Immunotherapy of High-Risk Acute Leukemia with a Recipient (Autologous) Vaccine Expressing Transgenic Human CD40L and IL-2 After Chemotherapy and Allogeneic Stem Cell Transplantation

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ABSTRACT

CD40L generates immune responses in leukemia-bearing mice, an effect that is potentiated by IL-2. We studied the feasibility, safety and immunologic efficacy of an IL-2- and CD40L-expressing recipient-derived tumor vaccine consisting of leukemic blasts admixed with skin fibroblasts transduced with adenoviral vectors encoding hIL-2 and hCD40L. Ten patients (including 7 children) with high-risk acute myeloid (n=4) or lymphoblastic (n=6) leukemia in cytologic remission (after allogeneic stem cell transplantation n=9 or chemotherapy alone n=1) received up to six subcutaneous injections of the IL2/CD40L vaccine. None of the patients were receiving immunosuppressive drugs. No severe adverse reactions were noted. Immunization produced a 10- to 890-fold increase in the frequencies of MHC-restricted T cells reactive against recipient-derived blasts. These leukemia-reactive T cells included both Tc/Th1 and Th2 subclasses, as determined from their production of granzyme-B, interferon-γ and interleukin-5. Two patients produced systemic IgG antibodies that bound to their blasts. Eight patients remained disease-free for 27-62 months after treatment (5-year overall survival, 90%). Thus, even in heavily treated patients, including recipients of allogeneic stem cell transplants, recipient-derived antileukemia vaccines can induce immune responses reactive against leukemic blasts. This approach may be worthy of further study, particularly in patients with a high risk of relapse.
INTRODUCTION

Acute leukemia cells can express a range of tumor-associated or tumor-specific antigens. Peptides derived from these antigens have shown promise in early-phase clinical studies of acute myeloid leukemia (AML). However, specific peptides may not be present in many subtypes of leukemia, or they may be restricted to particular HLA phenotypes, or the immune response may select cells that have lost the single peptide epitope to which the response is directed. An alternative approach is to use the entire leukemic cell as an immunogen. Both acute lymphoblastic leukemia (ALL) and AML are potentially well suited to this approach.

Cells from ALL patients express both Class I and Class II major histocompatibility complex (MHC) molecules, allowing the oncogene products to be directly presented to both CD8+ and CD4+ T cells. However, the leukemic cells generally do not express costimulatory surface molecules such as B7.1 (CD80) or B7.2 (CD86), which are necessary for induction of a T-cell response. Consequently, they induce specific T-cell anergy instead of specifically activating T cells. This lack of conventional costimulatory molecules notwithstanding, more than half of ALL cases express the CD40 antigen, the receptor for the CD40 ligand (CD40L), a potent costimulatory molecule in its own right. Engagement of CD40L augments the antigen-presenting function of normal and malignant B cells by upregulating the expression of intercellular adhesion molecules, as well as the costimulatory molecule B7.1, MHC Class I and II molecules and T-cell chemokines. Direct stimulation through the CD40-CD40L pathway activates dendritic cells and bypasses the classical CD4+ helper cell
mechanism in activating specific cytotoxic T cells (CTLs). Activation of CD40-positive leukemia and lymphoma cells by CD40L enables these cells to generate an antitumor immune response \textit{ex vivo}.\cite{5-7,12-16}

Costimulatory molecules also play an important role in generating a T-cell response against AML cells.\cite{17-19} CD40L directly stimulates CD4$^+$ and CD8$^+$ T cells that have become activated by engagement of tumor antigens on professional antigen-presenting cells (APCs), and cross-linking of CD40L on interleukin-2 (IL-2) -activated natural killer (NK) cells redirects their cytolysis to CD40L negative target cells.\cite{20-26} Hence, transgenic expression of the CD40L molecule on leukemic blast cells may improve the presentation of leukemia-specific antigens, so that antileukemic immune responses are produced through direct recruitment of effector T cells or activation of professional APCs. Human \textit{ex vivo} and murine \textit{in vivo} studies have shown that addition of IL-2 to these systems further enhances the effects of CD40L expression, most likely by expanding and sustaining the recruited effector cells.\cite{4,27,28}

Unfortunately, direct transduction of primary human leukemic cells remains inefficient and unpredictable, posing a major obstacle to adequate expression of any transgene. We therefore expressed the costimulatory molecule CD40L and transgenic IL-2 in accessory cells (syngeneic fibroblasts) and administered them with the patient’s own leukemic cells. In preclinical studies, this approach generated an antileukemic immune response \textit{in vivo},\cite{27} while usually avoiding unwanted immune reactions against normal host tissues.\cite{29} We now demonstrate the feasibility and safety of administering this
vaccine to patients in remission of high risk leukemia following allogeneic stem cell transplantation (n=9) or chemotherapy alone (n=1). We find leukemia reactive immune responses may be generated in the absence of autoimmune or graft versus host disease.
METHODS

Patients. The clinical protocol was approved by the Institutional Review Board of Baylor College of Medicine, by the Food and Drug Administration and by the Recombinant DNA Advisory Committee of the National Institutes of Health. Informed consent was provided according to the Declaration of Helsinki. Patients were eligible for leukemic cell collection and vaccine preparation if they were younger than 75 years at diagnosis and had primary or relapsed ALL (pre-B, B, T, B-cell precursor, or Burkitt-type if bone marrow blasts > 20%), or AML (M0 to M7) or myelodysplastic syndrome considered to be at high-risk of relapse (predicted relapse risk for this group >50% at 2 years). Patients were eligible for vaccine administration if their disease had entered complete or partial cytological remission (<20% blasts infiltrating the bone marrow) after a second or higher line of conventional and/or high-dose chemotherapy. Patients were enrolled a minimum of 100 days after allogeneic stem cell transplantation or a minimum of 1 week after their last course of chemotherapy and/or immunosuppressive drugs. They had recovered from the toxic effects of all prior chemotherapy before entering the study, and had an absolute neutrophil count (ANC) >500/μL, an absolute lymphocyte count (ALC) >200/μL, and a platelet count >50,000/μL. Exclusion criteria were: active infection at the time of entry, acute or chronic graft versus host disease (GvHD), disease progression (bone marrow blasts >20%), bilirubin >1.5 mg/dL, creatinine >1.5 mg/dL, ECOG performance status >2, and a life expectancy <10 weeks.

Adenoviral vectors. The hIL-2- and hCD40L-encoding adenoviral vectors were constructed by recombination with the Clal fragment of Ad-dl327, an E1a/b and E3
deletion mutant of human adenovirus serotype 5. Transgenes were under transcriptional control of the RSV (Rous sarcoma virus) promoter. The source of hCD40L cDNA was the pBluescriptII SK phagemid, pBS-hCD40L-6A9 (American Type Culture Collection, Manassas, VA) cloned by Gauchat and colleagues. The source of the hIL-2 cDNA is described elsewhere. Clinical grade adenoviral vectors were made, propagated and tested according to local and Federal requirements. The AdhIL2 and AdhCD40L vectors had titers of $6.6 \times 10^{11}$ IU/mL and $7.5 \times 10^{10}$ IU/mL, respectively. Both vectors had a VP:IU ratio of <30:1.

**Recipient-derived vaccine preparation.** All manufacturing procedures followed proposed Good Tissue Practices (GTP) and were approved by the Food and Drug Administration under IND#8243. Recipient-derived leukemic cells were enriched from peripheral blood or bone marrow mononuclear cells on a Lymphocyte Separation Medium gradient (ICN Pharmaceuticals Inc., Costa Mesa, CA) and stored at -170°C in liquid nitrogen until further needed. Blasts were also obtained by therapeutic leukapheresis. Enrichment was controlled by quantifying the clone with use of leukemia-associated surface markers and was considered adequate if >65% blast cells were present. Recipient-derived fibroblasts were collected by a 6-mm punch biopsy from the patient’s own skin prior to the study and were cultured in RPMI1640 medium (BioWhittaker, Walkersville, MD) with 10% fetal bovine serum (Hyclone, Logan, UT). After expansion of the fibroblasts to numbers adequate for the preparation of at least six injections, they were divided into two groups, each transduced with either Ad-hIL-2 at a multiplicity of infection (MOI) of 500 VPs/cell or Ad-hCD40L at an MOI of 1000 VPs/cell. Gene
expression was considered adequate if CD40L expression was >20% as measured by flow cytometry, and IL-2-production was >150 pg/10^6 cells/24 hr as measured by ELISA. Fibroblasts were frozen 24 hours after transduction and stored at -170°C in liquid nitrogen. All fibroblast preparations were negative for bacteria, fungi, mycoplasma, adventitious viruses, and replication-competent adenoviruses, and lacked measurable endotoxin. Neither irradiation (3000 cGy) nor a freeze-thaw cycle impaired transgene expression or fibroblast recovery/viability, but irradiated fibroblasts did not divide. Upon thawing, CD40L expression by fibroblasts was detected for up to 10 days, with a mean peak expression of 40% at 6 days after adenoviral gene transfer. Similarly, IL-2 secretion in the supernatant of transduced fibroblasts was detected for up to 10 days, with a mean peak secretion of 50,000 pg/10^6 cells per 24 hr on day 6.

Treatment. The treatment plan consisted of up to six subcutaneous injections of a fixed dose of irradiated (4000 cGy) recipient-derived leukemic blasts (2x10^7/injection), admixed with a fixed dose of IL-2-secreting irradiated (3000 cGy) recipient-derived skin fibroblasts (2x10^7/injection), and an escalating dose of CD40L-expressing irradiated (3000 cGy) fibroblasts, from 2x10^5/injection (dose-level 1) to 2x10^7/injection (dose-level 3). All injections were given subcutaneously in the upper arm in 1-ml volumes. The first three injections were given at weekly intervals, followed by a 2-week rest. If the first three injections were well tolerated, and if there was no evidence of tumor progression, patients received three additional injections of IL-2-secreting and CD40L-expressing fibroblasts admixed with leukemic blasts, separated by 2-week intervals.
Evaluation of toxicity and antitumor responses. Patients were monitored for local and systemic toxicity by physical examination and blood chemistry analysis at weekly intervals. Toxic reactions were graded according to National Cancer Institute criteria. Antitumor immune responses were assessed at weekly intervals for 10 weeks after the first injection, then every other week for 6 weeks, then monthly for 1 year. At 12 weeks and 6 months after the first injection, the disease status of patients was determined by clinical evaluation according to the WHO classification (WHO Handbook for Reporting Results for Cancer Treatment, Geneva 1979) and by bone marrow aspiration.

Phenotyping of local lesions. Injection-site skin biopsies were performed in all patients 1 week after the first and the second vaccine injections. Samples were immediately fixed in formalin and processed overnight. Immunohistochemical staining was done by a standard avidin/biotin technique used with the Optimax automated stainer (Biogenics, San Ramon, CA). The antibodies used included CD4 (clone OPD4, Dako, Carpinteria, CA) and CD8 (clone C8/144B, Dako) for lymphoid cells; S-100 (mono/polyclonal mix; Ventana, Tucson, AZ) for dendritic cells; and CD1a (clone O10, Immunotech, Westbrook, ME) for Langerhans cells. HLA-DR (clone TAL.1B5, Dako) was used as a marker for GvHD. CD68 (clone KP-1, Dako) and CD83 (clone 1H4b, Vision Biosystems, Norwell, MA) were used on biopsies from patients with discernible dendritic cell infiltrate as a marker of dendritic cell activation and maturation. Control skin biopsies were obtained from healthy volunteers on the research team.
Phenotyping of peripheral blood mononuclear cells. Fresh PBMCs were phenotyped before and after each immunization in all evaluable patients, by flow cytometric analysis (FACScan; Becton-Dickinson, San Jose, CA) with antibodies to: CD3, CD4, CD8, TCR-αβ, TCR-γδ, κ and λ chains, CD11b, CD15, CD16, CD19, CD20, CD25, CD27, CD45, CD45RA, CD45RO, CD56, CD57, CD69, HLA-DR (Becton-Dickinson) and LAG-3 (clone 17B4, Alexis Biochemical, San Diego, CA). The values for patients were compared with those for matched historical controls (a cohort of transplanted, non-vaccinated patients at our institution).

Assessment of T cytotoxic and helper-T profile by granzyme B (GrB), interferon-γ (IFN-γ) and interleukin-5 (IL-5) ELISPOT. When adequate numbers of PBMCs and target cells were available, we measured the profile of T cells responding to the immunizing blast cells using GrB, IFN-γ and IL-5 ELISPOT (Pharmingen/BD Bioscience, San Diego, CA). Frozen PBMC obtained pre- and postimmunization were thawed and seeded at 3x10^5 to 5x10^5 cells per well in a 96-well plate coated with monoclonal antibodies (mAbs) specific for GrB, IFN-γ or IL-5 in RPMI1640 (Biowhittaker) containing 5% heat-inactivated human AB serum (Gemini BioProducts, Woodland, CA) and 1% L-glutamine (Biowhittaker). When enough PBMCs were available, MHC restriction was determined by blocking CD4 or CD8 surface receptors on T cells by preincubating the PBMCs for 30 minutes at 37°C with either 10 μL of an anti-CD4 or an anti-CD8 azide-free mAb (Immunotech). Target cells consisted of unmodified recipient-derived leukemic blasts obtained prior to chemotherapy. Controls consisted of the NK-specific K562 myeloid cell
line, of recipient-derived skin fibroblasts and PHA blasts (obtained from each patient's prevaccination PBMCs). To control for nonspecific cytokine release during post-transplantation immune recovery, we assessed IFN-γ release by PBMCs from transplanted patients in an ELISPOT assay specific for pp65 (Jerini Bio Tools GmbH, Berlin, Germany), a potent CMV antigen. Frozen target cells were thawed and plated at 1x10^5 to 3x10^5 cells per well. Plates were incubated at 37°C, 5% CO2 for 24 (IFN-γ and GrB) or 48 hours (IL-5). Cells were then discarded and wells were washed thoroughly. Biotinylated secondary antibody to either GrB, IFN-γ or IL-5 was then added to the wells, with all subsequent steps following the manufacturer's recommendations. Spot analysis was performed with a Series 1 ImmunoSpot Image Analyzer® and software (Cellular Technologies Ltd, Cleveland OH). The frequency of positive (GrB-, IFN-γ- or IL-5-producing) cells compared to the total number of plated cells was calculated after the number of positive cells in the control wells had been subtracted from that of the experimental wells. These control wells contained nonstimulated PBMCs (spontaneous GrB, IFN-γ or IL-5 release), target cells alone or reagents alone (blanks).

Detection of circulating IgG against the immunizing cells. We studied the binding of antibodies produced after immunization with recipient-derived leukemic blasts and skin fibroblasts. Reactivity was also measured against several leukemia cell lines, including K562 (acute phase CML), CCL-120 (B-ALL, EBV positive), Daudi (Burkitt-like ALL), MOLT-3 (T-ALL) and an unrelated tumor cell line, IMR-32 (neuroblastoma, GD₂⁺). The cells were incubated with 5 µl or 50 µl of autologous plasma (diluted 1:2 in PBS) that
had been obtained immediately before vaccination, 1 to 2 weeks after the third and sixth vaccinations, or 6 and 12 months after the first vaccination. Negative controls consisted of pooled plasma obtained from multiple normal donors. The method used to detect circulating IgG is described elsewhere. In brief, bound IgG was detected with biotinylated F(ab’)2 (H+L) fragments of donkey antihuman IgG (Jackson Immuno Research, West Grove, PA) followed by Neutralite-Avidin-R-PE (Southern Biotechnology Associates, Birmingham, AL). All detection steps were repeated once at room temperature for 10 minutes. More than 10^5 cells were analyzed with a FACScan flow cytometer (Becton-Dickinson).

Statistical analyses. When warranted results of pre- and postimmunization assays were compared by 2-tailed paired t-test or Wilcoxon signed rank analysis and analysis of variance (one-way ANOVA) using SPSS software (SPSS, Inc., Chicago, IL).
RESULTS

Patients. The ages of the patients at study entry ranged from 4 to 56 years with a median of 15.5 years (Table 1). They had all suffered a relapse of their acute leukemia, except for patients 5, 8 and 9, who were eligible for vaccination because of pre-B ALL with the Philadelphia chromosome, myelodysplastic syndrome preceding AML, or secondary AML following treatment for Hodgkin disease. Of the 44 patients from whom blast cells were collected, 10 received their vaccines. Twenty-seven of the remaining 34 patients did not receive their vaccine because they lacked enough blasts for vaccine preparation, 5 chose another treatment option, and in 2, vaccines became available after the maximal accrual was met. Overall, the completion rate was 23%.

All but one patient were in complete cytological remission of their acute leukemia at the time of immunization. Patient 1, who was enrolled at dose-level 1 while in partial remission of Burkitt-like B leukemia, died of rapid disease progression before the third injection and was not evaluated for systemic immunological responses. Patient 5 had insufficient leukemic blasts after vaccine preparation for follow-up studies of immune responses to the vaccine; all other patients were evaluable for this endpoint. At the time of immunization, none of the patients had received any cytotoxic or radiation therapy for at least 4 weeks prior to enrollment. All other immunosuppressive drugs had been stopped an average of 71 days prior to the first vaccine administration (median 45 days; range 14-195 days) (Table 1). The mean neutrophil count at the time of the first vaccine injection was 322/μL (range, 171-609/μL) and the mean lymphocyte count was 521/μL (range, 158-1071/μL).
Toxicity. CD40L-expressing and IL-2-secreting skin fibroblasts produced clinically significant adverse effects in only 1 patient (no. 10), who developed a localized abscess at the site of injection 5. Surgical drainage revealed sterile pus; blood cultures were negative. There was no evidence of GvHD or autoantibodies in any of the post-transplant patients after immunization.

Local responses to vaccine injection. We observed mild, transient erythema at the injection sites in 3 patients (nos. 1, 6 and 9) after two or more injections. Twenty injection-site punch biopsies, performed on all 10 patients at 1 week after the first and second injections, were compared with skin biopsies from 2 healthy volunteers. None of the specimens contained leukemic cells. Inflammatory reactions were graded according to the highest density of lymphocytes in either the dermal perivascular or subcutaneous regions (mild, < 10 cells/hpf; moderate, 10-50/hpf; severe, > 50/hpf). Mild responses were noted in patients 8, 9 and 10; moderate in 1, 5 and 6; and severe in 2, 3, 4 and 7. The degree of inflammation increased from the first to the second biopsy in 5 patients: from moderate to severe in nos. 2, 3, 4 and 7 and from mild to moderate in no. 5. All patients showed a reversal of the normal 2:1 CD4 to CD8 ratio, with nos. 2, 9 and 10 having a marked CD8 infiltrate (from 1:17 to 1:60; Fig. 1G,H). Increased numbers of Langerhans cells in the perivascular areas of the dermis were noted in 8 of 10 patients (nos. 2-9; Fig. 1E,F). In the 6 biopsies from the 3 patients with the most discernible dendritic cell infiltrate, most cells were strongly CD1a positive. Perinuclear areas stained strongly for HLA-DR while cell membranes were less positive. Cytoplasmic staining revealed a fine diffuse granular cytoplasmic pattern for CD68 whereas CD83 was
negligible. These findings are consistent with activated, immature dendritic cells with no mature dendritic cells.

**Systemic responses to injection:** Mean (± SEM) peripheral blood cell counts (absolute and percentages) were determined in all 8 evaluable patients before and 7 days after the completion of inoculations. There was a modest but nonsignificant increase in the absolute number of circulating lymphocytes (Fig. 2; preimmunization 533±99/μL vs. postimmunization 722±139/μL, P>0.05), while the proportion of lymphocytes in the total leukocyte population did not change (preimmunization 28±5% vs. postimmunization 33±4%, P>0.05).

**Systemic responses to injection: NK cell and B- and T-lymphocyte populations.** There was a 1.5-fold increase in the CD3+ CD4+ lymphocyte population (Fig. 2; preimmunization 254±31/μL vs. postimmunization 392±61/μL, P=0.027), a 1.6-fold increase (Fig. 2; 227±72/μL vs. 362±49/μL, P=0.041) in the absolute number of circulating CD3+ CD8+ lymphocytes, and a 1.2-fold increase (Fig. 2; 229±61/μL vs. 284±53/μL, P=0.042) in the absolute number of circulating CD3+ DR+ activated lymphocytes. The mean (± SEM) proportions of CD4+ and CD8+ cells before and after treatment were not significantly different (5.51±0.98% and 5.16±1.81% vs. 6.90±0.78% and 6.34±0.73%, respectively; P>0.05), nor were the mean (± SEM) proportions of DR+ cells among the CD3+ population (46.75±7.36% vs. 41.82±6.51%; P>0.05). We did not observe any variation in the proportions of CD4+ CD25dim LAG-3+ regulatory T cells,
which remained within normal ranges before and after treatment (Fig. 2; 1.59±0.24% vs. 1.46±0.25%, \(P=0.664\)). The absolute numbers of NK and B cells, as well as their proportions, remained statistically unchanged during treatment (data not shown).

**Leukemia-reactive, T-cell-mediated cytolytic and helper responses.** We determined the cytotoxic and helper T-cell activities against recipient-derived blasts in all evaluable patients before and 7 days after completion of immunizations, measuring GrB, IFN-\(\gamma\) and IL-5 release after coculture with whole recipient-derived leukemia cells. Using the immunizing cell line (unmanipulated recipient-derived blasts) and freshly thawed, unmanipulated PBMCs collected before, during or after immunizations, we detected systemic cytotoxic effector cell activity against the immunizing, recipient-derived leukemic blasts in 5 of 8 patients (nos. 2, 3, 4, 9 and 10; Table 2 and Fig. 3A). The increase in GrB release was statistically significant after 3 immunizations compared to pre-vaccination values (\(p=0.046\)). We also observed T-helper type 1 (Th1) and Th2 leukemia-reactive immune effectors in 4 patients (nos. 2, 4, 7 and 9; Table 2 and Fig. 3B) and 3 patients (nos. 2, 4 and 7; Table 2 and Fig. 3C), respectively. Increases in IFN-\(\gamma\) and IL-5 secretion were statistically significant after 6 immunizations compared to pre-vaccination values (\(p=0.046\) and 0.043, respectively). The cytotoxic- and Th1-mediated responses were associated with both CD4\(^+\) and CD8\(^+\) T cells, as demonstrated by blocking experiments with monoclonal antibodies to these T-cell surface markers (Fig. 3A-C). As expected, the Th2 responses were mediated solely by CD4\(^+\) T cells (Fig. 3D). Limitations in the availability of patients’ PBMC at follow-up allowed only the assessment of IFN-\(\gamma\) release against recipient derived PHA (T cell)
blasts and fibroblasts. There was no significant increase in IFN-γ secretion after immunization compared to pre-vaccination values ($p>0.5$ for skin fibroblasts, $p>0.345$ for PHA blasts), and there was no correlation with the responses observed against recipient-derived leukemic blasts (Table 3). No patient developed autoimmunity clinically or from measurement of ANA or anti red cell antibodies. Of note, unstimulated controls or samples stimulated with allogeneic blasts showed no change in GrB or cytokine secretion following immunization (data not shown). In recipients of CMV-seropositive bone marrow donors, IFN-γ release by pp65-stimulated PBMCs collected before and after immunization remained unchanged ($p>0.26$ between pre- and post-immunization values), indicating that the appearance of leukemia-reactive effector cells was not simply a reflection of the tempo of immune recovery after bone marrow transplantation (Table 4).

**Leukemia-reactive humoral responses.** In 2 patients (nos. 6 and 7), T-helper cell activity was also demonstrated by the development of IgG antibodies specific for the immunizing cell line in plasma samples (Table 5). Even though increases in specific IgG did not reach statistical significance ($p>0.091$), patients nos. 6 and 7 had a 2- to 3-fold increase compared to controls. These IgG antibodies reacted only with the immunizing recipient-derived blasts, and did not cross-react against the unmodified recipient-derived skin fibroblasts, other leukemia cell lines (K562, CCL-120, MOLT-3 and Daudi) or other tumors (IMR-32) (data not shown). Pooled sera from normal donors showed no reactivity against leukemic or nonleukemic cells (data not shown).
Antileukemic responses. As summarized in Table 1, patient 1 had rapidly progressing Burkitt-like leukemia after the second immunization. He was taken off study and therefore was not eligible for evaluation of either tumor or systemic immunologic responses. He died of disease 33 days after entering the study (152 days after allogeneic bone marrow transplantation). Patient 3 relapsed 152 days after entering the study (470 days after allogeneic bone marrow transplantation). Relapse was isolated to the scalp. The patient was taken off study and is disease free 4 years after a third complete remission induced with conventional chemotherapy. All other patients remained free of measurable disease at the 6-month evaluation and are off all therapy without any evidence of disease at a median follow up of 41 months (range, 27-61.6 months; 5-year overall survival, 90%).
DISCUSSION

We have shown that immunogenic, recipient-derived leukemia vaccines can be prepared from unmanipulated leukemic blasts and IL-2- and CD40L gene-modified skin fibroblasts, and can be readministered to patients after allogeneic bone marrow transplantation. The engineered immunizing cells recruited CD8$^+$ and CD4$^+$ T cells, as well as CD1a$^+$ Langerhans cells, to the injection site. Systemically, there was a modest but consistent rise in circulating CD4$^+$ CD8$^+$ and activated CD3$^+$ DR$^+$ lymphocytes post-immunization. More importantly, ELISPOT analysis showed a 10 to 890-fold rise in leukemia-reactive cytotoxic CD4$^+$ and CD8$^+$ T cells as well as Th1 and Th2 helper T cells that persisted up to 3 months after the last immunization. Both cytotoxic and Th1 lymphocytes were found in the CD4$^+$ and CD8$^+$ T-cell populations, whereas Th2 lymphocytes were exclusively from the CD4$^+$ pool. Two patients made antibodies that bound to the recipient-derived leukemic blasts, reinforcing the concept that the vaccine had the ability to recruit and stimulate CD4$^+$ Th2 cells. There was no evidence for vaccine-induced tolerance. We did not observe any increase in CD4$^+$ CD25$^+$ regulatory T cells, and all but 2 patients developed measurable leukemia-reactive cellular responses. Of the 2 apparent cellular nonresponders, 1 developed a leukemia-reactive antibody. Local responses and DTH-like immune infiltrates at the sites of inoculation also suggest that anergy had been reversed.

Despite the generation of effector cells reactive to the recipient-derived leukemic blasts, immunization did not produce GvHD, even though none of the patients were receiving immunosuppressive drugs for a median time of 45 days prior to immunization. In the absence of a control group, we cannot exclude the possibility that local inflammatory
changes induced by injection of fibroblasts alone or by adenoviral proteins could have induced an equivalent local and systemic effect, but prior ex vivo and in vivo studies suggest such an effect is unlikely.\textsuperscript{5-7,35-37} By contrast, CD40-CD40L interactions play a key role in B-cell activation and differentiation, and engagement of CD40L augments the antigen-presenting functions of normal and malignant B cells and of professional APCs\textsuperscript{10,20,33} by upregulating the expression of intercellular adhesion molecules as well as the costimulatory molecule B7.1 and MHC Class I and II molecules.\textsuperscript{5-7} Consequently, ex vivo activation of CD40-positive leukemia and lymphoma cells by CD40L enables these cells to generate an antitumor response in vitro.\textsuperscript{5-7,12} This effect is complemented by the action of IL-2, which allows other cytotoxic precursor cells to bypass their requirement for DC- or Th1-derived helper signals in the generation of a CTL response.\textsuperscript{34,35} Animal models indicate that expression of transgenic IL-2 augments immunogenicity across a broad range of tumors, including leukemic cell lines.\textsuperscript{36} IL-2, unlike many other T-cell stimulatory cytokines, rarely induces leukemic cell proliferation,\textsuperscript{37} even when these cells express IL-2 receptors.\textsuperscript{38-40} Murine models from our own and other groups demonstrate that combinations of immunomodulatory molecules that act on different levels of the immune response enhance antitumor responses, and the combination of IL-2-secreting cells injected concomitantly with CD40L-expressing cells was shown to be superior to each component alone.\textsuperscript{27,28} Thus, our observation that the CD40L and IL-2 combination can induce local and systemic CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells specific to recipient-derived leukemic blasts is consistent with the known effects of this combination on normal as well as leukemic cells in vitro and in vivo.\textsuperscript{4,27,28,41-43}
We do not know whether the leukemia reactivity we detect is attributable to recognition of tumor specific or tumor associated antigen, but the absence of autoimmune disease, of clinical GvHD and of any correlation between leukemia reactivity and reactivity with patient PHA blasts or fibroblasts all suggest that the antigen(s) recognized are of restricted tissue distribution. While leukemic cells are susceptible to cytotoxic effector activities both \textit{in vitro} and \textit{in vivo}, it is yet not clear which components of the immune and innate responses are most critical for the effective destruction of the host leukemia \textit{in vivo}.\footnote{44-46} Experimental results confirm the pivotal role of CD4$^+$ T cells in providing helper effects to CD8$^+$ cytotoxic T lymphocytes or to B cells.\footnote{47} Activated B cells, in turn, can produce specific antileukemic antibodies.\footnote{48} Alternatively, activation of the CD40-CD40L pathway can cross-prime professional APCs and bypass the need for CD4 T-cell help.\footnote{11,49-51} At present, we do not know if the recruitment of the local and circulating T-cell effectors we observed is the result of a direct effect of enhanced antigen presentation by leukemic blasts stimulated through the CD40-CD40L pathway, as previously suggested,\footnote{15,43,49,52,53} or whether the favorable milieu generated by the gene-modified fibroblasts encourages cross-priming of intradermal professional APCs.\footnote{54} Previous observations from our group support the activation of CD40$^+$ leukemia cells when stimulated with CD40L-secreting fibroblasts.\footnote{16} Alternatively, APCs stimulated through the CD40-CD40L pathway may take up leukemic cells or apoptotic bodies and present the processed antigen to T cells stimulated by IL-2-producing fibroblasts.\footnote{55} Subsequently, the APCs may migrate to locoregional lymph nodes to recruit additional leukemia-reactive T cells.\footnote{56,57} Our histologic and systemic immunologic analyses are consistent with either or both processes. Ideally, skin biopsies should also be performed
within one or two days of immunization to assess the early events in the vaccination process, but this would remove the antigenic stimulus and likely prevent the production of the systemic effects we observed. Certainly by day 7, the injected tumor cells have disappeared, and this may explain why the residual infiltrating dendritic cells are activated but not yet mature.

Despite efforts to develop vectors that will reliably and effectively transduce primary human acute leukemia cells, none have yet shown sufficient promise to enter clinic testing. Our strategy of using skin fibroblasts as “transfection partners” appears immunologically effective, producing an antileukemic but not an unwanted autoimmune response. Nonetheless, it would be desirable to develop a means to directly transduce the leukemic cells themselves. The availability of such a process would substantially simplify the logistics of the vaccine preparation. An alternative consists of ex vivo generation of CD40L-stimulated autologous ALL vaccines, which was tested in a recent phase I trial. While these vaccines failed to produce immune responses in patients with relapsed, active disease, they were potent APCs capable of stimulating allogeneic and peptide-specific T cells in vitro. Despite the lack of activity in vivo in the chosen setting, this approach may prove logistically feasible for a larger study in patients in complete remission. Even so, the complexity of any whole cell tumor vaccine approach would greatly exceed that of alternative methods, particularly the use of peptides. For example, proteinase 1 (PR1) is a target for chronic myeloid leukemia-specific CTLs, and vaccination with a PR1 peptide induces prolonged partial as well as complete clinical and molecular remissions in patients with myeloid malignancies. However, peptide epitopes, including those derived from PR1, are not available across the HLA repertoire,
may be inadequate for lymphoblastic leukemia, and may lead to the selection of variants with loss of single-epitope tumor antigens. We would argue, therefore, that the added complexities of whole leukemia cell vaccines can be offset by their ability to elicit a more broad-based and more robust immune response.

Most clinical trials of cancer vaccines or tumor-derived peptides have been conducted in patients with bulky disease. For many well-established reasons, this strategy reduces the likelihood of observing prolonged or complete tumor responses. Murine CD40L gene-expressing autologous B-CLL cells injected intravenously have induced measurable antitumor responses \textit{in vivo} in patients, but much of this activity may be due to the direct induction of apoptosis of CD40-expressing endogenous tumor cells. We therefore chose to immunize patients with only minimal residual disease, in whom tumor-cell mediated immune inhibition would be less effective, and the selection of resistant tumor antigen-loss variants less likely. The size of our study and the intentional immunization of high-risk patients without measurable disease mean that we cannot directly link clinical outcome to the observed MHC-restricted cytotoxic as well as helper T-cell antileukemic immune responses. The recent observation that the memory T-cell compartment is the least affected by chemotherapy may allow for improved immunization by vaccinating prior to high-dose chemotherapy, subsequently using vaccine boosts to induce re-expansion of this memory T-cell compartment.

In conclusion, our results demonstrate the feasibility of generating a recipient-derived leukemia vaccine combining transgenic CD40L with IL-2. The vaccine is not only well tolerated, but also induces the same pattern of antileukemic immunity predicted from
our murine model.\textsuperscript{27} Although the results are consistent with a therapeutic benefit, any estimate of the vaccine’s true value will require more rigorous testing in a randomized trial.
ACKNOWLEDGMENTS

We would like to thank Tatiana Gotsolva for excellent technical assistance, Pat Alcoser and Sheryl Rodgers for their dedicated patient care, and Shannon Inman and Kimberly East for study coordination and data management. We are indebted to Persis Amrolia, Gian-Pietro Dotti, Régis Costello and Jean-Charles Soria for their careful reading of the manuscript and their helpful comments. We would also like to thank John Gilbert for scientific editing and Debbie Graustein for editorial assistance. We are grateful to all of our medical colleagues who referred patients to this study. Finally, we thank the patients and their families for their trust and their commitment to medical research and, hopefully, progress.
REFERENCES


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<th>Sex</th>
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<th>GvHD prophylaxis</th>
<th>Time from last treatment to first injection (days)</th>
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Table 3. IFN-γ responses to recipient-derived PHA blasts and skin fibroblasts before and after immunizations

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### Table 4. Interferon-γ response to pp65 stimulation before and after allogeneic bone marrow transplantation

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<th>6 months after first immunization</th>
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Table 5. Humoral immune responses to recipient-derived leukemic blasts before and after immunization.

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Figure 1

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Figure 2

A

lymphocytes

CD3⁺ HLA-DR⁺

CD3⁺ CD8⁺

CD3⁺ CD4⁺

x 1.3

P > 0.05

x 1.2

P = 0.042

x 1.6

P = 0.041

x 1.5

P = 0.027

Absolute counts per μL

B

CD4⁺ CD25⁺ Lag-3⁺

CD4⁺ CD25⁺

x 0.9

P > 0.005

x 1.2

P > 0.005
Figure 3

A

Granzyme B spots per 10^6 cells

PRE Injection 3 Injection 6

0 20 40 60 80 100 120 140

B

IFN-γ spots per 10^6 cells

PRE Injection 3 Injection 6

0 10 20 30 40 50

C

IFN-γ spots per 10^6 cells

PRE Injection 3 Injection 6

0 50 100 150 200 250 300

D

IL-5 spots per 10^6 cells

PRE Injection 3 Injection 6

0 100 200 300 400 500 600 700 800 900 1000
FIGURE LEGENDS

**Fig. 1.** Representative local responses to the autologous combination vaccine in punch biopsy specimens from the injection site. (A-D) Control tissues from healthy volunteers: A, CD1a (O10 staining, 20x); B, dendritic cells (S-100 staining, 20x); C, CD4 (CD45RO / OPD4 staining, 20x); D, CD8 (CD45RO / C8/144B staining, 20x). (E-H) Representative tissues from patients: E, abundant dermal perivascular infiltration of Langerhans cells (O10 staining, 20x) with F, retention of dendritic cell infiltrate in patient 5 (S-100 staining, 20x); G, paucity of CD4+ lymphocytes (CD45RO / OPD4 staining, 20x) with H, moderate infiltration of CD8+ lymphocytes in patient 2 (CD45RO / C8/144B staining, 20x). These studies were performed on all patients.

**Fig. 2.** Flow cytometric analysis of lymphocyte subpopulations before (open bars) or after (closed bars) administration of recipient-derived leukemia vaccines. (A) Absolute circulating lymphocyte counts showing cytotoxic (CD3+ CD8+), helper (CD3+ CD4+) and activated (CD3+ HLA-DR+) T-cell subpopulations. (B) Percentage of regulatory T-cells (CD4+ CD25dim Lag-3+) among all lymphocytes. Values are means ± SEM; fold values are given above P values.

**Fig. 3.** Representative ELISPOT results showing granzyme B, IFN-γ or IL-5 release from PBMCs obtained before or after 3 or 6 immunizations with recipient-derived leukemia vaccines. PBMCs were stimulated ex vivo against recipient-derived leukemic blasts alone (closed bars) or after preincubation with anti-CD4 (diagonal hatched bars) or anti-CD8 (horizontal hatched bars) monoclonal antibodies. (A) Granzyme B, patient 4; (B) IFN-γ, patient 4; (C) IFN-γ, patient 7; (D) IL-5, patient 2. All values are mean spot numbers for duplicate wells minus background per 10⁶ PBMCs.
FOOT NOTES

Table 1

*a* The dose-escalation schedule began at $2 \times 10^5$ CD40L-secreting skin fibroblasts per injection (dose-level 1), increasing in log increments to $2 \times 10^7$ (dose-level 3). IL-2-secreting skin fibroblasts and recipient-derived blasts were administered at a fixed dosage throughout the study ($2 \times 10^7$ per injection).

*b* ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; t, translocation; del, deletion.

c BMT, bone marrow transplantation; MRD, matched-related donor; MUD, matched-unrelated donor; MMUD, mismatched-unrelated donor; 5/6, 5 matches out of 6.

d TBI, total-body irradiation; ATG, antithymocyte globulin; AraC, cytarabine; Cyclo, cyclophosphamide.

e GvHD, graft-versus-host disease; CSA, cyclosporin-A; MTX, methotrexate.

f PR, partial remission; CR, complete remission.

g DOD, died of disease; NED, no evidence of disease.

Table 2

*a* The dose-escalation schedule began at $2 \times 10^5$ CD40L-secreting skin fibroblasts per injection (dose-level 1), increasing in log increments to $2 \times 10^7$ (dose-level 3). IL-2-secreting skin fibroblasts and recipient-derived blasts were administered at a fixed dosage throughout the study ($2 \times 10^7$ per injection).

*b* PBMCs, peripheral blood mononuclear cells; NA, not available.

c Increase in GrB secretion was statistically significant after 3 immunizations compared to pre-vaccination values ($p=0.046$).

d Increase in IFN-γ secretion was statistically significant after 6 immunizations compared to pre-vaccination values ($p=0.046$).

e Increase in IL-5 secretion was statistically significant after 6 immunizations compared to pre-vaccination values ($p=0.043$).
Table 3

a The dose-escalation schedule began at $2 \times 10^5$ CD40L-secreting skin fibroblasts per injection (dose-level 1), increasing in log increments to $2 \times 10^7$ (dose-level 3). IL-2-secreting skin fibroblasts and recipient-derived blasts were administered at a fixed dosage throughout the study ($2 \times 10^7$ per injection).

b PBMCs, peripheral blood mononuclear cells; SKF, skin fibroblasts; NA, not available; ND, not done. Differences in IFN-γ secretion before and after immunization were not statistically significant whether patient-derived skin fibroblasts ($p > 0.5$) or PHA blasts ($p > 0.345$) were used as targets.

c No PBMCs available prior to 1 week after the 1st immunization for patient 4.

Table 4

a The dose-escalation schedule began at $2 \times 10^5$ CD40L-secreting skin fibroblasts per injection (dose-level 1), increasing in log increments to $2 \times 10^7$ (dose-level 3). IL-2-secreting skin fibroblasts and recipient-derived blasts were administered at a fixed dosage throughout the study ($2 \times 10^7$ per injection).

b PBMCs, peripheral blood mononuclear cells; NA, not available. Differences in IFN-γ secretion upon stimulation with pp65 were not statistically significant ($p > 0.26$) whether PBMCs were collected before or after immunization.

c No PBMCs available prior to 1 week after the 1st immunization for patient 4.

Table 5

a The dose-escalation schedule began at $2 \times 10^5$ CD40L-secreting skin fibroblasts per injection (dose-level 1), increasing in log increments to $2 \times 10^7$ (dose-level 3). IL-2-secreting skin fibroblasts and recipient-derived blasts were administered at a fixed dosage throughout the study ($2 \times 10^7$ per injection).

b PE, phycoerythrin; NA, not available. Differences in mean fluorescence intensity were not statistically significant between patients ($p > 0.091$). Patients nos. 6 and 7 had a 2- to 3-fold increase of their post-immunization mean fluorescence intensity compared to normal controls.

c Controls consisted of pooled plasmas from normal donors; not shown are the responses of the patients’ plasmas against unrelated cells (no increase in the fluorescent signal).
Immunotherapy of high-risk acute leukemia with a recipient (autologous) vaccine expressing transgenic human CD40L and IL-2 after chemotherapy and allogeneic stem cell transplantation

Raphael F Rousseau, Ettore Biagi, Aurelie Dutour, Eric S Yvon, Michael P Brown, Tiffany Lin, Mei Zhuyong, Bambi Grilley, Edwina Popek, Helen E Heslop, Adrian P Gee, Robert A Krance, Uday Popat, George Carrun, Judith F Margolin and Malcolm K Brenner

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