Eradication of B-CLL by autologous and allogeneic host nonreactive anti–third-party CTLs

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Establishment of cell lines capable of killing leukemia cells, in the absence of alloreactivity against normal host cells, represents a most desirable goal in bone marrow transplantation (BMT) and cancer immunotherapy. By using a human → mouse chimeric model, we demonstrate that allogeneic anti–third-party cytotoxic T lymphocytes (CTLs) depleted of alloreactivity are endowed with a potent anti–B-cell chronic lymphocytic leukemia (B-CLL) reactivity. Likewise, CTL preparations generated from autologous T cells of the same patients with B-CLL exhibited comparable leukemia eradication, suggesting that the reactivity of allogeneic anti–third-party CTLs is not mediated by residual antihost clones. This specificity was also exhibited in vitro, and annexin staining revealed that B-CLL killing is mediated by apoptosis. While the CTLs killing of third-party cells could be blocked by anti-CD3 antibody, the lysis of the B-CLL cells was not inhibited by this antibody, suggesting a T-cell receptor (TCR)–independent cytotoxicity. The role of cell contact leading to apoptosis of B-CLL cells is shown in transwell plates and by anti–lymphocyte function-associated antigen-1 (LFA-1)–blocking antibody. Up-regulation of CD4 and the subsequent apoptosis of B-CLL cells depend on the initial LFA-1/ICAM-1 (intercellular adhesion molecule 1) interaction. Taken together, these results suggest that allogeneic or autologous host nonreactive anti–third-party CTLs may represent a new therapeutic approach for patients with B-CLL. (Blood. 2005;105:3365-3371) © 2005 by The American Society of Hematology

Introduction

The advent of allogeneic stem cell transplantation in elderly patients has opened the way for various cell therapy modalities in B-cell chronic lymphocytic leukemia (B-CLL). In this context, the search for immune cells endowed with graft-versus-leukemia activity in the absence of alloreactivity against normal cells represents a major challenge. It has been shown that anti–third-party cytotoxic T lymphocytes (CTLs) depleted of alloreactivity against the host by means of interleukin 2 (IL-2) deprivation are endowed with marked veto activity and, therefore, might potentially facilitate bone marrow allografting without graft-versus-host disease.1,2 Thus, as shown in our previous murine study, the stimulation against third-party stimulators in the absence of IL-2 leads to a selective growth of the anti–third-party clones and to the elimination of the other clones devoid of the ability to produce their own IL-2. The generation of host-nonreactive human CTLs comprises 2 major steps. First, mixed lymphocyte reaction (MLR) is carried out against third-party stimulators for 2 weeks in the absence of exogenous IL-2. In this step, IL-2 deprivation is used to induce apoptotic death of clones that are not stimulated, including antihost clones, which cannot be spared by making endogenous IL-2. Because mouse cells are very sensitive to IL-2 deprivation, the period of starvation in the mouse is only 5 days. Human T cells are more resistant and require about 2 weeks for effective induction of apoptosis by IL-2 starvation. Second, when IL-2 is added following the 2-week deprivation period, the restimulation leads to subsequent expansion of anti–third-party clones, which survived the IL-2 deprivation. Consequently, all other T-cell clones, including residual antihost clones, which might remain following the initial IL-2 deprivation period, are further diluted.

In the present study, we investigated the potential of such host-nonreactive human CTLs to eradicate fresh B-CLL pathologic cells in a human → mouse chimeric model previously established for the study of human B-CLL.3,4 In this model, engraftment of human peripheral blood mononuclear cells (PBMCs) in general is short term, and human lymphocytes survive effectively 3 to 6 weeks.3,5,6 Thereafter, their numbers decline and by the end of the second month can hardly be traced; therefore, we have made use of this model to investigate short-term effects.

Thus, PBMCs of patients with B-CLL were transplanted into the peritoneal cavity of human → mouse radiation chimera, and the engraftment of pathologic cells was monitored in the presence or absence of antileukemia treatment. When testing the effect of host-nonreactive anti–third-party CTLs on the engrafted B-CLL cells, marked killing of the latter cells was exhibited. In contrast, blasts from patients with acute myelogenous leukemia (AML) were spared and were not killed by the CTLs. A comparable anti–B-CLL
effect was observed following infusion of autologous anti–third-party CTLs substantiating the nonalloreactive nature of this effect. This selective killing of B-CLL was also validated in cocultures in vitro, enabling us to carry out studies on the mechanism of action. Thus, by using blocking antibodies we were able to investigate the role of T-cell receptor (TCR) recognition, lymphocyte function-associated antigen-1/intercellular adhesion molecule-1 (LFA-1/ICAM-1) binding and CD95/CD95L killing.

**Materials and methods**

**Preparation of human/mouse chimera**

Balgc/c mice were exposed to total body irradiation (TBI) at 3.5 Gy and 3 days later to 9.5 Gy. Preparation of bone marrow (BM) cells from nonobese diabetic/severe combined immunodeficient (NOD-SCID) donors was carried out as described. After one day after TBI, recipient mice were injected through the tail vein with 0.2 mL phosphate-buffered saline containing 3 to 4 × 10^6 Nod/Scid BM cells. All strains of mice were from the Weizmann Institute Animal Breeding Center (Rehovot, Israel).

**Fresh tumor cells**

Heparinized whole blood was drawn from patients with their written consent. PBMCs from patients with B-CLL at Rai stage IV and from patients with AML at stage M1 to M2 (French-American-Classification) were obtained by Ficoll density gradient centrifugation, as described elsewhere, and diluted to the desired cell concentration.

**Generation of nonalloreactive anti–third-party CTLs**

PBMCs were obtained by Ficoll density gradient centrifugation of buffy coats from healthy donors and then stimulated with allogeneic Epstein-Barr virus (EBV)–transformed B-cell line of an HLA background, different from that of the host HLA at a 40:1 ratio. For the preparation of autologous anti–third-party CTLs from patients with B-CLL, their PBMCs were subjected to T-cell enrichment by means of E-rosetting with sheep red blood cells as described.

After 10 days of IL-2 deprivation, cultured cells were mounted on Ficoll to discard dead cells and were restimulated with EBV stimulators at a 4:1 ratio. Four days later, the subpopulation of CD8^+ T cells was isolated by 1 round of positive selection with anti–CD8-coated magnetic beads, followed by a round of negative selection of CD4^+ CD56^- cells, after discarding labeled CD4^+ and CD56^+ cells, obtaining a population of more than 90% CD8^+ cells. Thereafter, the CD8^+ T cells were cultured in complete RPMI containing 300 HI/mL recombinant human IL-2 (rhIL-2; EuroCentrums, Amsterdam, The Netherlands) and EBV stimulators at the same 4:1 ratio. The cultures were restimulated once a week, and culture medium containing IL-2 was renewed every 2 to 3 days, for a period of 2 to 3 weeks after the initial addition of IL-2.

**Fluorescence-activated cell sorting (FACS) analysis**

Cells isolated from peritoneal washes of engrafted mice 7 to 10 days after the inoculation of CTLs were incubated with a mixture of labeled monoclonal antibodies for 20 minutes at 4°C. After washing off antibodies, 2- or 3-color analyses were performed with the use of an FACSscan analyzer (Becton Dickinson, Franklin Lakes, NJ). The lymphocytes were gated according to their size in forward and side scatters. The following labeled antihuman antibodies were used to recognize specific surface molecules: CD3-phycocerythrin (PE) and CD3–peridinin chlorophyll protein (Pan T cell) were purchased from Becton Dickinson; CD19–fluorescein isothiocyanate (FITC; Pan B cells/B-CLL) from Southern Biotechnology Associated (Birmingham, AL); CD20–FITC (B cells/some B-CLL), CD5–PE (T cell/B-CLL, phenotype), CD8–FITC (CTLs), CD45–PE (pan-leukocytes) and CD4–PE (non-CTL T cells), CD86–FITC and CD95–PE (activated B Cells) from Dako (Carpinteria, CA); CD40–FITC (B cells) and CD80–CY5 (cyanine 5; B7-1, activated B cells) from Serotec (Oxford, United Kingdom); and CD33–FITC, CD13–PE, (AML) and CD56–CyChrome (natural killer cells) from Pharmingen (San Diego, CA).

**Human IgG determination**

Sera from mice engrafted with human PBMCs with or without allogeneic anti–third-party CTLs were tested for human immunoglobulin G (IgG) as previously described. Briefly, human IgG was quantified by a sandwich enzyme-linked immunosorbent assay (ELISA) with the use of goat–F(ab')2–purified antihuman IgG (Zymed Laboratories, San Francisco, CA) as the capture antibody, and peroxidase-conjugated purified goat–antihuman IgG (Zymed Laboratories) as the detection reagent. Human IgG (Jackson Immunoresearch Laboratories, West Grove, PA) diluted at known concentrations was used as the standard. Fresh substrate solution of 2,2'-Azino-bis 3-ethylbenz-thiazoline-6-sulfonic acid was added, and the absorbance at 405 nm was quantified on an ELISA reader (Dynatech, Port Guernsey, Channel Islands, United Kingdom).

**Cell cultures**

Heparinized whole blood was drawn from patients with their written consent. PBMCs from patients with B-CLL at Rai stage IV were obtained by Ficoll density gradient centrifugation, as described elsewhere, and diluted to the desired cell concentration. B-CLL targets were cocultured with allogeneic or autologous CTL effectors previously generated as described in “Generation of nonalloreactive anti–third-party CTLs” in 6- or 24-well plates (NuncIon; Nunc, Roskilde, Denmark) at effector-target ratio of 1:5. At different time intervals, cells were recovered and analyzed for the presence of several surface markers by a FACSscan analyzer (Becton Dickinson).

**Inhibition of B-CLL killing by blocking antibodies**

To determine the inhibition of B-CLL killing by LFA-1–blocking antibody (Ab), CTLs and B-CLL cells were pooled together at effector-target ratio of 1:5 in 5-ml test tubes. LFA-1–blocking Ab (clones TSL18.1, 1.2 or 2F12) were added to pelleted cells at a final concentration of 10 μg/mL in minimal volumes, and samples were preincubated at 37°C for 10 minutes. Following preincubation, the samples were transferred to 6- or 24-well plates until assayed for annexin staining by a FACSscan analyzer (Becton Dickinson).

To determine the role of anti-CD3 Ab, the optimal blocking concentration (50 μg/mL) of anti-CD3 antibody (OKT-3; a kind gift from Dr G. Inghirami, New York University, New York) was initially defined by competition with a FITC-conjugated antibody with the use of FACS analysis (data not shown). Alloimmune anti–third-party CTLs (10^5 CTL/well) were incubated for 30 minutes at 4°C with or without anti-CD3. Anti–third-party CTLs (5 × 10^6 cells) treated or untreated with anti-CD3 were incubated separately in triplicate with 1.5 × 10^5 fresh B-CLL cells or EBV-transformed B cells that were used as third-party stimulators for these CTLs. Killing of tumor cells was evaluated by annexin staining. Blockade of CD95–CD95L–mediated apoptosis was carried out by incubation of CD95 blocking Ab (clone ZB4; Immunotech, Marseille, France; 300 ng/mL for 30 minutes at 4°C) with B-CLL cells before the establishment of cocultures with anti–third-party CTLs. Following the incubation, the samples were transferred to 6- or 24-well plates and incubated with anti–third-party CTL at effector-target ratio of 1:5. At different time intervals, cells were recovered and analyzed for the presence of several surface markers by a FACSscan analyzer (Becton Dickinson).

**Detection of apoptosis by annexin staining**

Samples from in vitro cultures were incubated with a mixture of selected monoclonal antibodies labeled with different fluorochromes for 20 minutes at 4°C. After washing off the unbound free antibodies, samples were incubated with 5 μL annexin-FITC (MBL, Nagoya, Japan) or annexin-CY5 (Pharmingen) for 10 minutes at room temperature. Subsequently, unbound annexin was washed out, and samples were analyzed by FACS as described in “Fluorescence-activated cell sorting (FACS) analysis.”
intraperitoneally with 100 to 150 bone marrow transplantation (BMT) from a SCID donor, chimeric mice were infused.

Figure 1. Anti–B-CLL reactivity of anti–third-party CTLs.

On day 2 or 3 following the infusion of CTLs, we prepared leukemia-bearing human PBMCs from patients with B-CLL or AML. On days 4 to 10 some of the leukemia recipients were intraperitoneally infused with PBMCs from patients with B-CLL or AML. On days 4 to 10 some of the leukemia recipients were intraperitoneally infused with 106 allogeneic (B,D) or autologous (F) CTLs. As can be seen in Table 1 and in Figure 1A-B, infusion of these anti–third-party CTLs, generated from 3 different healthy donors, led to a marked eradication of B-CLL cells obtained from 6 patients. FACS analysis 7 to 10 days after the infusion of CTLs revealed 49.1% to 98.7% inhibition of growth of CD19+20+CD5+ B-CLL cells. In contrast, no inhibition of AML could be detected upon infusion of the same number of CTLs (Table 2; Figure 1C-D), indicating that the CTLs are not reacting indiscriminately. The number of CTLs required for effective eradication with minimal dilution of the B-CLL cells is only one tenth of the numbers of infused B-CLL cells. Thus, it is unlikely that the observed reduction of the B-CLL signal detected by FACS could be attributed to a dilution by the CTLs. The lack of AML inhibition suggests a similar conclusion. Nevertheless, to rule out this possibility completely, in an initial experiment we tested the absolute cell number following recovery of the cells from the peritoneum. This analysis showed that the percentages in the FACS reflect a true reduction in the total cell number of B-CLL coengrafted with CTLs (the total number of CD19+CD5+ cells was 24.2 ± 2.4 × 10^4 in mice receiving B-CLL alone and 9.5 ± 5.7 × 10^4 in mice receiving both B-CLL and CTLs, P < .02).

Anti–third-party CTLs are depleted of alloreactivity against normal allogeneic PBMCs

To evaluate whether potentially residual host-reactive clones, which might be contaminating our anti–third-party CTLs preparations, mediate the anti–B-CLL effect, we examined the CTL reactivity against fully allogeneic normal B and T cells. Thus, anti–third-party CTLs were administered to mice, which were previously engrafted with allogeneic PBMCs from either healthy donors or from patients with B-CLL. As shown in Figure 2, allogeneic CTLs that succeed in eradicating B-CLL cells (Figure 2A-B) were unable to affect the engraftment of normal PBMCs (Figure 2C-D). Likewise, the human IgG secretion by normal B cells (5315±1025 μg/mL) was not affected significantly upon infusion of anti–third-party CTLs (5633±715 μg/L). It could be argued that these high levels of human IgG were already established prior to the administration of the CTLs. However, data from this model previously demonstrated a steady increase in IgG levels during the initial 3 weeks after implantation of human PBMCs.6 The failure of the CTLs to affect human immunoglobulin blood

Table 1. Anti-B-CLL reactivity of allogeneic anti–third-party CTLs

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Mice per group, n</th>
<th>% CD19/20+CD5+</th>
<th>Mice per group, n</th>
<th>% CD19/20+CD5+</th>
<th>% B-CLL eradication*</th>
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</thead>
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<tr>
<td>B-CLL</td>
<td>B-CLL + anti–third-party CTL</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>3.6</td>
<td>3</td>
<td>0.2</td>
<td>94.1†</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>5.7</td>
<td>5</td>
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</tr>
<tr>
<td>3</td>
<td>2</td>
<td>0.22</td>
<td>2</td>
<td>0.045</td>
<td>79.5 (NS)</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>54.9</td>
<td>2</td>
<td>0.7</td>
<td>98.7 (NS)</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>18.6</td>
<td>5</td>
<td>3.1</td>
<td>83.3‡</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>56.7</td>
<td>5</td>
<td>4.1</td>
<td>92.7§</td>
</tr>
</tbody>
</table>

NS indicates not significant.

*This value was calculated as follows: [% untreated – % treated/untreated] × 100.
†P < .0005.
‡P < 5 × 10^-6.
§P < 0.05.

Statistical analysis

Statistical significance was established by the Student t test.

Results

Allogeneic host-nonreactive anti–third-party CTLs are endowed with potent anti–B-CLL reactivity in vivo

To test whether anti–third-party CTLs have an anti–B-CLL reactivity, we prepared leukemia-bearing human → mouse chimera by intraperitoneal infusion into immune-deficient mice (prepared as described in "Materials and methods") of 100 to 150 × 10^6 PBMCs from patients with B-CLL or AML. On days 4 to 10 some of the leukemia recipients were intraperitoneally infused with 10 × 10^6 to 15 × 10^6 CTLs. As can be seen in Table 1 and in Figure 1A-B, infusion of these anti–third-party CTLs, generated from 3 different healthy donors, led to a marked eradication of B-CLL cells obtained from 6 patients. FACS analysis 7 to 10 days after the infusion of CTLs revealed 49.1% to 98.7% inhibition of growth of CD19+20+CD5+ B-CLL cells. In contrast, no inhibition of AML could be detected upon infusion of the same number of CTLs (Table 2; Figure 1C-D), indicating that the CTLs are not reacting indiscriminately. The number of CTLs required for effective eradication with minimal dilution of the B-CLL cells is only one tenth of the numbers of infused B-CLL cells. Thus, it is unlikely that the observed reduction of the B-CLL signal detected by FACS could be attributed to a dilution by the CTLs. The lack of AML inhibition suggests a similar conclusion. Nevertheless, to rule out this possibility completely, in an initial experiment we tested the absolute cell number following recovery of the cells from the peritoneum. This analysis showed that the percentages in the FACS reflect a true reduction in the total cell number of B-CLL coengrafted with CTLs (the total number of CD19+CD5+ cells was 24.2 ± 2.4 × 10^4 in mice receiving B-CLL alone and 9.5 ± 5.7 × 10^4 in mice receiving both B-CLL and CTLs, P < .02).

Table 2. Allogeneic anti–third-party CTLs do not exhibit reactivity against AML

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>% CD33/13+</th>
<th>% CD33/13+</th>
<th>% AML eradication*</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML</td>
<td>AML + anti–third-party CTL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>18.1</td>
<td>19.1</td>
<td>-0.05†</td>
</tr>
<tr>
<td>2</td>
<td>44</td>
<td>53</td>
<td>-20</td>
</tr>
<tr>
<td>3</td>
<td>41.7</td>
<td>39.58</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Three mice were used in each group.

*This value was calculated as follows: [% untreated – % treated/untreated] × 100.
†P > .1.
levels strongly indicates that engrafted B cells (which exhibit such low numbers that their analysis by FACS is prone to a high margin of error) are not affected. In view of the unchanged engraftment of autologous T cells in the presence of CTLs and considering that IgG secretion by normal B cells requires T-cell help, it is likely that both T- and B-cell subpopulations were not harmed by the allogeneic CTLs.

**Autologous CTLs stimulated against third-party stimulators are endowed with potent anti–B-CLL reactivity in vivo**

To rule out completely the possibility that the anti–B-CLL effect is mediated by residual alloreactivity, we tested similar CTL preparations generated from the patients’ own T cells. Thus, PBMCs were collected from the same 6 patients; their T cells were purified and subjected to the same protocol to which autologous T cells were exposed when preparing anti–third-party CTLs. Although T cells are rare in stage IV B-CLL, we were able to grow a sufficient number of cells in all 6 cases. Thus, in each of these experiments we implanted CTLs in 3 to 7 mice per group. Technical failures to maintain the mice caused poor harvest in only 2 of these 6 experiments. Taken together, the results (Table 3; Figure 1E-F) clearly show that autologous CTLs exhibit potent eradication of B-CLL pathologic cells in the chimeric mouse model.

**Eradication of B-CLL in vitro requires direct contact between CTL effectors and B-CLL targets**

To further study the anti–B-CLL reactivity of anti–third-party CTLs, we attempted to measure in vitro the survival of B-CLL cells following coculturing with autologous or allogeneic host nonreactive CTLs. Addition of autologous or allogeneic anti–third-party CTLs induced a significant decrease in the cell number of cocultured B-CLL cells from 5 different patients (from 68.3 ± 5.9 x 10^6 cells in B-CLL alone to 29 ± 2.3 x 10^6 cells in B-CLL + autologous CTLs and 25.3 ± 3.3 x 10^6 in B-CLL + allogeneic CTLs). Eradication of B-CLL cells was completely prevented if the anti–third-party CTLs were cocultured in transwell plates (80.3 ± 8.2 x 10^6 for B-CLL + autologous CTLs in transwell plates and 76.5 ± 8 x 10^6 in B-CLL + allogeneic CTLs in transwell plates), suggesting that cell contact is crucial for the eradication of the B-CLL cells.

**Allogeneic or autologous anti–third-party CTLs induce apoptotic death in B-CLL leukemic cells**

Accumulation of pathologic cells in the peripheral blood and in the lymph nodes of patients with B-CLL is attributed to a failure of these cells to undergo apoptosis rather than to enhanced proliferation. To test whether the deletion of leukemic cells by the CTLs is mediated by apoptosis, we tested whether the addition of anti–third-party CTLs leads to an increase in annexin-positive cells in the B-CLL population. As can be seen in Figure 3, showing a typical experiment, while there is no increase in apoptotic cells during the earliest hours of coculture with allogeneic (Figure 3A) or autologous (Figure 3B) CTLs, substantial increase occurs by the end of the second day. Again, in agreement with the in vivo

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**Table 3. Anti-B-CLL reactivity of autologous anti-third-party CTLs**

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>B-CLL</th>
<th>B-CLL + autologous CTL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mice per group, n</td>
<td>% CD19/20×CD5^a</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>3.6</td>
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</tr>
<tr>
<td>6</td>
<td>3</td>
<td>28.1</td>
</tr>
</tbody>
</table>

NS indicates not significant.

*This value was calculated as follows: (% untreated − % treated/untreated) x 100.
†P < .05.
‡P < 5 x 10^-5.
experiments, apoptosis induction is selectively induced in B-CLL cells but not in AML blasts. Figure 4 summarizes 3 different experiments with B-CLL and AML cells. While only 12.6% ± 2.7% of B-CLL cells were positive for annexin staining in the absence of CTLs, the level of annexin-positive B-CLL cells increased to 42.2% ± 3% in the presence of anti–third-party CTLs. In contrast, the level of apoptotic cells within the AML blasts was not changed significantly following their coculture with the same anti–third-party CTL. As can be expected from the results described in the previous paragraph, contact inhibition between B-CLL and anti–third-party CTL. As can be expected from the results described in the previous paragraph, contact inhibition between B-CLL and anti–third-party CTLs in transwell plates completely prevented the CTL induced apoptotic death of B-CLL cells (Figure 5).

Killing of B-CLL by anti–third-party CTLs is not inhibited by anti-CD3 blocking antibody

The observation that B-CLL cells are equally killed by allogeneic and autologous CTLs strongly indicates that this killing might be independent of TCR recognition. To further address this possibility we tested the potential inhibition of B-CLL killing by the anti-CD3 blocking antibody OKT3. In this experiment we monitored the survival of target cells, and we compared the inhibition of B-CLL killing to killing of EBV-transformed B cells originally used as the third-party stimulators of the CTLs. As can be seen in Figure 6A, the killing of the latter cells was markedly inhibited by the addition of OKT3, significantly increasing their survival from 3.4 ± 0.7 × 10^4 to 7.9 ± 1.5 × 10^4 (P < .02), similar to the survival level of B-CLL cells alone (9.0 ± 1.3 × 10^4). In contrast, addition of OKT3 did not reverse the killing of B-CLL pathologic cells by anti–third-party allogeneic CTLs (Figure 6B). Thus, the survival of B-CLL cells cocultured with anti–third-party CTLs in the presence or absence of OKT3 was not significantly different (7.0 ± 0.15 × 10^4 versus 9.4 ± 0.16 × 10^4, respectively). FACS analysis of OKT3-treated cells, testing the binding of fluorescent anti-CD3 antibody, shows that this molecule is down-modulated by the OKT3 treatment, but the CD8⁺ cells are not killed by the antibody. Likewise, intracellular levels of interferon-γ were not changed (data not shown).

B-CLL killing by anti–third-party CTLs is inhibited by anti-LFA-1 antibody

The key role of the LFA-1/ICAM-1 pair in the interactions between normal T and B cells is well recognized. Considering that TCR recognition is not operative in the interaction of allogeneic or autologous anti–third-party CTLs with B-CLL cells, we tested the potential interaction via LFA-1/ICAM-1 binding. Staining of the B-CLL cells with anti-CD54 antibody revealed that upon the addition of anti–third-party CTLs the level of CD54⁺ cells is markedly enhanced on the B-CLL cells (Figure 7). This observation suggested that the initial binding which may or may not be mediated via LFA-1/ICAM-1 interaction could be further strengthened following enhancement of CD54 levels so as to allow the prolonged association required for apoptosis induction. Further evidence for the key role of LFA-1 binding in the induction of CD54 up-regulation and in the initiation of events leading to apoptosis of B-CLL cells was provided by the use of LFA-1–blocking antibodies. Thus, a short preincubation of B-CLL/CTL cocultures with either of 2 different blocking antibodies against LFA-1 (clones TSI.18.1.1.2 and 2F12) inhibited significantly the
up-regulation of CD54 (Figure 7), as well as the induction of apoptosis by either autologous or allogeneic anti–third-party CTLs, measured by annexin staining (Table 4).

Cell surface expression of CD95, CD80, CD86, and CD40 following coculture of anti–third-party CTLs with B-CLL cells

The observation that CD54 is up-regulated on B-CLL cells upon the initial interaction with the CTLs, led us to investigate the potential role of the LFA-1/ICAM-1 interaction as described in "B-CLL killing by anti–third-party CTLs is inhibited by anti–LFA-1 antibody." Clearly, this initial interaction might be accompanied by other changes in cell surface molecules. Thus, B-CLL cells from 5 patients were incubated under the conditions described in "B-CLL killing by anti–third-party CTLs is inhibited by anti–LFA-1 antibody" either with allogeneic or autologous anti–third-party CTLs. After 72 hours samples divided into aliquots were tested by FACS for the expression of CD95, CD80, CD86, and CD40. Marked up-regulation of CD95 was induced both by allogeneic and autologous CTLs (from 7.425% ± 2.8% in B-CLL alone to 89.5% ± 13.2% and 74% ± 24.9%, in B-CLL cells obtained after coculture with allogeneic or autologous anti–third-party CTL, respectively). A minor, but significant, up-regulation of CD86 expression was also documented (from 45.9% ± 8% in B-CLL alone to 67.4% ± 5% and 69.5% ± 5.8% in B-CLL cells cultured with allogeneic or autologous anti–third-party CTL, respectively). Interestingly, the expression of another molecule of the B7 family, CD80, was not enhanced and remained at base level. In addition, the expression of another key molecule in B- to T-cell interactions, CD40, whose level in all B-CLL cells tested in this study was always high, was not altered.

B-CLL killing by anti–third-party CTLs is not inhibited by CD95–CD95L blockade

The marked induction of CD95 expression by the CTLs suggests that CD95–CD95L apoptosis could potentially mediate the killing of B-CLL cells by anti–third-party CTLs. In particular, this possibility is attractive, considering our previous finding that deletion of CD8 T cells, which recognize and bind to anti–third-party veto CTLs, is mediated through a CD95–CD95L apoptosis mechanism. Furthermore Husebekk et al,13 who previously suggested that autologous polyclonally activated CD8+ T cells can kill B-CLL cells, also showed that this killing can be inhibited by anti-CD95 antibody. However, as shown in Figure 8, while anti–LFA-1 antibody effectively blocks apoptosis as measured by annexin staining, the incubation with the same anti-CD95 antibody used by Husebekk et al13 did not inhibit annexin staining in the B-CLL pathologic cells. Thus, while the anti–LFA-1 antibody reduced the level of annexin-positive CD19+ cells from 82.0% to 46.3%, no such reduction could be found upon incubation with anti-CD95 antibody (82.0% and 85.7% with and without the antibody, respectively).

Discussion

Our data suggest that autologous or allogeneic anti–third-party CTLs are endowed with marked reactivity against the pathologic cells of patients with B-CLL while being nonreactive against AML blasts or against normal allogeneic B or T cells. This specificity was documented in vivo in a human → mouse chimeric model and in cocultures in vitro. The in vitro studies showed that the B-CLL cells are eradicated by apoptosis. This killing is progressive and is not seen when tested within the first 16 hours of incubation, ruling out potential cross-reactivity of the CTLs with the B-CLL cells. In addition, transwell experiments suggest that cell contact is essential.

The observation that this killing is mediated both by autologous, as well as by the allogeneic host-nonreactive CTLs, indicates that it is TCR independent. This suggestion is further substantiated by the finding that the killing of B-CLL cells is not inhibited by the anti-CD3 antibody OKT3. Thus, it is likely that nonspecific adhesion molecules bring about the initial binding. Indeed, the inhibition by anti–LFA-1 antibody of the B-CLL killing indicates that LFA-1/ICAM-1 contact plays a major role in the interaction of B-CLL cells with anti–third-party CTLs. Our results are consistent with several other studies that showed that CD8+ CTLs are capable of binding to potential targets in the absence of specific TCR.

**Table 4. Inhibition by anti-LFA-1 antibody of autologous or allogeneic anti–third-party CTL-induced apoptosis of B-CLL cells**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-CLL</td>
<td>13.4</td>
<td>6.2</td>
<td>9.3</td>
<td>12.5</td>
<td>21.0</td>
<td>9.0</td>
</tr>
<tr>
<td>B-CLL + autologous CTL</td>
<td>36.00</td>
<td>46.20</td>
<td>28.60</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>B-CLL + autologous CTL + anti-LFA-1</td>
<td>20.20</td>
<td>25.00</td>
<td>27.00</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>B-CLL + allogeneic CTL</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>41</td>
<td>54</td>
<td>37.18</td>
</tr>
<tr>
<td>B-CLL + allogeneic CTL + anti-LFA-1</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>23</td>
<td>29</td>
<td>23.55</td>
</tr>
<tr>
<td>% inhibition</td>
<td>66.81</td>
<td>84.13</td>
<td>39.19</td>
<td>63.16</td>
<td>75.76</td>
<td>48.37</td>
</tr>
</tbody>
</table>

*This value was calculated as follows: (B-CLL — indicates no data. anti–LFA-1/C50 allogeneic CTL) × 100.

![Figure 8. B-CLL killing by anti–third-party CTLs is not inhibited by CD95–CD95L blockade.](image-url)

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recognition. Such interactions are mediated by LFA-1/ICAM-1 binding, leading to the formation of an immune synapse.12,14

Our finding that the initial binding of LFA-1 is associated with further up-regulation of CD54 suggests an attractive working hypothesis in which the induction of apoptosis in B-CLL cells, by anti–third-party CTLs, might require a prolonged association between the cells, which can be brought about only if a certain high-affinity binding between the cells is initiated. Very recently, we showed that CD95L, together with CD8, both of which are present on the anti–third-party CTLs, play a crucial role in their ability to veto effector cells.15 However, while veto cells are recognized by the TCR of the effector T cells leading to the apoptosis of the latter cells, it should be emphasized that in our present study the anti–third-party CTLs cannot be recognized by their target B-CLL cells. Instead, the binding is likely brought about by LFA-1/ICAM-1 interaction. Thus, our present observation that CD95 is up-regulated upon the initiation of such binding is compatible with a CD95/CD95L apoptosis-based mechanism. Furthermore, this hypothesis has been supported by a recent demonstration that autologous T cells activated with anti-CD3 monoclonal antibody could kill B-CLL cells in vitro. In this study, a blocking anti-CD95 antibody inhibited the killing of B-CLL cells.13 However, this killing was tested on Cr51-labeled B-CLL cells that were activated artificially to enable effective Cr51 labeling. In contrast, in our study, using the same anti-CD95 antibody in the coculture of fresh B-CLL and anti–third-party CTLs, we did not find any inhibition of the apoptosis mediated by the CTLs.

Taken together, it is tempting to hypothesize that, upon binding of the CTLs to B-CLL cells via LFA-1/ICAM-1, death molecules other than FasL, which might be present on the activated CTLs (activated in culture against third-party stimulators), might trigger apoptosis in the tumor cells. Alternatively, perforin-mediated killing or signal transduction, bypassing the death receptor, might be triggered directly or indirectly upon the initial LFA-1/ICAM-1 binding. Clearly, further studies are required to investigate the role of other apoptotic pathways or molecules.

In addition, while AML blasts were not killed by the CTLs, preliminary results suggest that TCR-independent killing by such CTLs could be extended to EBV-transformed B-cell lines, the HLA of which was completely different from that of the original third-party stimulators (S.A., unpublished results, June 2001). Thus, further studies with other malignancies, and in particular with B-cell malignancies, are warranted.

Regardless of the mechanism, the results of our present study suggest that anti–third-party CTLs originating either from the patient or from an allogeneic donor exhibit potent reactivity against the pathologic cells in B-CLL, without adversely affecting normal T or B cells. Further preclinical evaluation of potential toxicity to other hematopoietic cells is still warranted prior to clinical attempts.

Although it could be argued that the primera model affords an imperfect simulation for the clinical situation since it utilizes adoptively transferred B-CLL cells and CTLs without evidence for survival benefit to the CTL-treated group, the demonstration of B-CLL eradication, which was duplicated both in vivo and in vitro, could potentially lead to 2 novel treatment modalities in B-CLL. First, if a matched sibling donor is available, a nonmyeloablative hematopoietic stem cell transplantation could be considered in conjunction with adoptive cell therapy using anti–third-party CTLs originating from the donor. It is anticipated that the latter cells, which were previously shown to be markedly depleted of graft-versus-host reactivity and to facilitate engraftment by virtue of their veto activity,16 will also contribute to the eradication of residual disease. If it is proved successful, it could also be considered in the setting of mismatched stem cell transplantation. Second, in patients who lack a matched sibling donor, infusion of autologous anti–third-party CTLs could prove effective. Such CTLs could be tested in the future as a single modality or in conjunction with other therapies.

References


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Eradication of B-CLL by autologous and allogeneic host nonreactive anti-third-party CTLs

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