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ORIGINAL ARTICLE

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Dendritic-cell-peptide immunization provides immunoprotection against *bcr-abl*-positive leukemia in mice

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Abstract Chronic myelogenous leukemia (CML) is a clonal disorder characterized by proliferation of cells that possess the *bcr-abl* fusion gene resulting in the production of one of two possible chimeric 210-kDa tyrosine kinase proteins. Since these chimeric proteins are expressed only in leukemic cells they have the potential to serve as tumor-specific antigens for cytotoxic T lymphocytes (CTL). Using the 12B1 murine leukemia cell line, derived by retroviral transformation of BALB/c bone marrow cells with the *bcr-abl* (b_3a_2) fusion gene, we have demonstrated that intravenous inoculation of 12B1 cells into BALB/c mice results in a disseminated acute leukemia analogous to human CML in blast crisis. Histological sections of liver and spleen and polymerase chain reaction analysis of peripheral blood, bone marrow, liver, spleen and lymph nodes confirmed the presence of *bcr-abl*$^+$ leukemia cells in these murine tissues, while Western blot data demonstrated the expression of the fusion protein in 12B1 cells. Immunization of mice with dendritic cells (DC) loaded with the synthetic *bcr-abl* chimeric nonapeptide, GFKQSSKAL, led to a 150 times higher frequency of *bcr-abl*-specific CTL precursors in the spleen than in mice immunized with peptide alone. In vitro re-stimulation of DC-peptide-primed splenocytes resulted in substantial secretion of interferon γ and augmented cytolytic activity against 12B1 targets. Finally, vaccination with peptide-loaded DC significantly prolonged survival of BALB/c mice that were challenged with 12B1 leukemia. The capacity to generate *bcr-abl*-specific CTL in vivo by DC-based immunization may have clinical implications in the treatment of CML.

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Introduction

Chronic myelogenous leukemia is a clonal disorder characterized by proliferation of cells that possess the Philadelphia chromosome, a reciprocal translocation between chromosomes 9 and 22 leading to the fusion of the breakpoint cluster region (*bcr*) on chromosome 22 with the Abelson (*abl*) oncogene on chromosome 9 [17]. This *bcr-abl* fusion results in the production of one of two possible chimeric 210-kDa tyrosine kinase proteins (b_2a_2 or b_3a_2 breakpoints). Since these chimeric proteins are expressed only on leukemic cells they could serve as tumor-specific antigens for cytotoxic T cells. In fact, in vitro studies using human cells have demonstrated that synthetic *bcr-abl* chimeric peptides can bind to certain HLA alleles on antigen-presenting cells and that the cytotoxic T lymphocytes (CTL) thus generated can lyse targets that have been pre-incubated with these peptides. However, reports are conflicting on whether CTL can lyse leukemic cells displaying endogenous *bcr-abl* peptides on their MHC class I molecules [5, 7, 31].

Murine studies have documented that peptides spanning the *bcr-abl* joining region have the appropriate motifs to bind the MHC class I molecule, H-2K^d [7]. CTL specific for peptides of the *bcr-abl* joining region were generated, but they failed to lyse murine leukemia cells expressing the p210 *bcr-abl* protein. This finding suggested that chimeric peptides were not naturally processed and presented in a form recognized by CTL. As a result, whether peptide-based tumor vaccines can induce in vivo immunoprotection against *bcr-abl*-expressing leukemia remains a critical unanswered question. We therefore established a murine model of *bcr-abl*$^+$ leukemia to determine whether immunization of mice with a *bcr-abl* peptide can impact survival. This clinically relevant model has many of the hallmarks of CML, such as the presence of *bcr-abl*$^+$ cells in the

peripheral blood, bone marrow and leukemic infiltration of other tissues resulting in hepatosplenomegaly and lymphadenopathy. Using dendritic cells (DC) loaded with the synthetic *bcr-abl* chimeric nonapeptide, GFKQSSKAL (lysine results from the *bcr-abl* fusion), we provide evidence that an immune response can be elicited that prolongs survival of mice bearing this aggressive leukemia. Moreover, using this approach we demonstrate that DC-peptide-primed splenocytes can be stimulated in vitro to become cytolytic against leukemia targets endogenously expressing *bcr-abl* gene products.

Materials 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 *bcr-abl* (b_3a_2) fusion gene and expresses the p210 *bcr-abl* protein [21]. This cell line was kindly provided by Dr. Wei Chen (Cleveland Clinic, Cleveland, Ohio). 12B1 cells were cultured at 37 °C under 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, 0.1 mM modified Eagle's medium non-essential amino acids, 1 mM sodium pyruvate, and 50 μM 2-mercaptoethanol all from Gibco/BRL (Gaithersburg, Md.).

Mice

Female BALB/c (H-2^d) mice (Harlan Sprague Dawley, Indianapolis, Ind), 6–10 weeks old, were used for the experiments. The animals were housed in a specific-pathogen-free facility and cared for according to the University of Arizona Institutional Animal Care and Use Committee guidelines.

Immunoblotting

The presence of p210 *bcr-abl* protein in the 12B1 cell line was confirmed by Western blotting and compared to results with the *bcr-abl*$^+$ human leukemia line, K562, and the *bcr-abl*$^-$ murine B cell line A20. An equivalent amount of protein from each cell line was loaded onto the gel. Following sodium dodecyl sulfate/polyacrylamide gel electrophoresis, the gels were electroblotted to nitrocellulose in an Idea Scientific electroblotter (Minneapolis, Minn.). Gels were transferred in 25 mM TRIS, 200 mM glycine, 20% methanol overnight at 60 V, stained with Ponceau Red and destained in 50 mM TRIS/Cl, 150 mM NaCl, 0.1% Tween 20, pH 7.4 (TBST). Blots were blocked in 3% non-fat dried milk in TBST for 20–60 min and then given three 5-min rinses in TBST. The protein of interest was identified, using the monoclonal antibody to *bcr* (AB-2) clone 7C6 (Oncogene Research Products, Cambridge, Mass.). Primary antibody solutions were prepared in blocking solution, and blots were incubated in primary antibody for 1 h at room temperature or 12 h at 4 °C, with three 5-min rinses in TBST. Peroxidase-conjugated goat anti-(mouse Ig) secondary antibody was applied for 1 h at room temperature or 12 h at 4 °C followed by chemiluminescent detection (Super Signal, Pierce, Rockford, Ill.).

Polymerase chain reaction (PCR)

The presence of the *bcr-abl* fusion gene (b_3a_2) was confirmed through PCR amplification and sequencing of the *bcr-abl* fusion region. DNA extracts from organs of BALB/c mice that had received injection of 12B1 cells were obtained, using a QIAamp DNA Mini Kit (QIAgen, Valencia, Calif.). A PCR cocktail of Gibco reagents, forward primer 5′-CCTCTGACTATGAGCGTG-3′ and reverse primer 5′-TCACACCATTCCCCATTG-3′ (Sigma Genosys, The Woodlands, Tex.), were thermocycled in an Eppendorf mastercycler gradient (Eppendorf, Westbury, N.Y.) under the following conditions: 1 min at 94 °C; 1 min at 54 °C, and 2 min at 72 °C for 35 cycles. The resultant 456-bp fragment was purified by a QIAquick PCR purification kit (QIAgen, Valencia, Calif.) and sequenced on an ABI 377 (Perkin Elmer Corp., Foster City, Calif.) instrument (Laboratory of Molecular Systematics and Evolution, in the Division of Biotechnology at the University of Arizona).

Immunohistochemistry

Mice with advanced leukemia were humanely killed. Immediately upon sacrifice, the livers and spleens were harvested. Frozen blocks were prepared by arranging tissues in an aluminum foil cup, using O.C.T. compound (Miles Inc., Elkhart, Ind.) as an embedding medium and snap-freezing in liquid nitrogen. Blocks were stored at −70 °C.

Serial frozen sections were cut 4 μm thick on an IEC microtome cryostat, thaw-mounted onto glass slides and fixed for 5 min in acetone. One section from each block was stained with hematoxylin and eosin for morphological and pathological assessment. Other sections were blocked with 10% normal goat serum (Sigma) and then incubated with biotinylated mAb from PharMingen: CD4 (clone RM4-5), CD8 (clone 5H10-1), and Mac-1 (clone M1/70). Staining by an immunoperoxidase procedure was performed as described previously [4] using avidin-biotin blocking reagents, avidin-biotin complex and diaminobenzidine as chromogen (Kirkegaard and Perry, Gaithersburg, Md.).

Flow cytometry

Cells were washed in phosphate-buffered saline (PBS) containing 2% heat-inactivated fetal bovine serum and 0.1% sodium azide (Sigma). Samples containing 2×10^5 cells were placed in each well of 96-well U-bottomed microtiter plates. Surface expression of distinct molecules was determined by incubating the cells first with an Fc-receptor-blocking antibody for 5 min (PharMingen, San Diego, Calif.) and then with saturating amounts of monoclonal antibodies for 30 min at 4 °C. Except where otherwise indicated, antibodies used were from PharMingen, San Diego Calif., and included anti-H-$2K^d$ (clone SF1-1.1, mouse IgG2a), anti-H-$2D^d$ (clone 34-2-12, mouse IgG2a), anti-I-A^d (clone AMS-32.1, mouse IgG2b), anti-Mac-1 (clone M1/70, rat IgG2b), anti-CD11c (clone HL3, hamster IgG), F4/80 (clone C1:A3-1, rat IgG2b; Serotec, Indianapolis, Ind.), anti-Gr-1 (clone RB6-8C5, rat IgG2b), anti-CD45R/B220 (clone RA3-6B2, rat IgG2a), anti-CD54 (clone 3E2, hamster IgG), anti-CD80 (clone 16-10A1, hamster IgG), anti-CD86 (clone GL1, rat IgG2a), anti-CD4 (clone GK1.5, rat IgG2b), and anti-CD8 (clone 53-6.7, hamster IgG). The cells were then washed three times and fixed with PBS containing 1% formaldehyde (Polysciences, Warrington, Pa.). Samples comprising 10^4 cells were analyzed by a Becton Dickinson flow cytometer (FACScan).

Generation of bone-marrow-derived dendritic cells

Murine bone marrow DC were generated by a slightly modified protocol from that has been previously described [12]. Bone marrow was harvested from femurs and tibiae and filtered through a Nytex screen. Red blood cells were lysed in a hypotonic buffer and the marrow was cultured in AIM V medium (therapeutic grade, Gibco BRL), which contains L-glutamine, human serum albumin, 50 μg/ml streptomycin sulfate, and 10 μg/ml gentamicin sulfate. Murine granulocyte/macrophage-colony-stimulating factor (GM-CSF), 10 ng/ml; kindly provided by Immunex, Seattle, Wash.) and interleukin-4 (IL-4, 10 ng/ml; Peprotech, Rocky Hill, N.J.) were

added to the culture. After 4 days, the non-adherent and loosely adherent cells were harvested and layered onto a metrizamide gradient (14.5% metrizamide solution, Sigma Chemical Co, St. Louis, Mo.) and centrifuged. Enriched DC were washed and cultured with GM-CSF, IL-4 and murine CD40L trimer (1 μg/ml, Immunex) for an additional 24 h. The DC were then washed and used for in vitro or in vivo experiments.

Murine immunizations

In all experiments BALB/c mice were immunized subcutaneously in the left and right groin, the base of the tail and both hind foot pads with peptide, empty DC, or peptide-pulsed DC. The synthetic peptides used for loading the DC were (a) GFKQSSKAL, a *bcr-ab*l peptide and (b) HYLSTQSALSK, a green fluorescent protein peptide (both from Sigma-Genosys). These peptides were added to 10^6 DC at a concentration of 100 μg/ml with 5 μg/ml β2-microglobulin, incubated at 37° for 3 h and washed in PBS prior to injection into mice. Mice were given a total of $(0.5–1) \times 10^6$ DC twice with an interval of 1 week (i.e., day −14 and day −7).

Interferon γ (IFNγ) and IL-2 production by splenocytes of immunized mice

Splenocytes from naïve or immunized mice were placed in microtiter plates and serially diluted, and in some cases they were stimulated with 5 μg/ml *bcr-abl* peptide. Culture supernatants were collected and assayed for IFNγ, using an enzyme-linked immunosorbent assay kit (R & D Systems, Minneapolis, Minn.).

The IL-2-dependent murine T cell line, CTLL-2 (American Type Culture Collection) was used to determine the IL-2 bioactivity present in the supernatants [13]. Samples were added in triplicate to 96-well flat-bottom plates. CTLL-2 cells (5,000 cells/well), washed free of IL-2, were then added. The microtiter plates were incubated for 24 h at 37 °C and 5% CO_2, then pulsed for another 24 h with 1 μCi [^{3}H]thymidine (ICN Pharmaceuticals). The cells were then harvested with a 96-well Packard cell harvester and the radioactivity measured on a Packard beta counter. The IL-2 bioactivity was calculated by comparison with an IL-2 standard curve.

Cytotoxicity assay

^{51}Cr-release assays were performed as described previously [16]. In brief, 12B1 target cells (1×10^6–2×10^6 in 0.5 ml tissue culture medium) were incubated with 500 μCi $Na^{51}CrO_4$ (5 mCi/ml, ICN Pharmaceuticals, Irvine, Calif.) for 1 h at 37 °C. Targets were washed three times and resuspended at a concentration of 10^5 cells/ml. Fifty microliters (5,000 targets/well) were then added in triplicate to 96-well V-bottomed microtiter plates, to which effectors had been previously added, and serially diluted to yield effector-to-target ratios from 20:1 to 2:1. Spontaneous-release wells contained only tissue culture medium and ^{51}Cr-labeled targets; maximal-release wells contained 5% Triton X-100 (Sigma) and ^{51}Cr-labeled targets. The microtiter plates were centrifuged at 200 *g* for 2 min and incubated at 37 °C and 5% CO_2 for 4 h. The plates were then centrifuged at 200 *g* for 10 min, after which 75-μl aliquots of supernatant were harvested and added to 150 μl scintillation fluid; the radioactivity (cpm) was measured on a Packard beta counter. Cytotoxicity was determined by the formula:

$$\text{Cytotoxicity (\%)} = 100 \times \frac{\text{mean experimental release} - \text{mean spontaneous release}}{\text{mean maximal release} - \text{mean spontaneous release}}$$

One lytic unit was defined as the number of effectors required to lyse 20% of targets; cytotoxicity is presented as lytic unity/10^6 effector cells.

Estimation of CTL precursor (CTLp) frequencies

Splenocytes from naïve or immunized mice were placed in microtiter U-bottomed plates in a limiting-dilution fashion (titrations of 200,000–823 cells/well) with 30 replicate wells for each dilution, and stimulated with 5 μg/ml of *bcr-abl* peptide. On days 3 and 6 of culture, 20 U/ml IL-2 (Chiron, Emeryville, Calif.) were added to the wells. After 8 days in culture, wells were tested for cytotoxicity against ^{51}Cr-labeled 12B1 pulsed or not for 1 h with *bcr-abl* peptide (5 μg/ml). Wells were considered positive when cytotoxicity (i.e. ^{51}Cr release) exceeded three standard deviations over the mean value of control wells without responder cells. The CTLp frequency estimations were calculated using the Poisson equation [30].

In vivo immunoprotection experiments

Seven days following the last immunization (day 0), the mice were challenged via tail vein injection with 66 viable 12B1 leukemia cells, using a 27-gauge needle. 12B1 cells used for the challenge were harvested 2–3 h prior to i.v. injection from mice bearing subcutaneous tumors. These solid tumors were removed under sterile conditions, placed in PBS, finely minced with a scalpel, gently crushed with the piston of a syringe, gathered in a pipette, filtered through a 100-μm Nytex mesh, washed twice, resuspended in PBS and injected intravenously in a volume of 0.2 ml.

Statistical analysis

The Kaplan-Meier product-limit method was used to plot the survival of mice inoculated with the *bcr-abl*$^+$ leukemia and the log-rank statistic to test differences between groups [15, 23].

Results

Murine model of *bcr-abl*-positive leukemia

12B1 was derived by transducing BALB/c bone marrow cells with the *bcr-abl* (b_3a_2) fusion gene [21]. Western blot analysis was used to confirm the presence of *bcr-abl* protein in the 12B1 cell line (Fig. 1). These cells express substantial quantities of the *bcr-abl* fusion protein when compared to another *bcr-abl*$^+$ human leukemia line, K562. We established the 12B1 leukemia model in BALB/c mice to study whether peptide-loaded DC can induce immunoprotection. This is an aggressive leukemia, a cell dose as low as 66 cells i.v. being uniformly lethal and resulting in a median time to death of 22 days. Mice receiving 12B1 cells by i.v. injection display leukemic infiltration of the spleen, liver, lymph nodes, bone marrow and blood, as detected by PCR analysis of tissues for the *bcr-abl* joining region (Fig. 2). Histological evaluation of tissues revealed marked leukemic cell infiltration of the spleen resulting in destruction of its normal architecture

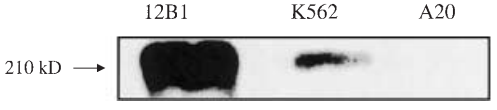


Fig. 1 The presence of p210 *bcr-abl* protein in the 12B1 cell line confirmed by Western blotting using the monoclonal antibody to *bcr* protein (AB-2) as described in Materials and methods. The K562 human leukemia cell was used as a positive control and the murine B cell leukemia line A20 as a negative control

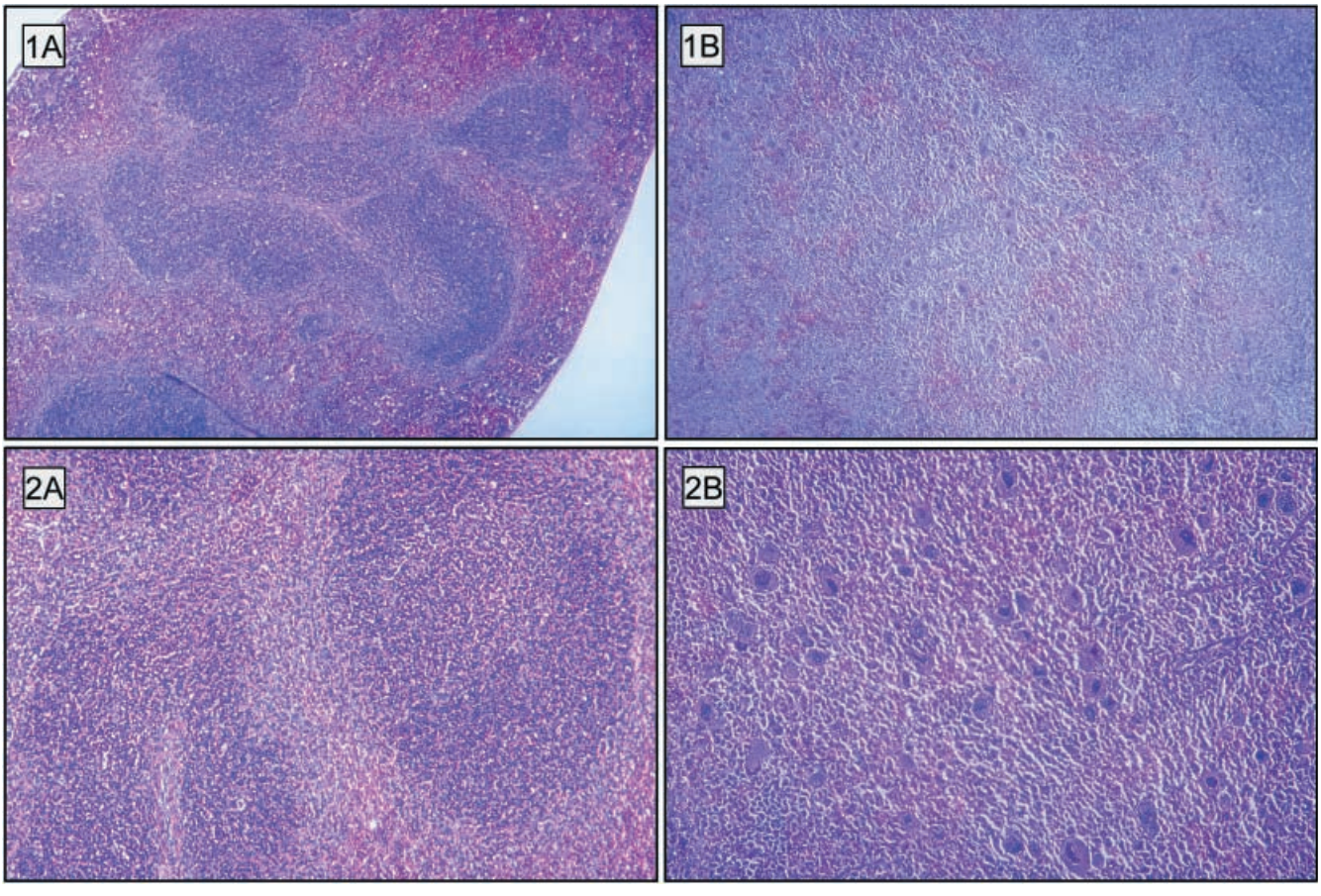
Fig. 2 Presence of the *bcr-abl* fusion gene (b_3a_2) in tissues of mice inoculated with 12B1 confirmed by polymerase chain reaction amplification of the *bcr-abl* fusion region. *S* spleen, *L* liver, *M* bone marrow, *B* blood, *N* lymph nodes. *Bold type* tissues from mice inoculated i.v. with 12B1 cells; *outline type* tissues from mice without tumor

(Fig. 3). There was evidence of increased extramedullary hematopoiesis with large numbers of megakaryocytes present throughout the spleens. 12B1 leukemia cells were also evident albeit less prominent in the livers (data not shown). Immunohistochemistry demonstrated a diffuse dense macrophage infiltration (Mac-1^+) in spleens of leukemia-bearing mice. $CD4^+$ (Fig. 3) and $CD8^+$ (data not shown) cells were decreased in number and dispersed throughout the leukemic mouse spleens instead of concentrated in the white pulp.

Use of peptide-loaded DC for immunization of BALB/c mice

The GFKQSSKAL chimeric peptide spanning the *bcr-abl* joining region has previously been shown to bind to the MHC class I allele, H-$2K^d$ [7]. 12B1 cells have high expression of H-$2K^d$ (data not shown) and consequently can serve as targets of CTL if sufficient endogenous peptides are presented on their cell surfaces. The 12B1 system therefore provides an in vivo model to study whether DC pulsed with GFKQSSKAL can generate leukemia-specific CTL with high-affinity T cell receptors able to recognize potentially low concentrations of the *bcr-abl* presented peptides. As a control peptide we used a green fluorescent protein peptide (HYLSTQSALSK). This 11-mer is processed by DC and the resulting 9-mer

Fig. 3A, B Histological sections of spleen from (**A**) healthy and (**B**) 12B1 leukemia-bearing BALB/c mice. Sections stained with hematoxylin and eosin (*1* 50×, *2* 100×). **A** *1*, *2* Normal spleen histology with clearly defined white and red pulp. **B** *1*, *2* Diffuse leukemic infiltration of spleen resulting in loss of normal splenic architecture. Evidence of increased extramedullary hematopoiesis with large numbers of megakaryocytes. **A**, **B** *3* Immunohistochemical control staining; secondary antibody only (blue is from the counterstain used). **A** *4* Immunoreactive brown-staining Mac-1^+ cells predominately in red pulp of spleen. **B** *4* Increased numbers of Mac-1^+ cells diffusely infiltrating spleen. **A** *5* $CD4^+$ T cells concentrated in white pulp of normal spleen. **B** *5* Scattered infiltration of splenic parenchyma with $CD4^+$ T cells in leukemia-bearing mice



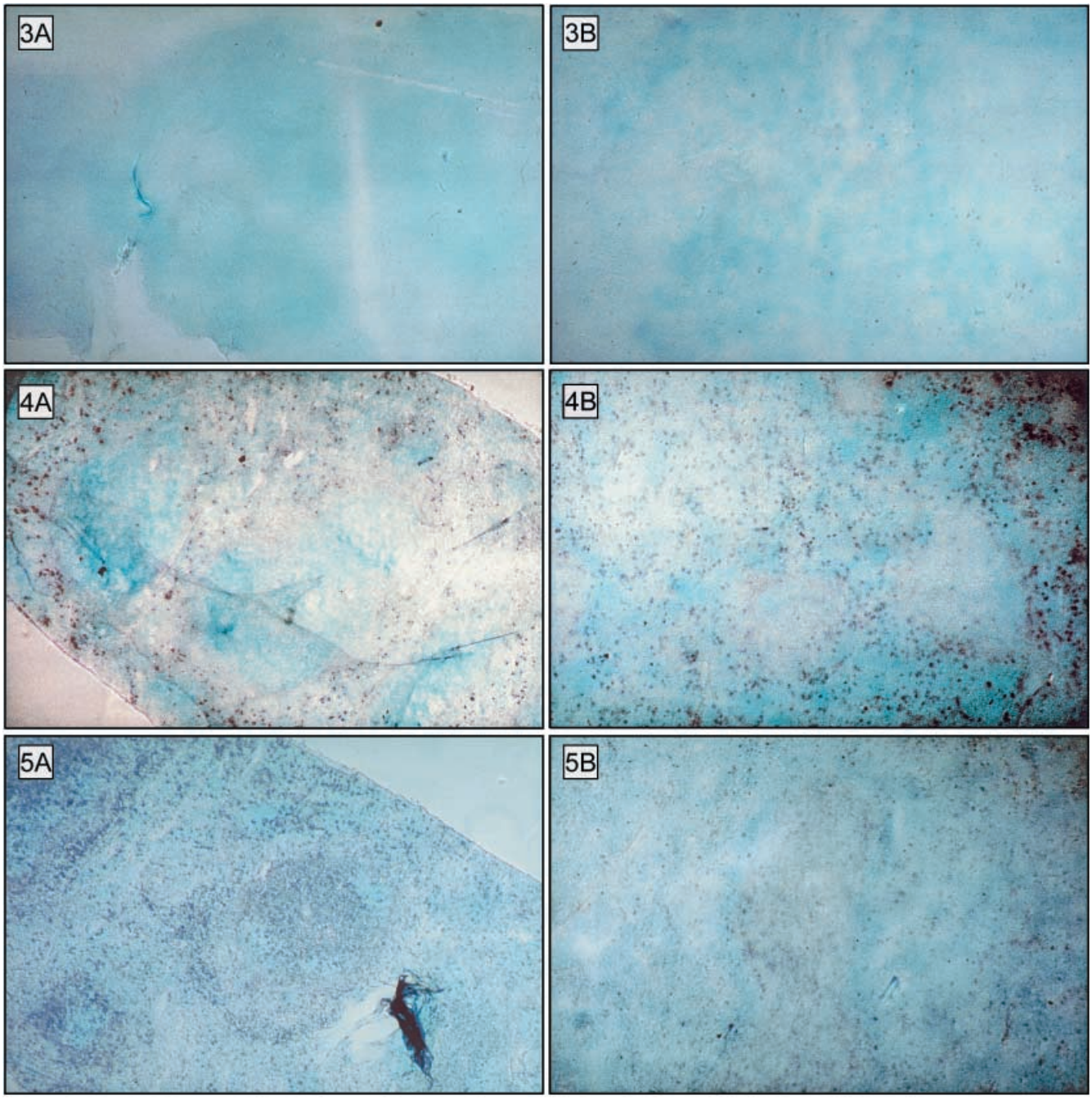


Fig. 3 (Continued)

HYLSTQSAL is predicted to bind with high affinity to H-2K^d [26].

The DC used for our studies were generated from BALB/c mouse bone marrow in serum-free medium (as described in Materials and methods). These antigen-presenting cells reproducibly expressed high levels of MHC class I (H-2K^d, 96%; H-2D^d, 90%; data not shown), MHC class II (I-A^d, 96%), CD54 (ICAM-1, 95%), CD80 (B7-1, 94%), CD86 (B7-2, 87%), CD11c (96%), CD11b (Mac-1, 96%) and had low expression of the macrophage marker, F4/80 (9%), and the granulocyte marker, Gr-1 (16%).

DC-peptide-primed splenocytes secrete IFNγ and have augmented cytolytic activity against 12B1 leukemia targets

BALB/c mice were immunized twice (days −14, −7), their spleens were harvested (day 0) and the splenocytes stimulated in vitro with GFKQSSKAL peptide as described in Materials and methods. The production of IFNγ was highest in DC-GFKQSSKAL-primed splenocytes (Fig. 4A). Surprisingly, in vivo exposure to unpulsed DC resulted in substantial IFNγ production by cultured spleen cells. In vivo priming with

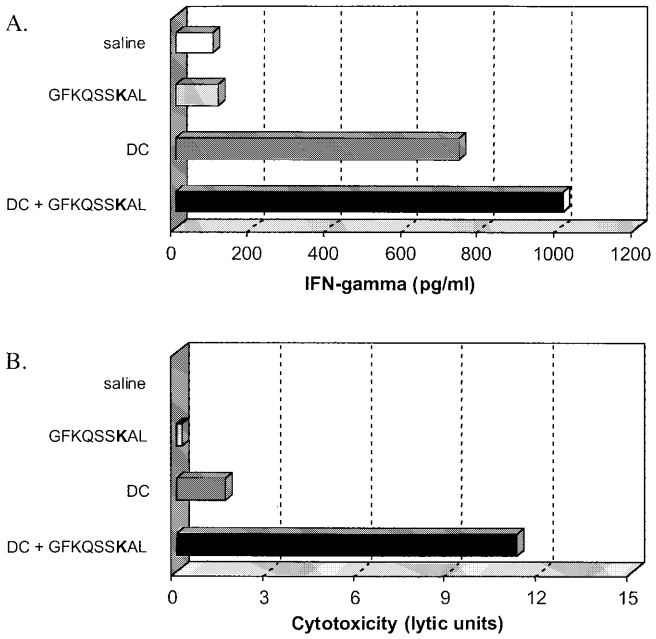


Fig. 4 **A** Effect of *bcr-abl*-loaded dendritic cell (*DC*) vaccination on interferon γ (IFNγ) production by splenocytes. Mice were immunized twice (days −14, −7), their spleens were harvested (day 0) and the splenocytes stimulated in vitro with *bcr-abl* peptide. Culture supernatants were collected after 24 h and assayed for IFNγ using by enzyme-linked immunosorbent assay. **B** Effect of *bcr-abl*-loaded DC vaccination on cytotoxicity. Mice were immunized twice (days −14, −7) and their spleens were harvested on day 0. The in vivo primed splenocytes were cultured for 8 days with *bcr-abl* peptide and low concentrations of interleukin-2 as described in Materials and methods. Stimulated effector cells were tested for cytolytic activity against 12B1 cells using ^{51}Cr-release assays. One lytic unit was defined as the number of effectors required to lyse 20% of targets; cytotoxicity is presented as lytic units/10^6 effector cells. The figure depicts representative data from one of three similar experiments

GFKQSSKAL or saline failed to elicit significant IFNγ secretion.

In additional experiments we examined fresh spleen cells of mice previously given saline or unpulsed DC in order to analyze further how immunization with "empty" DC may stimulate IFNγ production. Mice received saline or 10^6 DC subcutaneously and their spleens were harvested 7 days later. The spleens of DC-treated animals were clearly larger in size (data not shown) and contained more CD25$^+$, CD4$^+$, CD8$^+$ T cells, CD11$^+$, and Mac-1$^+$ cells (Table 1), while the numbers of B220$^+$ and Gr-1$^+$ cells were comparable to those in spleens of saline-treated controls. Splenocytes from DC-treated mice, cultured without additional stimulation for 24 h, produced detectable amounts of IFNγ and IL-2, while spleen cells from saline-treated mice did not (Table 1). These observations were reproducible in two additional mouse strains, namely C3H/HeJ and C57BL/6, and were uniformly present irrespective of the route of inoculation of unpulsed DC (s.c., i.v. or i.p.).

Further experiments were performed to evaluate the cytolytic function of in vivo primed splenocytes restimulated in vitro with GFKQSSKAL. Splenic effectors from GFKQSSKAL-pulsed DC were more potent killers of 12B1 leukemia cells (Fig. 4B). Similar to the cytokine production assays, effector cells generated from DC-primed mice also had some cytolytic activity against the *bcr-abl*$^+$ target, albeit less than that seen in DC-GFKQSSKAL-immunized mice. GFKQSSKAL immunization without DC was ineffective in priming cytotoxic effectors. Taken together these data indicate that immunization of mice with DC, cultured under the conditions described in Materials and methods, appears to direct T cells toward a Th1 phenotype which, in turn, produces cytokines such as IFNγ and IL-2 inducing nonspecific cytolytic activity.

Immunization of mice with chimeric *bcr-abl*-peptide-loaded DC increases the frequency of specific CTLp

Limiting-dilution assays were done next to determine whether DC-based immunizations would increase the *bcr-abl*-specific CTL precursor frequencies. Mice were immunized as described in Materials and methods. Groups of mice received saline, irrelevant green fluorescent protein peptide HYLSTQSALSK, *bcr-abl* peptide GFKQSSKAL, unpulsed DC or DC loaded with HYLSTQSALSK or GFKQSSKAL. Immunization with unpulsed DC or DC loaded with irrelevant peptide nonspecifically increased cytolytic activity of splenocytes, resulting in an increase in "CTLp frequencies" that were 31 and 29 times higher respectively than those of the saline controls (Fig. 5). However, immunization with GFKQSSKAL-pulsed DC resulted in a 367-fold increase in CTLp. GFKQSSKAL peptide immunization without DC did not augment CTLp any more than did the saline immunized controls. As with previous experiments, immunization with unpulsed DC stimulated cytotoxicity by nonspecific lymphoid effectors presumably through stimulation of IFNγ and IL-2 production. These studies clearly demonstrate

Table 1 Characteristics of splenocytes from mice treated with saline and unpulsed dendritic cells (*DC*). Phenotype and cytokine data are derived from pooled spleens from three mice in each group. *IFNγ* interferon γ, *IL-2* interleukin-2 (cytokines produced by 10^6 cells/ml in 24 h)

Characteristic	Saline	DC
10^{-6} × No. cells per spleen	33.2 ± 7.2	55.6 ± 4.7
CD4$^+$	3.7	13.4
CD8$^+$	1.6	4.1
CD25$^+$	0.3	3.9
B220$^+$	21.7	26.0
Mac-1$^+$	4.2	7.1
F4/80	6.2	8.7
CD11c	0.6	2.4
Gr-1$^+$	4.4	4.2
IFNγ (pg/ml)[2]	< 1	132
IL-2 (U/ml)	< 1	25

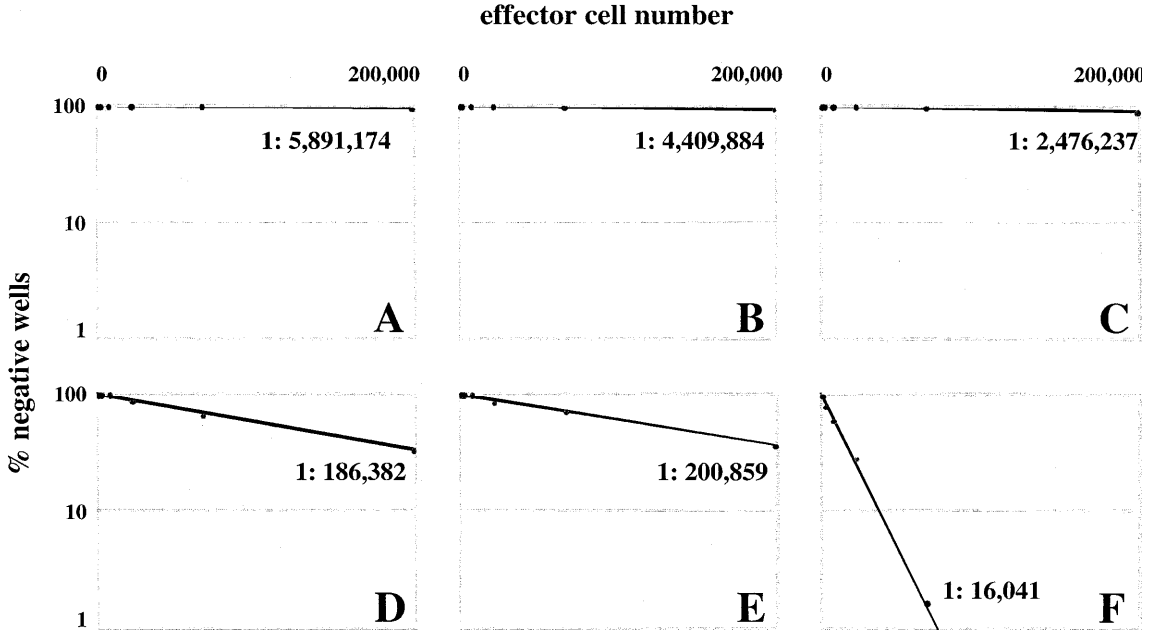


Fig. 5A–F Effect of immunization of mice with *bcr-abl* peptide, GFKQSSKAL, and peptide-pulsed dendritic cells on cytotoxic T lymphocyte precursor (CTLp) frequency estimation. BALB/c mice were immunized with (**A**) saline, (**B**) irrelevant green fluorescent protein peptide HYLSTQSALSK, (**C**) *bcr-abl* peptide GFKQSSKAL, (**D**) unpulsed DC, (**E**) DC loaded with HYLSTQSALSK or (**F**) DC pulsed with GFKQSSKAL peptide on days −14 and −7. On day 0 responder splenocytes from immunized mice were placed in microtiter plates in a limiting-dilution fashion and stimulated with 5 μg/ml *bcr-abl* peptide and a low concentration of interleukin-2 as described in Materials and methods. After 8 days in culture, wells were tested for cytotoxicity against ^{51}Cr-labeled 12B1 pulsed or not for 1 h with *bcr-abl* peptide (5 μg/ml). Wells were considered positive when cytotoxicity exceeded three standard deviations over the mean value of control wells without responder cells. The CTLp frequency estimations were calculated from the Poisson equation. The figure depicts representative data from one of two similar experiments

that DC-GFKQSSKAL significantly increased CTLp more effectively than empty DC or DC loaded with irrelevant peptide. Interestingly, susceptibility to lysis was not substantially higher in 12B1 cells incubated with exogenous *bcr-abl* peptide than in 12B1 alone, suggesting that the effector cells generated may recognize endogenously processed *bcr-abl* fusion peptides (data not shown).

Efficacy of *bcr-abl*-peptide-pulsed DC in generating protective immunity

To determine whether or not DC pulsed with the *bcr-abl* fusion peptide, GFKQSSKAL, are effective in stimulating protective immunity, DC-peptide-immunized mice were challenged i.v. with 12B1 cells 1 week after their second immunization. Since DC-HYLSTQSALSK was no better than unpulsed DC in generating cytotoxicity in vitro, this irrelevant peptide was not included in the in vivo survival experiments. Figure 6 depicts survival data comparing the effect of GFKQSSKAL-loaded DC vaccination to that of DC or GFKQSSKAL alone. Mice immunized with *bcr-abl*-GFKQSSKAL-loaded DC survived significantly longer than mice immunized with GFKQSSKAL or unpulsed DC.

To determine whether the addition of exogenous peptide can increase the susceptibility of 12B1 to lysis by the in vivo generated CTL, we challenged groups of naïve and immunized mice with 12B1 cells that had been previously incubated with the *bcr-abl* peptide, GFKQSSKAL. Animals challenged with "peptide-coated" 12B1 cells did not survive longer than those receiving 12B1 cells alone (data not shown). This would indicate that exogenous peptide did not increase the susceptibility of 12B1 cells to killing in vivo by effector

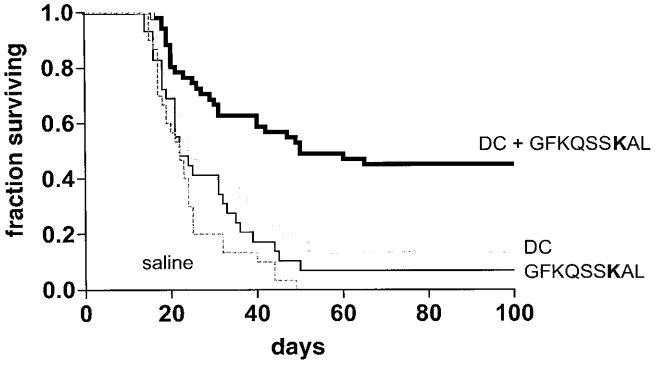


Fig. 6 Effect of *bcr-abl*-loaded DC vaccination on survival following 12B1 leukemia challenge. Mice were immunized s.c. (using the protocol described in Materials and methods) on days −14 and −7 and challenged on day 0 with 66 12B1 cells given i.v. Data represent pooled results from three experiments (n = 26–30 mice/group). P values versus saline control: GFKQSSKAL NS, DC 0.01, DC + GFKQSSKAL <0.0001; versus GFKQSSKAL: DC 0.29, DC + GFKQSSKAL 0.0001; versus DC: DC + GFKQSSKAL 0.001

cells generated following DC-GFKQSSKAL immunization.

Additional experiments were performed to assess whether immunization of mice bearing 12B1 leukemia would result in elimination of preexisting disease. Mice were given 12B1 cells on day 0 and immunized with saline, DC-HYLSTQSALSK or DC-GFKQSSKAL on days +2 and +4. This regimen failed to prolong survival of leukemic mice significantly (data not shown).

Discussion

12B1 is a murine leukemia cell line derived by retroviral transduction of BALB/c bone marrow cells with the *bcr-abl* (b_3a_2) fusion gene and consequently expresses the p210 *bcr-abl* protein [21]. The susceptibility of this cell line to lysis by CTL was first described by Chen et al. [6]; however, an in vivo model of 12B1 has not previously been described. The experiments presented herein demonstrate that intravenous inoculation of 12B1 *bcr-abl*$^+$ cells into BALB/c mice results in a disseminated acute leukemia analogous to human chronic myelogenous leukemia in blast crisis. Histological sections of liver and spleen and PCR analysis of peripheral blood, bone marrow, liver, spleen and lymph nodes confirmed the presence of *bcr-abl*$^+$ cells in these murine tissues, while Western blot data demonstrated the expression of the fusion protein in 12B1 cells.

Previous reports have documented that *bcr-abl*-specific CTL clones can be generated in BALB/c mice, but these effectors could only lyse 12B1 cells that had been incubated with synthetic peptides (i.e. "coating" their surfaces) [7]. In those experiments, priming mice with a mixture of 21 *bcr-abl* peptides emulsified with complete Freund's adjuvant failed to generate effective killers. It was speculated that such priming yielded low-affinity CTL with an inability to lyse leukemic cells displaying low levels of endogenously processed peptide on their surface. In our studies, we used DC loaded with a single *bcr-abl* chimeric nonapeptide GFKQSSKAL, which has the highest binding affinity for H-2K^d (Dr. Kenneth Parker, http://www-bimas.dcrt.nih.gov/molbio/hla_bind/index.html). We found that GFKQSSKAL-loaded DC can generate cytolytic effectors capable of lysing 12B1 cells, suggesting that when the most effective antigen-presenting cells are pulsed with a suitable peptide they can elicit high-affinity effector cells that may recognize endogenous *bcr-abl* peptides on leukemic cells. Moreover, we have provided new in vivo evidence that the cellular response generated by DC-peptide immunization was potent enough to prolong survival of mice challenged with 12B1 leukemia. We were not able, however, to demonstrate efficacy of a therapeutic vaccination using DC-GFKQSSKAL. This is likely due to the fact that, in an established leukemia setting, the generation of an immune response is overwhelmed by the rapidly progressing leukemia.

Immunization with unpulsed DC has been previously described to provide some protection against tumor challenge [24]. This is secondary to tissue-culture calf serum components, which may be processed and presented to T cells in vivo resulting in the generation of CTL against serum proteins. If immunized mice are challenged with tumor cells propagated in vitro, anti-(calf serum) CTL may cross-react with tumor cells carrying bovine serum epitopes on their MHC molecules. Porgador et al. reported that induction of CTL responses to ovalbumin peptides were obscured by the high levels of nonspecific lysis [24]. They attributed this to concurrent generation of CTL against fetal calf serum antigens. In order to avoid these nonspecific T cell responses, we grew our DC in serum-free medium. Furthermore, in all the experiments described herein, mice were challenged with 12B1 cells grown in vivo as subcutaneous tumors, processed and washed in serum-free medium prior to i.v. leukemia induction. Despite the precautions taken to minimize nonspecific responses, exposure to unpulsed DC increased the number of splenic T cells in mice. These cells produced considerable amounts of Th1-type cytokines, IFNγ and IL-2, and had cytolytic activity against 12B1. It is feasible that the production of IFNγ and IL-2 may augment anti-leukemia responses by inducing lymphokine-activated killer (LAK) activity in vivo. Earlier studies have documented that antigen-presenting cells such as DC or macrophages can promote LAK activity mediated by T cells [2, 28, 29]. In vitro studies demonstrated somewhat increased lytic activity of splenocytes from mice receiving unpulsed DC. This effect was also evident in vivo, with mice treated with empty DC surviving somewhat longer than those receiving saline.

The importance of T cells in preventing relapse following allogeneic stem cell transplantation has been well documented in CML [1, 11, 14, 20]. Relapse rates are higher in syngeneic transplants than in allogeneic transplants, and higher in related matched transplants than in unrelated. T cell depletion has decreased the incidence and severity of graft-versus-host disease at the expense of increasing relapse rates [14]. The role of T cells in eliciting a graft-versus-leukemia effect in CML is further supported by induction of sustained remissions following donor leukocyte infusions in patients relapsing after allogeneic bone marrow transplantation (BMT) [3, 10, 18, 25, 27]. The capacity, therefore, to generate leukemia-specific CTL may have clinical implications in CML. Several laboratories have documented that human CD4$^+$ and CD8$^+$ T cells can be elicited against *bcr-abl* peptides [5–8, 19, 22, 31]. Moreover, DC from patients with CML, which constitutively express *bcr-abl* protein and thus present *bcr-abl* peptides, have been shown to be capable of stimulating anti-leukemic CTL responses in vitro [8].

Our data indicate that vaccination with DC pulsed with a chimeric peptide spanning the *bcr-abl* joining region can induce significant immunoprotection against leukemia in vivo. Our model more closely resembles

blast crisis that is typically difficult to treat. As a result we are optimistic that this form of immunotherapy may be even more effective in chronic-phase CML. We are, however, aware that the relevance of the murine immune response is unclear. Whether this strategy will be effective in human CML remains to be determined and may depend on additional factors. For example, the ability of a *bcr-abl* peptide to stimulate an immune response is dependent on its binding affinity to certain MHC class I molecules, thus restricting this treatment to a subset of patients. Moreover, as opposed to mice, patients with CML may be tolerant to *bcr-abl* fusion protein. Despite these potential limitations, a DC-based peptide vaccine appears to be a promising new immunobiological approach that warrants further investigation.

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References

1. Appelbaum FR, Clift R, Radich J, Anasetti C, Buckner CD (1995) Bone marrow transplantation for chronic myelogenous leukemia. Semin Oncol 22: 405
2. Atzpodien J, Gulati SC (1993) T cells and monocytes regulate the generation and functional activity of natural killer-derived lymphokine-activated killer cells. Stem Cells 11: 511
3. Bacigalupo A, Soracco M, Vassallo F, Abate M, Van Lint MT, Gualandi F, Lamparelli T, Occhini D, Mordini N, Bregante S, Figari O, Benvenuto F, Sessarego M, Fugazza G, Carlier P, Valbonesi M (1997) Donor lymphocyte infusions (DLI) in patients with chronic myeloid leukemia following allogeneic bone marrow transplantation. Bone Marrow Transplant 19: 927
4. Bausero MA, Panoskaltsismortari A, Blazar BR, Katsanis E (1996) Effective immunization against neuroblastoma using double-transduced tumor cells secreting GM-CSF interferon-gamma. J Immunother 19: 113
5. Bocchia M, Korontsvit T, Xu Q, Mackinnon S, Yang SY, Sette A, Scheinberg DA (1996) Specific human cellular immunity to *bcr-abl* oncogene-derived peptides. Blood 87: 3587
6. Bosch GJ ten, Joosten AM, Kessler JH, Melief CJ, Leeksma OC (1996) Recognition of BCR-ABL positive leukemic blasts by human $CD4^+$ T cells elicited by primary in vitro immunization with a BCR-ABL breakpoint peptide. Blood 88: 3522
7. Chen W, Qin H, Reese VA, Cheever MA (1998) CTLs specific for *bcr-abl* joining region segment peptides fail to lyse leukemia cells expressing p210 *bcr-abl* protein. J Immunother 21: 257
8. Choudhury A, Gajewski JL, Liang JC, Popat U, Claxton DF, Kliche KO, Andreeff M, Champlin RE (1997) Use of leukemic dendritic cells for the generation of antileukemic cellular cytotoxicity against Philadelphia chromosome-positive chronic myelogenous leukemia. Blood 89: 1133
9. Cullis JO, Barrett AJ, Goldman JM, Lechler RI (1994) Binding of BCR/ABL junctional peptides to major histocompatibility complex (MHC) class I molecules: studies in antigen-processing defective cell lines. Leukemia 8: 165
10. Drobyski WR, Keever CA, Roth MS, Koethe S, Hanson G, McFadden P, Gottschall JL, Ash RC, Tuinen P van, Horowitz MM (1993) Salvage immunotherapy using donor leukocyte infusions as treatment for relapsed chronic myelogenous leukemia after allogeneic bone marrow transplantation: efficacy and toxicity of a defined T-cell dose. Blood 82: 2310
11. Enright H, Daniels K, Arthur DC, Dusenbery KE, Kersey JH, Kim T, Miller WJ, Ramsay NK, Vercellotti GM, Weisdorf DJ, McGlave PB (1996) Related donor marrow transplant for chronic myeloid leukemia: patient characteristics predictive of outcome. Bone Marrow Transplant 17: 537
12. Fields RC, Osterholzer JJ, Fuller JA, Thomas EK, Geraghty PJ, Mule JJ (1998) Comparative analysis of murine dendritic cells derived from spleen and bone marrow. J Immunother 21: 323
13. Gillis S, Ferm MM, Ou W, Smith KA (1978) T cell growth factor: parameters of production and a quantitative microassay for activity. J Immunol 120: 2027
14. Goldman JM, Gale RP, Horowitz MM, Biggs JC, Champlin RE, Gluckman E, Hoffmann RG, Jacobsen SJ, Marmont AM, McGlave PB (1988) Bone marrow transplantation for chronic myelogenous leukemia in chronic phase. Increased risk for relapse associated with T-cell depletion. Ann Intern Med 108: 806
15. Kaplan EL, Meier P (1958) Nonparametric estimation from incomplete observations. J Am Stat Assoc 53: 457
16. Katsanis E, Xu Z, Anderson PM, Dancisak BB, Bausero MA, Weisdorf DJ, Blazar BR, Ochoa AC (1994) Short-term ex vivo activation of splenocytes with anti-CD3 plus IL-2 and infusion post-BMT into mice results in in vivo expansion of effector cells with potent anti-lymphoma activity. Bone Marrow Transplant 14: 563
17. Klein A de, Kessel AG van, Grosveld G, Bartram CR, Hagemeijer A, Bootsma D, Spurr NK, Heisterkamp N, Groffen J, Stephenson JR (1982) A cellular oncogene is translocated to the Philadelphia chromosome in chronic myelocytic leukaemia. Nature 300: 765
18. Kolb HJ, Schattenberg A, Goldman JM, Hertenstein B, Jacobsen N, Arcese W, Ljungman P, Ferrant A, Verdonck L, Niederwieser D (1995) Graft-versus-leukemia effect of donor lymphocyte transfusions in marrow grafted patients. European Group for Blood and Marrow Transplantation Working Party Chronic Leukemia (see comments). Blood 86: 2041
19. Mannering SI, McKenzie JL, Fearnley DB, Hart DN (1997) HLA-DR1-restricted *bcr-abl* (b_3a_2)-specific $CD4^+$ T lymphocytes respond to dendritic cells pulsed with b_3a_2 peptide and antigen-presenting cells exposed to b_3a_2 containing cell lysates. Blood 90: 290
20. McGlave P, Bartsch G, Anasetti C, Ash R, Beatty P, Gajewski J, Kernan NA (1993) Unrelated donor marrow transplantation therapy for chronic myelogenous leukemia: initial experience of the National Marrow Donor Program. Blood 81: 543
21. McLaughlin J, Chianese E, Witte ON (1987) In vitro transformation of immature hematopoietic cells by the P210 BCR/ABL oncogene product of the Philadelphia chromosome. Proc Nat Acad Sci USA 84: 6558
22. Pawelec G, Max H, Halder T, Bruserud O, Merl A, Silva P da, Kalbacher H (1996) BCR/ABL leukemia oncogene fusion peptides selectively bind to certain HLA-DR alleles and can be recognized by T cells found at low frequency in the repertoire of normal donors. Blood 88: 2118
23. Peto R, Peto J (1972) Asymptotically efficient rank invariant procedures. J R Stat Soc A135: 185
24. Porgador A, Gilboa E (1995) Bone marrow-generated dendritic cells pulsed with a class i-restricted peptide are potent inducers of cytotoxic t lymphocytes. J Exp Med 182: 255
25. Porter DL, Roth MS, McGarigle C, Ferrara JL, Antin JH (1994) Induction of graft-versus-host disease as immunotherapy for relapsed chronic myeloid leukemia. N Engl J Med 330: 100
26. Rammensee HG, Friede T, Stevanoviic S (1995) MHC ligands and peptide motifs: first listing. Immunogenetics 41: 178
27. Rhee F van, Lin F, Cullis JO, Spencer A, Cross NC, Chase A, Garicochea B, Bungey J, Barrett J, Goldman JM (1994) Relapse of chronic myeloid leukemia after allogeneic bone marrow transplant: the case for giving donor leukocyte transfusions before the onset of hematologic relapse. Blood 83: 3377

28. Sawada H, Abo T, Sugawara S, Kumagai K (1988) Prerequisite for the induction of lymphokine-activated killer cells from T lymphocytes. J Immunol 140: 3668
29. Silvennoinen O, Vakkila J, Hurme M (1988) Accessory cells, dendritic cells, or monocytes, are required for the lymphokine-activated killer cell induction from resting T cell but not from natural killer cell precursors. J Immunol 141: 1404
30. Taswell C, MacDonald HR, Cerottini JC (1980) Clonal analysis of cytolytic T lymphocyte specificity. I. Phenotypically distinct sets of clones as the cellular basis of cross-reactivity to alloantigens. J Exp Med 151: 1372
31. Yotnda P, Firat H, Garcia-Pons F, Garcia Z, Gourru G, Vernant JP, Lemonnier FA, Leblond V, Langlade-Demoyen P (1998) Cytotoxic T cell response against the chimeric p210 BCR-ABL protein in patients with chronic myelogenous leukemia. J Clinical Invest 101: 2290