In Vitro Engagement of CD3 and CD28 Corrects T Cell Defects in Chronic Lymphocytic Leukemia

Mark Bonyhadi, Mark Frohlich, Angela Rasmussen, Christophe Ferrand, Laura Grosmaire, Eric Robinet, Jose Leis, Richard T. Maziarz, Pierre Tiberghien and Ronald J. Berenson

*J Immunol* 2005; 174:2366-2375; doi: 10.4049/jimmunol.174.4.2366
http://www.jimmunol.org/content/174/4/2366

References

This article cites 36 articles, 14 of which you can access for free at:
http://www.jimmunol.org/content/174/4/2366.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts


In Vitro Engagement of CD3 and CD28 Corrects T Cell Defects in Chronic Lymphocytic Leukemia

Mark Bonyhadi,* Mark Frohlich,* Angela Rasmussen,** Christophe Ferrand,‡
Laura Grosmaire,*,§ Eric Robinet,‡ Jose Leis,‖ Richard T. Maziarz,‖ Pierre Tiberghien,‡ and
Ronald J. Berenson*

Chronic lymphocytic leukemia (CLL) is characterized by the gradual accumulation of CD19+CD5+ malignant B cells along with immune cell dysfunction (1). Immune response and homeostatic control defects have been reported in the T cell compartment as well as the leukemic B cell compartment (2). Abnormalities in these compartments may contribute to the inability of the immune system to recognize and destroy the leukemic cells. For example, decreased expression of CD40L (CD154) and reduced signaling via the TCR CD3ζ signaling element have been demonstrated in laboratory studies to compromise the ability of T cells to respond to and eliminate leukemic cells from CLL patients (3–5). In addition, decreased expression of the important effector molecules CD80 and CD86 and insensitivity to CD95 (FAS)-dependent death pathways have all been observed in leukemic B cells (2, 6). Furthermore, current therapeutic agents used to treat CLL, such as fludarabine and alemtuzumab, damage both T and B cell compartments, leading to additional deficits in immune function that increase the risk for infections (7). Therapeutic approaches capable of stimulating the immune systems of CLL patients and reversing these T cell and B cell deficits would have the potential to provide an antitumor effect as well as prevent infections.

We have developed the Xcellerate Process, in which T cells are activated ex vivo using Xcyte Dynabeads, which are superparamagnetic beads to which anti-CD3 and anti-CD28 mAbs have been covalently attached (herein referred to as CD3/CD28 beads) (8). The process is initiated by mixing CD3/CD28 beads with PBMC followed by cell culture over 1–2 wk. Clinical studies in patients with non-Hodgkin’s lymphoma, multiple myeloma, kidney cancer, and prostate cancer have demonstrated that activated T cells can be grown to large numbers for clinical applications where preliminary evidence of safety has been demonstrated (8–10). In contrast to these types of cancer, the blood of CLL patients is characterized by the presence of large numbers of tumor cells and a very low percentage of T cells. Therefore, in the present study, we modified culture conditions to obtain a process that effectively expands T cells and eliminates leukemic cells. We also demonstrated the ability of CD3/CD28 bead activation and expansion to reverse CLL patients’ T cell deficits. Finally, we assessed the antitumor activity of the expanded T cells. The data support the concept of administering autologous activated and expanded T cells to treat patients with CLL.

Materials and Methods

Abs, kits, and CD3/CD28 beads

Abs and reagents used for flow cytometric analyses or for functional studies include the following: FITC conjugates anti-CD3, -CD4, -CD8, -CD25...
Flow cytometric analysis

Staining was performed to analyze as described previously (11). After staining, cells were washed, resuspended in 1% paraformaldehyde, and analyzed by FACSCalibur (BD Biosciences) using CellQuest software or FCS Express version 1.0 software (De Novo Software).

T lymphocyte preparation and storage

Following Institutional Review Board approval at Oregon Health and Science University, and California Cancer Care (Greenbrae, CA), blood was collected from CLL patients under written informed consent. Samples were obtained from patients at various stages of disease (Rai stages I–IV). Many patients had received prior therapies including treatment with cyclophosphamide, fludarabine, prednisone, rituximab, or vincristine. PBMC were obtained by leukapheresis or venipuncture. For blood collected by venipuncture, PBMC were isolated by Ficoll-Paque PLUS (Amersham Biosciences) density gradient centrifugation. Cells were analyzed by flow cytometry for T cell content and leukemic B cell content. Leukapheresis products were washed by a COBE 2991 or a Cytomate (Baxter) cell washer. PBMC were cryopreserved and stored in the vapor phase of liquid nitrogen until the initiation of cell culture.

T lymphocyte selection and culture

Thawed PBMC containing CD3+ T cells were combined with Xcye Dynabeads at a 3:1 bead-to-T cell ratio in V-Xivo 15 culture medium containing gentamicin (100 µg/ml), 5% heat inactivated human serum (BioWhitaker), 20 mM HEPES buffer (Invitrogen Life Technologies), 2 mM L-glutamine (Invitrogen Life Technologies), and 100–200 IU/ml IL-2 (Chiron) (complete medium). Beads and cells were rotated at 1 rpm at room temperature for 2 h, and then bead-bound cells were magnetically selected. Selected cells were incubated at small scale in culture medium at 37°C and 5% CO2. Samples were removed for cell counting, cytokine analysis, and flow cytometric analysis at various time intervals during the cultures. Culture splitting was performed to keep CD3 flow cytometric analysis at various time intervals during the cultures. Culture splitting was performed to keep CD3 flow cytometric analysis at various time intervals during the cultures. Culture splitting was performed to keep CD3 flow cytometric analysis at various time intervals during the cultures.

Cytokine measurements

 Supernatants were collected from cell cultures at various time points, and cytokine levels were quantified by performing a capture-sandwich immunoassay using the MultiAnalyte Profiling microsphere technology on a Lumex 100 as per manufacturer’s instructions. Unlabelled carboxylated beads (Lumex) were loaded with Abs specific for GM-CSF (R&D Systems), IL-10, TNF-α, and IFN-γ (BD Pharmingen).

RNA extraction, cDNA synthesis, PCR amplification, and electrophoresis

RNA was prepared from snap-frozen cell pellets, and total RNA was reverse transcribed with random hexanucleotides as previously described (12). Each TCR Vβ segment was amplified with one of the 24 TCR Vβ subfamily-specific primers and a TCR Cβ primer recognizing both constant regions Cβ1 and Cβ2 of the TCR β-chain. An aliquot of cDNA synthesis reaction was amplified as previously described (13). Two microliters of each of the 24 first-run PCR products was subjected to three cycles of elongation (run off) under the same conditions, except that the Cβ primer was now labeled with the FAM fluorescent dye. Run-off PCR products were loaded on a 6% acrylamide sequencing gel on a 377 automatic sequencer (Applied Biosystems) for size and fluorescence intensity determination.

TCR repertoire data analysis

ImmunoScope 3.1e software was used to analyze, from the sequencing gel, the fluorescent intensity of peaks representing products of differing nucleotide lengths (14). To analyze the large raw data set generated by ImmunoScope software (~2400 CDR3 profiles), the raw data were compiled in a Excel database, using the ISEAppeaks software package (15). The skewing, or perturbation of TCR Vβ repertoire was measured as previously described (16).

Allogeneic response: CD25 induction

CD3+ T cells from CLL donors were purified by depletion using a Human T Cell Enrichment kit (R&D Systems). Autologous T cells were cultured for 12–14 days using the Xcellerate Process. Either unmanipulated or bead-expanded T cells (1 × 105 cells) were combined with irradiated (4500 rad) pooled allogeneic PBMC in 2 ml of culture. Positive controls for stimulation contained T cells and CD3/CD28 beads at a 1:5 bead-to-T cell ratio. T cells without stimulation were used as negative controls. Cultures were incubated at 37°C. Cells were analyzed by FACS for surface expression of CD25 after 72 h in culture.

Restimulation of expanded T lymphocytes

For T cell function studies, on the final day of culture (days 12–15), beads were removed by magnetic selection, and cells were washed and resuspended in complete medium. Cells were added to culture plates coated with anti-CD3 and anti-CD28 Abs followed by overnight incubation at 37°C and 5% CO2.

Redirected lysis assay

A standard 4-h 51Cr release assay was performed using various E:T ratios. JY cells (EBV-transformed B cell line) were pulsed with 51Cr (200 µCi for 1 h). Xcelerated T Cells from CLL patients served as effector cells. Cells were incubated at 37°C/5% CO2 in round-bottom 96-well plates (Corning). All wells were transferred into a LumaPlate (PerkinElmer) and left on the bench to dry overnight. Radioactivity was measured using a scintillation counter (TopCount; PerkinElmer).

Tumor-specific T cell evaluation: ELISPOT

At the end of the initial CD3/CD28 bead expansion process, residual beads were removed with a MOPS- or M-PCL magnet (Dynal Biotech), and serial dilutions (100 µl/well) of Xcelerated T Cells were added to separate wells of a polystyrene microplate (R&D Systems), which was precoated with a mAb specific for human IFN-γ. A fixed number (1 × 104 cells/well) of autologous PBMC target cells (80–95% CLL cells) was added to wells to give graded E:T ratios. The target CLL cells were prepared from cryopreserved PBMCs. Once thawed, the cells were incubated for 2 h at 37°C in complete medium and filtered with a 70-µm cell strainer (BD Biosciences) to remove cell debris aggregates. All assay media contained human rIL-2 at a final concentration of 100 IU/ml. The mitogens PMA/ ionomycin were added at final concentrations of 0.5 ng and 0.2 µg per milliliter, respectively, to positive control wells. The plate was incubated for 24 h at 37°C in a 5% CO2 incubator. All reagents and buffers were provided in the R&D Systems human IFN-γ ELISPOT kit, and plate processing and IFN-γ detection were performed as per the manufacturer’s instructions. An automated ImmunoSpot Analyzer and associated software (CTL Analyzers) was used to enumerate IFN-γ-positive spots.

CD3/CD28 bead-expanded T cell and leukemic B cell coculture

T cells that were bead-expanded for 12–15 days were mixed with freshly thawed autologous PBMC from CLL patients at PBMC-to-T cell ratios indicated in the figures. After 24 h, cultures were harvested for staining of activation markers, and after 48 h, for analysis of apoptosis markers. For Transwell cultures, T cells were either cultured with autologous leukemic B cells or in separate chambers of a Costar Transwell Plate (Corning and Life Sciences). For CD154 blocking experiments, anti-CD154, clone 24-31 (Alexis Platform), was used at 20 µg/ml. For experiments involving the addition of FAS-L, cells were cocultured for 24 h, and FAS-L was then added to the cocultures at 2.5 ng/ml. Cultures were harvested for staining 24 h later. Induction of surface markers on B cells in coculture was measured by flow cytometry. Cells were stained with annexin FITC, CD19-PE, and propidium iodide (PI; 1 µg/ml). A CD19 gate was used for analysis of B cells.

Results

Cell expansions

In contrast to our previously reported procedure for activating and expanding T cells, which was developed for PBMC with a relatively normal representation of T cells (range, 36–78%), we developed a procedure optimized for PBMC with low T cell content (≤10%) and high leukemic burden (≥50%) (Table I) (10). In
brief, PBMC from CLL patients were incubated with CD3/CD28 beads at a 3:1 bead-to-CD3+ T cell ratio at total nucleated cell densities ranging from 0.5 × 10^6 to 3 × 10^6 total nucleated cells/ml. Cells and beads were incubated for 2 h while slowly mixing to facilitate maximal contact between beads and T cells. Bound cells were then magnetically selected and placed into culture. In contrast to T cells from healthy donors and patients with other types of cancer, which begin dividing within the first 3–4 days of culture after coming in contact with CD3/CD28 beads, initiation of cell division is delayed for T cells from CLL patients, and logarithmic growth is not apparent until ~6 days after culture initiation (8, 10). To achieve a minimum 200-fold expansion of T cells, CD3/CD28 bead-activated T cell cultures were conducted over 9–14 days, in contrast to the 8–10 days required to achieve similar levels of expansion for tissues from normal donors or patients with other types of cancer in small-scale cultures. Using this procedure, T cells expanded 1439 ± 1252-fold (mean ± SD) (Fig. 1a). T cells from CLL patients at different Rai disease stages expanded to varying degrees with a trend for slightly reduced fold expansion for Rai stage IV tissues (M. Bonyhadi and R. J. Berenson, unpublished observations). Concomitant with the expansion of T cells, a rapid decrease in leukemic B cells (CD19+CD5+) was observed (Fig. 1b). The final cell product contained predominantly T cells (93.2 ± 2.3%) and few residual leukemic B cells (0.1 ± 0.06%). Cell viability was high at the end of culture (94 ± 4%; range, 85–100%).

**Phenotype and kinetics: T cells**

The expression of key T cell surface markers was measured for CD3/CD28 bead-activated T cells generated from CLL patients. Various receptors were examined during culture, including the following: phenotypic markers, such as CD4, CD8, and CD28; activation markers, such as IL-2R (CD25), CD40L (CD154), 41BB (CD137), and OX40 (CD134); as well as adhesion and homing receptors such as ICAM (CD54) and L-selectin (CD62L).

Initial PBMC samples from CLL patients contained both CD4+ and CD8+ T cells at CD4:CD8 ratios ranging from 0.4 to 4.7 (1.7 ± 1.2) (M. Bonyhadi and R. J. Berenson, unpublished observations). By the end of culture, both CD4+ and CD8+ T cells were still present, with CD4:CD8 ratios ranging from 0.5 to 10.9 (2.7 ± 2.8) (Fig. 2a). CD4+ T cells uniformly expressed high levels of CD28 throughout the expansion process (Fig. 2b). Starting CD8+ T cell populations were predominantly CD28+, but contained both CD28+ and CD28– T cells at various ratios in different patients. By the end of culture, the CD8+ T cell population was almost completely CD28+ (M. Bonyhadi and R. J. Berenson, unpublished observations).

The kinetics and pattern of expression of several activation markers, including CD25 (IL-2R), CD54, CD134, CD137, and CD154, were similar during T cell expansion (Fig. 2b). These markers, as measured by flow cytometry, were not detectable or were present at low levels on T cells at culture initiation. Expression increased within several days after CD3/CD28 bead activation, generally reaching peak levels between days 4 and 9 of culture, reflecting a state of cell activation that was concomitant with initiation of cell division. Subsequently, a gradual overall reduction in expression of each of these markers was observed; however, levels did not return to baseline for all cells, with subpopulations of T cells maintaining increased marker expression at culture termination. CD25 and CD54 were expressed at high levels on both activated CD4+ and CD8+ T cells. High levels of CD154 and CD134 were found only on activated CD4+ T cells. Conversely, lower levels of CD154 and CD134 were observed on their CD8− counterparts. In contrast, higher expression of CD137 was detected on CD8+ T cells than on CD4+ T cells. The kinetics and patterns of expression of all of these activation markers closely parallel those observed for healthy donors under similar culture conditions (M. Bonyhadi and R. J. Berenson, unpublished observations); however, induction of expression of markers in CLL patients’ T cells is delayed by several days compared with healthy donors.

**Figure 1.** CLL patients’ T cells expand, and leukemic B cells disappear following CD3/CD28 bead activation. T cell expansion and loss of leukemic B cells were measured, respectively, by flow cytometric quantitation of CD3+ T cell and CD19+ B cell populations in culture and by performing live cell counts using the trypan-blue exclusion method. a. The kinetics of CD3/CD28 bead-activated T cell expansion using culture conditions described in Materials and Methods. b. T cell and leukemic B cell content as a percentage of the total nucleated cell count during the expansion process.

**Table 1.** T cell and B cell composition of PBMC from CLL patients in comparison to healthy donor tissues

<table>
<thead>
<tr>
<th></th>
<th>%CD3+</th>
<th>%CD4+</th>
<th>%CD8+</th>
<th>%CD19+</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLL patients (mean)</td>
<td>5.9</td>
<td>3.1</td>
<td>1.7</td>
<td>85.5</td>
</tr>
<tr>
<td>SD</td>
<td>2.2</td>
<td>1.1</td>
<td>1.2</td>
<td>8.1</td>
</tr>
<tr>
<td>Range (n = 11)</td>
<td>0.6–27.2</td>
<td>0.3–10.5</td>
<td>0.1–5.1</td>
<td>58.3–96.8</td>
</tr>
<tr>
<td>Healthy donors (mean)</td>
<td>57.9</td>
<td>36.3</td>
<td>16.7</td>
<td>8.3</td>
</tr>
<tr>
<td>SD</td>
<td>8.8</td>
<td>7.1</td>
<td>4.4</td>
<td>4.0</td>
</tr>
<tr>
<td>Range (n = 123)</td>
<td>36.4–78.0</td>
<td>17.8–53.8</td>
<td>6.8–33.8</td>
<td>2.3–21.8</td>
</tr>
</tbody>
</table>

* Blood samples from CLL patients as well as healthy donors were analyzed by flow cytometry for T cell and B cell content. CD4+ and CD8+ T cells were also enumerated.
Cytokine profiles

At several time points during the CD3/CD28 bead activation and expansion process (days 3–5, 6–8, and 12–14), supernatants were collected to measure the types and amounts of cytokines present (Fig. 2c). Very low amounts of cytokines such as GM-CSF, IFN-γ, IL-10, and TNF-α were detected in control cultures using nonactivated PBMC from CLL patients. In contrast, supernatants collected from patients’ T cells that were bead-activated contained high levels of GM-CSF from day 3 onward, with peak levels observed at days 6–8. IFN-γ was produced earlier in the activation process with peak levels observed at days 3–5. Similar to IFN-γ, TNF-α was detected at the earliest time points (days 3–5), with expression diminishing to low levels thereafter. Little IL-10 could be detected in the supernatants collected at any time point during the primary expansion process. We also measured the ability of patients’ CD3/CD28 bead-activated T cells to produce cytokines upon restimulation. For these experiments, bead-activated and expanded T cells, which had exited log-phase growth, were restimulated with CD3/CD28 beads at a 1:1 bead-to-T cell ratio and supernatants were collected after 48 h of culture. Higher levels of IFN-γ and TNF-α were observed compared with the levels measured during primary activation and expansion. IL-10 levels were found at significant levels in the supernatants following T cell restimulation.

TCR repertoire analysis

Previous studies have reported skewing of the TCR repertoire in CLL patients. TCR families have been shown to be overrepresented and/or underrepresented, with variation from patient to patient (17). We performed spectratype and flow cytometric analyses to measure the TCR repertoire before and after CD3/CD28 bead expansion of the T cells from CLL patients.

We evaluated TCR Vβ region (Vβ) usage by surface staining of T cells from several CLL donors using Abs specific for different TCR Vβ chains followed by flow cytometric analysis. Both CD4+ T cells and CD8+ T cells from CLL patients exhibited skewed TCR Vβ usage profiles (Fig. 3, a and b). In comparison to healthy donors, CD4+ T cells expressing several different TCR Vβ receptor families, such as Vβ2, Vβ8, Vβ14, and Vβ17, were underrepresented. Following CD3/CD28 bead expansion, expression of most of the underrepresented Vβ families normalized. Similar observations were made for CD8+ T cells, where the effect appeared

FIGURE 2. Flow cytometric analysis of CD4+ and CD8+ T cell distribution and expression of key cell surface markers on T cells and leukemic B cells during bead activation and expansion. a, Flow cytometric analysis of CD3/CD28 bead-activated and -expanded T cells from CLL PBMC showing maintenance of both CD4+ and CD8+ T cells in this representative plot. Numbers represent the percent of the PBMC that expressed either or both of the CD4 and CD8 markers. Cells were analyzed using a live cell gate (forward light scatter vs side light scatter). b, Expression of various T cell surface activation, adhesion, and costimulatory molecules for a representative CLL tissue analyzed by flow cytometry at various time points. Cells were stained with Abs specific for CD3, CD4, or CD8, and one of the following: CD25, CD137, CD28, CD154, CD134, or CD54. Nonstimulated cells were analyzed on day 4 of culture, whereas activated cells were analyzed on day 4 of culture, whereas activated cells were analyzed on day 4 of culture, whereas activated cells were analyzed on day 4 of culture. Cells were analyzed using both a live cell gate (forward light scatter vs side light scatter) and a CD3 gate. c, The levels of several cytokines were measured in culture supernatants at time points indicated in the figure, both during the primary activation and expansion process and at 24 h after restimulation of day 12–14 expanded T cells. Day 3–5 supernatants were collected from control wells containing T cells from the same donor that had not been activated (n = 6). Control values are shown by the left-most bar of the bar graphs for each cytokine measured.
to be even more striking. In the CLL patients' CD8⁺ T cell populations, not only were several TCR Vβ families underrepresented in comparison to healthy donors (Vβ1, Vβ2, Vβ5, Vβ8, and Vβ14), but many TCR Vβ families were overrepresented (Vβ2, Vβ14, Vβ21.3). Similar to CD4⁺ T cells, the CD8⁺ T cell TCR Vβ composition returned to a more normal pattern following CD3/CD28 bead
expansion. There was an increase in the number of T cells expressing underrepresented TCR Vβ families, as well as a reduction in the number of T cells expressing overrepresented TCR Vβ families for both CD4+ and CD8+ T cell subsets.

We also examined the TCR repertoire both before and after expansion using the spectratype analysis method. Most CLL patient displayed skewed TCR Vβ repertoire before activation and expansion (Fig. 3, a and b). Some TCR Vβ families exhibited profiles suggesting a predominance of oligoclonal or monoclonal T cells. Only a few of the 24 Vβ families exhibited the normal Gaussian distribution pattern found in healthy donor tissues (M. Bonyhadi, M. Frohlich, and R. J. Berenson, unpublished observations for healthy donor tissues). Following activation and expansion, skewing was significantly decreased in most patients, resulting in TCR Vβ expression patterns typical of healthy individuals.

We used the Gorochov method of analysis to determine the degree of skewness, or relative perturbation of the repertoire away from the norm (16). In 11 of the 13 CLL patients evaluated, TCR Vβ repertoire skewing was reduced following expansion (Fig. 3c). In 10 of the 11 patients with skewed repertoire, the repertoire returned to normal as measured by the Gorochov index. In 1 of the 13 patients, the skewness of their repertoire was mildly increased, and in 1 patient, the repertoire did not appear skewed before or after expansion. Overall, normalization of the TCR repertoire was highly statistically significant, as measured by Student’s t test (p = 0.000144).

Restoration of alloresponsiveness to T cells from CLL patients

To evaluate the general immune function of expanded T cells to a physiologically relevant stimulus, we examined the responses of the patients’ T cells to allo-Ags. Nonstimulated T cells or CD3/CD28 bead-expanded T cells were cocultured with irradiated pooled allogeneic PBMC. After 72 h in culture, T cells were analyzed for induction of CD25 expression on the CD8+ T cell subset. Control cultures containing pre- and post-CD3/CD28 bead-expanded T cells did not show induction of CD25 expression in the absence of stimulator cells (Fig. 4). Only a small fraction of the starting T cells from CLL patients expressed CD25 when stimulated with allogeneic PBMC (1% CD25+). In contrast, 37% of the patient’s CD3/CD28 bead-expanded T cells expressed CD25 after incubation with the same pool of allogeneic PBMC. Under similar experimental conditions, the T cell proliferative response to allostimulation was measured in a [3H]thymidine incorporation assay. Low levels of proliferation were observed for T cells that had not been CD3/CD28 bead-expanded, whereas their CD3/CD28 bead-expanded counterparts exhibited high levels of thymidine incorporation (M. Bonyhadi and A. Rasmussen, unpublished observations). In a standard redirected lysis assay, efficient killing of target cells was observed by bead-expanded T cells, reflecting functional lytic capabilities of the expanded T cells (M. Bonyhadi, unpublished observations).

Frequency of tumor-reactive T cells

To measure the frequency of tumor-reactive T cells present in the blood of patients with CLL, we used an IFN-γ ELISPOT assay. We measured the frequency of tumor-reactive T cells before and after CD3/CD28 bead expansion. In most patients, tumor-reactive T cells were undetectable in the starting PBMC samples. In contrast, tumor-reactive T cell frequencies were observed following expansion, ranging from 1 in 200 to 1 in 2500 (mean, 1 in 787 ± 665; n = 13 patients). The limit of detection for this assay varied between 1:1,000 and 1:13,000, depending upon the percentage of T cells in the initial PBMC pool.

Effect of CD3/CD28 bead-activated and -expanded T cells on leukemic B cells

We compared the effects of T cells from CLL patients on the expression of key leukemic B cell markers, such as CD54, CD80, CD86, and FAS before and following CD3/CD28 bead expansion. Overnight coculture of patient CD3/CD28 bead-expanded T cells with autologous leukemic B cells resulted in the rapid up-regulation of CD54, CD80, CD86, and FAS as measured by flow cytometry (Fig. 5a). In contrast, purified, but not CD3/CD28 bead-expanded, T cells isolated from the PBMC of a CLL patient failed to mediate this effect (M. Bonyhadi, L. Grosmaire, and R. J. Berenson, unpublished observations). Previous studies have shown that, under different culture conditions, FAS-mediated cell death can be enhanced or inhibited in the leukemic B cells from CLL patients (6, 18, 19). Therefore, we determined whether FAS induced on leukemic B cells by CD3/CD28 bead-activated T cells was functional. CD3/CD28 bead-activated T cells were cultured with autologous leukemic B cells, which induced FAS expression on the B cells. Recombinant FAS-L was used to cross-link FAS. After an additional 24 h in culture, CD19+ B cells were analyzed by flow cytometry using annexin and PI to measure apoptosis and death of the leukemic B cells (Fig. 5b). Control cultures containing only leukemic B cells that had not been cocultured with activated T cells showed that ~34% of leukemic B cells were positive for annexin (including PI-positive and PI-negative compartments), and 29% were positive for both annexin and PI. Leukemic B cells cocultured with the patient’s CD3/CD28 bead-expanded T cells resulted in a marked increase in the percentage of both annexin and annexin-PI double-positive cells to 62 and 47%, respectively. Moreover, the entire leukemic B cell population shifted toward increased annexin positivity, suggesting that all leukemic B cells were entering the programmed cell death pathway. When FAS-L was added to these cultures, these levels further increased to 90 and 68%, respectively. Addition of the cross-linking reagent, FAS-L, to control wells containing only leukemic B cells that had not been cocultured with activated T cells resulted in little change in these values (39% annexin-positive and 32% annexin and PI positive). These data demonstrate that CD3/CD28 bead-activated T cells are potent inducers of functional FAS on leukemic B cells.
This experiment was repeated using tissues from three individual CLL donors with similar results. Analyzed for expression of CD95, CD80, CD86, and CD54. Controls were thawed autologous PBMC from the same patient without addition of T cells. After gating on live cells based upon forward and side light scatter, B cells expressing CD19, but negative for CD3, were analyzed by flow cytometry. Cultures continued for 48 h and then cells were stained with annexin-FITC, and PI. Stained cells were analyzed by flow cytometry after setting gates on coculture with activated and expanded T cells. Cocultures were identical with those described above, except that some cultures had FAS-L added at 24 h.

Thirteen days after activation with CD3/CD28 beads, T cells from a CLL patient were cocultured at various ratios with freshly thawed autologous PBMC containing >90% leukemic B cells. After 24 h, cultured cells were stained for surface expression of CD3, CD19, and CD95, CD80, CD86, or CD54 and analyzed by flow cytometry. After gating on live cells based upon forward and side light scatter, B cells expressing CD19, but negative for CD3, were analyzed for expression of CD95, CD80, CD86, and CD54. Controls were thawed autologous PBMC from the same patient without addition of T cells.

We investigated whether direct cell-cell contact was necessary for the antileukemic effects of the bead-activated T cells and which molecules were responsible for the antitumor activity. Patient’s bead-expanded T cells and their leukemic B cells were cultured together or separated by a Transwell culture system. Under conditions of direct cell-cell contact were CD54, CD86, and FAS strongly up-regulated on the leukemic B cells, thereby implying that soluble factors were not required for this effect (Fig. 6a). Additional experiments were conducted to identify the cell surface molecule(s) responsible for this activity. In a series of coculture experiments, addition of a neutralizing anti-CD154 Ab abrogated most of the induction of CD54, CD86, and FAS expression on autologous leukemic B cells by CD3/CD28 bead-activated T cells (Fig. 6b). These results suggest that much of the up-regulation of these key receptors is dependent upon engagement of CD40 on leukemic B cells by the CD154 molecule expressed on the bead-activated and -expanded T cells.

**Discussion**

Progressive T cell dysfunction in most patients with CLL tends to parallel the progression of this disease (1, 2). The infusion of functionally competent T cells could improve immune responses against pathogens as well as the leukemic cells, and thus represents an attractive approach for treating this disease. The concept of administering healthy T cells generated from CLL patients has been previously proposed, but has yet to be tested in the clinic (20, 21). Previous methods for ex vivo T cell expansion, such as the use of anti-CD3 Abs (OKT3) and IL-2, have resulted in only modest growth of T cells from CLL patients and do not provide adequate elimination of leukemic B cells without additional depletion steps (21). Furthermore, previous approaches resulted in expansion of CD8+ T cells and only limited numbers of CD4+ T cells (21).

We have developed a simple and efficient ex vivo process to activate and expand T cells and eliminate leukemic cells from the blood of CLL patients. Similar processes using CD3/CD28 bead activation technology have been used to expand T cells from healthy donors, as well as from patients with HIV infections and a variety of cancers (9, 10, 22). T cells grown using this method have been well tolerated and demonstrated preliminary evidence of therapeutic effects in clinical trials (9, 10, 23).

The blood of patients with leukemia contains large numbers of tumor cells, which might serve as a barrier for interaction of Ab-coated beads and T cells. However, despite high leukemic cell numbers in blood samples from CLL patients, T cells from patients at all Rai disease stages were effectively activated and expanded using CD3/CD28 beads, albeit with a trend toward reduced expansion for tissues from Rai disease stage IV patients. Additional patient samples will need to be analyzed to determine whether this trend is statistically significant. During the expansion process, leukemic B cell numbers dropped, suggesting that this approach might be used clinically to generate a T cell product with few, if any, leukemic B cells, thereby reducing the risk of infusing leukemic cells back into the patient. Compared with T cells grown using a similar process from healthy donors and patients with other types of cancer, there was a delay in the onset of activation and expansion of CLL patient T cells.

In contrast to other T cell expansion methods (21), both CD4+ and CD8+ T cells are increased in number using CD3/CD28 beads. The expansion of CD4+ T cells may be clinically important based on preclinical studies as well as a recent clinical study indicating that CD4+ T cells play a critical role in generating antitumor effects (24, 25).

During CD3/CD28 bead expansion, both CD4+ and CD8+ T cell subsets expressed an array of key effector molecules that have
been shown to contribute to potent immune responses, including CD54, CD134, CD137, and CD154. For example, CD154 expression was observed at high levels on the CD4+ T cells and at significant levels on the CD8+ T cells following CD3/CD28 bead activation. Previous studies have shown that the expression of CD154 is reduced in T cells from CLL patients, decreasing the ability of the T cells to engage CD40 on leukemic B cells (3, 26). Without CD40 engagement, CD80 and CD86 expression on leukemic B cells is decreased, thereby limiting their ability to costimulate the T cell arm of the immune system. Gene therapy has been used as one approach to increase expression of CD154 and thus CD40 engagement of leukemic B cells in CLL patients. In this ex vivo approach, CD154 is transduced into leukemic B cells and the transduced cells are infused into patients to engage CD40 on the patients’ leukemic B cells in vivo, thereby rendering them more visible to the patient’s immune system (27). This approach has recently been tested in a clinical trial, which demonstrated reductions in leukemic counts and lymph nodes concomitant with increases in circulating T cell counts and levels of several cytokines in the circulation (27). CD3/CD28 bead-activated T cells appear to express much higher levels of CD154 than have been achieved with the gene therapy approach described above. In addition, CD3/CD28 bead-activated T cells produce many other molecules, such as TNF-α, granzyme A, and granzyme B, which may facilitate antileukemic activity (M. Bonyhadi, unpublished observations). Furthermore, compared with the number of transduced B cells generated by the gene therapy approach, a much higher number of T cells can be produced using the CD3/CD28 bead process. Therefore, it is possible that CD3/CD28 bead-activated T cells produced from CLL patients may have increased therapeutic activity compared with the gene therapy approach.

During primary CD3/CD28 bead activation (days 3–8), T cells from CLL patients secrete large amounts of type 1 cytokines, such as IFN-γ and TNF-α. The absence or low levels of type 2 cytokines, such as IL-10, further suggests that the CD3/CD28 bead activation process favors the generation of Th1 and T cytokotoxic (Tc)1 T cells as opposed to Th2 and Tc2 T cells. Type 1 cytokines are produced by Th1 and Tc1 T cells that are responsible for cell-mediated immune responses that are thought to play a major role in antitumor immunity. Although cytokine levels decreased significantly during later phases of the expansion process, restimulation at the end of the process resulted in production of very high levels of cytokines, perhaps reflecting improved immune function and/or transition from a predominantly naïve T cell phenotype to a memory T cell phenotype. In addition, GM-CSF was produced at high levels during the entire activation and expansion process, which even further increased upon restimulation. GM-CSF plays an important role in recruiting and promoting the differentiation of APCs. Therefore, CD3/CD28 bead-activated and -expanded T cells from CLL patients may help enhance immune responses by increasing the activity of APCs in vivo.

The rapid induction of cytokine secretion upon restimulation, as well as the induction of alloresponsiveness in MLR assays, suggests that CD3/CD28 bead activation can facilitate or restore important T cell responses. The improved responsiveness of CD3/CD28 bead-expanded T cells to allostimulation may reflect the correction of T cell activation defects, which have been reported in the setting of CLL (1, 2). Alternatively, it may be that the CD3/CD28 bead activation process drives the pool of naïve T cells into a memory T cell phenotype, which would then be more sensitive to the relatively weak activation signals delivered by nonactivated allostimulator cells. The first scenario is supported by the observation that similar allo-MLR cultures using nonactivated T cells from healthy donors resulted in rapid induction of high levels of CD25 expression on responding CD8+ T cells (M. Bonyhadi and A. Rasmussen, unpublished observations).

Using IFN-γ secretion as an indication of tumor specificity, ELISpot studies revealed that before activation and expansion, few, if any, tumor-reactive T cells were detectable in the blood of CLL patients. Following CD3/CD28 bead activation and expansion, there was an increase in the number of tumor-reactive T cells.
as measured by ELISPOT in all patient samples tested. The increase in tumor-reactive T cells could be the result of preferential expansion of these cells during culture, enhanced perhaps by the presence of tumor Ags present during culture initiation. Alternatively, the tumor-reactive cells may have been present before expansion, but were unable to respond due to functional defects. The activation and expansion process may have helped correct defects in T cell function, thereby restoring the IFN-γ secretion potential of tumor-reactive T cells. In either case, the rapid induction of apoptotic death observed in leukemic B cells during the CD3/CD28 bead activation process appeared to exceed what one might expect with tumor-specific T cell frequencies of <1:200 (<0.5%). Based on these observations, we evaluated CD3/CD28 bead-activated T cells for their ability to elicit antitumor effects that were independent of specific Ag recognition.

We have demonstrated that CD3/CD28 bead-activated T cells rapidly up-regulate CD95 on the B cells in a cell-cell contact-dependent manner. Experiments have also shown that induction of these markers is primarily mediated via the CD40L-CD40 pathway, consistent with prior studies (28). Typically, the phenotype of CLL B cells is similar to normal resting B cells, with little or no expression of CD54, CD80, CD86, and CD95. The induction of functional CD95 on leukemic B cells by CD3/CD28 bead-expanded T cells appears to reverse what has been reported to be a relative insensitivity of leukemic B cells to endogenous pathways of programmed cell death (29). Addition of FAS-L to leukemic B cells following coculture with CD3/CD28 bead-activated T cells led to marked induction of leukemic B cell apoptosis. However, even in the absence of cross-linking FAS-L, coculture of activated T cells with autologous leukemic B cells appears to drive the entire B cell population toward the apoptotic pathway. The infusion of CD3/CD28 bead-activated T cells could have similar effects in vivo, thereby enabling FAS/FAS-L interactions between leukemic B cells and FAS-L-bearing tissues, both in the circulation and in lymphoid spaces. This may be particularly important in the setting of residual disease in which leukemic cells are often found in secondary lymphoid spaces such as lymph nodes, where a significant percentage of follicular dendritic cells express FAS-L (30). Thus, CD3/CD28 bead-activated T cells could provide an antitumor effect in a manner that would not require recognition of tumor Ags.

The induction of CD54, CD80, and CD86 on leukemic B cells by CD3/CD28 bead-activated T cells also suggests that the APC function of leukemic B cells may be improved, thereby improving their visibility to tumor-reactive T cells. There have been previous reports that the TCR repertoire is skewed in CLL patients (17, 31). We also observed significant skewing in a majority of peripheral blood samples obtained from CLL patients, measured by both cell surface TCR Vβ expression analysis and molecular analysis of TCR Vβ diversity by the spectratype method. One of the most intriguing observations in this study was the normalization of the TCR repertoire in CLL patients following CD3/CD28 bead activation. Conventional methods for expanding T cells, such as anti-CD3 Ab plus IL-2, have been reported to induce or exacerbate TCR repertoire skewing (12, 32). Although it has been shown that expansion of T cells using bead-immobilized anti-CD3 and anti-CD28 preserves repertoire, at least for healthy donors (33), these studies demonstrate that this approach can be used in the setting of CLL to restore a skewed repertoire toward normal, which we and others have recently demonstrated using CD3/CD28 bead expansion in the setting of HIV and multiple myeloma (23). By spectratype analysis, it is not possible to determine whether overrepresented T cell families or clones are deleted during the expansion process, or if they are simply outgrown by “underrepresented” T cell families. However, analysis of cell surface expression by flow cytometric analysis of TCR Vβ usage demonstrated that both processes probably take place during CD3/CD28 bead activation and expansion. The biological and clinical significance of these phenomena is uncertain. T cells bearing the “overrepresented” TCR Vβs could represent tumor-specific T cells or pathogen-specific T cells, and reduction in their relative numbers during the expansion process could diminish the potential antitumor activity and/or antiviral capabilities of the T cell infusion product. However, recent data argue against the hypothesis that these overexpressed populations are rich in tumor-specific T cells. Mackus et al. reported that, in CLL, the absolute number of CD8+ T cells is often increased, and much of this can be attributed to the increased frequency of CTL specific for persistent viruses, such as CMV (34). In contrast, many studies in other settings suggest that skewed repertoires represent deficits in T cells that correlate with other measures of T cell dysfunction (35, 36). Correction of repertoire skewing in other settings such as HIV is associated with improved clinical outcome (16). Therefore, the infusion of a large number of T cells that exhibit a broad repertoire of Ag specificities could reduce infectious complications associated with CLL. Clinical studies should help to address these questions.

Finally, in many disease settings, such as cancer, infectious disease, and autoimmunity, a variety of immune deficits, including T cell and APC dysfunction, have been well characterized. Normalization of immune function may be a viable approach for treating many of these diseases, and the infusion of T cells generated by the process described herein may provide the means for correcting some of these deficits. The potent Ag-independent effects of CD3/CD28 bead-activated T cells on leukemic B cells in CLL suggests that this approach may be equally effective in other B cell malignancies, such as lymphoma. Based upon the preclinical data presented in this study, clinical trials have been initiated to test the potential therapeutic activity of CD3/CD28 bead-activated T cells in patients with CLL.

References


