

A non-coding cationic lipid DNA complex produces lasting anti-leukemic effects

Nikki Keasey,¹ Zachary Herse,¹ Stella Chang,² Denny H. Liggitt,³ Marla Lay,² Jeffery Fairman² and David F. Claxton^{1,*}

¹Penn State College of Medicine; Hershey, PA; Department of Medicine; Hematology/Oncology Division; ²JuvarisBiotherapeutics; Burlingame, CA USA;

³University of Washington School of Medicine; Department of Comparative Medicine; Seattle, Washington

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Abbreviations: APC, antigen presenting cell; CLDC, cationic lipid DNA complex; GVL, graft versus leukemia; GFP, green fluorescent protein; IFN γ , interferon- γ ; IV, intravenous; NK, natural killer; PBS, phosphate-buffered saline; SC, subcutaneous; TLR-9, toll-like receptor 9

Cationic lipid DNA complex (CLDC) is an immunostimulatory preparation that has significant anti-leukemic effects in multiple murine models of leukemia: BCR-ABL⁺ myelogenous leukemia in C3H/HeJ animals and myelomonocytic leukemia in BALB/c mice. Following leukemic challenge, CLDC treatment inhibits tumor cell growth in vivo and extends survival, sometimes resulting in apparent eradication of tumor cells. CLDC induces multiple cytokines including interferon-gamma (IFN γ), and intravenous treatment results in a more rapid and robust response than subcutaneous treatment. IFN γ is induced in a dose-dependent manner, and tachyphylaxis results from repeated doses of CLDC. Tachyphylaxis of therapeutic effects is exacerbated at higher doses, thus the optimal survival benefits are seen at intermediate doses. Animals whose leukemia has been successfully treated with CLDC exhibit a survival advantage when faced with a secondary leukemic challenge, suggesting the existence of an adaptive anti-leukemic response. This work demonstrates the effectiveness of CLDC in multiple experimental leukemias and is consistent with a stimulation of a lasting TH₁ anti-leukemic immune response.

Introduction

CLDC has been shown to be a potent activator of innate immunity and to induce natural killer (NK) cells, IFN γ , IL-12 and other cytokines. This immune activation is dependent upon CpG motifs present in the non-coding plasmid DNA, as non-CpG plasmids have been shown in other models to fail to elicit a protective immune response.¹ The CpG plasmid stimulates immune activation via the Toll-like Receptor 9 (TLR-9), but evidence suggests the involvement of unknown pathway(s) independent of TLR-9.²⁻⁴ TLR-9 cues an immune response via myeloid differentiation factor 88 (MyD88),⁵ mimicking the body's response to bacterial DNA. Either component alone (cationic lipid or CpG plasmid DNA) is incapable of invoking this immune response, as the lipids likely provide protection from degradation as well as a delivery vehicle enabling cellular entry of the plasmid DNA, but in themselves have no therapeutic effects.^{1,6} The cationic liposomal components may also direct the plasmid DNA subcellularly, favoring endosomal retention over lysosomal degradation.⁷ When combined with soluble protein or peptide antigens, CLDC has been shown to induce cross-priming and a potent T-cell response, potentially contributing to the control of a neoplastic condition.⁵

Acute leukemias have long been recognized as suitable targets for immunotherapy.⁸ Allogeneic blood and marrow transplantation is an active form of such treatment frequently used in younger patients.^{9,10} Graft versus leukemia (GVL) effects have been documented in animal and human settings to be mediated by both antigen specific cytotoxic lymphocytes^{11,12} and by NK cells.¹³ As might be expected, these activities operate most efficiently when the burden of residual leukemia in patients is small, as in patients who are in complete remission but are expected to experience disease relapse. More recently, non-myeloablative conditioning regimens have enabled investigators to extend GVL effects to older patients with positive results.^{14,15} Autologous immunotherapy has also been studied by a number of investigators and a significant body of experimental data suggests that in multiple animal models of acute myelogenous leukemia, the autologous immune system can control the leukemia effectively. Several investigators have documented the ability of HLA restricted and antigen specific cytotoxic lymphocytes to contribute to this control.^{16,17} Chaudhury et al. clearly showed cytokine induced differentiation of chronic myelogenous and acute myelogenous leukemia cells into dendritic antigen presenting cells (APCs) that could induce leukemia-specific cytotoxicity in autologous lymphocytes.¹⁸⁻²⁰ Other investigators have demonstrated the potential

*Correspondence to: David F. Claxton; Email: dclaxton@hmc.psu.edu

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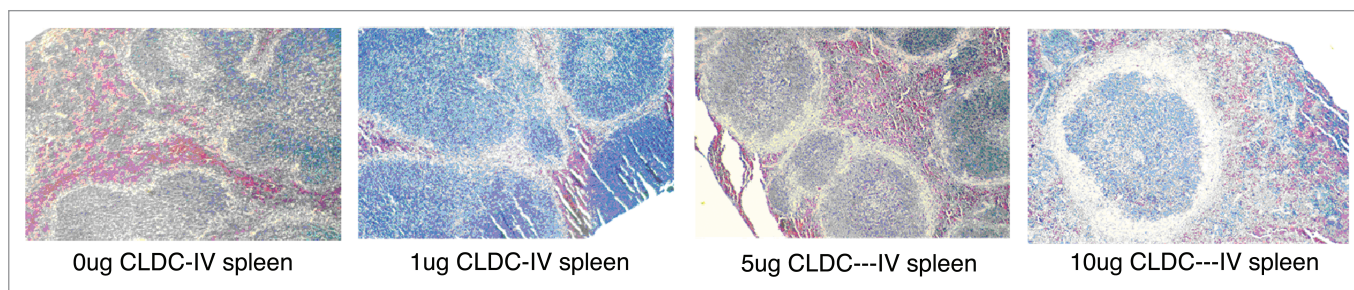


Figure 1. Single IV doses of CLDC were associated with accumulation within the splenic marginal zones of numerous pale-staining macrophages, consistent with innate immune activation (7 days post IV administration).

of antigen-specific immunotherapy in leukemia. Molldrem et al. have shown that chronic myelogenous leukemia specific cytotoxicity may be mediated by cytotoxic lymphocytes specific for the PR-1 antigen.²¹⁻²⁴ His group has also shown anti PR-1 cytotoxicity induced by vaccination may yield anti-leukemic responses in a group of myeloid leukemia patients.²⁵ There is also evidence that the WT1 protein, known to contribute to leukemogenesis, may be immunogenic both in mice and humans.^{26,27}

Previous studies have demonstrated that CLDC is effective in the treatment of mouse solid tumors⁶ and the treatment of canine patients with spontaneous malignancies.²⁸ The current studies were designed to establish the efficacy of CLDC immunotherapy in multiple murine models of leukemia and to gauge the contribution of the adaptive response in a situation where a specific antigen is not identified or administered therapeutically. A further goal of these studies is to evaluate the dose-response relationship of CLDC, so as to investigate appropriate dosing schedules to avoid tachyphylaxis and maximize benefit in clinical trials.

Results

Leukemogenic cell lines result in predictable leukemic death. Leukemic challenge of C3H/HeJ mice with BCR-ABL transfected 32D cells (32D/BCR-ABL) regularly produced death of animals between days 26 and 34 and the 32D/BA-GFP cells (an independently derived BCR-ABL clone carrying a fluorescent marker) yielded deaths somewhat earlier (day 18–26), as has been reported previously.³⁰ In both of these clones, animals either ill with leukemia or succumbing to leukemia showed considerable splenomegaly and histological infiltration with blastic immature progenitor cells (data not shown). The 32D/BA-GFP cells yielded circulating GFP-expressing cells detectable by flow cytometry (described below). WEHI-3B cells were similarly lethal, yielding splenic enlargement and leukemic deaths in challenged syngenic BALB/c mice between days 21 and 41, consistent with previous reports.³¹

CLDC causes innate immune activation. CLDC was administered subcutaneously (SC) or intravenously (IV) with no evidence of toxicity. Histologically, IV administration of CLDC was associated with accumulation of numerous pale-staining macrophages within the splenic marginal zones, consistent with innate immune activation (Fig. 1) as identified by previous authors.⁶

In an effort to maximize the immune response following administration of CLDC, we compared innate immune activation using CLDC given by SC and IV administration. An IV dose of only 5 μg of CLDC results in rapid and robust response, with $\text{IFN}\gamma$ levels peaking at 2,600 pg/ml at 6 hours following administration (Fig. 2A and Table 1). The systemic $\text{IFN}\gamma$ response following SC administration of 20 μg CLDC peaks at 18 hours at a level of 70 pg/ml (Table 1). Substantially elevated levels of other cytokines such as IL-12, $\text{IFN}\alpha$ and CCL-2 were also detected following IV administration of CLDC, but not with SC administration of CLDC (Table 1). IV administration of CLDC at lower doses results in lower levels of systemic cytokines as expected, with at least a 100-fold range of response (0.05 μg –5 μg CLDC, data not shown). The cytokine release profile of IV administration as well as previous GLP toxicology in non-human primates³² and prior human experience with CLDC given by IV administration (unpublished data) suggested further evaluation of IV dosing of CLDC.

To examine the effect of multiple CLDC doses on cytokine response, the time between repeated doses was altered. $\text{IFN}\gamma$ levels were measured following a second CLDC dose that came 1, 3, 7, 10 or 14 days after the initial dose in unchallenged mice (Fig. 2B). When a second dose of CLDC is administered only one day after the first, no $\text{IFN}\gamma$ induction is apparent indicating that CLDC induces a refractory period. The $\text{IFN}\gamma$ response returns as the time between multiple doses is lengthened, and by 14 days, the response has returned to the levels induced in a naïve mouse. These data indicate that timing of multiple CLDC doses is critical to achieving optimal $\text{IFN}\gamma$ response.

Doses greater than 5 μg were also tested by IV injection. Because $\text{IFN}\gamma$ levels peak at 6 hours following IV CLDC, a 6-hour time point was used for all subsequent measurements. C3H/HeJ mice were challenged with 32D/BA-GFP and weekly CLDC treatments were started on day 3. Following the initial dose of 10, 20 or 30 μg IV CLDC, serum $\text{IFN}\gamma$ levels increased in a dose-dependent manner (Fig. 3A). However, upon repeated weekly treatments, a trend of tachyphylaxis became apparent, with higher doses exhibiting a more dramatic decrease in $\text{IFN}\gamma$ (Fig. 3B).

IV treatment with CLDC is associated with a dose-dependent survival benefit. To test the efficacy of CLDC via IV administration and to determine a dose response relationship with efficacy, 32D/BA-GFP was tested in a CLDC dose titration

experiment. Mice were treated with CLDC on days 2 and 9 following 32D/BA-GFP challenge and then monitored for survival. This treatment conferred a significant survival benefit in a dose-dependent manner (Fig. 4).

Larger IV doses (10, 20 and 30 μg) were also tested in the 32D/BA-GFP model, and GFP tumor burden was followed closely to assess the kinetics of tumor cell growth in each treatment group. In the absence of treatment, circulating GFP-positive cells were measurable by flow cytometry as early as 11 days after challenge (Fig. 5A and B). This population expanded aggressively, resulting in death in 24–26 days (Fig. 5C). Weekly IV treatments with CLDC beginning on day 3 significantly inhibited growth of the GFP-positive population at all doses tested (Fig. 5A and B). The 10 μg dose resulted in the best control of tumor burden and the greatest number of long-term surviving (apparently cured) animals (Fig. 5C). Treatment with 20 μg CLDC significantly improved survival over control mice, but was not as beneficial as 10 μg . The 30 μg dose yielded mixed results, with some animals dying before or concurrently with the untreated controls, and others surviving until >80 days. This dose-response relationship indicates a threshold effect, where an intermediate dose is more beneficial than higher or lower doses.

The IV dose-response relationship was also examined in the myelomonocytic leukemia induced by WEHI-3B in BALB/c mice. In this model, weekly administration of 20 μg IV CLDC is more effective than 10 μg (Fig. 6), indicating that the survival benefit of CLDC is not specific to the C3H/HeJ leukemia model, and that dose must be determined empirically for individual cancer types and animal strains.

Successful treatment with CLDC extends survival following secondary challenge. We then addressed the question of immunological memory after CLDC therapy of established leukemia. This work exploited two versions of BCR-ABL-transformed 32D cells: non-fluorescent 32D/BCR-ABL (challenge 1) and fluorescent 32D/BA-GFP (challenge 2). Animals that received challenge 1 without treatment died within 34 days (Fig. 7). Animals in Group 1 receiving challenge 2 without treatment died on day 16–19 (Fig. 7). CLDC treatment did not affect survival when given well in advance of challenge 2, as is seen by the death of Group 2 animals on days 14–19, similar to Group 1. Group 3 animals received challenge 1 on day-60 with 32D/BCR-ABL and were successfully treated with CLDC given on day 46 and 32. These animals then received challenge 2 on day 0 and survived significantly longer than groups 1 and 2 ($p < 0.01$).

Discussion

CLDC has been shown to be a systemic immunostimulant, activating effector cells of the innate and adaptive immune systems.⁶ This formulation has shown therapeutic activity for experimental solid tumors.⁶ Available data suggests that this activity stems in part, but likely not fully, from stimulation through TLR-9. Our results presented here clearly show significant *in vivo* activity for this material in therapy of three murine models of acute leukemia. Both subcutaneous and intravenous routes of administration are active, and available evidence suggests that some animals

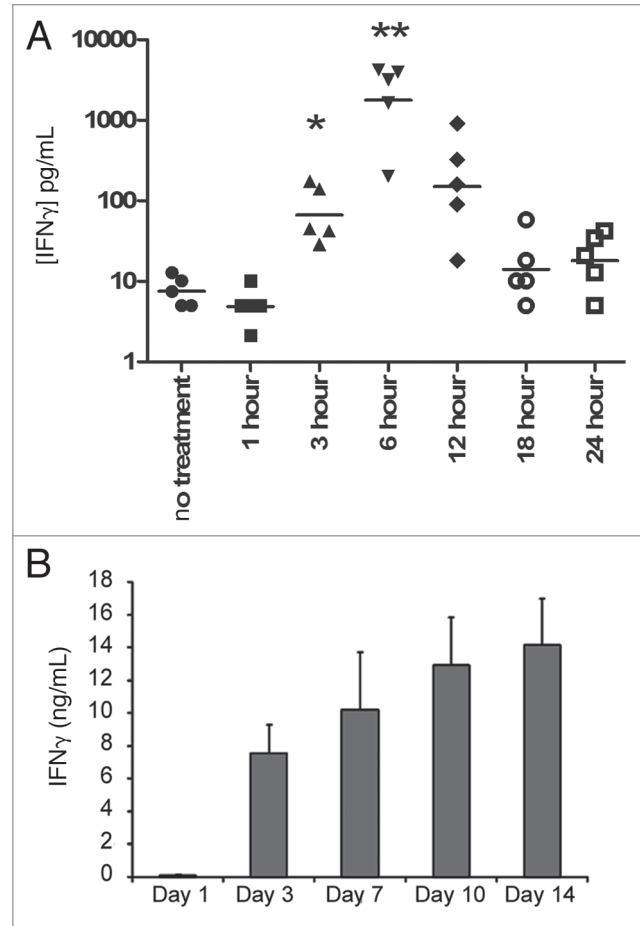


Figure 2. IFN γ is induced following IV CLDC treatment. Unchallenged BALB/c mice ($n = 5$ per group) were treated with 5 μg CLDC by IV injection (A) and serum was collected at indicated time points for ELISA analysis. Unchallenged BALB/c mice ($n = 5$ per group) were given 5 μg CLDC by IV injection (B). The IV dose was repeated 1, 3, 7, 10 or 14 days later and IFN γ levels were measured from serum samples taken 6 hours later.

Table 1. Cytokine levels following IV or SC CLDC treatment

Cytokine*	IV		SC	
	Peak level pg/mL**	Time of maximum	Peak level pg/mL	Time of maximum
TNF α	220 (41)	1 hour	nd***	
IFN α	850 (530)	3 hour	nd	
CCL-2****	9600 (500)	3 hour	nd	
IL-6	650 (130)	6 hour	40 (25)	18 hour
IFN γ	2600 (760)	6 hour	70 (25)	18 hour
IL-12p70	3000 (1100)	6 hour	nd	

*The following cytokines were undetectable following IV and SC treatment: IL-10, IL-15, IL-23 and GM-CSF. **Cytokine levels were assayed by ELISA at 0, 1, 3, 6, 12, 18 and 24 hours following IV treatment with 5 μg CLDC by IV injection or 20 μg CLDC by SC injection. Cytokine levels are shown at peak levels, with standard deviation noted in parentheses. ***nd, not detectable. ****CCL-2 baseline levels were 20 pg/mL. All other cytokines were not detectable before treatment.

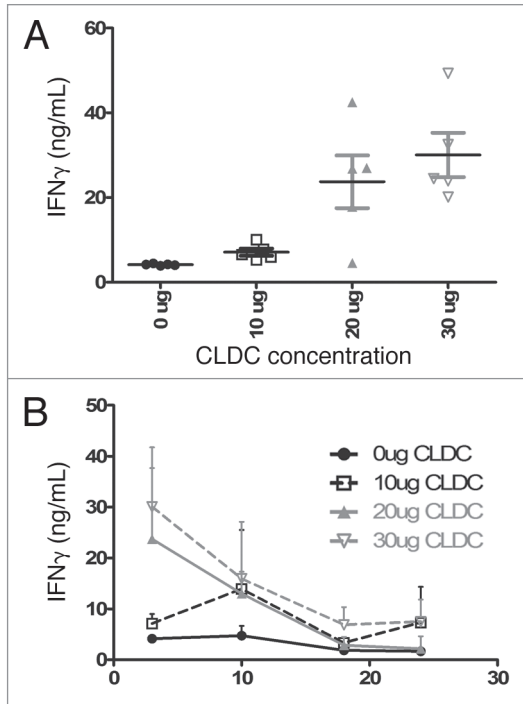


Figure 3. CLDC administration results in a dose-dependent induction of IFN γ that exhibits tachyphylaxis. (A) IFN γ levels of 32D/BA-GFP-challenged C3H/HeJ mice display a dose-dependent induction. Serum samples were drawn six hours after the indicated IV dose of CLDC. Individual measurements are displayed for each mouse following the day 3 treatment. $p < 0.01$ for 0 μ g vs. 10 μ g, 0 μ g vs. 30 μ g and 10 μ g vs. 30 μ g. $p < 0.05$ for 0 μ g vs. 20 μ g and 10 μ g vs. 20 μ g. 20 μ g vs. 30 μ g not significantly different. (B) Average and standard deviation values from treatments on days 3, 10, 18 and 24 are graphed, revealing a decrease in IFN γ levels following repeated CLDC treatment. Significant differences are noted: 10 μ g Day 3 vs. 18: $p < 0.01$. 20 μ g Day 3 vs. 18, 10 vs. 18, 3 vs. 24 and 10 vs. 24, $p < 0.01$. 30 μ g Day 3 vs. 10 and 3 vs. 24: $p < 0.05$, Day 3 vs. 18 $p < 0.001$.

have been cured of this leukemia via CLDC therapy. Given the effectiveness, reproducibility and therapeutic range of the IV route, it may prove optimal for future study.

In this report we show the dose dependency of CLDC treatment of leukemic neoplasms. Among CLDC doses tested, higher doses yielded higher IFN γ levels but showed tachyphylaxis of both IFN γ levels and anti-leukemic effect. The optimal dose also seems to vary from one leukemia model to another (Figs. 5 and 6). In the C3H/HeJ model, the 10 μ g dose provided the best tumor burden control and survival benefit while in the WEHI-3b model, 20 μ g was best. This difference may result from differences in genetic background in the two mouse strains. Alternatively, the type of leukemia induced by the myeloid 32D/BCR-ABL cells and the myelomonocytic WEHI-3B cells may respond differently to CLDC dosing. It is clear that clinical application of this therapeutic preparation will require careful attention to dosing, as genetic and leukemic differences will also be present in human patient populations.

Timing of treatment administration also appears to be critical for optimization of therapeutic benefit. IFN γ measurements on mice given repeated CLDC treatments suggest the induction

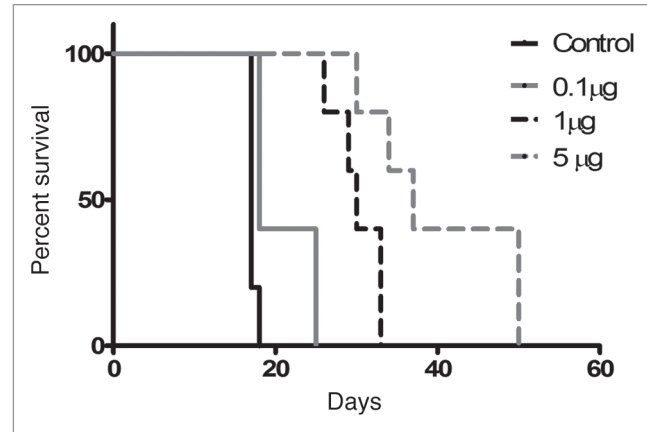


Figure 4. CLDC therapy shows dose dependent anti-leukemic effects. Animals received IV CLDC at doses of 0.1 μ g, 1 μ g or 5 μ g on days 2 and 9 following 32D/BA-GFP challenge. $p < 0.05$ for control vs. 0.1 μ g and 1 μ g vs. 5 μ g. $p < 0.01$ for all other pair-wise comparisons.

of a refractory period following treatment. Together, these data suggest that optimization of therapeutic effects may be expected to require some care and attention to the amount and timing of CLDC doses. It is notable however that there is no identified correlation between optimal IFN γ increase and therapeutic effect.

Previous data in other experimental systems shows involvement of CTLs and NK cells in biological responses to CLDC,⁵ but important questions remain regarding the mechanism(s) of the CLDC anti-leukemic therapeutic effect. In particular, the possible role of adaptive, antigen-specific immune response merits investigation.

Work involving multiple subsequent leukemic challenges is useful in probing the possibility of immunological memory. In Figure 7, mice that were treated successfully following an initial challenge with 32D/BCR-ABL fared better when faced with an additional challenge with 32D/BA-GFP than mice being challenged for the first time with 32D/BA-GFP. This suggests a lasting anti-leukemic benefit results from successful treatment with CLDC. Immunological memory provides a logical possible explanation for this lasting anti-leukemic effect. In this experiment, successful therapy of 32D/BCR-ABL cells contributed to in vivo inhibition of growth of 32D/BA-GFP. This is consistent with persistence of cellular effectors with cross reactivity for both clones. These results do not however exclude the possibility of participation of humoral immunity or macrophage mediated cytotoxicity in the therapy. Furthermore, the relative contributions of NK cells including NKT cells and antigen specific CTLs remain unclear. This latter issue is of particular importance because of the usefulness of long term immunity associated with the development of antigen specific responses.

Anti-leukemic immunotherapy utilizing CLDC is demonstrated here in multiple transplantable murine models. CLDC induces a robust IFN γ response and results in control of tumor burden and extension of survival. The treatment is highly effective, reproducible and provides a lasting survival benefit in the face of secondary leukemic challenge. The application of these

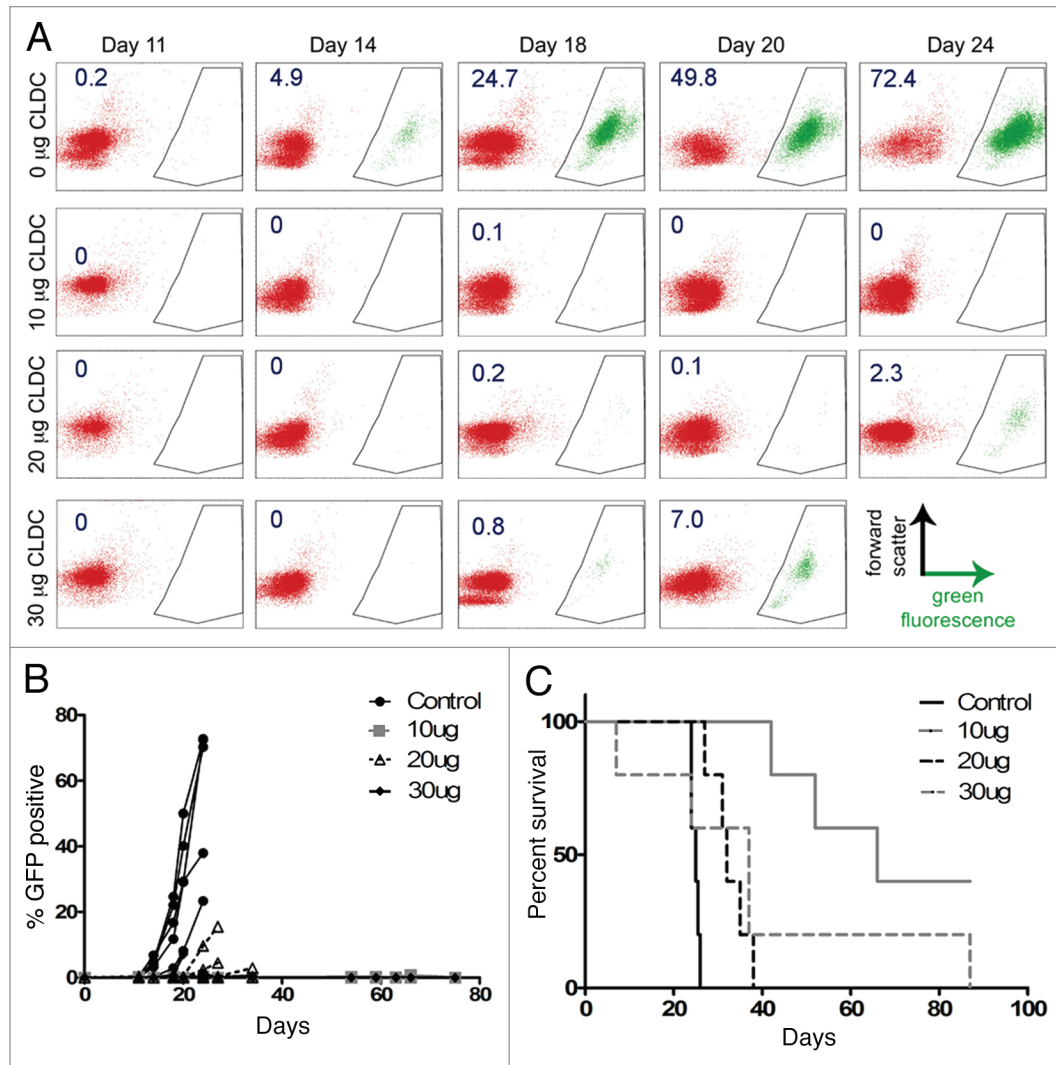


Figure 5. CLDC treatment inhibits tumor burden growth and extends survival. Mice were challenged with 32D/BA-GFP and treated with weekly IV injections of 0, 10, 20 or 30 µg CLDC from days 3 through 24 after challenge. Mice were periodically monitored for circulating tumor burden by flow cytometry. (A) Flow cytometric analysis of a representative mouse is shown from each treatment group. GFP-positive cells are displayed in green, with % GFP-positive cells noted for each plot. (B) Percent GFP-positive cells in individual mouse is graphed versus time (n = 5 per group). (C) Survival of CLDC-treated mice depends on IV dose administered; p < 0.01 for control vs. 10 µg, control vs. 20 µg and 10 µg vs. 20 µg.

observations to the therapy of human neoplasms is clearly of interest.

Materials and Methods

Leukemic clones. Three mouse models were studied. 32D cells transformed with BCR-ABL (32D/BCR-ABL) and congenic with C3H/HeJ mice were obtained from Dr. S.S. Clark, University of Wisconsin as a kind gift.²⁹ A similar clone of 32D derived cells independently transformed with BCR-ABL but also transfected to stably express the green fluorescent protein molecule (32D/BA-GFP) was obtained as a kind gift from Dr. Ling.³⁰ WEHI-3B cells (congenic with BALB/c mice) were obtained from ATCC. Animals received leukemia infusions via IV tail vein injection. Injection of 1 x 10⁶ cells yielded reliable leukemic death and thus was administered for all subsequent experiments.

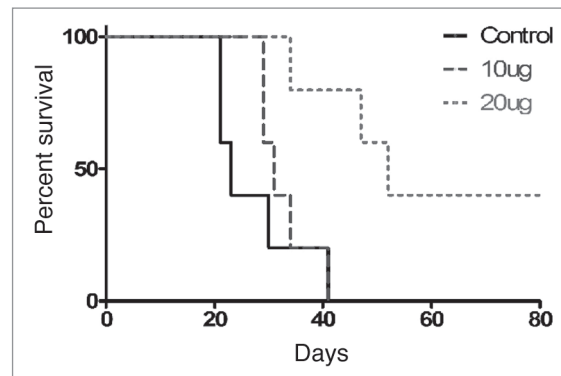


Figure 6. CLDC extends survival in BALB/c mice in a dose-dependent manner. WEHI-3B-challenged BALB/c mice were treated on days 3, 10, 17 and 24 with IV injections of 0, 10 or 20 µg CLDC and followed for survival. Control vs. 20 µg and 10 µg vs. 20 µg, p < 0.01.

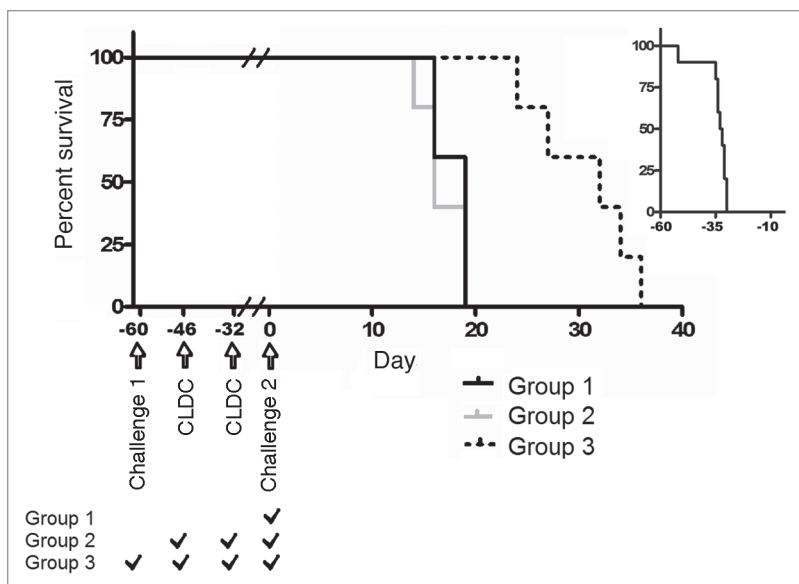


Figure 7. Successful treatment with CLDC provides benefit during secondary challenge. Mice challenged with 32D/BCR-ABL die within 34 days if left untreated (upper right corner insert, $n = 10$). Group 1 mice received 32D/BA-GFP challenge on Day 0 and died by day 19 ($n = 5$ per group). Group 2 mice were treated with 10 μg IV CLDC on Day-46 and Day-32 relative to 32D/BA-GFP and also died by day 19. Group 3 mice received an initial challenge with 32D/BCR-ABL on day-60, were treated with CLDC as in Group 2 and survived significantly longer in response to 32D/BA-GFP challenge (10–17 day increase in survival, $p < 0.01$).

Mice. Male C3H/HeJ and BALB/c mice were obtained from Charles River Laboratories at 7–8 weeks of age. Animals were kept in conventional animal facilities using minimum conventional feed and water and were monitored for illness and survival. Using the models described here, lethargy and inability to feed preceded death by less than 24 hours. Thus, the primary experimental endpoint was death or near moribund status requiring sacrifice. Some animals were subjected to necropsy after sacrifice or spontaneous death. Tissues were processed by routine technique after formalin fixation and hematoxylin and eosin staining of sections. All procedures were reviewed and approved by the Penn State Institutional Animal Care and Use Committee.

Analysis of peripheral blood. To analyze circulating tumor burden in some experiments, blood was subjected to flow cytometric analysis for quantification of 32D/BA-GFP cells. Blood (15 μL) was drawn via tail snip and red blood cells were lysed in 200 μL RBC lysis solution (5 Prime, Gaithersburg, MD). White blood cells were pelleted by centrifugation for 2 min in an Eppendorf 5415 C centrifuge at 300 $\times g$, washed in 1 ml phosphate-buffered saline (PBS) and resuspended in 400 μL PBS. Analysis was performed on a FACSCalibur instrument. List-mode multiparameter data files

(each file with forward scatter, side scatter and 4 fluorescent parameters) were analyzed using CellQuest Pro Software. Files were gated to exclude red cell debris. A minimum of 20,000 gated events was acquired.

For IFN γ analysis, mice were treated as indicated with CLDC and serum was collected after the specified timepoint. Cytokine levels were assayed using ELISA reagents per manufacturer's instructions (eBioscience, San Diego, CA).

CLDC treatment. CLDC was prepared from liposomes of the cationic lipid DOTIM, the neutral lipid cholesterol and non-coding bacterial plasmid DNA. The pMB75.6 plasmid was produced from non-pathogenic *E. coli* DH5 α cells and is 4,242 base-pairs in length. Concentrations of CLDC components were verified by HPLC analysis and spectrophotometry. The DNA was found to be 100% circular plasmid DNA and the product was manufactured and tested under Good Manufacturing Practice regulations and found to be sterile and free from endotoxin and particulate matter. Treated animals received CLDC either SC (subcutaneous base of tail injection) or IV (intravenous tail vein injection). CLDC dosage was defined by the content of DNA in the delivered dose. SC administration volume was adjusted to 200 μL while IV administration volume was 100 μL .

Statistical analysis. Animal survival was documented by the method of Kaplan and Meier and statistical significance of survival data was analyzed by the Mantel-Cox log-rank test using GraphPad Software version 5.00 for Windows, San Diego California USA, www.graphpad.com. This software was also used to plot and analyze data using the Student's t-test.

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