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IMATINIB MESYLATE EFFECTIVELY COMBINES WITH CHAPERONE-RICH CELL LYSATE-LOADED DENDRITIC CELLS TO TREAT *bcr-abl*$^+$ MURINE LEUKEMIA

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Imatinib mesylate has become an effective agent for the treatment of chronic myeloid leukemia (CML). However, the development of drug resistance has led to examination of combination therapies. In this study, we investigated the effects of combining imatinib with immunotherapy against a murine *bcr-abl*$^+$ leukemia, 12B1. We have previously shown that multiple chaperone proteins may be enriched into a vaccine form from tumor cell lysates by a free-solution isoelectric focusing method. We refer to these vaccines as chaperone-rich cell lysates (CRCLs) and have found that they are potent immunologic agents against a variety of murine tumors, including 12B1. We now demonstrate that the combination of imatinib with dendritic cells loaded with 12B1-derived CRCL yields high activation of anti-12B1-specific T cells and potent antitumor activity, resulting in tumor-free survival in up to 63% of mice with *bcr-abl*$^+$ 12B1 tumors. Our data suggest that immunotherapy can be effectively combined with imatinib for the treatment of CML.

Key words: *imatinib mesylate; chaperone/heat shock proteins; dendritic cells; chronic myeloid leukemia*

Chronic myelogenous leukemia (CML) is a clonal myeloproliferative disorder genetically characterized by the presence of cells that carry the Philadelphia (Ph) chromosome.[1] The Ph chromosome results from a reciprocal translocation between chromosomes 9 and 22—t(9;22)(q34;q11)—leading to the fusion of the breakpoint cluster region (bcr) on chromosome 22 with the Abelson (abl) oncogene from chromosome 9.[2] This fusion results in the production of 1 of 2 chimeric 210 kD proteins (b_2a_2 or b_3a_2 breakpoints, although other breakpoints are possible) with tyrosine kinase activities that are necessary and sufficient for transformation. Of the available drug therapies against CML (*e.g.*, hydroxyurea, busulfan, interferon α), only imatinib mesylate (STI571, Glivec, or Gleevec) specifically targets the p210 BCR-ABL proteins. Although imatinib can achieve remissions, allogeneic stem cell transplantation remains the only curative therapy for CML.[3]

The remarkable record of imatinib mesylate against CML in chronic phase is tempered by its reduced effectiveness against the disease in the accelerated phase or during blastic transformation[4–6] or by the increasing number of cases where drug resistance develops.[7,8] These situations have led to drug combination approaches to augment the activity of imatinib via alternative targeting of either the p210 proteins or other important downstream signal transduction molecules.[9–13] However, there have been few attempts to combine imatinib with immunotherapy,[14] and there are no published reports on the utilization of vaccine therapy in conjunction with imatinib either in human trials or in animal models. On the other hand, imatinib mesylate has been reported to be able to enhance the antigen-presenting capacities of dendritic cells (DCs), suggesting that imatinib may be used in the immunotherapy of cancer.[15] Our previous reports have shown that multiple chaperone proteins may be enriched into a vaccine form from tumor cell lysates by a free-solution isoelectric focusing (FS-IEF) method.[16] We refer to these vaccines as chaperone-rich cell lysates (CRCLs) and have reported that they are potent immunologic agents against a variety of murine tumors,[16,17] including the 12B1 BCR-ABL$^+$ leukemia.[17,18] In this current work, we show that the combination of imatinib with cellular vaccines of DCs pulsed with 12B1-derived CRCLs yields anti-12B1-specific T cells and potent therapeutic antitumor activity resulting in tumor-free survival in a high percentage of mice. This report suggests that CRCL vaccine may be effectively combined with imatinib mesylate to treat *bcr-abl*$^+$ leukemia.

MATERIAL AND METHODS

Bcr-abl-*positive leukemia cell line*

12B1 is a murine leukemia cell line derived by retroviral transformation of BALB/c bone marrow cells with the human *bcr-abl* (b3a2) fusion gene. This is an aggressive leukemia, with the 100% lethal dose (LD_{100}) being 10^2 cells after tail vein injection (i.v.) and 10^3 cells after subcutaneous injection (s.c.).[17,19] The 12B1 cell line was kindly provided by Dr. Wei Chen (Cleveland Clinic, Cleveland, OH). The cell line was tested monthly and found to be free of mycoplasma contamination. A20 cells are leukemia/lymphoma cells syngeneic to BALB/c mice.[20]

Mice

Six- to 10-week-old female BALB/c ($H2^d$) mice (Harlan Sprague Dawley, Indianapolis, IN) were used for the experiments. The animals were housed in microisolation in a dedicated pathogen-free facility and cared for according to the University of Arizona Institutional Animal Care and Use Committee guidelines.

Preparation of imatinib mesylate for murine use

Commercially available imatinib capsule contents (Gleevec/Glivec, Norvartis Pharmaceutical, Basel, Switzerland) were dissolved in distilled water (Sigma Chemical, St Louis, MO) at desired concentrations, aliquoted and stored at −20°C for future *in vitro* and *in vivo* experiments.

Imatinib mesylate treatment of in vitro *grown cells*

Tissue-cultured 12B1 or A20 cells were plated in 96-well flat-bottom plates (50,000/well) in the presence of increasing concen-

Abbreviations: APC, antigen-presenting cell; CML, chronic myeloid leukemia; CRCL, chaperone-rich cell lysate; CTL, cytotoxic T lymphocyte; DC, dendritic cell; FITC, fluorescein isothiocyanate; FS-IEF, free solution-isoelectric focusing; GM-CSF, granulocyte-macrophage colony-stimulating factor; LD_{100}, 100% lethal dose; HSP, heat shock protein; IFN-γ, interferon γ; IL, interleukin; LU, lytic unit; MLR, mixed lymphocyte reaction; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl-sulfate-polyacrylamide gel electrophoresis; TUNEL, terminal deoxynucleotidyltransferase dUTP nick-end labeling.

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trations of imatinib for 24 hr. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma), stock solution 5 mg/ml, at 10 μl per well was added for an additional 4 hr. The supernatant was aspirated and the formazan crystals were solubilized in dimethylsulfoxide, followed by determination of optical densities at 560 and 690 nm using a microtiter plate reader (BioMetallics, Princeton, NJ).

Terminal deoxynucleotidyltransferase dUTP nick-end labeling (TUNEL) assay

Apoptosis induction of 12B1 cells *in vivo* was determined by TUNEL assays using an Apo-Direct kit (BD Pharmingen, San Diego CA). BALB/c mice were s.c. injected with 3×10^3 viable 12B1 cells in the right groin on day 0. When tumor diameters reached around 2–3 mm, mice were treated with phosphate-buffered saline (PBS) or single dose of 900 mg/kg imatinib by gavage. Tumors were removed 24 hr later, embedded in OCT (Sakura Finetek, Torrance, CA), frozen at −80°C, cut to 5 μm thick sections, then mounted on microscope slides and stored at −80°C. Frozen slides were fixed in freshly prepared 1% paraformaldehyde (Sigma) in PBS for 15 min on ice. They were then rinsed with PBS once and submerged in 70% cold ethanol for 30 min on ice. Following 2 rinses in PBS, slides were allowed to dry. TUNEL staining solution was added to the slides and they were incubated in a humidified chamber at 37°C for 60 min followed by washing with rinsing buffer for 3 times. Ribonuclease/propidium iodide solution was then added to the slides and they were incubated in the dark for 10 min. After 2 washes with PBS, they were mounted using Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL) and covered with coverslips. Samples were examined using a BioRad 1024 MRC confocal imaging system (BioRad Laboratories, Hercules, CA).

Annexin V staining of imatinib mesylate-treated cells

12B1 cells in culture were treated with 2 μM of imatinib for 6 or 20 hr. Cells were then collected and stained with annexin V-fluoroscein isothiocyanate (FITC) and propidium iodide (PI) using the Annexin-V-Fluos staining kit (Roche Molecular Biochemicals, Indianapolis, IN) followed by flow cytometric analysis of cells (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Western blotting of 12B1 cell lysates for BCR-ABL protein and phosphotyrosine content

12B1 cells were treated *in vitro* with 2 μM of imatinib for time points from 0 to 24 hr. Cells were harvested by centrifugation and lysed in TNES protein lysis buffer (0.05 M Tris, 1% NP-40, 2.5 mM EDTA, 0.1 M sodium chloride; Sigma) containing 2 mM sodium orthovanadate (Sigma). Protein concentrations were determined by BCA assay (Pierce Endogen, Rockford IL) and 30 μg lysate from each time point was loaded onto 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, electrophoresed and electroblotted to nitrocellulose. The membranes were probed with antibodies to c-abl (2 μg/ml, Ab-3; Oncogene Research Products, San Diego, CA) or to phosphotyrosine-containing proteins (clone 4G10; Upstate Biotechnology, Lake Placid, NY) followed by appropriate secondary antibodies. Positive control for tyrosine-phosphorylated proteins was an EGF-stimulated A431 cell lysate provided by Upstate Biotechnology. Membranes were developed with enhanced chemiluminescent substrates (Pierce Endogen).

Heat shock protein (HSP) 70 and 60 expression in imatinib mesylate-treated 12B1 cells

12B1 cells were treated *in vitro* with 2 μM of imatinib overnight. HSP70 and -60 expression on 12B1 cell surface and the total HSP70 and -60 expression in 12B1 cells were examined by flow cytometry and Western blotting, respectively, as described previously[21] with a different purified anti-HSP70 (clone N27F3-4, mouse IgG_1; StressGen, Victoria, Canada) and a different secondary antibody: Alexa Fluor 488 $F(ab')_2$ fragment goat antimouse IgG (H+L; Molecular Probes, Eugene, OR).

Imatinib mesylate treatment of mice

Mice were treated with the indicated doses of imatinib every morning and every evening from days 2 to 9 by gavage. Imatinib was administered in a volume of 300 μl sterile water by means of straight animal feeding needles (Popper and Sons, New Hyde Park, NY). The treatment regimens were well tolerated.

Tumor generation

All tissue/cell culture reagents were purchased from Gibco/Invitrogen (Gaithersburg, MD). 12B1 and A20 cells were cultured at 37°C and in 5% CO_2 in RPMI medium containing 10% heat-inactivated fetal calf serum and supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, 0.025 μg/ml amphotericin B, 0.5 × minimal essential medium nonessential amino acids, 1 mM sodium pyruvate and 50 μM 2-mercaptoethanol. Cells were prepared for injection by washing and resuspending in Hanks' balanced salt solution. The cells were counted and adjusted to a concentration of 25×10^6 cells/ml. Female BALB/c mice were injected with 0.2 ml (5×10^6 cells) subcutaneously in both flanks and were monitored for tumor development. Tumors greater than 1 cm in diameter were harvested from euthanized mice. *In vivo* passaging of tumors for biochemical preparations or for tumor challenge involved harvesting and mincing the tumor to produce a cell suspension. The cell suspension was filtered through a Falcon 100 μm nylon strainer (Becton Dickinson Labware, Franklin Lakes, NJ) to remove debris and centrifuged. The cell pellet was resuspended, washed, counted and injected into mice.

FS-IEF for CRCL generation

Tumor tissue grown *in vivo* was harvested from mice and homogenized at 4°C in a motor-driven glass/Teflon homogenizer; the buffer was 10 mM Tris-Cl (pH 7.4), 10 mM NaCl, 0.1% Triton X-100, 0.1% Triton X-114, 0.1% Igepal CA-630 (equivalent to Nonidet P-40; all detergents were from Sigma) with the following protease inhibitors (Roche Molecular Biochemicals): leupeptin (2 μg/ml), pepstatin A (1 μg/ml), Perfabloc (0.5 mM) and a Complete protease inhibitor cocktail tablet. This buffer was chosen for its low ionic strength and ability to solubilize membranes. The homogenate was centrifuged at 10,000*g* for 30 min at 4°C to obtain a low-speed supernatant. That supernatant was centrifuged at 100,000*g* for 60 min at 4°C to obtain a high-speed supernatant. This was then dialyzed against 5 mM Tris, Cl (pH 7.4), 5 mM NaCl, 0.05% Triton X-100, 0.05% Triton X-114, 0.05% Igepal CA-630. Protein concentration of this dialysate was determined by the BCA method (Pierce Endogen) using bovine serum albumin as a standard. This dialysate was frozen in aliquots containing 25 mg of total protein. To generate vaccine, one aliquot was filtered through a 0.8 μm filter and prepared for isoelectric focusing by adding urea to 6 M, the detergents Triton X-100, Triton X-114 and Igepal each to 0.05%, and a mixture of pH gradient buffer pairs (5 ml of each member of each pair): pH 3.9–5.6, 200 mM MES (2-[4-morpholino]-ethane sulfonic acid) and 200 mM glycyl-glycine (Fisher Scientific, Fair Lawn, NJ); pH 4.5–6.1, 200 mM MOPSO (3-[n-morpholino]-2-hydroxy-propanesulfonic acid and 200 mM β-alanine) (Sigma); pH 5.1–6.8, 200 mM TAPS (2-hydroxy-1,1-bis[hydroxymethyl]ethyl)amino]-1-propanesulfonic acid; Fisher) and 200 mM ϵ-amino-n-caproic acid (Sigma). Water was added to a total volume of 50–60 ml. FS-IEF was carried out in a Rotofor device (BioRad Laboratories). Isoelectric focusing was conducted for 5 hr at 15 W constant power while the apparatus was cooled with recirculating water at 4°C; the anode compartment contained 0.1 M H_3PO_4, while the cathode compartment contained 0.1 M NaOH. Twenty fractions were harvested; the pH of each fraction was determined with a standard pH meter, and the protein content was analyzed by SDS-PAGE and Western blotting as previously described.[22] SDS-PAGE and Western blot results

indicated that following FS-IEF, several fractions ranging from pH 5.1 to 6.0 contained HSP70, HSP90, GRP94/gp96 and calreticulinwithin them.[18,23] Fractions selected to be pooled for vaccines were those that contained all 4 of the above HSPs. FS-IEF utilizes small amounts of starting material to yield relatively large amounts of tumor-derived chaperone proteins. In general, 1 g of tumor can yield 1,000 μg CRCL vaccine, while from the same amount of tumor only 30–50 μg of individual chaperone protein such as HSP70 can be generated using conventional purification strategies. Endotoxin level of the CRCL is lower than 0.01 endotoxin units/μg of CRCL as examined by Limulus Amebocyte Lysate assay (QCL-1000; BioWhittaker, Walkersville, MD).

Fractions from FS-IEF that contained substantial amounts of 4 chaperone proteins (HSP70, HSP90, GRP94/gp96 and calreticulin) as determined by SDS-PAGE and Western blotting were pooled and dialyzed stepwise out of urea and detergents (starting in 0.1 × PBS, 2 M urea and 0.025% detergents, ending with 0.1 × PBS). Pooled fractions were then concentrated using Centricon devices (Millipore, Bedford, MA) and reconstituted in PBS. Vaccines were then passed onto an Extracti-gel D column (Pierce Endogen) to remove detergent. Protein concentrations were determined by the BCA method and the concentrated proteins were diluted to appropriate concentration for *in vivo* and *in vitro* experiments.

Generation of bone marrow-derived DCs

BALB/c mouse bone marrow DCs were generated using a slightly modified protocol from that described previously.[24] Bone marrow was harvested from femurs and tibiae and filtered through a Falcon 100 μm nylon cell strainer (Becton Dickinson Labware). Red blood cells were lysed in a hypotonic buffer and the marrow was cultured in complete RPMI medium (therapeutic grade; Gibco/Invitrogen), which contained 10% fetal calf serum, L-glutamine, 50 μg/ml streptomycin sulfate and 10 μg/ml gentamicin sulfate. Murine granulocyte-macrophage colony-stimulating factor (GM-CSF; 10 ng/ml; Peprotech, Rocky Hill, NJ) and interleukin (IL)-4 (10 ng/ml) were added to the culture. After 6 days, the nonadherent and loosely adherent cells were harvested, washed and used for *in vivo* and *in vitro* experiments. Less than 10% of these cells were contaminated by macrophages ($CD14^+$ cells) as determined by flow cytometry.

In vivo *tumor growth experiments*

Mice were injected with 3×10^3 (LD_{100}) viable 12B1 cells in the right groin on day 0. 12B1 cells were obtained from one single *in vivo* passage. On day 2, mice began treatment with imatinib by gavage (200, 300, or 400 mg/kg) twice per day for 7 days. In experiments with DCs, day 5 DCs were incubated with 50 μg/ml CRCL vaccines in the presence of 10 ng/ml murine GM-CSF and 10 ng/ml murine IL-4 for 24 hr, then the DCs were washed with PBS 3 times and resuspended in PBS followed by s.c. injection into the left groin of mice. A total of 5×10^5 DCs were injected per mouse. Tumor size was measured every other day with calipers once the tumors became palpable. Tumor volume was calculated using the formula length × width^2 × (π/6). Differences in mean tumor volume between groups were evaluated using an unpaired *t*-test.

Interferon γ (IFN-γ) secretion, IL-2 production and mixed lymphocyte reaction (MLR) of splenocytes from treated mice

BALB/c mice were injected with 3×10^3 viable 12B1 cells on day 0. Mice were then treated as indicated above. On day 12, splenocytes were harvested. IFN-γ and IL-2 production and MLR of splenocytes were examined as described previously.[18]

Cytotoxicity assay

BALB/c mice were treated as indicated above. On day 14, splenocytes were harvested and cultured in RPMI complete media in the presence of 10 μg/ml CRCL and 20 U/ml IL-2 for 7 days. Viable cells were then harvested by Ficoll density centrifugation (Sigma) and used as effector cells. Stimulated effector cells were tested for cytolytic activity against 12B1 or A20 cells using a Cytotox 96 Non-Radioactive Cytotoxicity Assay kit (Promega, Madison, WI) following the instructions provided. The percentage of cytotoxicity was determined according to the formula provided in the kit instructions by the manufacturer. One lytic unit (LU) was defined as the number of effectors required to lyse 40% of targets; cytotoxicity is presented as LU per 10^6 effector cells.

RESULTS

12B1 cells show in vitro *and* in vivo *sensitivity to imatinib mesylate*

Since 12B1 cells are murine bone marrow-derived cells transduced with the human bcr-abl cDNA encoding the (b_3a_2) p210 BCR-ABL protein, we anticipated that these cells would be sensitive to imatinib exposure. 12B1 cells grown for 24 hr in increasing concentrations of imatinib showed a dose-dependent reduction in viability as measured by MTT assay (Fig. 1*a*). A20 cells, a syngeneic murine leukemia/lymphoma (*i.e.*, lacking the *bcr-abl* transgene), were insensitive to the drug at all concentrations tested.

We further demonstrated that imatinib induced apoptosis of 12B1 cells both *in vitro* and *in vivo*. 12B1 cells exposed to 2 μM imatinib stained with prominent amounts of annexin V on their cell surface with increasing exposure to the drug (Fig. 1*b*). Flow cytometric analysis indicated that after 6-hr incubation, about 30% of 12B1 cells were annexin V-positive, which increased to over 55% by 20 hr, while half of these cells were moving into secondary necrosis (PI- and annexin-positive). Comparable proportions of 12B1 cells underwent apoptosis when treated with 4 μM imatinib (data not shown). Apoptotic induction of 12B1 cells *in vivo* was further confirmed by TUNEL staining of tumors harvested from mice receiving imatinib treatment (Fig. 1*c*).

Imatinib mesylate inhibits 12B1 bcr-abl tyrosine kinase phosphorylation in vitro

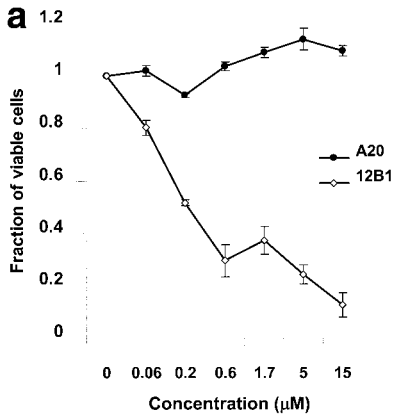
To examine whether the 12B1 susceptibility to imatinib may lie in the phosphorylation status of p210, 12B1 cells were exposed to 2 μM of imatinib for 0–24 hr, and the cells were harvested at the time points shown (Fig. 2). Cell lysates were prepared and separated on SDS-PAGE, followed by transfer to nitrocellulose for Western blotting. We observed that the p210 BCR-ABL protein was present to an appreciable extent in cells at all of the time points chosen (as demonstrated by probing of the blot with an antibody for c-abl; Fig. 2, top). However, the p210 protein displayed progressively less tyrosine phosphorylation (when probed with antiphosphotyrosine antibodies; Fig. 2, bottom). The reduced phosphorylaton status of p210 may result in reduced activation of downstream signaling molecules, which may override antiapoptotic mechanisms employed by BCR-ABL-positive leukemic cells.

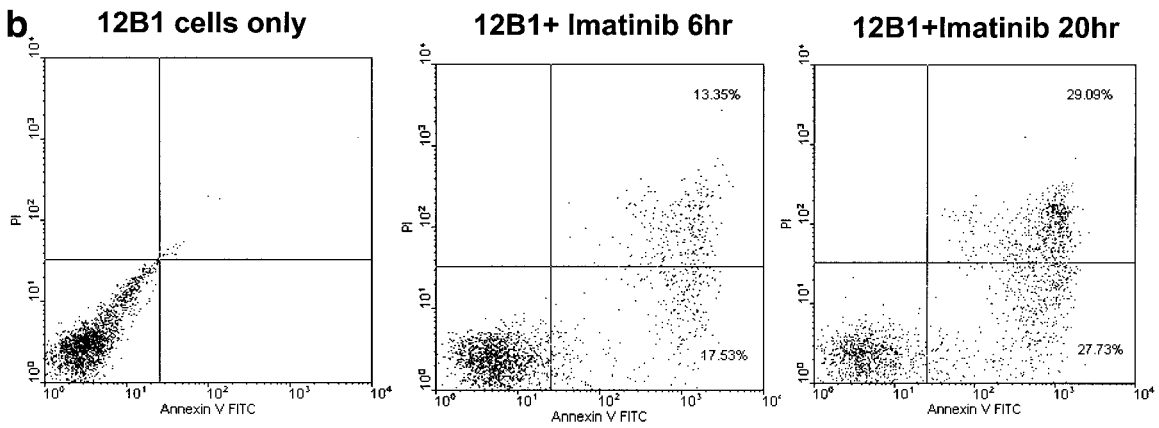
Imatinib mesylate reduces tumor burden and prolongs survival but does not cure mice of disease

We next examined the efficacy of imatinib treatment of mice bearing 12B1 tumors. In dose escalation studies, we found that increasing amounts of imatinib resulted in significant retardation of tumor growth (Fig. 3*a*). However, all animals eventually succumbed to disseminated disease or to large tumor masses (Fig. 3*b*). For future treatments, we chose 300 mg/kg of imatinib twice daily for 7 days, since the effects were comparable to higher drug doses, and this was already a relatively high dose for mice when compared to other reports.[25]

Combination therapy of imatinib mesylate with DCs pulsed with 12B1-derived CRCL can be curative against mice bearing 12B1 tumor

We wished to examine whether combination of specific chemotherapy with specific immunotherapy may offer an advantage over monotherapy. Since imatinib is not particularly myelosuppressive and may actually enhance antigen-presenting cell (APC) activity,[15] combining it with an immunostimulatory vaccine such as CRCL-





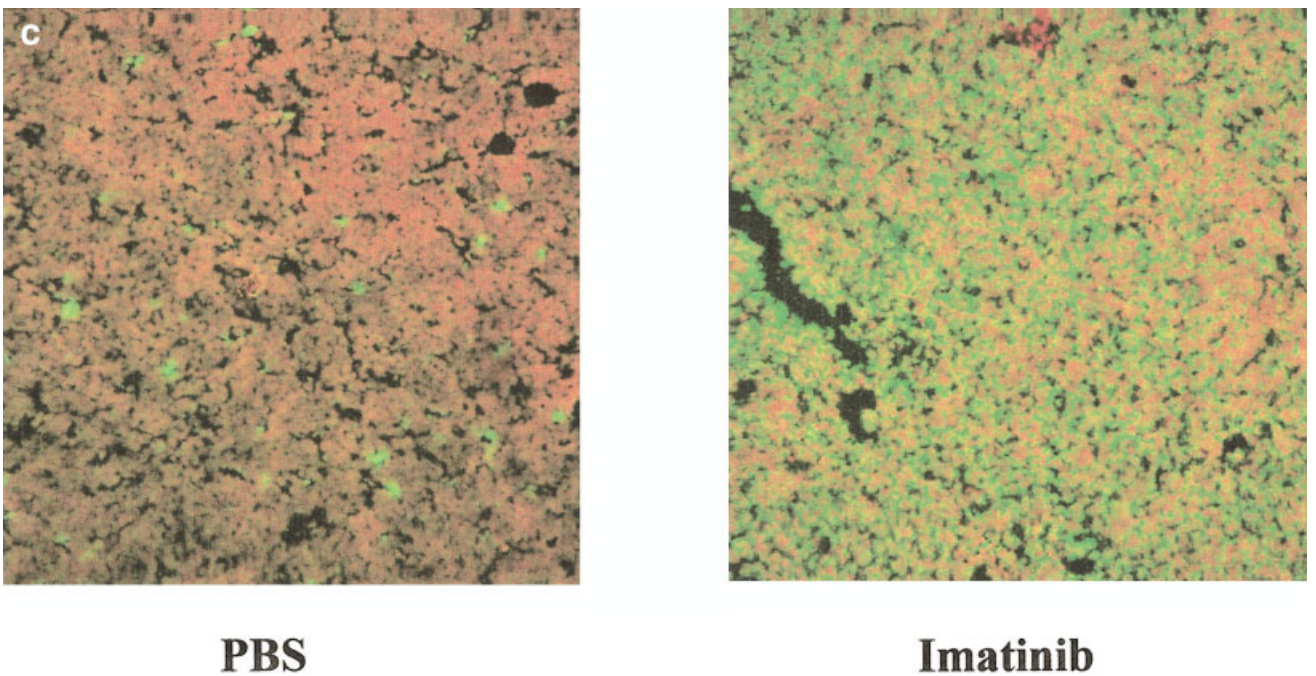


FIGURE 1 – 12B1 cells are sensitive to imatinib mesylate. (*a*) *In vitro* sensitivity of 12B1 cells to increasing concentrations of imatinib. 12B1 cells or A20 cells (5×10^4) were grown in indicated concentrations of imatinib for 24 hr. Cell viability was evaluated by MTT assays. (*b*) Imatinib mesylate induces apoptosis of 12B1 cells *in vitro*. 12B1 cells were exposed to 2 μM imatinib for 6 or 20 hr. Apoptosis induction was measured by Annexin V and PI staining followed by flow cytometric analyses. (*c*) Imatinib mesylate induces apoptosis of 12B1 cells *in vivo*. BALB/c mice were s.c. injected with 3×10^3 viable 12B1 cells on day 0. When tumor diameters reached 2–3 mm, mice were treated with PBS or a single dose of 900 mg/kg imatinib by gavage. Tumors were removed 24 hr later. Apoptotic induction of 12B1 cells was detected by TUNEL staining followed by examination under confocal laser microscopy. Original magnification × 20. Representative data from 3 experiments are shown.

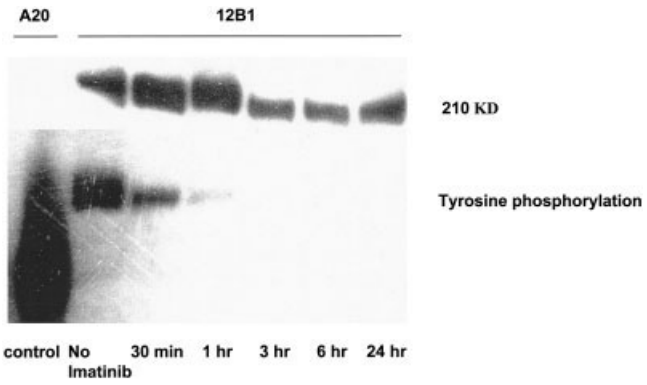


FIGURE 2 – Imatinib mesylate inhibits 12B1 BCR-ABL tyrosine kinase phosphorylation. 12B1 cells were exposed to 2 μM of imatinib mesylate for 0–24 hr, and cells were harvested at the time points shown. Cell lysates were prepared and separated on SDS-PAGE, followed by transfer to nitrocellulose for Western blotting. Presence of p210 BCR-ABL protein was probed using an antibody for c-abl. A20 cell lysate was served as a negative control. The same membrane was further probed for tyrosine phosphorylation with an antiphosphotyrosine antibody. Positive control (indicated as control in the lower panel) for phophorylated tyrosine is EGF-stimulated A431 cell lysate provided by the manufacturer. Representative data from 3 experiments are shown.

pulsed DCs seemed like a feasible approach, particularly in light of the success we previously had using this vaccination approach alone.[17,18] Mice were inoculated with tumor on day 0 and were treated with imatinib as above (*i.e.*, 300 mg/kg gavage twice daily) from days 2 to 9. DCs pulsed with 12B1-derived CRCL (DC/CRCL, 5×10^5 cells/mouse) were injected s.c. on day 2. Tumor volume was thereafter monitored. Figure 4 shows individual tumor growth curves of mice in each treatment group. All mice in the PBS control or imatinib-alone groups developed tumor. However, DC/CRCL alone or combination therapy resulted in eradication of tumor growth in 3/8 and 4/8 mice, respectively. The mean tumor volume of each group was further analyzed. Each of the 3 treatment regimens (imatinib alone, DC/CRCL alone and combination) significantly delayed the mean tumor growth when compared to saline-treated controls. Moreover, treatment with imatinib + DC/CRCL was superior to imatinib or DC/CRCL alone ($p < 0.05$). These data were reproduced in an additional experiment (data not shown) with survival analysis of the pooled data demonstrating a survival of 63% of mice in the combination therapy group, which compared favorably to 44% for the group treated with immunotherapy alone and 6% in the imatinib group.

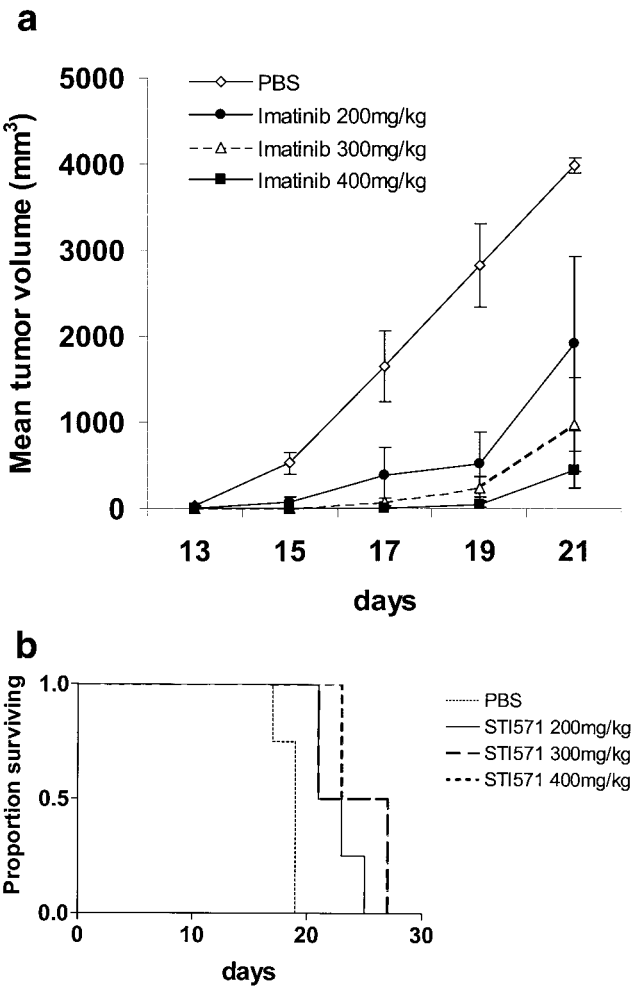


FIGURE 3 – Treatment with imatinib mesylate reduces tumor burden and prolongs survival of mice bearing 12B1 leukemia. (*a*) BALB/c mice were injected with 3×10^3 12B1 cells subcutaneously in the right groin on day 0. Mice were given 200–400 mg/kg imatinib via gavage twice a day from days 2 to 9. Tumor volume was measured (PBS *vs.* all imatinib treatment groups, $p < 0.05$; no significant difference between the imatinib treatment groups). (*b*) Survival of imatinib-treated mice was monitored (PBS *vs.* all doses of imatinib, $p < 0.05$; no significant differences between the imatinib treatment groups).

Combination therapy of imatinib and DC/CRCL induces superior IFN-γ secretion and cytotoxic T lymphoctye (CTL) activity in vivo

We have previously shown that vaccination with DC/CRCL induced T-cell-dependent immunity.[18] In addition, splenocytes from vaccinated mice displayed tumor antigen-specific CTL activity and secreted higher amounts of IFN-γ following *in vitro* restimulation with tumor antigen or antigenic peptide-bearing chaperones.[17,18] In this study, we investigated the IFN-γ secretion by splenocytes and the generation of tumor-specific CTL activity following treatment of mice with imatinib and/or DC/CRCL. Mice were inoculated with tumor on day 0 and received either DC/CRCL alone, imatinib alone, or both. On day 14, spleens were harvested, and splenocytes were restimulated with 12B1-derived CRCL. IFN-γ ELISPOT assays were performed. Only splenocytes from mice receiving DC/CRCL (with or without imatinib) showed IFN-γ production following restimulation. Interestingly, there was a sharp increase in IFN-γ production when mice received the imatinib + DC/CRCL combination. Quantitatively, there was a 2-fold increase in IFN-γ-producing cells in spleens of mice receiving combination therapy *versus* those receiving DC/CRCL alone (Fig. 5*a*). Moreover, we found increased IL-2 production and T-cell proliferation in splenocytes from mice receiving combination therapy when compared to those receiving imatinib or DC/CRCL alone (data not shown).

CTLs play important roles in controlling tumor growth. To measure specific CTL activity, splenocytes from treated mice were restimulated *in vitro* with 12B1 CRCL for 7 days and then tested for cytolytic function against 12B1 tumor or against syngeneic control A20 tumor targets. DC/CRCL alone and combination therapy elicited comparable CTL activity against 12B1 tumor target but not against A20 tumor cells (Fig. 5*b*). In contrast, imatinib alone or saline failed to generate CTL activity.

To assess more accurately the increase in cytotoxic activity taking place *in vivo*, we therefore determined the total cytotoxic activity per spleen and presented this as lytic units. Mice treated with imatinib + DC/CRCL had a 2-fold increase in lytic units per spleen when compared to mice treated with DC/CRCL alone and yielded a 7-fold increase when compared to imatinib (Fig. 5*c*).

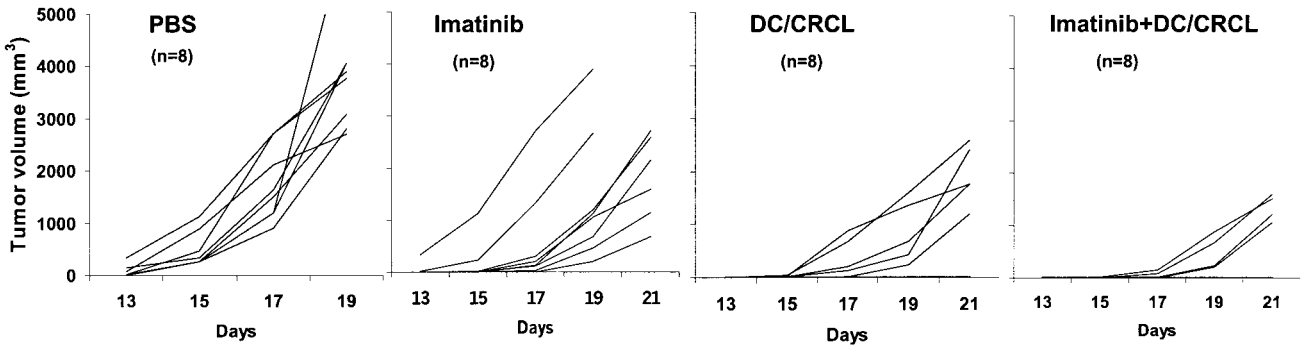


FIGURE 4 – Combining imatinib mesylate with DCs pulsed with tumor-derived CRCL delays 12B1 tumor growth. BALB/c mice were injected with 3×10^3 12B1 cells subcutaneously in the right groin on day 0. Mice were then treated with 300 mg/kg imatinib twice daily for 7 days beginning on day 2, or with a single DC/CRCL vaccination on day 2, or with imatinib + DC/CRCL. Tumor growth curves of individual mice in each treatment group are shown (representative data from 1 of 2 experiments are shown; for mean tumor volume of each treatment: PBS *vs.* all treatment groups, $p < 0.05$; imatinib *vs.* DC/CRCL, $p < 0.05$; imatinib *vs.* combination therapy, $p < 0.05$; DC/CRCL *vs.* combination therapy, $p < 0.05$ from day 21 on).

DISCUSSION

Combining imanitib mesylate with other effective agents appears vital since drug resistance to imatinib is clearly an emerging problem in CML.[7,8] In addition, the ability of imatinib mesylate to enhance the function of APCs makes it an ideal drug for the combination therapy with immunotherapy.[15] In this study, we found that adding an effective vaccine approach to imatinib treatment can significantly improve survival of mice bearing 12B1 tumors. 12B1 is an aggressive murine *bcr-abl*$^+$ leukemia; inoculation of mice with as few as 10^2 cells i.v. or 10^3 cells s.c. is uniformly lethal with a median survival of 20–25 days.[17–19] Combination treatment with imatinib and DCs pulsed with 12B1-derived CRCL was able to cure up to 63% of mice with 12B1 tumors. Moreover, mice receiving combination therapy were found to have higher splenic IFN-γ production and increased CTL activity when compared to those receiving imatinib or DC/CRCL alone.

Imatinib has been shown to induce apoptosis in *bcr-abl*$^+$ leukemia cells.[26–28] In our study, imatinib induced apoptosis of 12B1 cells both *in vitro* and *in vivo* as demonstrated by annexin V staining and TUNEL assay; however, no measurable immune responses were generated by these apoptotic cells. Our data are consistent with a recent study demonstrating the failure of imatinib in inducing PR1-specific CTLs in CML patients.[29] We have previously reported that apoptotic 12B1 cells are not immunogenic.[21,30] Heat shock treatment, which leads to increased cell membrane HSP60 and inducible HSP70 expression, converted nonimmunogenic apoptotic 12B1 cells into immunogenic ones. Imatinib upregulated total cellular HSP70 expression as measured by Western blotting. However, we observed only a minimal increase in membrane HSP70 as determined by flow cytometry. HSP60 levels were not affected by imatinib treatment (data not shown). These data may partly explain the lack of immune responses in imatinib-treated mice. We have previously shown that CRCL derived from liver could act as a potent adjuvant if utilized in conjunction with apoptotic tumor cells.[30] It is therefore likely that the imatinib-induced apoptosis of 12B1 releases tumor antigens and, when given in combination with CRCL that has both antigenic as well as adjuvant effects, leads to an enhanced antitumor immune response.

The use of tumor-derived chaperone proteins (often called heat shock proteins) as anticancer vaccines has reached clinical trial stages[31,32] after numerous reports of the immunizing efficacy of these proteins in animal models.[33] The operational paradigm for the immunologic effects of tumor-derived chaperone proteins involves both innate and adaptive responses. The innate responses are derived from the proinflammatory (cytokine-like) effects that exogenous, extracellular chaperone proteins have on professional APCs, particularly dendritic cells, presumably due to interactions with APC surface receptors for chaperone proteins.[34–39] These effects on DCs include the upregulation costimulatory molecules such as CD40, CD80 and CD86, as well as increased expression of MHC class I and II molecules and activation of the NF-κB pathway.[18,40–42] Also, APCs secrete cytokines such as TNF-α and IL-12 following encounters with chaperone proteins, further enhancing APC stimulation of T cells.[18,41,42] The ensuing adaptive responses are those generated against antigenic peptides that are bound by the chaperone proteins as part of their escort duties. Chaperoned peptides experience privileged access to the antigen processing pathway and thus end up being presented to T cells for antigen selection. It is assumed that at least some of the tumor-derived peptides will be antigenic, leading to a T-cell antitumor response that is enhanced by the chaperone protein-induced activated state of the APC.

It has been reported that imatinib significantly enhances antigen presentation by bone marrow-derived APCs.[15] We reasoned that in mice treated with combination therapy, both imatinib and CRCL enhanced APC activity, whereby more potent immune responses were generated as demonstrated by the increased IFN-γ production and superior CTL activity. Other formal possibilities may exist to explain the enhanced response; for instance, imatinib may reduce leukemia burden sufficiently to overcome an associated leukemia-induced immunosuppression, which would be evidenced in the increased IFN-γ output by immune effector cells in tumor-bearing mice treated with imatinib and DC/CRCL vaccines. Imatinib may also suppress regulatory T cells that can be envisioned as tolerizing entities, presumably to avoid autoimmune responses. Breaking this tolerance may result in greater activation of T cells that recognize near-self in the tumor. We are actively pursuing mechanistic answers to the question of how the combination therapy improves treatment outcomes via the immune system.

We should point out that we have not attempted to optimize imatinib therapy in this very aggressive murine leukemia model. The 7-day, 300 mg/kg b.i.d. regimen was chosen to represent essentially a round of chemotherapy and to enable us to observe the effects of adjuvant immunotherapy in conjunction with the drug. Since our scheme with imatinib was very rarely curative outright, we were able to discern the additional benefits of our DC/CRCL vaccines in a therapeutic setting. It is certainly possible that further manipulation of the dosage and duration of imatinib use would improve survival outcomes for drug-treated mice (and therefore for the combination therapy-treated mice as well). However, the point of this work was to demonstrate the feasibility and efficacy of anticancer vaccines in conjunction with an effective

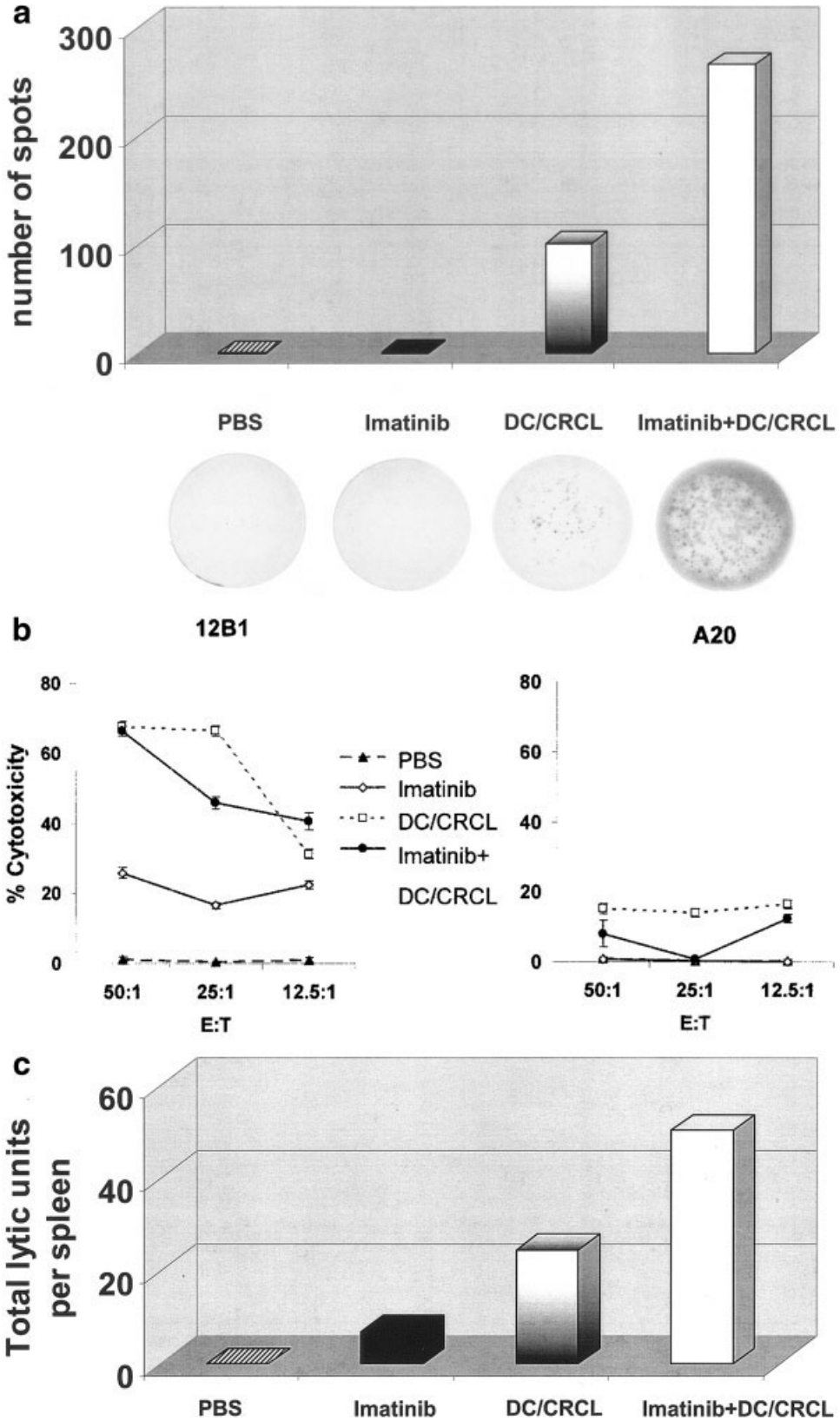


FIGURE 5 – Imatinib mesylate and DC/CRCL combination therapy stimulate IFN-γ secretion and CTL activity. BALB/c mice were injected with 3×10^3 viable 12B1 cells on day 0. Mice were then treated with 300 mg/kg imatinib twice daily for 7 days, or with DC/CRCL on day 2, or with imatinib + DC/CRCL. Splenocytes were harvested on day 14. (*a*) IFN-γ secretion by splenocytes was measured using ELISPOT assays (representative data from 3 experiments are shown). PBS or imatinib *vs*. DC/CRCL or combination therapy, $p < 0.05$; DC/CRCL *vs*. combination therapy, $p < 0.05$. (*b*) Splenocytes were cultured in complete RPMI in the presence of 10 μg/ml 12B1 CRCL and 20 U/ml IL-2 for 7 days. Viable cells were then harvested by Ficoll density centrifugation and used as effector cells. Effector cells were tested for cytolytic activity against 12B1 or A20 cells using a nonradioactive cytotoxicity assay (representative data of 2 experiments are shown). (*c*) Total lytic units were calculated and plotted (representative data of 2 experiments are shown; PBS *vs*. all treatment groups, $p < 0.05$; imatinib *vs*. DC/CRCL, $p < 0.05$; imatinib *vs*. combination therapy, $p < 0.05$; DC/CRCL *vs*. combination therapy, $p < 0.05$).

drug that in and of itself is not immunosuppressive and may even be immunosupportive.[15] The value of these results is in the implication that one may be able to control, if not cure, CML with chemo- and immunotherapeutic combinations.

We feel our data provide compelling evidence for the utilization of chaperone protein-based anticancer vaccines in combination with specific chemotherapy, in this case imatinib mesylate. While the search for better drug combinations to pair with imatinib continues,[12] we suggest that immunotherapy be given a higher priority in that endeavor. CRCL vaccines, especially when used as an immunogen for pulsing DCs, may represent a novel form of immunotherapy ideally suited for augmenting the targeted imatinib chemotherapy.

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