

Responses to Human CD40 Ligand/Human Interleukin-2 Autologous Cell Vaccine in Patients with B-Cell Chronic Lymphocytic Leukemia

Ettore Biagi,¹ Raphael Rousseau,¹ Eric Yvon,¹ Mary Schwartz,² Gianpietro Dotti,¹ Aaron Foster,¹ Diana Havlik-Cooper,¹ Bambi Grilley,¹ Adrian Gee,¹ Kelty Baker,¹ George Carrum,¹ Lawrence Rice,¹ Michael Andreeff,³ Uday Popat,¹ and Malcolm Brenner¹

Abstract Purpose: Human CD40 ligand activates the malignant B-cell chronic lymphocytic leukemia cells and enhances their capacity to present tumor antigens. Human interleukin-2 further potentiates the immunogenicity of human CD40 ligand in preclinical murine models.

Experimental Design: We prepared autologous B-cell chronic lymphocytic leukemia cells that expressed both human CD40 ligand (>90% positive) and human interleukin-2 (median secretion, 1,822 pg/mL/10⁶ cells; range, 174-3,604 pg). Nine patients were enrolled in a phase I trial, receiving three to eight s.c. vaccinations.

Results: Vaccinations were administered without evidence of significant local or systemic toxicity. A B-cell chronic lymphocytic leukemia-specific T-cell response was detected in seven patients. The mean frequencies of IFN- γ , granzyme-B, and IL-5 spot-forming cells were 1/1,230, 1/1,450, and 1/4,500, respectively, representing a 43- to 164-fold increase over the frequency before vaccine administration. Three patients produced leukemia-specific immunoglobulins. Three patients had >50% reduction in the size of affected lymph nodes. Nonetheless, the antitumor immune responses were observed only transiently once immunization ceased. High levels of circulating CD4⁺/CD25⁺/LAG-3⁺/FoxP-3⁺ immunoregulatory T cells were present before, during and after treatment and *in vitro* removal of these cells increased the antileukemic T-cell reactivity.

Conclusions: These results suggest that immune responses to B-cell chronic lymphocytic leukemia can be obtained with human CD40 ligand/human interleukin-2-expressing s.c. vaccines but that these responses are transient. High levels of circulating regulatory T cells are present, and it will be of interest to see if their removal *in vivo* augments and prolongs the antitumor immune response.

The malignant B-cell chronic lymphocytic leukemia (B-CLL) cells express a range of tumor-associated and tumor-specific antigens, including immunoglobulins. These cells also express high levels of MHC class I and II molecules but lack costimulatory molecules and are ineffective antigen-presenting cells. Their immunogenicity can be increased by manipulation of the CD40/CD40 ligand (CD40L) pathway, in which CD40L interacts with CD40 on B-CLL target cells to increase their antigen-presenting

capacity through up-regulation of the costimulatory molecules CD80 and CD86 and adhesion molecules, such as CD54 (1-5). CD40L also induces dendritic cell maturation *in vivo*, increasing their ability to take up and process antigens (6-9). These effects have encouraged studies of CD40L as immunotherapy for human B-cell malignancies, with tumor responses and disease stabilization noted in both preclinical and clinical settings (1-5, 10-16). Using *in vitro* and *in vivo* models, we have shown that the immunostimulatory effect obtained with human CD40L (hCD40L) can be further increased with use of leukemic cells modified to produce transgenic human interleukin-2 (hIL-2) in the microenvironment in which they present their tumor-associated antigens (1, 4, 5). We therefore combined s.c. injections of hCD40L and hIL-2-expressing B-CLL cells in an immunotherapy study in which the dose of hIL-2-secreting cells was fixed at 2×10^7 per injection, whereas the dose of hCD40L-expressing cells was escalated from 2×10^5 (level 1) to 2×10^7 (level 3) per injection.

These vaccines produced no serious adverse effects, and specific T-cell and humoral anti-B-CLL immune responses were detected in the peripheral blood of seven of nine treated patients. However, these responses were only transient and not accompanied by sustained clinical responses in any patient. Of note, high levels of regulatory T (T-reg) cells were also present

Authors' Affiliations: ¹Center for Cell and Gene Therapy, Baylor College of Medicine, The Methodist Hospital and Texas Children's Hospital; ²Department of Pathology, The Methodist Hospital; and ³Department of Blood and Marrow Transplantation, Section of Molecular Hematology and Therapy, University of Texas M.D. Anderson Cancer Center, Houston, Texas
Received 3/7/05; revised 7/4/05; accepted 7/12/05.

Grant support: NIH grant CA78792 and The Methodist Hospital Foundation Award and Chao Scholar Grant (G. Dotti).

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Requests for reprints: Ettore Biagi, Center for Cell and Gene Therapy, Baylor College of Medicine, 6621 Fannin Street, Houston, TX 77030. Phone: 832-824-4725; Fax: 832-825-4668; E-mail: etttore.biagi@pediatrionmonza.it.

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doi:10.1158/1078-0432.CCR-05-0484

in patient peripheral blood before immunization and persisted throughout the study, and their *in vitro* removal augmented the T-cell-mediated reactivity toward the autologous leukemia target.

Materials and Methods

Patient characteristics. The clinical protocol was approved by the Institutional Review Board of Baylor College of Medicine, the Food and Drug Administration, and the Recombinant DNA Advisory Committee of the NIH after the risks and benefits of the proposed experimental protocol were thoroughly evaluated. An informed consent was obtained from each subject enrolled in the study. Patients were eligible for peripheral blood collection and vaccine preparation if they had a diagnosis of B-CLL (in any stage of disease) but were not in Richter's transformation. Patients were eligible for vaccine administration if they had (a) manipulated B-CLL cells sufficient for at least three injections, (b) a life expectancy of ≥ 10 weeks, (c) an absolute neutrophil count of $\geq 500/\mu\text{L}$, (d) an absolute normal T-cell count of $\geq 200/\mu\text{L}$, (e) hemoglobin of ≥ 8 g/dL, (f) a platelet count of $\geq 50,000/\mu\text{L}$, (g) had not received treatment with other investigational agents within the last 4 weeks before the first vaccine injection, (h) had hepatic and renal functions not exceeding thrice the upper limit of normal, (i) had not received any immunosuppressive drugs and lacked autoimmune diseases (including immune thrombocytopenia or autoimmune hemolytic anemia), and (j) had no history of treatment for B-CLL-related infections.

Table 1 shows the clinical characteristics of the nine patients enrolled. The average WBC count at the time of collection was 40,200/ μL (range, 8,900-101,200). The mean percentage of circulating normal T cells was 8.5% (range, 3.6-18.7%).

Vaccine preparation and injection. All manufacturing procedures followed proposed Good Tissue Practice guidelines. Peripheral blood (50-100 mL) was collected and peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation. The percentage of leukemic cells was determined by CD5/CD19/CD20 staining and was always $\geq 90\%$. PBMCs were then frozen in DMSO freezing medium and stored in liquid nitrogen for further use. A certified MRC-5 (a human embryonic lung fibroblast cell line; American Type Culture Collection, Rockville, MD) cell bank (17) was established, and at the time of vaccine preparation, aliquots were thawed and irradiated at 30 Gy. One portion was cultured in RPMI 1640 (Hyclone, Perbio Science, Logan, UT) supplemented with 10% heat-inactivated FCS (Hyclone) and 2 mmol/L L-glutamine (BioWhittaker, Walkersville, MD). The remainder was transduced with a clinical-grade adeno-hCD40L vector at 12.5×10^3 viral particles per cell and

then cultured. Details of vector construction and the coculture system are provided elsewhere (16).

After 1 to 5 days, when the MRC-5 cells were $>50\%$ confluent, B-CLL cells were seeded onto both the adeno-hCD40L-transduced MRC-5 cells and the nontransduced MRC-5 cells and maintained in coculture for a further 24 hours at 37°C in 5% CO₂. The B-CLL cells cocultured with hCD40L-expressing MRC-5 cells were then harvested, analyzed for CD40L transfer and CD80, CD86, and CD54 expression (BD Bioscience, San Jose, CA), and frozen for further use (16, 18). The B-CLL cells cocultured on nonmanipulated MRC-5 were then collected and transduced with a clinical-grade adeno-hIL-2 vector at 5.3×10^3 viral particles per cell. An aliquot of these cells was kept in culture, and hIL-2 secretion was measured at 72 hours by ELISA. The remaining cells were frozen for further use (4).

At the time of vaccination, aliquots of manipulated cells were thawed, irradiated at 30 Gy, and resuspended in 0.5 mL of $1 \times$ PBS solution containing human albumin 10% and then injected s.c. into the upper arm of such patient.

Study design. The treatment consisted of three to eight s.c. injections of a fixed dose of irradiated hIL-2-secreting autologous B-CLL cells (2×10^7) with an escalating dose of irradiated hCD40L-expressing leukemia cells (2×10^5 - 2×10^7). The first three injections were given at weekly intervals and the remaining at 2-week intervals. Three patients were enrolled at each of the three planned dose levels. The differences in number of vaccine doses received by each patient (Table 1) reflect the variable numbers of viable manipulated B-CLL cells collected after vaccine preparation.

Patients were monitored for local and systemic toxicity according to the grading system of the National Cancer Institute (see <http://ctep.cancer.gov/reporting/index.html>). Peripheral blood was also drawn weekly for the first 10 weeks to monitor the antileukemia immune response, then every other week for 6 weeks, then monthly.

Evaluation of the response after vaccine administration. Injection site punch skin biopsies were done 1 week after the first and second injections, and samples were immediately fixed in formalin. Methods of analysis of the cellular infiltrates are described elsewhere (19). Briefly, the immunohistochemical studies were done on formalin-fixed, paraffin-embedded sections using the DAKO Envision system and the DAKO autostainer (DAKO Cytomation, Glostrup, Denmark). 3,3'-Diaminobenzidine was used as the chromagen. CD3 (clone CD3p, Novocastra Laboratories Ltd., Burlingame, CA), CD4 (clone 4B12, Vision BioSystems, Inc., Norwell, MA), CD5 (clone CD5/54/F6, Novocastra Laboratories), CD8 (clone C8, DAKO Cytomation), CD20 (clone KS20-8, DAKO Cytomation), Pax-5 (clone 24, Transduction Laboratories, Lexington, KY), and CD43 (clone DF-T1, DAKO Cytomation) antibodies were used to distinguish normal T-cell and B-cell infiltrates from B-CLL

Table 1. Characteristics of the patients and the vaccines administered

Patient no. (study level)	Gender/age (y)	Stage (RAI)	Previous treatment	Vaccine CD40L expression	Vaccine IL-2 secretion	No. vaccines
1 (1)	Male/46	3	Yes	89	1,831.5	8
2 (1)	Male/49	4	No	97	3,389.6	6
3 (1)	Male/65	2	No	94	2,412.3	6
4 (2)	Female/51	3	Yes	95	1,021.3	5
5 (2)	Male/71	1	No	94	3,604	5
6 (2)	Male/55	4	No	69	1,616.6	3
7 (3)	Female/63	2	No	92	174.16	8
8 (3)	Male/67	2	No	90	1,349.46	8
9 (3)	Male/53	2	Yes	94	1,006	6

NOTE: CD40L expression is reported as the percentage of CD40L⁺ cells gated on viable CD5⁺/CD19⁺ cells and IL-2 secretion is reported as pg/mL/10⁶ leukemic cells measured at 72 hours after transduction.

cells. The CD25 antibody (clone 4C9, Novocastra Laboratories) was also used to detect the expression of CD25 on infiltrating T cells.

Fresh PBMCs were phenotyped before and after each immunization, with use of the following antibodies (all purchased from BD Bioscience): CD3, CD4, CD5, CD8, CD19, CD20, CD25, and CD56.

Humoral and cellular antileukemic responses were measured at time 0 (before the first injection), at 1 to 2 weeks after the third injection, and at 1 to 2 weeks and 2 to 3 months after the last immunization. Briefly, autologous T cells were obtained from PBMCs by positive selection with CD4 and CD8 magnetic beads and MACS technology (Miltenyi Biotech, Bergish Gladbach, Germany). Purity was always >90%. These T cells were cocultured in a 36-hour ELISPOT assay with nonmanipulated autologous leukemic cells, collected, and frozen before vaccination, at a 1:5 T-cell/leukemic cell ratio, in AIM-V serum-free medium (Life Technologies, Gaithersburg, MD). Control analyses were done with each cell population alone and T cells cocultured with allogeneic B-CLL cells and (in four of nine patients) with autologous T-phytohemagglutinin blasts and autologous EBV-transformed B-lymphoblastoid cell lines. To confirm whether the antitumor T-cell activity was MHC class I or II restricted, we incubated target cells with human HLA-A, HLA-B, and HLA-C (clone W6/32) or HLA-DP, HLA-DQ, HLA-DR (clone CR3/43) antibodies (DAKOCytomation) or with isotype control antibody, all at 20 µg/mL, for 30 minutes at room temperature, a concentration and set of conditions shown previously to be optimal (16). Effector T cells were then added to the ELISPOT plates.

Humoral immune responses were evaluated before, during, and after immunization by diluting autologous plasma 1:10 with 1× PBS and adding it to autologous, unmodified, leukemia cells. Control plasma samples were obtained from three nonvaccinated B-CLL patients and three healthy donors. A FITC-conjugated anti-human IgG (BD Bioscience) was used to detect antibodies bound to the leukemia cells, and fluorescence-activated cell sorting analysis was done to evaluate increases of mean fluorescence intensity as described previously (19).

Analysis of regulatory T cells. T-reg cells in peripheral blood were studied both with the CD4/CD25 antibody combination and with an anti-LAG-3 FITC-conjugated antibody (clone 17B4, Alexis Biochemicals, Axxora, LLC, San Diego, CA; ref. 20). The analysis of CD25 expression on CD4-gated cells was also extended to unvaccinated CLL patients ($n = 8$) and normal donors ($n = 5$). T-reg cells in treated patients were also characterized by FoxP-3 mRNA reverse transcription-PCR quantification on CD4-selected PBMCs (Miltenyi Biotech; ref. 21). Total RNA was extracted with a RNeasy kit (Qiagen, Valencia, CA). Reverse transcription-PCR was done sequentially in the same tube with the Qiagen QuantiTect SYBR Green RT-PCR kit. All samples were analyzed in triplicate. The reactions were done with the DNA Engine Opticon System. FoxP-3 message levels were calculated after the data were normalized with glyceraldehyde-3-phosphate dehydrogenase. CD4⁺/CD25⁻ cells from normal donors served as the reference sample.

LAG-3 blocking experiments and CD4/CD25 depletion/positive selection were additionally done to assess the immunosuppressive properties of each putative T-reg population. PBMCs were positively selected for CD4⁺/CD8⁺ T cells using antibody-coated beads (CD3 positivity, >95%; Miltenyi Biotech) and incubated with an unconjugated blocking anti-LAG-3 antibody (clone 17B4, Alexis Biochemicals) at 30 µg/mL (20) or with an isotype control antibody for 30 minutes at room temperature. They were then cocultured for 5 days with 40 Gy-irradiated hCD40L-expressing autologous leukemia cells (1:1 ratio in AIM-V medium). At the end of the coculture, antileukemia reactivity was analyzed in a 24-hour IFN-γ ELISPOT assay using CD19-selected unmanipulated autologous B-CLL cells as targets. In other experiments, the PBMCs were subjected to a double round of CD19 depletion (Miltenyi Biotech), and the CD19-depleted cells were then selected according to CD25 positivity by use of the Miltenyi Biotech system. The CD25⁺ fraction was ≥98% CD4⁺/CD25⁺ and the CD25-depleted fraction was <3% CD25⁺. The CD25-depleted and nondepleted T cells were cocultured for 5 days with 40 Gy-irradiated hCD40L-expressing autologous leukemia cells (as described before), and their reactivity

was analyzed in a 24-hour IFN-γ ELISPOT assay after restimulation with CD19-selected unmanipulated B-CLL cells. Cytokine production in the supernatants of these cultures was measured using the cytokine bead array system (BD Bioscience). The CD25⁺ enriched and depleted CD4 cells were also tested in a standard mixed lymphocyte reaction with allogeneic healthy donor's PBMC. The cells were incubated at a 1:1 ratio in 96-well plates (1×10^5 cells per well for each population) with 40 Gy-irradiated stimulator cells. Proliferation was measured on day 5 by [³H]thymidine incorporation (22).

Statistical analysis. Paired Student's *t* tests were used to determine the statistical significance of experimental results, which are presented as means and SD or ranges.

Results

Vaccine preparation. We successfully prepared vaccines for all nine patients, and the percentage of hCD40L-expressing transduced MRC-5 cells always exceeded 90% (data not shown). Flow cytometry analysis (Table 1; Fig. 1A and B) showed that there was a statistically significant up-regulation of CD40L ($P < 0.005$) on B-CLL cells, attributable to the transfer of this molecule during coculture with hCD40L-expressing MRC-5 cells (16, 18). The resultant CD40-mediated activation of the B-CLL cells caused them to up-regulate their expression of the costimulatory molecules CD80 and CD86 ($P < 0.005$; Fig. 1A and B). CD40 engagement also induced a significant increase of the mean fluorescence intensity of the cell adhesion molecule CD54, from an average value of 35 to as high as 1,900 after stimulation ($P < 0.001$; see also Fig. 1B).

The mean level of hIL-2 secretion (measured at 72 hours after transduction) was 1,822 pg/mL/ 10^6 cells (range, 174-3,604; Table 1).

Local and systemic toxicity. Grade 1 toxicity (local pain, redness, and swelling) was observed at the site of vaccine administration and was accompanied by transient muscle aches and low-grade fever in three patients. These reactions were mild and self-limiting and were the only systemic toxicities observed. In particular, there was no evidence of autoimmune anemia, cytopenia, or hepatic dysfunction, such as that associated with systemic administration of the murine CD40L molecule (3).

Local and systemic immune responses after vaccine administration. Eighteen skin biopsies were done. A minimal to moderate inflammatory reaction was observed, consisting of focal superficial lymphocytic perivascular infiltration with mild dermal edema (Fig. 2). Phenotyping showed that the cellular infiltrates were predominantly CD3⁺ T cells, with absent to rare B-CLL cells (data not shown). Most of the infiltrating T cells were CD4⁺ (average percentage, 65%; range, 30-90%), with an average of 32% of T cells being also CD25⁺. Insufficient B-CLL cells remained to assess continued *in vivo* expression of CD40L, but previous *in vitro* experiments showed that CD40L expression lasts for at least 7 days (16).

Changes in T cells, natural killer cells, or normal B-cell counts were not observed in any treated patient (data not shown).

ELISPOT assays for IFN-γ (a cytokine typical for a Th-1 profile), granzyme-B (a molecule typical of cytotoxic T-cell functions), and IL-5 (a cytokine distinctive for a Th-2 profile) showed a significant increase in leukemia-reactive T cells in seven of nine patients tested after the last vaccine injection (Fig. 3A). The mean frequencies of IFN-γ, granzyme-B, and IL-5 spot-forming cells among 1×10^3 T cells were 1/1,230, 1/1,450, and 1/4,500, respectively, representing a 43- to

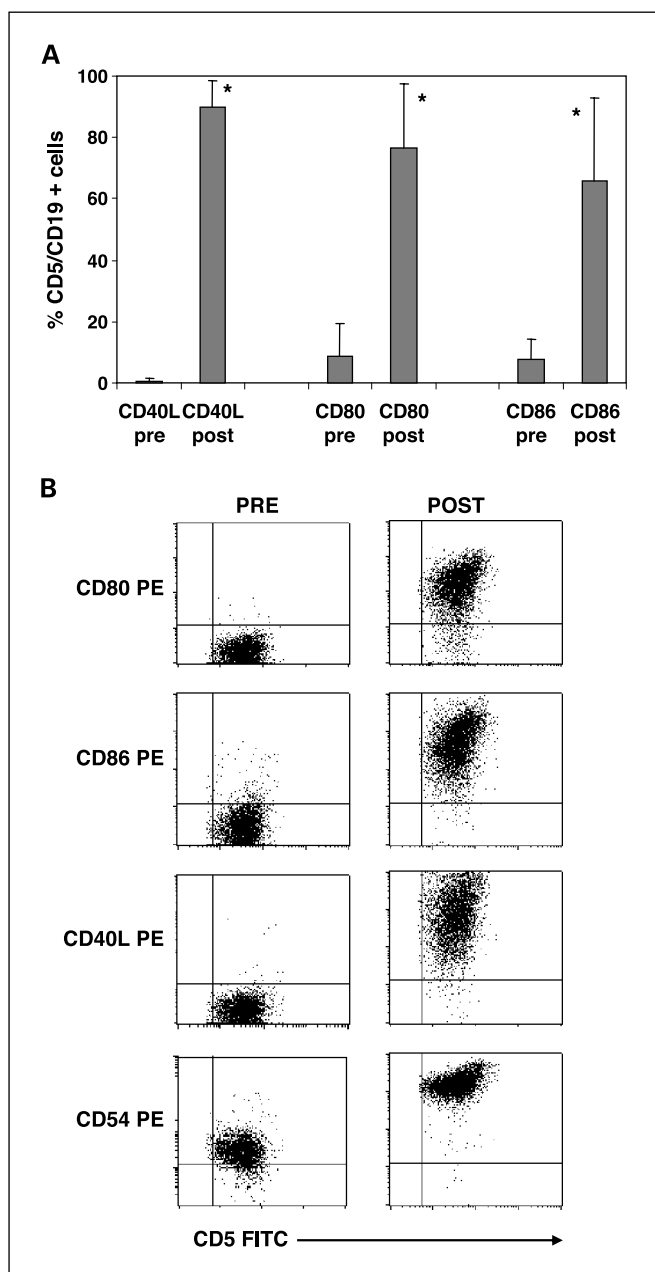


Fig. 1. Coculture of B-CLL cells with hCD40L-expressing MRC-5 induces efficient up-regulation of CD80 and CD86 and transfer of hCD40L on leukemia cells. **A**, columns, mean of surface expression of CD40L, CD80, and CD86, before (*pre*) and after (*post*) manipulation ($n = 9$), on CD5⁺/CD19⁺ viable leukemia cells; bars, SD. *, $P < 0.005$. **B**, representative sample of B-CLL cells showing expression of CD80, CD86, CD40L, and CD54 before and after manipulation. *X* axis, CD5 FITC; *Y* axis, each analyzed molecule [phycoerythrin (PE) conjugated].

164-fold increase over the frequency before vaccine administration ($P < 0.01$ for all molecules). The reactivity detected was specific for the autologous leukemia cells, because it was inhibited by antibodies to MHC molecules (Fig. 3B) and reactivity could not be detected against allogeneic B-CLL cells, autologous T-phytohemagglutinin blasts, or EBV-transformed autologous lymphoblastoid B-cell lines (frequency, $<1/10,000$) for all molecules. However, these tumor-reactive T cells were not persistent, becoming undetectable by 2 to 3 months after the last vaccine administration (Fig. 3A).

In addition, IgG antibodies reactive against autologous leukemia cells were detected in the plasma of three patients after vaccination, but levels were only 2- to 3-fold higher than pretreatment (data not shown). No reactivity was observed with plasma from three nonimmunized patients or from three normal donors (data not shown), and there was no cross-reactivity of plasma between different B-CLL samples. Leukemia-specific antibodies became undetectable 2 to 3 months after the last vaccination (data not shown).

There was no evident dose-response relationship between the dose of CD40L-expressing cells and any of the above immune responses. Nonetheless, as shown by Fig. 3A, the magnitude of the immune response seemed to progressively increase with the number of vaccines administered, being maximal after the last immunization.

Clinical responses. At the 6-week evaluation, three of the patients (nos. 4, 5, and 7) with a higher ELISPOT response, with regard to IFN- γ and granzyme-B release, showed a $>50\%$ reduction of affected lymph nodes, but these nodes returned to their previous size within 12 weeks. A decrease in the leukemic cell count was not observed in any treated patient. Eight of the nine evaluable patients had stable disease (RAI classification) after 6 to 23 months of follow-up (median, 15).

High levels of regulatory T cells before and during vaccination. These data indicate not only that B-CLL patients retain the ability to respond immunologically to autologous tumor cells but also that the response is of brief duration and that sustained tumor regression does not occur. One possible explanation for the transience of the antitumor immune response to the hCD40L/hIL-2 vaccine might lie in the effects of T-reg cells (23–26). Immunostaining of peripheral blood lymphocytes revealed an abnormally large percentage of CD4⁺/CD25⁺-expressing cells before the first vaccine administration, which persisted during and up to 3 months after treatment in eight of the nine patients studied (Fig. 4A). The T-reg identity of this CD4⁺/CD25⁺ population in vaccinated patients was phenotypically confirmed by studies of FoxP-3 mRNA and surface LAG-3 expression (Fig. 4B and C; refs. 20, 21). Analysis of T-reg CD4⁺/CD25⁺ cells in nonvaccinated CLL patients ($n = 8$) confirmed an abnormally large percentage of this population in peripheral blood (mean percentage CD4⁺/CD25⁺ cells of total CD4, 49%; range, 28–65%; Fig. 4D). Analysis of PBMCs from five healthy donors indicated a significantly lower percentage of CD4⁺/CD25⁺ cells than was found in the B-CLL patients (average of CD4⁺/CD25⁺ cells out of CD4, 9%; range, 5–15%; Fig. 4D).

CD4⁺/CD25⁺ cells in vaccinated patients also had regulatory function, because blocking or depleting the CD25⁺ fraction produced an increased number of IFN- γ -releasing T cells in ELISPOT assays with autologous leukemic cells (Fig. 5A and B). Cytokine measurements confirmed that antitumor reactivity of patient T cells was increased by depletion of the CD25⁺ regulatory subset. Higher levels of IFN- γ (Fig. 5C) were observed (mean, 5,625 pg/mL; range, 5,250–7,500) in CD25⁺-depleted than in unselected fractions (mean, 1,315 pg/mL; range, 47–2,500; $P < 0.05$). Levels of tumor necrosis factor- α , IL-2, and IL-5 were similarly increased (Fig. 5C).

The activity of these regulatory CD4⁺/CD25⁺ T cells was not specific for antitumor responses; they also showed potent inhibitory activity in standard mixed lymphocyte reaction assays (Fig. 5D).

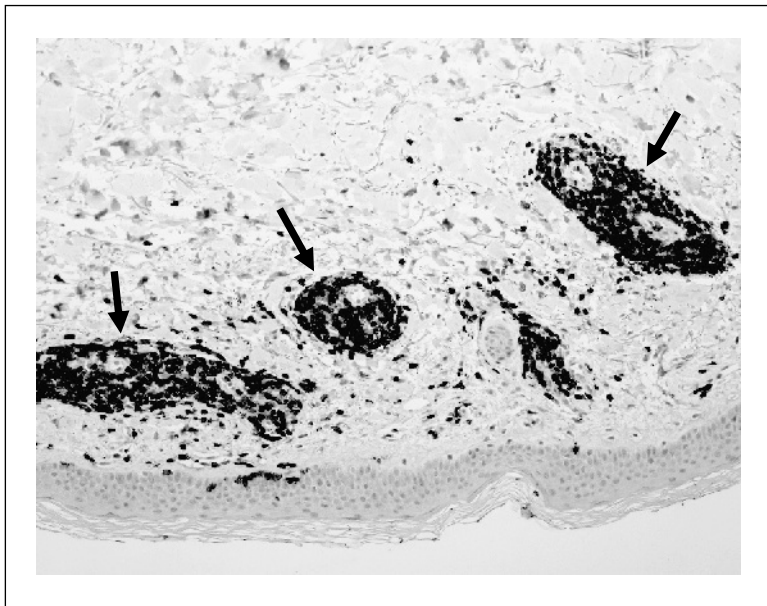


Fig. 2. T-cell perivascular infiltrate after vaccine administration in punch biopsy specimen from the injection site. A perivascular T-cell infiltration (*black arrows*, CD3 immunostain; original magnification, $\times 100$) detected at the site of vaccine administration. An Olympus microscope (model BX41) (Olympus America Inc., Melville, NY) and UPlan Apo $\times 10$ objectives were used. The numerical aperture was 0.40. The camera and software used were Olympus DP70 and Olympus DP controller, respectively.

Discussion

Our findings show the feasibility of using hCD40L and hIL-2 as an autologous cell combination vaccine in patients with B-CLL. These molecules did not produce adverse events and had clear immunomodulatory effects, eliciting CD4⁺ and CD8⁺ effector cells reactive with autologous B-CLL cells. Nevertheless, the immunologic and antitumor effects were transient. We also observed the presence and persistence of an expanded subset of T-reg cells, whose *in vitro* removal augmented the T-cell anti-leukemia reactivity, thus suggesting a possible role of these cells in hampering the vaccine-induced immune responses *in vivo*.

Expression of CD40L by B-CLL cells, together with the subsequent up-regulation of B7 molecules, can augment the antigen-presenting capacity of leukemia cells, breaking the anergic state of T cells circulating in peripheral blood and thereby inducing the generation of leukemia-reactive T lymphocytes (1–5, 16). This observation has been used as a rationale for developing a B-CLL “vaccine” expressing either murine CD40L or hCD40L (3, 16). Kipps et al. conducted a phase I clinical trial with murine CD40L-transduced B-CLL cells, which showed that i.v. injections of high doses of these cells can produce a transient but consistent decrease of circulating leukemic cells and a reduction in lymph node size (3).

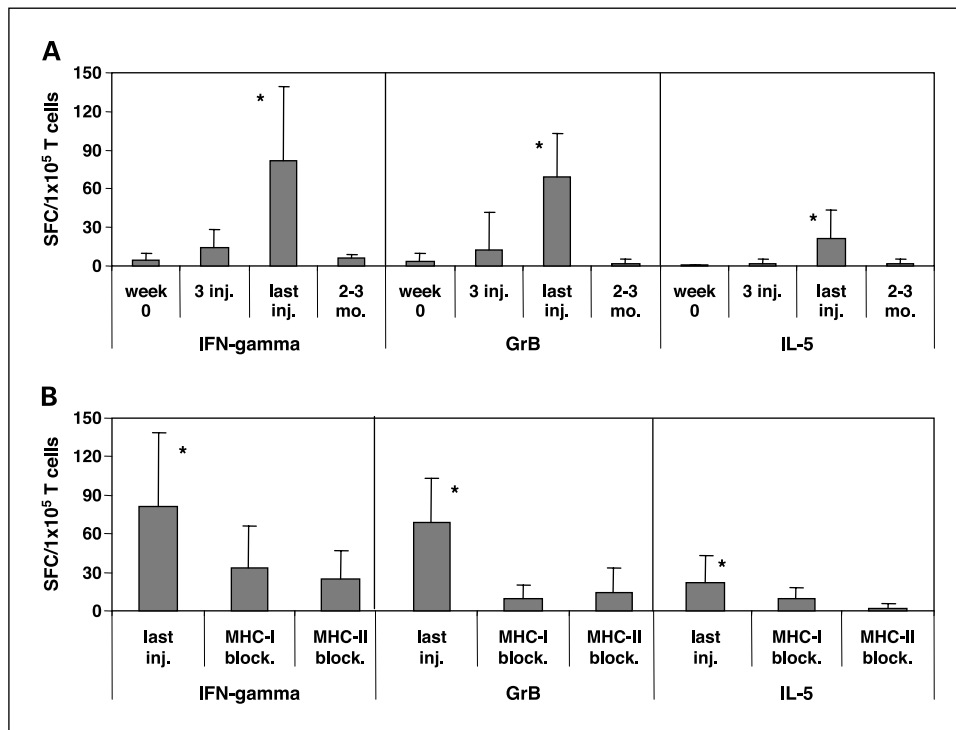


Fig. 3. Vaccine administration induces an antileukemia CD4/CD8-mediated immune response. **A**, mean number of IFN- γ , granzyme-B (*GrB*), and IL-5 spot-forming cells (*SFC*; $n = 7$) among 1×10^5 T cells isolated from peripheral blood at different times before and after the third and last vaccine injections; *bars*, SD. These cells were cocultured with autologous leukemia cells at a 1:5 T-cell/leukemic cell ratio. Cytokine release after exposure to allogeneic B-CLL cells or autologous T-phytohemagglutinin blasts or EBV-transformed lymphoblastoid B-cell lines was not significant (data not shown). *, $P < 0.01$. **B**, mean spot-forming cell number per 1×10^5 T cells after exposure to autologous leukemic cells ($n = 7$) before and after MHC class I and II blocking. *, $P < 0.05$. With regard to IL-5 secretion, only MHC class II blocking gave a statistically significant result as anticipated.

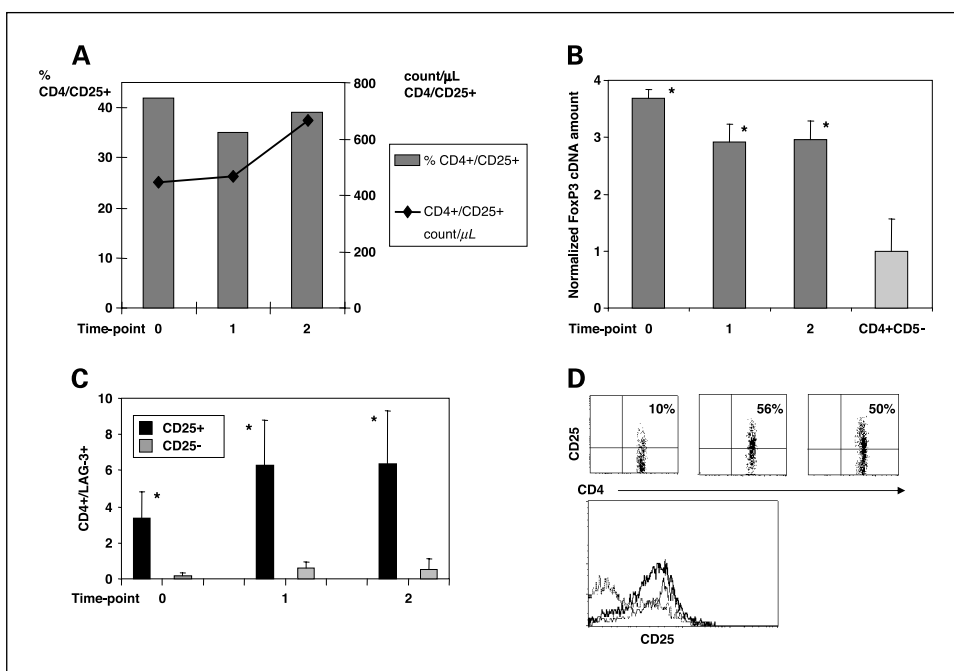


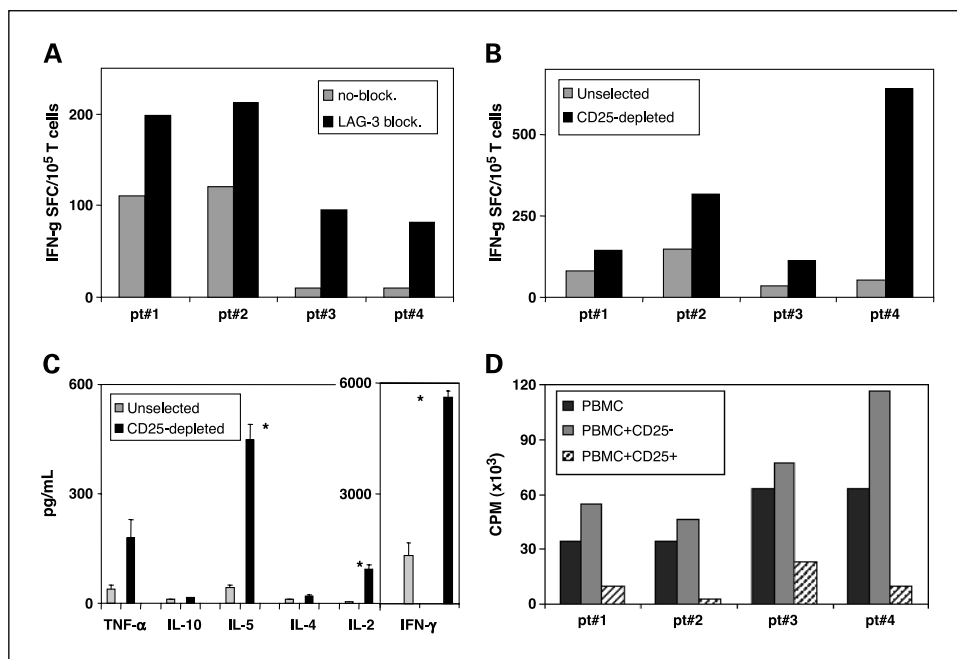
Fig. 4. High levels of immunoregulatory CD4⁺/CD25⁺ T cells are present in the peripheral blood of treated patients. *A*, mean percentage (CD25⁺CD4⁺ cells/all CD4⁺ cells) and absolute number of CD25⁺/CD4⁺ cells detected in the peripheral blood immediately before the first vaccination (time point 0), at 1 to 2 weeks after the third injection (time point 1), and at 2 to 3 months (time point 2) after the last vaccination (*n* = 8). *B*, expression of FoxP-3 cDNA in CD4-selected T cells (dark gray columns) before, during, and after the vaccine schedule compared with the values from CD4⁺/CD25⁻ cells (light gray columns; *n* = 4). Levels of FoxP-3 expression were significantly higher. *, *P* < 0.05. *C*, mean percentages of LAG-3⁺ cells in CD4⁺/CD25⁺ (black columns) and CD4⁺/CD25⁻ (gray columns) cells before, during, and after the vaccine schedule (*n* = 8); bars, SD. A significantly higher percentage of CD4⁺/CD25⁺ cells compared with CD4⁺/CD25⁻ cells expressed LAG-3 at each of the time points tested. *, *P* < 0.05. *D*, representative dot plot and histogram analysis of CD4⁺/CD25⁺ cells of a control healthy donor (top left and dotted line at the bottom) compared with a nonvaccinated CLL patient (top middle and bold line at the bottom) and a vaccinated CLL patient (top right and solid line at the bottom). The analysis shows CD25 expression on CD4⁺ cells after gating on CD3⁺ cells. Numbers indicate the percentages of CD25⁺ cells out of CD4.

These effects seemed initially to be related to CD95-mediated apoptosis after CD40 ligation on B-CLL cells (27), but longer follow-up showed that five patients had stable disease, with a median time to further treatment of 42 months. Two of those patients had required no additional intervention after several

years of follow-up, but it is not clear if this effect is immune mediated (28).

We devised an alternative approach to immunotherapy for B-CLL patients based on our previous *in vitro* observation that the combination of hCD40L with human IL-2 is superior to

Fig. 5. T-reg cells decrease the anti-B-CLL autologous T-cell reactivity and inhibit allogeneic proliferation. *A*, patients' selected T cells (*n* = 4) were incubated with CD40L-expressing autologous leukemia cells for 5 days in the presence of a LAG-3 blocking antibody (black columns) or an isotype control (gray columns), and their antileukemia reactivity was measured in an IFN- γ ELISPOT assay against unmanipulated autologous B-CLL cells. *B*, experiments in (*A*) were repeated with unselected T cells (gray columns) versus CD25-depleted T cells (black columns). *C*, mean levels of cytokine production (pg/mL) from the experiments described in (*B*) were measured by cytokine bead array; bars, SD. Higher levels of IFN- γ , tumor necrosis factor- α (TNF- α), IL-2, and IL-5 were found in the cultures with CD25-depleted T cells (black columns) compared with unselected T cells (gray columns). *, *P* < 0.05. *D*, CD4⁺/CD25⁺ selected T cells (dashed columns) were compared with CD25-depleted T cells (gray columns) in an allogeneic primary mixed lymphocyte reaction with PBMCs from healthy donors. Baseline proliferation of PBMCs alone (black columns). Proliferation was measured on day 5 by [³H]thymidine incorporation [counts per minute (CPM)].



either molecule alone in eliciting the generation of autologous T-cell-mediated antileukemic reactivity (4). Moreover, this combination vaccine induced prolonged protection against tumors in a murine leukemia-lymphoma model established with the A20 cell line (1). We elected to administer transduced autologous tumor cells by the s.c. rather than i.v. route using the rationale that a smaller number of cells would produce therapeutic benefit attributable to the induction of antitumor immunity rather than to large-scale CD40L-CD95-mediated apoptosis (27).

One of the major concerns in using autologous tumor cells as vaccines is the induction of immune responses that are generally autoreactive rather than predominantly confined to the tumor cells. The use of CD40L is of particular concern because it can break the anergic state of autologous T-cell toward self-antigens and because of experimental models confirming that autoimmunity can indeed be induced by tumor vaccines expressing this molecule (29). Our patients lacked clinical manifestations of autoimmunity, including autoimmune anemia or cytopenias (to which B-CLL patients are especially susceptible), and their Coombs tests remained negative. I.v. injection of many murine CD40L-expressing B-CLL cells has been associated with an increase in hepatic transaminase levels and prolongation of prothrombin times, likely reflecting CD95-mediated apoptosis of hepatic parenchymal cells (3, 27). Such toxic effects were not observed in our patients probably because of the s.c. administration of a much lower cell dose than used in previous studies.

In the absence of significant adverse effects, we did find evidence for an antitumor immune response. Flu-like mild-grade symptoms (low-grade fever and muscle ache) were observed in some patients together with local signs of inflammation at the injection site. These signs were of short duration and self-limited and were likely related to the immune response after the injection. Skin biopsies showed local infiltration of T cells, and systemically, there was a CD4⁺/CD8⁺ T-cell-mediated response in seven of nine patients as measured in ELISPOT assays for IFN- γ , granzyme-B (which detect antigen-specific T cells with a Th-1 profile and cytotoxic properties), and IL-5 (which identifies antigen-specific CD4⁺ T cells with Th-2 characteristics). Three patients produced tumor-binding antibodies, a further demonstration of the recruitment of Th-2 T cells. We did not observe a correlation of the magnitude of the immune response detected with the cell dose administered, but an association was observed with the number of vaccine injections done, implying that, above a cell number threshold, continued administration of vaccines may be preferable to individual dose escalation. Nevertheless, all responses in this study were only transient, becoming undetectable by 3 months after the last vaccination. Many immune defects that might explain this outcome have already been identified in B-CLL patients (30–34). Soluble factors released by the leukemic clone may be responsible for a profound defect in circulating dendritic cells, which do not express the maturation marker CD83 or the costimulatory molecule CD80, and are unable to drive a type 1 T-cell response (30). Circulating T cells in such patients possess several abnormalities. They frequently show a skewed expression of the T-cell receptor repertoire with low levels of the T-cell receptor ζ chain, CD28, and CD152 (31). Moreover, B-CLL cells express high levels of

Fas ligand and may induce the apoptotic death of plasma cells, with subsequent hypogammaglobulinemia (32). B-CLL cells also live in protected niches, resulting in a markedly reduced apoptotic rate due to a microenvironment high in tumor necrosis factor- α , IL-4, and IL-8, all of which have an antiapoptotic effect on B-CLL cells (33, 34). All of these factors act during the induction phase of the immune response and may not be as important in B-CLL as thought previously, because patients with this disease retain the capacity to generate an antitumor immune response as indicated by the present study.

Our findings suggest that increased levels of circulating T-reg cells should be added to the above list (23–26). We found that CLL patients have high levels of T-reg cells circulating in peripheral blood before vaccination. Analysis of skin biopsies from the injection site showed a predominant infiltration of CD4 T cells. Around 30% of infiltrating T cells were also CD25⁺, with wide variability in intensity of CD25 staining. This reflects an admixture of activated and T-reg cells, but because immunohistochemical analysis does not allow definitive functional assessment, the presence of T-reg cells versus activated T cells remains unknown. Nonetheless, IL-2 secreted locally by the vaccine does not produce systemic proliferation of T-reg cells, because flow cytometry analysis of nonvaccinated CLL patients confirmed the presence of higher levels of CD4⁺/CD25⁺ T-reg cells in peripheral blood, and the levels of putative T-reg in the circulation of treated patients did not increase after immunization. This observation is in accordance with recently published data, in which high levels of T-reg cells were found in a population of 73 CLL patients (35). It is not clear why B-CLL patients have increased numbers of T-reg cells, although the presence of many B-CLL cells lacking costimulatory molecules may favor induction of T-reg over effector T cells as described in other models (26).

Can the effects of T-reg cells be overcome by administering a sufficiently large number of CD40L-expressing cells by an alternative route? Kipps et al. reported that i.v. infusion of murine CD40L-transduced cells lowered B-CLL cell counts in some patients while sustaining disease stability in others (3, 27, 28), effects that were not clearly observed in our investigation. This apparent difference in outcome is difficult to interpret, because phase I studies lack the power to enable comparisons of efficacy, and measurements of T-reg cell number or function were not included in the study by Kipps et al. If CD4⁺/CD25⁺ T-reg cells can indeed compromise the generation or maintenance of antileukemic T-cell responses, their removal will be required for optimal immune stimulation by hCD40L/hIL-2 vaccines regardless of the dose or the route of administration. The availability of CD25 antibodies capable of depleting regulatory subsets *in vivo* should allow this notion to be tested experimentally (36, 37).

Acknowledgments

We thank Dr. John Carwile, Dr. Edward Lee, Tatiana Gotsolva, Zhuyong Mei, Seanda Blocker, Gloria Obi, Helen Huls, Karen Nunez-Wallace, Joyce Ku, April Durett, Amanda Bowling, and David Rodney for their expert advice and technical support. E. Biagi thanks the «Comitato Maria Letizia Verga per lo studio e la cura della leucemia del bambino» (Monza, Italy) and «Fondazione Cassa di Risparmio di Genova ed Imperia-Associazione Cristina Bassi contro le leucemie acute dell'adulto» (Genova, Italy) for their generous support.

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