C. The high-molecular-weight region of the gel where the FITC label migrated was excised. To remove the protein sample from the agarose, a 1.7 ml tube with a small hole punctured in the bottom was packed with glass wool and the agarose band was set atop the glass wool. The 1.7 ml tube was placed inside a larger tube to collect the liquid sample following centrifugation. The sample was now concentrated using a 10 kDa cut-off membrane filter (Vivaspin 500, ISCBioexpress). The sample was then separated by SDS-PAGE under reducing conditions and further stained with Sypro Ruby (Molecular Probes, Eugene, OR, USA) for band detection. The bands detected were excised and submitted to the Arizona Proteomics Consortium Core Facility at the University of Arizona for mass spectrometry analysis.

Liquid chromatography-mass spectrometry (LC-MS/MS) analysis

Analysis of excised, in-gel digested bands was carried out using a quadrupole ion trap LCQ Classic mass spectrometer from ThermoFinnigan (34). The LCQ Classic was equipped with a Michrom MAGIC 2002 HPLC and a nanoelectrospray ionization source (University of Washington, Seattle, WA, USA). Peptides were eluted from a 15 cm pulled-tip capillary

column (100 μm I.D. \times 360 μm O.D.; 3–5 μm tip opening) packed with 8–9 cm Vydac C18 material (5 μm , 300 Å pore size), using a gradient of 0–65% solvent B (98% methanol/2% water/0.5% formic acid/0.01% trifluoroacetic acid) over a 60 min period at a flow rate of 200–300 nl min^{-1} . The sequences of individual peptides were identified using the Turbo SEQUEST algorithm to search and correlate the MS/MS spectra with amino acid sequences in the nonredundant protein database (35).

Immunoblotting for HSC70

The protein identified by mass spectrometry as a peptide binding target was confirmed by Western blotting. CRCL derived from liver was used as a positive control using a standard protocol (32). The protein of interest was identified using a rat monoclonal antibody to HSC70 (Stressgen, Vancouver, British Columbia).

Fluorometric measurement of FITC-peptide incorporation

FITC-labeled peptide was incorporated into CRCL as described above, and peptide retention in CRCL was measured using a Fluoroskan Ascent Microplate Fluorometer (Thermo Electron Corporation) with an excitation at 488 nm and an emission at 527 nm. Quantitation of peptide incorporation was done by generating a fluorescence standard curve with known amounts of FITC-peptide.

Flow cytometry

Dendritic cells were experimentally treated with CRCL, FITC-peptide, or FITC-peptide-embedded CRCL. The cells were washed in PBS, fixed, and analyzed using a FACScan (Becton Dickinson Immunocytometry, San Jose, CA, USA) with gating on CD11c⁺ cells as a marker for DCs (CD11c-PE, Pharmingen, San Jose, CA, USA).

Confocal microscopy

Dendritic cells were experimentally treated with either Texas Red-labeled (Molecular Probes) CRCL (following the manufacturer's protocol) or FITC-peptide, or with (labeled) peptide-embedded CRCL. The cells were washed in PBS and then fixed with PBS containing 4% paraformaldehyde (Polysciences, Warrington, PA, USA). Cells were then washed and transferred by Cytospin (Thermo Shandon, Pittsburgh, PA, USA) onto microscope slides, followed by examination at 100 \times magnification using a Nikon TE300 microscope (Tokyo, Japan) and Bio-Rad 1024 MRC Confocal Imaging System (Bio-Rad). Images depicted are single confocal sections scanned and obtained with appropriate filters for the particular fluorochrome. The images were digitally overlaid to provide coincident display of the two colors separately or in a merged image where colocalized green and red fluorochromes are seen as yellow.

IL-2 reporter bioassay

IL-2 production by antigen-stimulated B3Z T cell hybridomas was measured in surrogate via a Beta-Galactosidase Production Assay using kit from Novagen (Madison, WI, USA).

ELISPOT assay

Enzyme-linked immunospot (ELISPOT) assays were performed to assess IFN- γ production of splenocytes from vaccinated mice following *in vitro* stimulation with CRCL or peptides. Splenocytes (10^6) were cultured with 50 $\mu\text{g}/\text{mL}$ CRCL, 50 $\mu\text{g}/\text{mL}$ peptide-embedded CRCL, or 5 $\mu\text{g}/\text{mL}$ CRCL peptides for 48 h on Millipore MultiScreen-HA 96-well plates (MAHA S45; Millipore), and ELISPOT plates probed and developed as described previously (32). Wells of interest were photographed with a microscope-mounted Leica DFC480, and images captured with Leica Fire Cam DFC Twain software (Leica Microsystems, Bannockburn, IL, USA). The image of each well was electronically optimized to visualize the maximum number of spots.

Animal studies

Female BALB/c (H-2^d) and C57BL/6 (H-2^b) mice (National Cancer Institute, Frederick, MD, USA) 6- to 10-weeks old were used for the experiments. The animals were housed in a dedicated pathogen-free facility and cared for according to the University of Arizona Institutional Animal Care and Use Committee guidelines. Following tumor injection on Day 0, the mice were subjected to different treatments regimens on days 1 and 3. Mice were given subcutaneous injections (groin opposite the tumor site) with BCR/ABL peptide-embedded liver CRCL (20 $\mu\text{g}/\text{injection}$ in 100 μl of PBS). Other groups consisted of mice injected with PBS, BCR/ABL peptide alone (without adjuvant), or liver CRCL alone. Tumor growth was monitored as described (32).

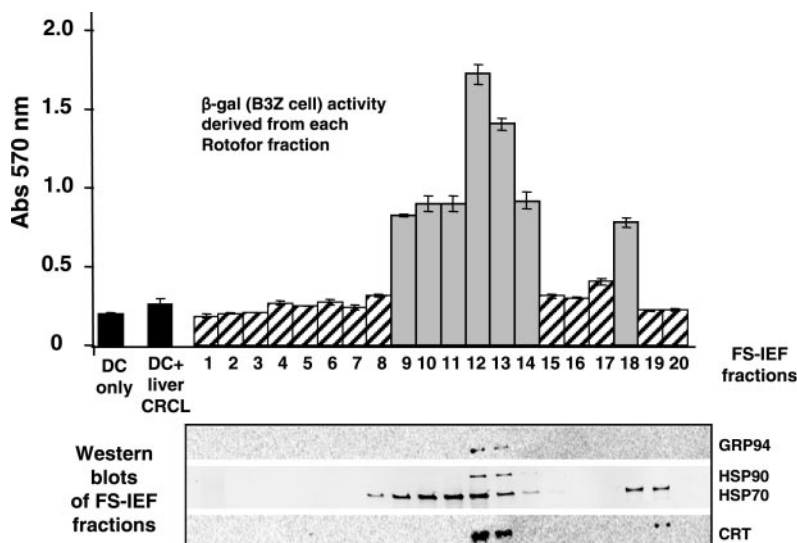
RESULTS

Exogenous peptide may be efficaciously incorporated into CRCL vaccine during free solution-isoelectric focusing (FS-IEF)

We had previously reported that leukemia-derived CRCL has antigenic peptides (*e.g.*, BCR/ABL fusion peptide) associated with it (26), although the mechanism for this association is unclear. We speculated that

we could exploit CRCL's peptide-binding capacity by adding exogenous peptide to the unrefined lysate preparation (*i.e.*, prior to the isofocusing procedure). We would then have to determine whether the specific peptide coseparated with the chaperone proteins in the fractions that would be pooled to make the CRCL vaccine. To that end we chose the 9-mer ovalbumin peptide, SIINFELK (OVA₂₅₇₋₂₆₄), which was added to C57BL/6 mouse liver homogenate before subjecting the mixture to FS-IEF. We chose SIINFELK as a model antigen in this setting because we could readily assay for its presence upon DC presentation using specific T cell clones (B3Z cells, see below). Following FS-IEF, protein samples from the 20 harvested fractions were probed for the four main chaperone proteins of interest in generating CRCL vaccine: HSP/HSC70, HSP90, GRP94, and calreticulin. One or more of those four chaperone proteins was present in fractions 9–14 and 18. The 20 fractions were separately prepared as vaccines, and each of the 20 fraction “vaccines” was individually pulsed onto 20 different groups of C57BL/6 mouse DCs overnight. The pulsed DCs were incubated with B3Z T cell hybridomas, which produce beta-galactosidase following T cell receptor stimulation with the H2K^b-presented SIINFELK peptide. Thus, IL-2 secretion was measured in surrogate by beta-galactosidase output as a readout for the fractionation of the SIINFELK peptide. This output was found to be significantly increased in B3Z cells that were incubated with DCs that had been pulsed with proteins from fractions 9–14 and 18 (Fig. 1), which coincided with the fractions containing the four aforementioned chaperones. These results indicated that SIINFELK peptide localized to chaperone-containing fractions corresponding to the fractions ordinarily chosen to generate the CRCL vaccine. Furthermore, these fractions had the ability to donate the SIINFELK peptide to DCs, resulting in the MHC presentation of the peptide by DCs that lead to specific T cell stimulation. SIINFELK peptide alone

Figure 1. Peptide loading of homogenate via FS-IEF elicits T cell activation in fractions containing chaperone proteins of interest. SIINFELK peptide loaded into C57BL/6 liver lysate was subjected to FS-IEF; the subsequent 20 fractions were harvested individually and each produced into vaccines. The 20 fractions were pulsed onto C57BL/6 mouse DCs and further incubated with B3Z T-cells to determine T cell activation through β -galactosidase output. After processing all 20 FS-IEF fractions separately, it was determined that fractions 9–14 and 18 supplied SIINFELK peptide to be presented by APCs to B3Z cells, which lead to IL-2 secretion/ β -galactosidase activity. These fractions also corresponded to the chaperones of interest (containing HSP70, HSP90, GRP94, and calreticulin, by Western blot), are typically harvested to produce the CRCL vaccine. Data are representative of three independent experiments.



subjected to FS-IEF and vaccine preparation did not elicit B3Z activity from any of the 20 fractions (data not shown), nor did liver CRCL prepared without addition of exogenous SIINFEKL peptide (Fig. 1).

High levels of exogenous peptide may be incorporated into, and functionally relayed to dendritic cells by CRCL—a role for HSC70?

As a model, SIINFEKL peptide demonstrated colocalization into the chaperone-containing fractions selected to produce CRCL vaccine. It was further determined that peptide incorporation could be accomplished by simply combining CRCL as an end product with a peptide of interest and allowing it to incubate at room temperature (refer to Materials and Methods). However, it was critical to know how much peptide, postincorporation, remained bound to CRCL (or cell lysate as a control). We used a fluorescein-labeled BCR/ABL (FITC-GFK) peptide to measure the amount of peptide bound postincubation to 50 μ g 12B1 leukemia-derived CRCL compared with 12B1 lysate, mouse IgG, or peptide alone. This peptide was chosen for its relevance as an antigen against chronic myelogenous leukemia (CML), and 12B1 CRCL vaccine has GFK peptide (or precursor) as a component (26). Thus, we could make some assessment of additional peptide antigen capacity of the vaccine that we presume already has GFK peptide in it. We also measured peptide incorporation in 50 μ g liver-derived CRCL *vs.* liver lysate, mouse IgG, or peptide alone. The addition of peptide into an “antigen-free” carrier would essentially produce a “designer” vaccine. Protein/peptide complexes were formed and handled as described in Materials and Methods. We determined that a readily detectable 1–2 μ g of peptide bound to both 12B1 CRCL (Fig. 2A) and liver-derived CRCL (Fig. 2B) vaccines, while only 0.2–0.3 μ g of peptide bound to either lysate. Since the amount of CRCL vaccine used was 50 μ g total, this suggests that between 1–2 μ g of peptide, or ~3% of total polypeptide content, may be incorporated into every 50 μ g of CRCL vaccine, indicating a 5–10 fold increased peptide carrying capacity when compared to standard lysate. Also, despite the inherent presence of GFK peptide in 12B1 CRCL, additional FITC-GFK peptide was readily incorporated into tumor-derived CRCL.

To address the issue of antigen delivery from peptide-embedded CRCL to DCs leading to T cell stimulation, we recapitulated the DC pulsing experiments from Fig. 1, this time using the facile mixing of liver CRCL and SIINFEKL peptide (with controls peptide-embedded liver lysate, peptide-IgG, or peptide alone). The readout was β -galactosidase output from B3Z cells, chosen again for assay simplicity (Fig. 2C). SIINFEKL-embedded liver CRCL was more effective than any of the other peptide delivery methods at engendering a T cell response, thus proving that simple mixing of peptide and CRCL yielded a functional vaccine in terms of DC/T cell interactions.

The tight binding of peptide within CRCL following mixing begs the question of what protein or proteins are responsible. After incorporation of FITC-GFK into CRCL, we presumed we could track the peptide if it found a binding partner by tracking the fluorescence. Following mixing of FITC-GFK peptide with liver CRCL, chromatographic methods of protein isolation proved difficult and ineffective. We thus chose to separate the FITC-GFK embedded liver CRCL on an agarose gel system run under native conditions. The fluorescence was detectable in a high-molecular-weight region of the agarose gel. Following the excision of a band (Fig. 2D, top panel), the protein(s) associated with the fluorescence was/were separated from the agarose gel piece by SDS-PAGE and internal peptide sequences were determined by proteomic techniques. The database search identified two peptide sequences that matched murine heat-shock cognate 70, a main component in the CRCL vaccine (Fig. 2D, middle panel). To confirm that the 70 kDa protein was HSC70, we performed Western blot analysis using a specific rat monoclonal anti-HSC70 antibody (Fig. 2D, bottom panel). These results strongly imply that HSC70 is one of the major peptide binding proteins in liver CRCL (or at least is one of the most abundant), and this fits well with the colocalization data for the SIINFEKL peptide into fractions containing HSP/HSC70 (Fig. 1).

Peptides incorporated into CRCL can effectively transfer to dendritic cell surfaces

The data above indicated that peptides localizing into chaperone-containing fractions during FS-IEF, and mixed after FS-IEF, could effectively be presented by DCs to T-cells. To verify the transfer and DC surface presentation of the exogenously added peptides incorporated into CRCL, we used unlabeled 12B1 CRCL and unlabeled liver-derived CRCL embedded with FITC-GFK peptide. Peptide-embedded 12B1 (or liver) CRCL, peptide-embedded 12B1 (or liver) lysate, or 1 μ g of peptide alone (the approximate amount of peptide retained in 50 μ g of CRCL), were pulsed onto BALB/c mouse dendritic cells overnight. The cells were washed, fixed, and analyzed by flow cytometry. The dendritic cell population (gated for CD11c⁺) pulsed with FITC-GFK peptide-embedded CRCL (whether tumor-derived or liver-derived CRCL) showed significantly higher mean fluorescence intensity values than did DCs pulsed with peptide embedded into lysate or FITC-peptide alone (Fig. 3).

CRCL enhances peptide uptake into dendritic cells

From the flow cytometry studies (above) it was unclear if peptide that had been incorporated into CRCL was dissociated from CRCL and onto DC surfaces, or if there was still colocalization of CRCL and the peptide. To assess this, liver-derived CRCL and its corresponding lysate were labeled with Texas Red. This labeled CRCL was embedded with FITC-labeled GFK peptide

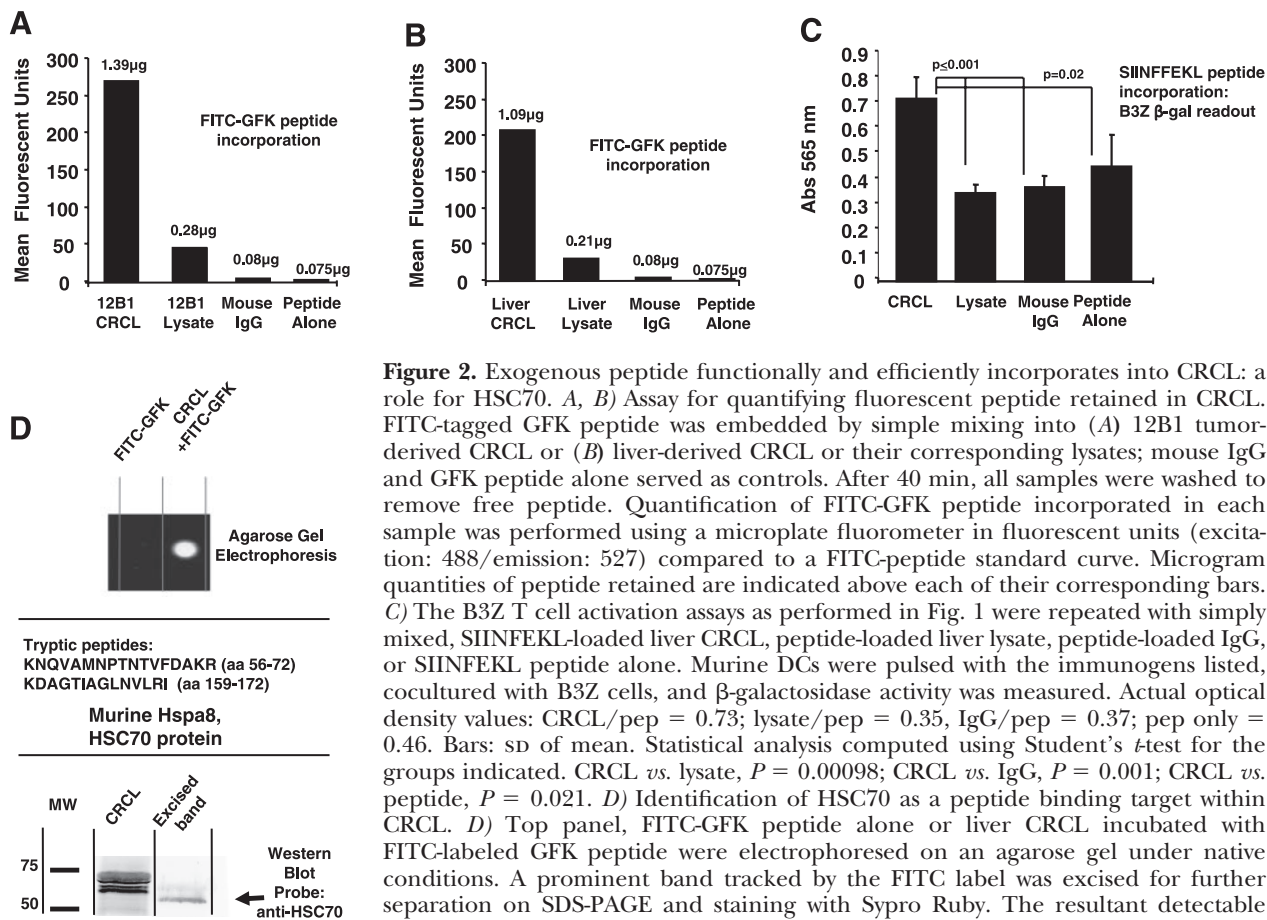


Figure 2. Exogenous peptide functionally and efficiently incorporates into CRCL: a role for HSC70. *A, B*) Assay for quantifying fluorescent peptide retained in CRCL. FITC-tagged GFK peptide was embedded by simple mixing into *(A)* 12B1 tumor-derived CRCL or *(B)* liver-derived CRCL or their corresponding lysates; mouse IgG and GFK peptide alone served as controls. After 40 min, all samples were washed to remove free peptide. Quantification of FITC-GFK peptide incorporated in each sample was performed using a microplate fluorometer in fluorescent units (excitation: 488/emission: 527) compared to a FITC-peptide standard curve. Microgram quantities of peptide retained are indicated above each of their corresponding bars. *C*) The B3Z T cell activation assays as performed in Fig. 1 were repeated with simply mixed, SIINFEKL-loaded liver CRCL, peptide-loaded liver lysate, peptide-loaded IgG, or SIINFEKL peptide alone. Murine DCs were pulsed with the immunogens listed, cocultured with B3Z cells, and β -galactosidase activity was measured. Actual optical density values: CRCL/pep = 0.73; lysate/pep = 0.35; IgG/pep = 0.37; pep only = 0.46. Bars: SD of mean. Statistical analysis computed using Student's *t*-test for the groups indicated. CRCL vs. lysate, $P = 0.00098$; CRCL vs. IgG, $P = 0.001$; CRCL vs. peptide, $P = 0.021$. *D*) Identification of HSC70 as a peptide binding target within CRCL. *D*) Top panel, FITC-GFK peptide alone or liver CRCL incubated with FITC-labeled GFK peptide were electrophoresed on an agarose gel under native conditions. A prominent band tracked by the FITC label was excised for further separation on SDS-PAGE and staining with Sypro Ruby. The resultant detectable band was analyzed by trypsin-digest peptide mass fingerprinting by LC-MS/MS. Database searching identified the peptides as from murine heat shock cognate 70 kDa protein (HSC70) (*D*, middle panel). This was verified by Western blotting with a monoclonal antibody specific for HSC70 (*D*, bottom panel). Liver CRCL is run next to the excised band as a positive control. Data are representative of three independent experiments.

and pulsed onto dendritic cells overnight. The cells were then washed, fixed, and imaged by confocal microscopy to determine peptide delivery and uptake by dendritic cells with respect to CRCL proteins. As shown in **Fig. 4**, CRCL proteins and labeled peptide show high degrees of internalization into the pulsed DCs, along with colocalization of protein and peptide signals. Labeled lysate, while bound and internalized by DCs, did not enhance exogenous peptide uptake to any detectable level in this assay, perhaps because lysate retains little exogenously added peptide (**Fig. 2**).

Immunization of mice with peptide-embedded CRCL induces antigen-specific IFN- γ secretion

The *in vitro* assays suggested that peptide-embedded CRCL could stimulate T cell activation from a T cell hybridoma via DC presentation of peptide. To determine whether specific peptide antigenicity is maintained *in vivo*, BCR/ABL peptide (GFK) was embedded into liver CRCL, and BALB/c mice were immunized with GFK-embedded liver CRCL, liver CRCL alone, or PBS as a control, on days 0 and 2. Liver CRCL was chosen as the "matrix" as it is devoid of tumor antigens. On day 7, splenocytes were harvested and restimulated

in vitro with peptide-embedded liver CRCL, liver CRCL, GFK peptide, an irrelevant peptide (HYLSTQSALS), or media alone as background, and ELISPOT assays were performed. As expected, mice immunized with liver CRCL or PBS showed insignificant IFN- γ secretion with any restimulation because no GFK peptide was present in the vaccine (**Fig. 5**). Splenocytes from mice primed with GFK peptide-embedded liver CRCL produced significant amounts of IFN- γ when restimulated with the GFK peptide, and an even higher level of secretion when restimulated with GFK peptide-embedded liver CRCL. These findings indicated that exogenous GFK peptide, which is not inherently found in liver CRCL alone, when embedded in the vaccine, is a major antigenic component of this "designer" CRCL. No primed splenocytes secreted IFN- γ when restimulated with an irrelevant peptide or with liver CRCL alone. We should point out that in numerous previous experiments we have never been able to measure any immune output (ELISPOT, ELISA, or tumor growth inhibition) from mice that received only GFK peptide (without adjuvant) as a vaccine (data not shown; see also **Fig. 6**). These findings confirm that *in vivo* GFKQSSKAL retains potent immunogenicity in GFK peptide-embedded liver CRCL.

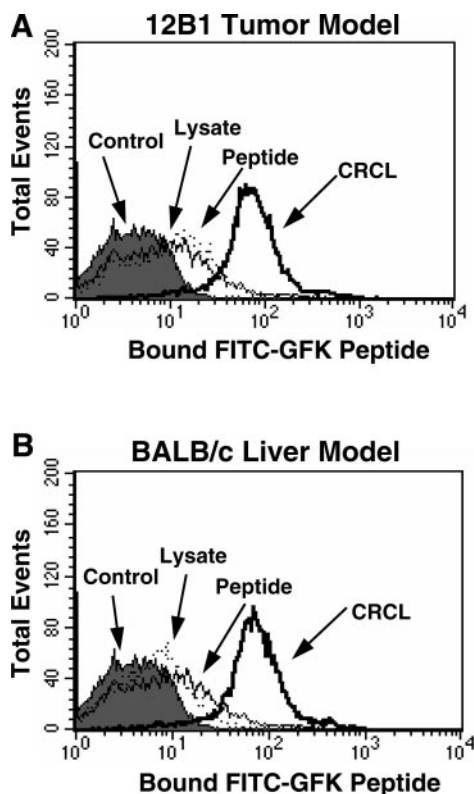


Figure 3. Peptides embedded into CRCL effectively transfer to dendritic cell surfaces. *A, B*) BALB/c mouse dendritic cells (DCs) were pulsed with FITC-GFK-embedded, or with FITC-GFK peptide embedded CRCL or lysates. Following overnight pulsing, DCs were fixed and stained with anti-CD11c-PE for gating, and flow cytometry analyses were performed. Mean fluorescence intensities were measured for (A), control (cells only), 5.99; 12B1 lysate+FITC-GFK, 14.7; FITC-GFK alone, 13.0; 12B1 CRCL+FITC-GFK, 86.0. MFI for (B); control, 5.99; liver lysate+FITC-GFK, 8.02; FITC-GFK alone, 13.0; liver CRCL+FITC-GFK, 83.1. Data are representative of three independent experiments.

CRCL vaccine embedded with exogenous BCR/ABL peptide has enhanced antitumor activity

In an earlier study (32), we showed that 12B1 leukemia-derived CRCL, especially when combined with DCs, could delay tumor growth when compared with individual heat-shock proteins as vaccines in treating pre-existing 12B1 tumors. Given the *in vitro* and *in vivo* data indicating that peptide-embedded CRCL could generate specific T cell responses, we asked whether animals vaccinated with CRCL embedded with GFK could reject 12B1 tumors. Thus, we treated mice with liver CRCL embedded with GFK peptide on days 1 and 3 following subcutaneous injection of 12B1 tumor cells. To avoid any complicating issues surrounding the use of DCs, we chose to immunize animals directly with the peptide-embedded CRCL vaccines. The addition of GFK peptide to liver-derived CRCL vaccine profoundly delayed tumor growth compared with groups of mice immunized with liver CRCL alone, GFK peptide alone, or saline, with at least half of the GFK/liver CRCL-treated mice rejecting their visible tumors (Fig. 6A). Since

12B1 is a highly disseminating and metastatic tumor, and mice may die with little evidence of subcutaneous tumor growth, we also followed survival of treated mice. The GFK/liver CRCL vaccine also significantly prolonged survival in immunized animals, curing up to 50% of the leukemia-bearing mice (Fig. 6B). Tumor-free animals remained disease-free (>90 d post-tumor inoculation).

DISCUSSION

As “danger signals”, certain chaperone proteins are capable of potent innate immune modulation (19, 28, 36, 37), and those that have been purified from a tumor source have been reported as effective vaccines, culminating in tumor-specific cytotoxic T-lymphocyte responses, and antitumor immunity in several *in vivo* models (13, 32, 38, 39). Yet, a frequent disadvantage to single chaperone vaccines is the lack of total purified protein obtained from tumor sources to generate the vaccine, as well as the labor necessary for vaccine production (40). We found that CRCL vaccine has a more pronounced immunological effect per unit material of protein than any of the individual chaperone proteins used as a vaccine alone (13, 27, 32). Moreover, we have demonstrated that the antigenicity and antitumor efficacy of CRCL can be augmented by loading it onto dendritic cells. CRCL has been shown to mature and activate DCs (13), thus enhancing antigen presentation and T cell stimulation.

The basis for antigen-specific active immunotherapy is provided by the identification of tumor-specific and tumor associated antigens expressed by different human tumors (41, 42, 43). The BCR/ABL protein is an excellent model to study tumor-specific antigens as it is found in the tumor cells of chronic myelogenous leukemia but not normal tissue. This fusion oncoprotein contains several specific peptides, including the fusion peptide itself, GFKQSSKAL, which has been previously shown to bind to MHC class I and II molecules in mice and humans (44, 45, 46). It is clearly relevant as a therapeutic target from both chemo- and immunotherapy standpoints (47).

Due to the fact that chaperone proteins purified from tumor or other pathological sources associate with antigenic peptides (36) and that CRCL vaccine is enriched for multiple chaperones (27), we sought to determine if CRCL has the ability to bind exogenously added specific peptides of choice, which might enhance the overall antigenic effect of the vaccine; in essence, we wanted to create a “designer” CRCL. Such approaches have been difficult and sometimes inefficient when using purified chaperones in *in vitro* systems designed to bind antigens (peptides or proteins) (12, 48–50). As proof of principle, SIINFEKL peptide was loaded into clarified liver lysate to determine if and where peptide binding would occur during the FS-IEF process for the making of CRCL. Liver lysate was utilized since it is devoid of ovalbumin, there was a

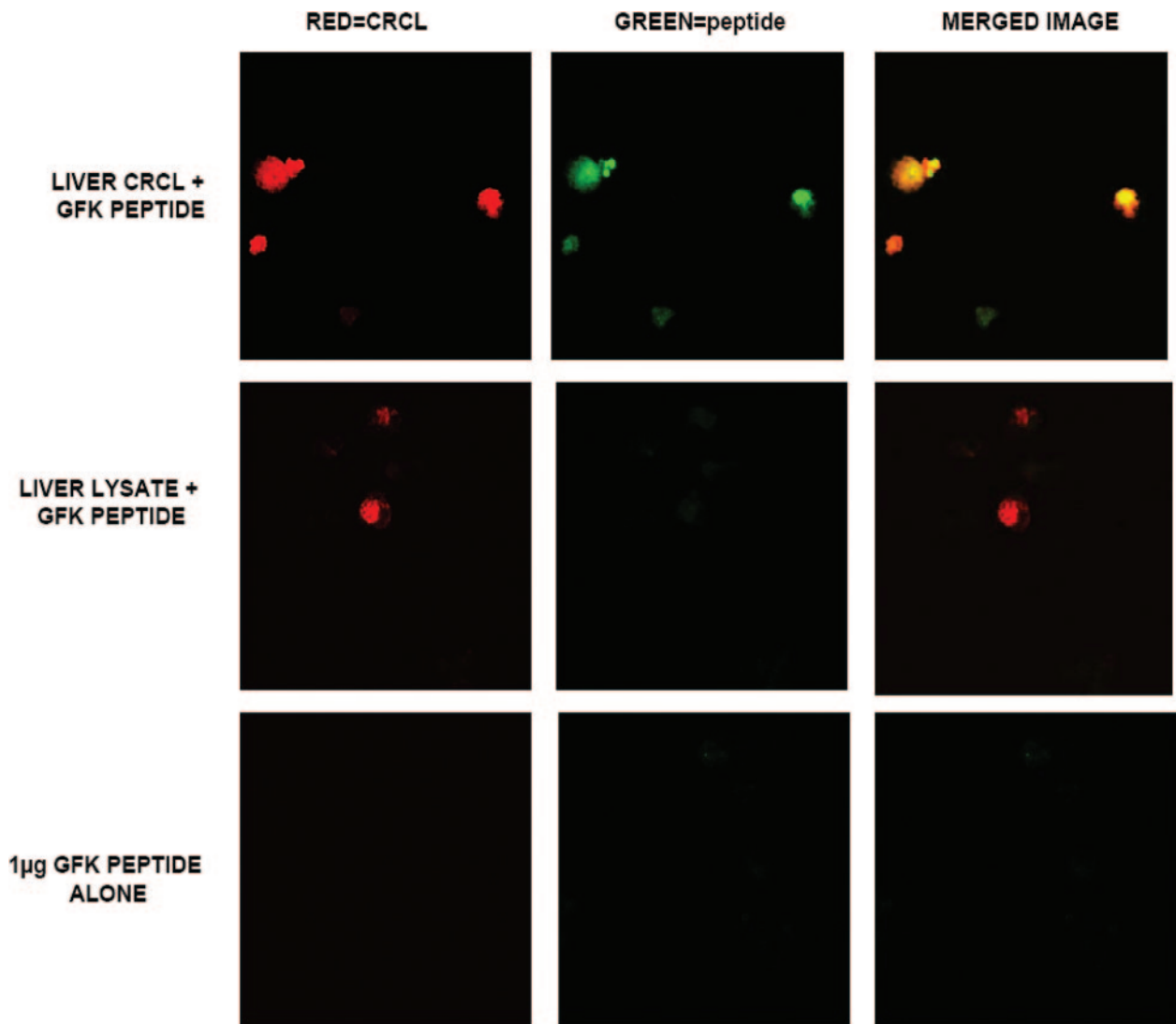


Figure 4. Effective peptide/CRCL uptake by dendritic cells. DCs were experimentally treated with FITC-GFK peptide-embedded liver CRCL, FITC-GFK embedded liver lysate, or FITC-GFK peptide alone overnight. CRCL and lysate were labeled with Texas Red before peptide incorporation for dual-label imaging by confocal microscopy. All images were acquired at the same 100 \times magnification using the oil immersion objective. Images were collected from the same (confocal) plane and were digitally overlaid to reveal red, green, or yellow (colocalized) fluorescence. Data are representative of three independent experiments.

straightforward readout for the fractionation/localization of SIINFEKL peptide, and liver CRCL served to demonstrate that nontumor sources could be useful as chaperone-based methods of antigen delivery. Following separation and harvest, each individual fraction was handled as we would normally treat pooled fractions for vaccine preparation; that is to say, each fraction was dialyzed using 10 kDa cut-off cassettes, concentrated using a 10 kDa cut-off membrane, and passed over a column used to remove detergents. Any of these steps should have resulted in the loss of peptide that was not tightly associated with the focused liver proteins within a given fraction. Not only did the peptide localize to chaperone-containing fractions chosen to generate CRCL vaccine, these fractions had the ability to donate the SIINFEKL peptide to the DCs in a manner that led to specific T cell stimulation (Fig. 1) likely via cross-

presentation pathways. This was important because it strongly suggests that the chaperones somehow play a role in the accumulation of the peptides during FS-IEF. It is also important to note that those chaperones could functionally deliver the peptide to DCs for presentation to T-cells. While this method of peptide incorporation into CRCL was essentially no more difficult than the normal preparation of CRCL vaccines, we later discovered that simply mixing peptide with preformed CRCL (i.e., CRCL that had already been processed into a vaccine form) resulting in surprisingly high levels of peptide incorporation, again in a functionally deliverable form for DC-based T cell activation. From Fig. 1 it can be seen that T cell stimulation tracks with HSP/HSC70 content in the FS-IEF fractionation. Indeed, we have shown herein that one of the key components in CRCL, which appears to bind to the exogenous pep-

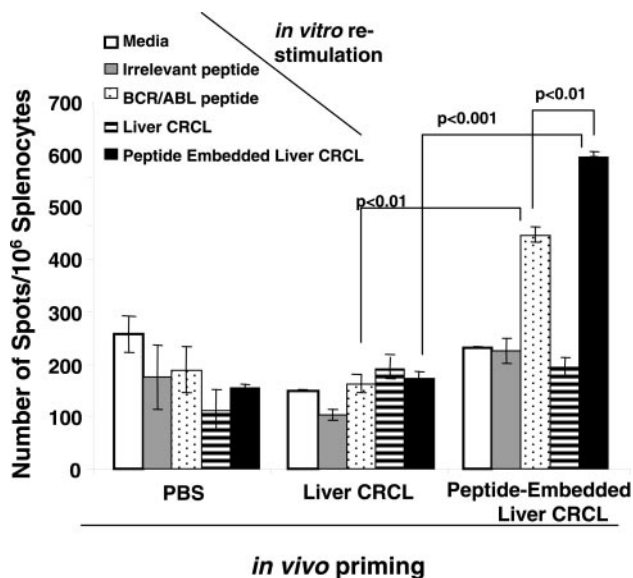


Figure 5. Immunization of mice with GFK-peptide-embedded CRCL induces BCR/ABL-specific IFN- γ secretion from primed and restimulated splenocytes. Mice were immunized with PBS, liver CRCL, or GFK (BCR/ABL peptide)-embedded liver CRCL. All immunizations took place on days 0 and 2. On day 7, splenocytes were collected and restimulated with indicated peptides or CRCLs for 48 h. IFN- γ production was determined by ELISPOT assay. Error bars represent SEM. Statistical analysis computed using Student's *t* test; groups compared are indicated.

tide, is heat-shock cognate 70 protein (HSC 70) (Fig. 2). Curiously, binding of specific peptides to purified HSP or HSC70 has not been as easily accomplished

previously (48). While this work does not shed much light into the controversy of how chaperone-based cancer vaccines acquire antigen (5, 6, 8, 15), it strongly implies that the antigenic component can be manipulated.

Our data here have shown that a significant amount of peptide of interest can be incorporated into tumor-derived or liver-derived CRCL as opposed to the nominal amount detected following incorporation into their corresponding lysates (Fig. 2). Furthermore, we determined that the considerably higher amount of FITC-peptide retained in both liver and tumor-derived CRCL was effectively cell-surface localized on dendritic cells, contrary to the minimal amount of FITC-peptide that was retained in both lysates (Fig. 3). Moreover, this phenomenon was visually apparent via confocal microscopy (Fig. 4). Thus, even if the peptide of interest is added exogenously to CRCL, the peptide still traffics to DCs and is effectively presented by them. The DC stimulatory effect of CRCL over lysate or peptide alone likely contributes to this (13). Pulsing DCs with preformed CRCL incorporated with peptide also resulted in stimulation of peptide specific T-cells both *in vitro* and *in vivo* (Figs. 5 and 6). These findings directly compared the peptide-carrying potential of both lysate and CRCL vaccine along with their ability to elicit a strong T cell activation. All results in this study conclude that CRCL vaccine has an extraordinary peptide carrying capacity and, when embedded with specific peptide, leads to powerful specific T cell stimulation. No such enhancement was observed with peptide-embedded lysate or peptide alone.

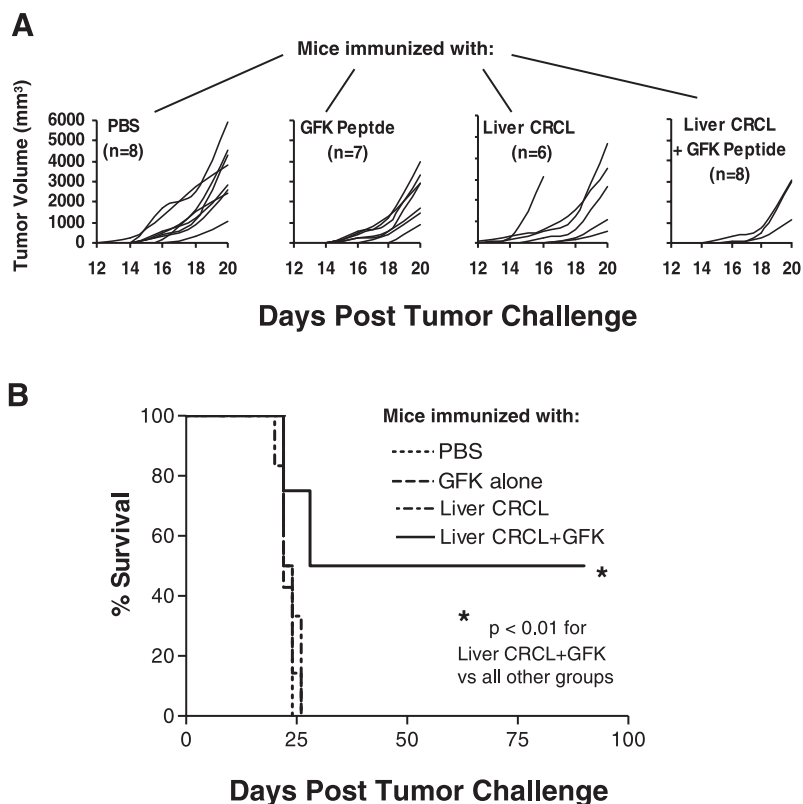


Figure 6. Antitumor efficacy: treatment of pre-existing 12B1 tumors with GFK-embedded CRCL. Mice were injected subcutaneously (s.c.) with 3000 *in vivo* passed 12B1 tumor cells on day 0. On days 1 and 3, mice were injected with 20 μ g of GFK peptide-embedded liver CRCL. Other groups of mice were also injected s.c. with either PBS, 1 μ g GFK peptide alone or 20 μ g liver CRCL alone. A) Tumor volume measurements of individual mice per group. Total numbers of mice per group are listed in parentheses; thus, for the group treated with Liver CRCL+GFK peptide, 3 of 8 animals developed measurable tumors during the observation period. Animals were sacrificed when tumor volume reached 3000 mm³. B) Kaplan-Meier survival plots of the mice shown in (A). Of the mice vaccinated with GFK peptide-embedded liver CRCL, 50% remained tumor-free at day 90. Data are representative of two independent experiments and Log-Rank was used to determine statistical significance.

Importantly, we demonstrated through *in vivo* anti-tumor growth assays that BCR/ABL peptide-embedded CRCL, when administered therapeutically, considerably delayed growth of subcutaneous 12B1 leukemia when compared to CRCL alone or peptide alone (Fig. 6A). This treatment also resulted in a 50% survival rate for afflicted animals, which is a remarkable outcome against this aggressive leukemia. It is likely that the *in vitro* phenomenon of enhanced specific T cell activation that we observed with the peptide-embedded vaccines plays a role in the tumor rejection observed *in vivo*. It should be pointed out that this leukemia model is nonimmunogenic despite its expression of the human p210 BCR/ABL protein; all untreated animals receiving tumor inoculation (100 cells intravenously, 1000 cells subcutaneously) will succumb to disseminated disease (26, 28, 31, 32). It is also resistant to imatinib treatment (51) and ultimately unresponsive to total body irradiation and bone marrow transplant (52).

Antigenic peptides or tumor lysate in combination with DCs are currently used as vaccine strategies (45, 53–55). Although tumor-derived lysate has been determined to contain antigenic peptides, at the same time it can also contain inhibitors, such as TGF- β , which may reduce the therapeutic outcome of the vaccine (56, 57). A further potential problem of tumor-derived lysate as a therapy may be the lack of access to tumor tissue required from the patient to make enough vaccine. The process of making CRCL requires little starting material and yields a larger quantity of vaccine when compared to individual chaperone purifications [5–30 fold more (27, 32)]. This line of thinking also brought about the concept of producing peptide-embedded CRCL deriving from normal, nontumor (and potentially nonautologous) tissue, such as human placenta [which is also rich in chaperones (58)], from which chaperones may be enriched by FS-IEF (Graner, unpublished data), in the event that minimal or no tumor tissue is available. Therefore, in this study, given the difficulty of obtaining mouse placental tissue, murine liver tissue was utilized alongside 12B1 tumor-derived CRCL as a model for designing peptide-embedded CRCL. In a scenario where a vaccine is made from normal tissue, clearly one would have to add in antigens artificially and exogenously, as we did with BCR/ABL peptide incorporation into liver CRCL. Another possibility would be the use of tumor cell lines (Li *et al.*, in press) as a source of CRCL, with subsequent supplementation of the CRCL vaccine with appropriate antigenic peptide. Thus, a designer vaccine could be made from scratch, possibly with a cocktail of known antigenic peptides specific for the disease, including forms of cancer or perhaps infectious diseases. It is also conceivable to use peptides of unknown antigenicity, such as those acid-stripped from the cell surfaces of patient tumors (53, 55), thus recapitulating the personalized components of an autologous vaccine. In addition, given the current state of the art in clinical trial immune monitoring, known antigenic peptides would

offer a means of tracking and assessing the efficacy of the vaccine.

Studies are ongoing to determine the carrying capacity of CRCL vaccine incorporated with multiple peptides. Ideally, in a clinical setting this peptide-embedded vaccine would include a cocktail of human tumor peptides to strategically target various epitopes in an attempt to prevent immune escape by the tumor. Taken together, the results of this study lead us to postulate that the novel concept of creating a peptide-embedded CRCL using a wide range of known human peptides will offer the ability to effectively administer high doses of various peptides that can be loaded onto MHC molecules on antigen presenting cells. Furthermore, this procedure could personalize effective vaccines to those afflicted with cancers containing known antigens, such as the BCR/ABL fusion peptide. These encouraging results with peptide-embedded CRCL may offer a practical and effective alternative for anticancer immunotherapy, and perhaps other vaccine formulations for infectious diseases. FJ

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