Cytotoxic CD4+ T cells in patients with B cell chronic lymphocytic leukemia kill via a perforin-mediated pathway.

Nino Porakishvili[1,2]
Lela Kardava[1]
Andrew P. Jewell[4]
Kwee Yong[3]
Martin J. Glennie[5]
Arne Akbar[2]
Peter M. Lydyard[2]

[1] Department of Immunology, Tbilisi State University, Georgia
[2] Department of Immunology & Molecular Pathology, Royal Free & University College Medical School, London, UK
[3] Department of Haematology, Royal Free & University College Medical School, London, UK
[4] School of Life Sciences, Kingston University, UK
[5] Tenovus Research Laboratories, Cancer Sciences Division, University of Southampton

This is an electronic version of an article published in Haematologica, 89 (4). pp. 435-443, April 2004. Haematologica is available online at:

http://www.haematologica.org/journal/2004/890435.pdf

[haematologica]
2004;89:435-443

Cytotoxic CD4+ T cells in patients with B cell chronic lymphocytic leukemia kill via a perforin-mediated pathway

NINO PORAKISHVILI
LELA KARDAVA
ANDREW P. JEWELL
KWEE YONG
MARTIN J GLENNIE
ARNE AKBAR
PETER M LYDYARD

A B S T R A C T

Background and Objectives. B-cell chronic lymphocytic leukemia (B-CLL) is a clonal expansion of $CD5^+$B cells that accumulate due to their uncontrolled growth and resistance to apoptosis. We have previously shown that up to 50% of blood $CD4^+$ T cells in B-CLL patients have a cytotoxicity-related $CD28^-$ $CD57^+$ phenotype and high content of both granzyme B and perforin (PF). In this study we investigate the cytotoxic potential of these cells against autologous B-CLL cells.

Design and Methods. Blood $CD4^+$ or $CD8^+$ T cells were positively isolated from B-CLL patients and cultured under a range of conditions with autologous purified B-CLL cells and with bispecific [anti-CD3 × anti-CD19] antibodies. Apoptosis of labeled B-CLL cells was assessed using the change of mitochondrial membrane potential with the fluorescent dye $DiOC_6$ and confirmed by annexin V binding.

Results. There was time- and dose-dependent killing of B-CLL cells by both $CD8^+$ and $CD4^+$ T cells and this ranged from 6.6 - 68.0% for $CD4^+$ cells and 6.4 - 57.8% for $CD8^+$ cells. Almost complete inhibition by concanamycin A suggests that $CD4^+$ T cells like $CD8^+$ T cells induced apoptosis through a perforin-mediated pathway, but not via Fas/FasL (as indicated by lack of blocking with brefeldin A), tumor necrosis factor α or TRAIL.

Interpretation and Conclusions. This study shows that blood $CD4^+PF^+$ T cells enriched in B-CLL patients, are able to kill autologous B-CLL cells *ex vivo*, through bispecific antibodies via a perforin mediated mechanism.

Key words: B-CLL, CD4-cytotoxicity, perforin.

Department of Immunology, Tbilisi State University, Georgia (NP, LK); Department of Immunology and Molecular Pathology (NP, AA, PML) and Hematology (KY), Royal Free and University College Medical School, London UK; School of Life Sciences, Kingston University, UK (APJ): Tenovus Research Laboratories, Cancer Sciences Division, University of Southampton, UK (MJG).

*Correspondence:
Nino Porakishvili, School of Biosciences, University of Westminster, 115 New Cavendish Street, W1W 6UW, London, UK. E-mail:
n_porakishvili@hotmail.com
n.porakishvili@wmin.ac.uk*

B-cell chronic lymphocytic leukemia (B-CLL) is a clonal expansion of $CD5^+$ B cells that accumulate in the peripheral blood due to their resistance to apoptosis.[1,2] Despite the marked phenotypic as well as chromosomal heterogeneity of leukemic cells from individual patients, the cells characteristically show prolonged survival and over-express the anti-apoptotic Bcl-2 protein.[3] A number of attempts have been made to overcome the resistance of B-CLL cells to apoptosis *in vitro*, including treatment with antibodies to the B-cell receptor,[4] CD5,[3] CD20,[5] and various drugs known to induce death through apoptosis *in vivo*.[6,7]

That T cells can be induced to kill autologous and allogeneic B-CLL cells has been shown using T-cell superantigens[8] and following culture of autologous T cells with B-CLL supernatant pulsed dendritic cells.[9] Effector cytotoxic T lymphocytes (CTL) generated for 7 days in the presence of anti-CD28 were able to kill autologous B-CLL cells through an [anti-CD3 × anti-CD19] bispecific antibody (BsAb).[10] More recently it was shown that normal effector CTL generated for 24 days can successfully kill allogeneic B-CLL cells, the activity being partially blocked by inhibitors of granule exocytosis and therefore thought to be attributed to perforin (PF).[11] In addition, B-CLL cells transfected with CD154 (CD40L) can induce both proliferative and cytotoxic responses by autologous T cells.[12,13] However, the ability of *ex vivo* T cells to utilize the PF-mediated pathway to kill autologous leukemic cells has not been shown.

It is well documented that $CD8^+$ CTL are the main cell type that mediate cytotoxicity through both Fas/FasL and PF/granzyme pathways.[14,15] We have recently shown a considerable expansion of $CD4^+$ T cells with

a cytotoxicity-related $CD28^-CD57^+$ phenotype and high content of both granzyme B and PF in the blood of patients with B-CLL, in some cases accounting for up to 50% of all circulating $CD4^+$ T cells, as well as increased expression of PF by $CD8^+$ cells.[16] Since B-CLL is often accompanied by humoral autoimmunity[17] or immunodeficiency,[18] expansion of $CD4^+PF^+$ cells in blood of B-CLL patients may reflect the immunoregulatory function of these cells. $CD4^+PF^+$ T cells have also recently been documented in several autoimmune disorders.[19,20] Regulatory T cells (Tregs) have been identified as an anergic $CD4^+CD25^+CD45RB^{dim}$ population.[21]

The aim of this study was to determine whether the *ex vivo* expanded $CD4^+PF^+$ cells were able to kill autologous B-CLL cells via a PF-mediated pathway. We also investigated whether $CD4^+PF^+$ cells expressed a regulatory phenotype.

Design and Methods

Patients

With the approval of the local Ethical Committee we studied 26 B-CLL patients (3 women and 23 men) attending the outpatients clinics at UCLH Trust. Patients in disease stages Rai 1-4 were studied (Table 1). Twenty-three patients were deemed *untreated* since they had received no treatment for at least 4 months prior to investigation. Two patients were treated with chlorambucil and one with steroids. The white blood cell (WBC) counts varied from 2.4 to 90.4×10^9/L.

Cell preparation

Peripheral blood mononuclear cells (PBMC) were separated from heparinized blood on a Lymphoprep gradient (Gibco), washed twice and resuspended in RPMI-1640 (Gibco) supplemented with 10% fetal calf serum (FCS, Gibco) and streptomycin/penicillin (Gibco). The cell concentration was adjusted to 5×10^6cells/mL.

Enrichment of $CD4^+$ and $CD8^+$ cells

Cells were enriched for $CD4^+$ and $CD8^+$ populations using positive isolation kits (Dynal) according to the manufacturer's instructions. Beads were coated with anti-CD4 or anti-CD8 mouse IgM monoclonal antibodies (mAbs) and then attached beads were removed using Detachabead (Dynal) according to the manufacturer's instructions. The purity of the isolated $CD4^+$ or $CD8^+$ cells was evaluated by staining with anti-CD4 or anti-CD8 mAbs, conjugated to CyC (Pharmingen) and analyzed by flow cytometry. Only cell suspensions with a purity of > 95% were used for cytotoxic assays.

Enrichment of B-CLL cells

B cells were obtained by negative depletion of peripheral blood T cells and monocytes using anti-CD4, anti-CD8 (Sigma), anti-CD14 and anti-CD3 (Pharmingen) mAbs and Dynal beads according to the manufacturer's instructions. The enriched B cells were washed twice and used for cytotoxicity assays. The purity of the isolated B-CLL cells was checked by staining with CyC-conjugated anti-CD19 and FITC-conjugated anti-CD5 mAbs, (Pharmingen) and analyzed by flow cytometry. Only cell suspensions containing > 98% B-CLL cells were used for cytotoxic assays. The proportion of $CD19^+CD5^-$ cells in the enriched B-cell population was negligible. The viability of the isolated cells was monitored using trypan blue, and only viable cell suspensions (>95%) were used for the assays.

Cytotoxicity assay

Purified target B-CLL cells (10^7 cells/mL) were labeled with the red fluorescent dye PKH26 (Sigma) according to the manufacturer's instruction. The cells were washed and adjusted to 10^5 cells/mL in RPMI-1640. The $CD4^+$ and $CD8^+$ cell suspensions (effector cells) used to kill the target B cells were adjusted to 2.0×10^6 cells/mL in RPMI-1640 and diluted so that addition of 100 μL of each would give effector/target (E/T) cell ratios of 20:1, 10:1, 5:1 and 1:1 when added to 100 mL of target cells in 96-well round-bottomed microplates (Nunc). Bispecific anti-CD3 × anti -CD19 antibody (OKT3×RFB9) was added at a final concentration of 2 μg/mL. Control wells were without BsAb and contained the highest E/T ratio (20:1) or without effector cells at 1.0×10^5 cells/mL in 200 μL of RPMI-1640 to control for spontaneous apoptosis. Cell mixtures were incubated for 4, 24 or 48 hours at 37°C and the cytotoxicity detected only in the red fluorescent dye marked target B-CLL cells (in FL-2) as described below.

Bispecific antibodies were prepared using a chemical cross-linker to couple mAb Fab' fragments via their hinge-region SH groups[22] and the optimal concentration of BsAb for cytotoxicity was determined in preliminary studies using allogeneic B-CLL target cells and control $CD8^+$ cells. Apoptosis was measured primarily by the loss of mitochondrial membrane potential as detected by decreased emission from the dye 3,3'

Table 1. Patient's data.

Stage (Rai)	*Number of patients*	*WBC×10⁹/L*	
		Range	*Mean ± SD**
0	None	–	–
1	7	7.1-20.7	12.8±5.0
2	9 (1 - Chlorambucil)	19.2-90.4	46.0±28.1
3	6 (1 - Chlorambucil)	9.1-61.5	28.0±19.1
4	4 (1 - Steroids)	2.4-10	5.4±2.7

*The patients were untreated unless otherwise stated. *Standard deviation.*

dihexyloxacarbocyanine iodide ($DiOC_6$, Sigma), a generally accepted method for the evaluation of effector cell induced apoptosis.[11] $DiOC_6$ was added at a final concentration of 40 nM, 15' prior to the end of the incubation period and the green fluorescence of the dye measured by flow cytometry by gating on the red-linker marked target B-CLL cells. The results were expressed as percentages of apoptotic cells (dull green fluorescence) over the total including viable cells (bright green fluorescence) minus spontaneous apoptosis seen in the same gated population, unless indicated otherwise. To confirm our apoptosis results obtained with $DiOC_6$, in some experiments apoptosis was measured by cell binding of annexin V. Briefly, the labeled B-CLL cells from 8 patients following incubation with $CD4^+$ T cells were washed twice with $Ca2^+$-free binding buffer and stained with annexin V-FITC (APO-1 kit, Sigma) for 10 minutes at room temperature. Green fluorescence staining of PKH26-labeled cells was measured and the results expressed as percentages of apoptotic annexin V binding cells.

Mechanism of induction of apoptosis by $CD4^+$ T cells

To determine whether the apoptosis of target cells involved PF and/or Fas-mediated pathways, $CD4^+$ or $CD8^+$ cells (0.2×10^6 in 200 μL of RPMI-1640) were pretreated for 2 hr at 37°C with either 100 nM of concanamycin A (CMA, Sigma), which causes the degradation of PF-containing granules[23] or 10 μM of brefeldin A (BFA, Sigma) which disrupts Fas/FasL-mediated interactions.[24] The cells were then washed three times and co-cultured with target cells at the highest E/T cell ratio (20:1) for 48h in the presence of BsAb. To study the possible bystander killing through TNFα, anti-TNFα mAb (Upstate, clone 2C8) was added at a final concentration of 500 ng/mL (as recommended by the manufacturer), at the beginning of the culture together with BsAb at a 20:1 E/T cell ratio for 48h.

Expression of perforin by T cells with a regulatory phenotype

Peripheral blood mononuclear cells were prepared as described above and stained with optimal concentrations of CyC-conjugated anti-CD4 and PE-conjugated anti-CD25 mAbs or CyC-conjugated anti-CD4 and PE-conjugated anti-CD45RB mAbs (Pharmingen) previously titrated to give optimum conditions. They were then fixed, permeabilized and treated with FITC-anti-PF mAb (Ancell) as described previously.[16] In additional experiments PBMC from 12 B-CLL patients and 8 controls were stained with anti-CD4, anti-PF and PE-anti-IL-10 mAbs (Pharmingen). The cells were analyzed by flow cytometry and the results expressed as the mean fluorescence intensity (MFI) of CD45RB in PF^+ and PF^- $CD4^+$ T cells and percentages of $CD4^+$ PF^+ $CD25^+$ cells or $CD4^+$ PF^+ $IL10^+$ cells.

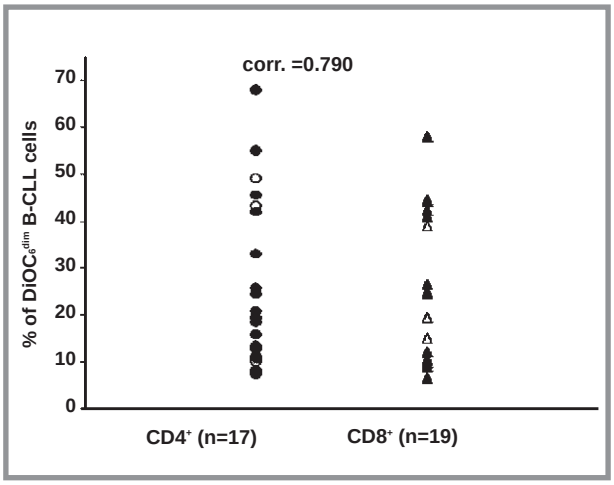

Figure 1. Apoptosis of B-CLL leukemic cells induced by $CD4^+$ and $CD8^+$ autologous effector cells. Effector CD4 and CD8 cells were cultured with autologous B-CLL cells labeled with a red fluorescent linker (RFL) at different effector/target (E/T) cell ratios with BsAb for 4-48 hours. Apoptosis was measured by addition of the mitochondrial dye $DiOC_6$ to effector/target cell mixtures for 15 mins and the decrease in green fluorescence determined by flow cytometry in B-CLL cells as described in the Materials and methods. The results represent the population gated on RFL^+ B-CLL cells only. This Figure show the maximum levels of apoptosis induced by CD4 cells (17 patients) and CD8 cells (19 patients) with spontaneous levels of apoptosis of the B-CLL cells subtracted. Data from two patients treated with chlorambucil and one treated with steroids are shown by open circles and triangles. A correlation coefficient was used to compare the levels of cytotoxicity mediated by $CD4^+$ and $CD8^+$ cells from individual patients.

Statistical analysis

The data were analyzed using Student's t-test, correlation coefficient and Mann-Whitney non-parametric test where appropriate. Data shown in the figures represent means ± standard deviations.

Results

Both $CD4^+$ and $CD8^+$ cells induce apoptosis of autologous B-CLL cells

Both $CD4^+$ and $CD8^+$ cells isolated from the blood of B-CLL patients induced apoptosis of autologous leukemic cells in the presence of anti-CD3 × anti-CD19 BsAb at an E/T ratio in the range of 20:1 - 5:1 (Figure 1). The results show that $CD4^+$ T cells induce apoptosis of autologous B-CLL cells. $CD8^+$ T cells, as expected, also induce apoptosis. The level of apoptosis was different for each patient's tumor cells and varied between 6.6 and 68.0% of $DiOC_6^{dull}$ cells for $CD4^+$ cells (17 out of

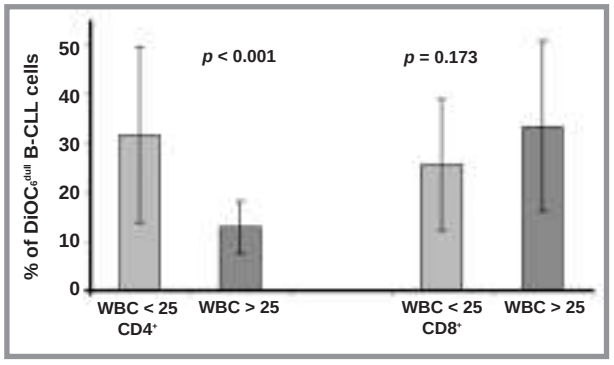

Figure 2. Cytotoxicity by CD4+ but not CD8+ cells is related to the white blood cell (WBC) count. Apoptosis data from Figure 1 is plotted for two groups of patients arbitrarily defined by a WBC count higher (CD4+ n= 9; CD8+ n= 6) and lower (CD4+ n=15; CD8+ n=15) than 25×10⁹/L.

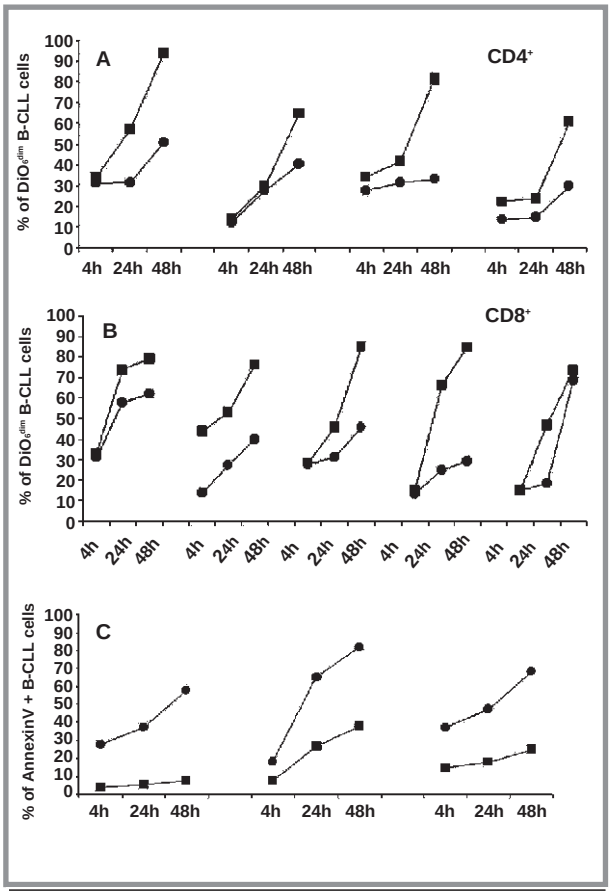

Figure 3. Time course for induction of apoptosis of B-CLL cells by CD4+ and CD8+ cells. B-CLL target cells were cultured with CD4+ or CD8+ cells at E/T cell ratios of 20:1 or 10:1 in the presence of BsAb for 4, 24 and 48hr as described in Materials and methods. Apoptosis was measured by green mitochondrial dye DiOC6 by gating on B-CLL cells labeled with the red fluorescent linker as described in Figure 1. CD4+ cells from 4 individual patients (3A) and CD8+ cells from 5 individual patients (3B) are shown (square symbols) together with the spontaneous apoptosis (round symbols) by individual target cells. Figure 3C shows the level of binding of annexin V-FITC to the target cells in similar experiments with CD4+ cells from 3 representative patients as effector cells.

22 patients) and 6.4 - 57.8% for CD8+ cells (19 out of 21 patients). Only negligible amounts of cytotoxicity (<5%) were seen in the majority of the cases without BsAb. There was no correlation between the level of cytotoxicity induced by CD4+ or CD8+ T cells and stage of the disease (*data not shown*). However, there was a significant negative correlation (p=0.004) between the white blood cell (WBC) count and cytotoxic activity of CD4+ (but not CD8+ cells), the effector cells from the patients with a WBC of more than 25×10^9 showing decreased cytotoxic activity (Figure 2, pooled data). The level of apoptosis induced by the CD4+ cells was directly related to the percentages of cells containing perforin (correlation coefficient of 0.75): 19.8±11.9% of CD4+PF+ cells and 49.9±20.7% of CD8+PF+ cells.

CD4+ mediated cytotoxicity is time-dependent

Apoptosis of autologous B-CLL cells induced by both CD4+ and CD8+ cells increased with time in culture (Figure 3). Data are shown for patients from whom sufficient CD4+ T cells (4 patients, Figure 3A) and CD8+ T cells (5 patients, Figure 3B) could be obtained for a complete time course at an E/T ratio of 20:1 or 10:1. Spontaneous apoptosis of the B-CLL cells alone is also shown. A time-related increase in apoptosis appeared to be a characteristic of CD4+ cells whereas that induced by CD8+ cells had generally plateaued by 24 hours. We did not study cytotoxicity beyond 48h, since B-CLL cells from the majority of the patients had been killed by this time point. To confirm these data we studied annexin V binding by B-CLL target cells in eight patients who had shown increased apoptosis as measured by the $DiOC_6$ assay. The results for three representative patients show similar profiles for a time-related induction of CD4+-induced apoptosis measured by annexin-V-FITC binding to the cell surface of apoptotic B-CLL cells (Figure 3C).

Apoptosis of autologous B-CLL cells induced by CD4+ and CD8+ cells was dependent on effector cell number

A representative experiment shows the effect of increasing the E/T ratio on apoptosis of B-CLL cells measured by $DiOC_6$ staining after 24 and 48 hours in culture (Figure 4). Data from three individual patients show the level of CD4+ T-cell-induced apoptosis of B-CLL cells decreased with a decreasing E/T ratio with some minimal cytotoxicity detectable at an E/T ratio of 1:1; Figure 5A). Pooled data from patients tested at different E/T ratios show the same trends in dependence of cytotoxicity on E/T ratios (Figure 5B). Interestingly, at 10:1 and 20:1 E/T ratios CD8+ cells were approaching plateau levels for cytotoxicity as shown for individual patients (n=3: Figure 5C) and pooled patient data (Figure 5D).

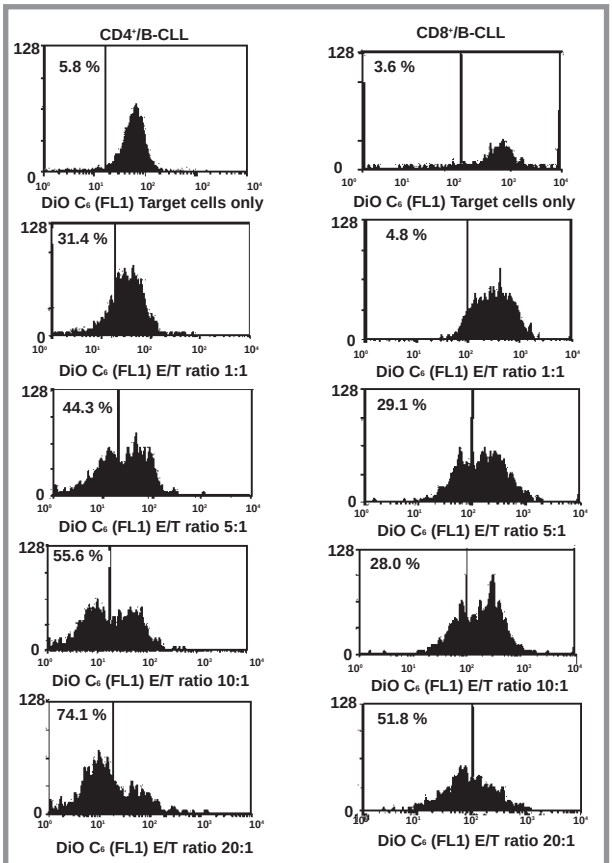

Figure 4. Representative profiles showing an increase in apoptosis with E/T cell ratio for both CD4+ and CD8+ effector cells. B-CLL target cells were cultured with CD4+ or CD8+ cells at E/T cell ratios of 20:1, 10:1, 5:1 and 1:1 in the presence of BsAb for 48 hr. Apoptosis was measured as described in Figure 1. The values shown have spontaneous apoptosis subtracted. Only the statistical differences with *p* values <0.05 are indicated.

CD4+ T cells kill autologous B-CLL cells via a perforin-mediated pathway

CD8+ T cells have been shown to induce apoptosis primarily through a PF-mediated pathway.[14,15] In order to determine whether the apoptosis of B-CLL cells induced by *ex vivo* CD4+ cells from B-CLL patients was also mediated through a PF-dependent mechanism, effector cells were treated with CMA prior to addition of target leukemic cells. Cytotoxicity mediated by CD4+ cells from the majority of patients (8 out of 11) was blocked by concanamycin (Figure 6A: $p \leq 0.025$). In only three patients, did concanamycin fail to completely block the cytotoxic effect of CD4+ cells. As expected, the cytotoxicity mediated by CD8+ cells at 48 hours was completely blocked by this drug (Figure 6B, $p \leq 0.029$).

To investigate the role of the Fas/FasL pathway in induction of apoptosis by CD4+ cells, the cells were precultured with brefeldin. In addition, to test whether TNFα had a role in cytotoxicity we included anti-TNFα antibodies in some cultures. Neither of these treatments interfered with cytotoxicity mediated by either CD4+ or CD8+ T cells. (Figures 6A and 6B).

CD4+PF+ cells do not belong to the terminally differentiated Treg population, but are enriched with CD25+ and IL10+ cells compared with CD4+PF- cells

We found that PF+ CD4+ cells were enriched in CD45RB^{bright} cells as measured by the mean fluorescence intensity (MFI) of CD45RB, whilst the PF- CD4+ cells contained significantly more CD45RB^{dim} cells (Figure 7A, p=0.005). This suggests that the proportion of terminally differentiated Treg cells within the PF+ CD4+ cell

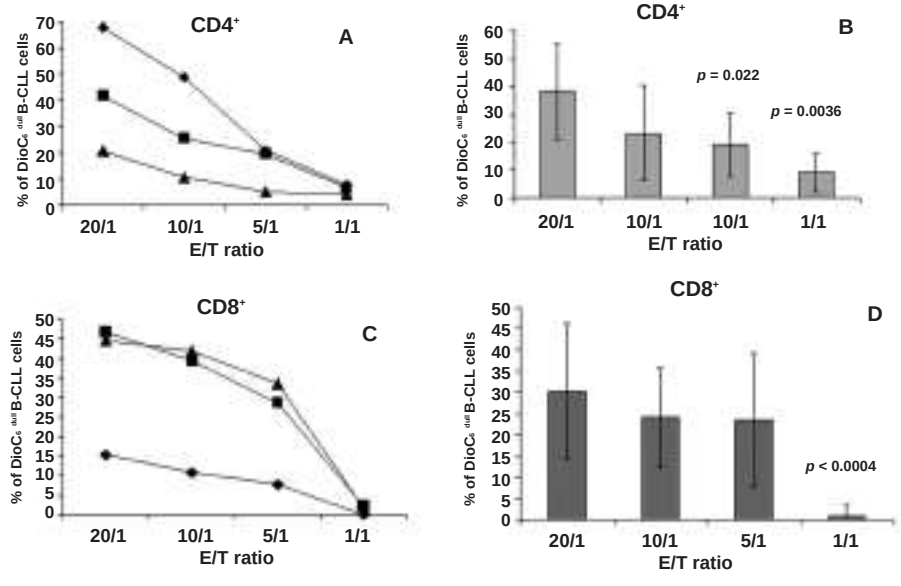

Figure 5. Dose-dependence of effector cells on the cytotoxic activity of CD4+ and CD8+ cells. B-CLL target cells were cultured with CD4+ or CD8+ cells at E/T cell ratios 20:1, 10:1, 5:1 and 1:1 in the presence of BsAb for 48hr. Apoptosis was measured as described in Figure 1. Data are shown for individual patients (n=3) for CD4+ effector cells (5A) and pooled data for CD4+ cells (5B): *p* values show the differences with the highest 20:1 E/T ratio. Individual patient data (n=3) for CD8+ effector cells (5C) and pooled data for CD8+ cells (5D) are also shown: the *p* value shows the difference between 1:1 and all other E/T ratios used.

population in B-CLL patients is low, as defined by expression of CD45RB. However, significantly more PF^+ $CD4^+$ cells from B-CLL patients expressed CD25 (p=0.011) than did their control counterparts, whilst the proportion of $CD25^+$ cells in the PF^- $CD4^+$population was similar in both controls and B-CLL patients (Figure 7B). In addition, more $CD4^+$ PF^+ cells in patients with B-CLL constitutively expressed IL-10 (Figure 7C), than did the PF^- population (8.4 ± 9.6 versus 1.5 ± 2.3, p=0.009). This value was significantly higher than that of the $CD4^+$ PF^+ cells in healthy controls (2.1±0.9, p=0.015) suggesting that in B-CLL patients the $CD4^+$ PF^+ population is enriched with immunoregulatory T cells as determined by constitutive expression of IL10.

Discussion

Although a property previously attributed to $CD8^+$ T cells, in this study we show that $CD4^+$ T lymphocytes were able to kill autologous B-CLL cells through a bispecific antibody directed to CD3 and CD19.[10] We have recently found a significant expansion of $CD4^+$ T cells with the cytotoxicity-associated phenotype, expressing perforin and granzymes in patients with B-CLL.[16] In this study we specifically investigated whether *ex vivo* $CD4^+$ T cells induce apoptosis of autologous B-CLL cells through the cytotoxicity-associated machinery that they contain. In addition we studied whether these cells belonged to the T regulatory population with $CD4^+$ $CD25^+$ $CD45RB^{dim}$ phenotype.

The major finding reported here is that *ex vivo* $CD4^+$ cells, like $CD8^+$ cells, were able to induce significant apoptosis of autologous B-CLL cells in the presence of anti-CD19×CD3 BsAb (Figure 1). There was some correlation between the ability of both $CD4^+$ and $CD8^+$ cells in individual patients to induce cytotoxicity (0.790), although the individual values for apoptosis of B-CLL cells varied from 6.6% to 68.0% for $CD4^+$ cells and from 6.4% to 57.8% for $CD8^+$ cells. In our study, $CD4^+$ cells, like $CD8^+$ cells, induced apoptosis of the target cells in a dose-dependent manner (Figures 4 and 5), a characteristic feature of classical cytotoxic cells.[11] Since we pre-labeled the target B-CLL cells with a red membrane linker we were able to specifically detect apoptosis in this population and ignore any apoptosis of non-CLL B cells. Previous studies by others have also shown killing of malignant B-CLL cells through BsAb to CD3 and CD19 together with anti-CD28 in a 4 hr cytotoxicity assay.[10] However, in their studies, blood lymphocytes were activated for 9 days prior to addition of B-CLL target cells and the pathway utilized was believed to be Fas/FasL. In our experiments we anticipated that since the patient's $CD4^+$ T cells (and $CD8^+$T cells) had the $CD28^-$ $CD57^+$ phenotype of mature cytotoxic cells,[16] they would directly kill the autologous tumour cells in the presence of BsAb linking them to the target cells upon activation via CD3.

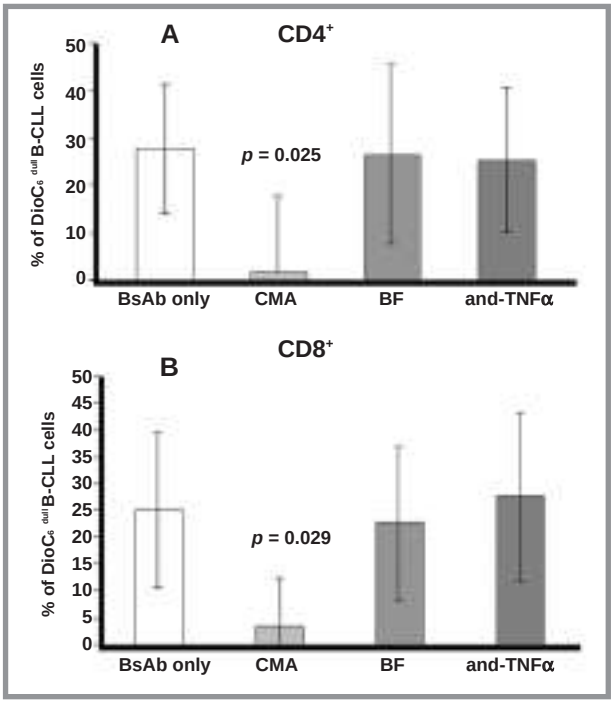

Figure 6. $CD4^+$ and $CD8^+$-mediated apoptosis is blocked by CMA, but not BF or anti-TNFα. $CD4^+$ or $CD8^+$ cells were pretreated for 2h with either 100 nM of CMA or 10 μM of BF and mixed with B-CLL target cells as described in Materials and methods. The cells were co-cultured for 48h with BsAb at an effector/target ratio of 20:1. In some cultures anti-TNFα mAb was added at the beginning of the culture. Apoptosis of the target cells above that of spontaneous was expressed by the percentages of $DiOC_6^{dim}$ B-CLL cells, marked with a red fluorescent linker. CMA: concanamycin A; BF: brefeldin.

Although there was some limited cytotoxicity by the $CD4^+$ (and more so by the $CD8^+$ T cells) measured at 4 hours, in the majority of cases the cytotoxicity by $CD4^+$ cells was higher at 24 and 48 hours (Figures 3A and 3B). After more prolonged exposure to the effector cells the majority of B-CLL cells had reached the later stages of apoptosis (ie. cell shrinkage, *data not shown*) indicating irreversibility of the process. Induction of target B-CLL apoptosis was confirmed by the binding of annexin V (Figure 3C). Thus, unlike assays using pre-generated cytotoxic T cells[11] or NK cells, there was a time requirement to obtain optimum killing. This could account for the lack of evidence in previous studies of *ex vivo* $CD4^+$-mediated cytotoxicity measured in classical 4h cytotoxicity assays.[25]

The reason for the delay in cytotoxicity is unclear. One possibility is that $CD4^+$ cells need to be activated to induce apoptosis. However, both $CD4^+$ and $CD8^+$ cells from two patients with high levels of perforin expression induced up to 41.8% of apoptosis of target Ramos

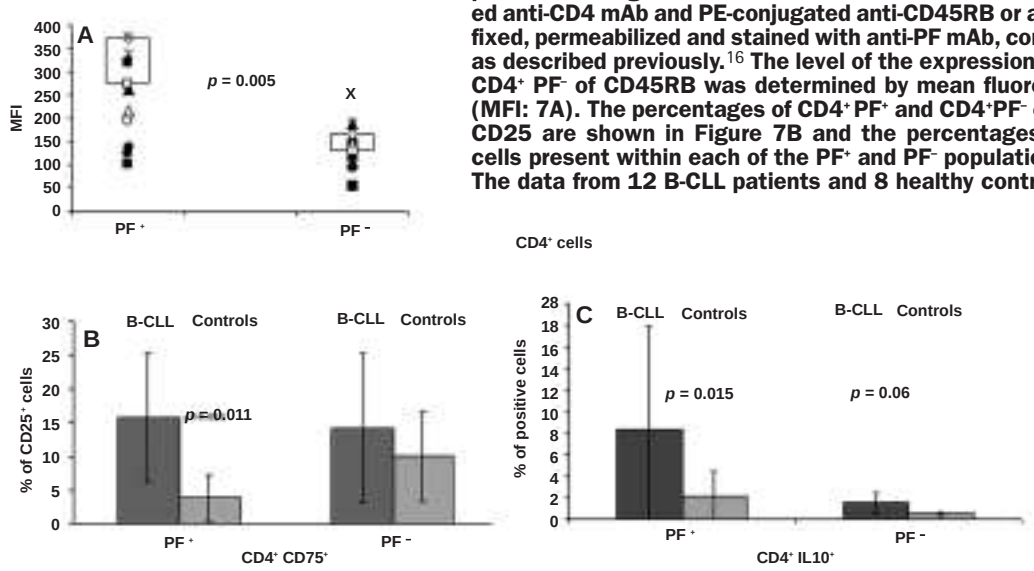

Figure 7. Expression of CD45RB (A) and CD25 (B) by CD4+ PF+ and CD4+ PF- cells in peripheral blood of B-CLL patients. PBMC from B-CLL patients and age-matched controls were stained with CyC-conjugated anti-CD4 mAb and PE-conjugated anti-CD45RB or anti-CD25 mAbs, fixed, permeabilized and stained with anti-PF mAb, conjugated to FITC as described previously.[16] The level of the expression by CD4+PF+ and CD4+ PF- of CD45RB was determined by mean fluorescent intensity (MFI: 7A). The percentages of CD4+ PF+ and CD4+PF- cells expressing CD25 are shown in Figure 7B and the percentages of CD4+ IL-10+ cells present within each of the PF+ and PF- populations in Figure7C. The data from 12 B-CLL patients and 8 healthy controls are shown.

and DG75 B cells after only 4h in the presence of BsAb (*data not shown*). It therefore seems likely the CD4+ T cells are mature cytotoxic cells *ex vivo*. In further support of the concept that the CD4+ T cells are mature cytotoxic cells *ex vivo*, CD4+ PF+ cells from control and virus-infected individuals showed killing of EBV-infected target cells mediated through Staphylococcal enterotoxin B within 4 hours, demonstrating that these cells are capable of releasing perforin and killing immediately.[26]

Another possibility is that the target B-CLL cells themselves are not susceptible *ex vivo* to the action of perforin and granzymes but acquire this susceptibility during culture. In fact, a previous study by others indicated that cytotoxicity mediated by T cells is enhanced by pre-activation of the target B-CLL cells by phorbol ester.[27] Consistent with the necessity for the target B cells to be *activated*, we found that some molecules were up-regulated on B-CLL cells in culture with the effector CD4+T cells. Expression of CD86 was increased on B-CLL cells (in 5 patients) from 4.9±3.0% when cultured alone for 48 hrs to 13.7±4.4% in the presence of autologous CD4+ T cells and BsAb (p=0.0039). B-CLL cells cultured with autologous CD8+ cells and BsAb also up-regulated CD86, although to a lesser extent (8.8±6.6%, p=0.082). Another possibility is that the effector T cells might recognize leukemic cells dying in culture and aid in their demise. Previous data from a variety of sources have indicated that CD8+ T cells primarily induce cytotoxicity through perforin-mediated mechanisms[14,15] and have suggested that the main mechanism by which CD4+ T cells induce apoptosis is through the Fas/FasLpathway.[14,28] Since the frequency of CD4+ T cells in B-CLL patients containing perforin is significantly higher than in normal controls[16] it was important to determine whether CD4+ T-cell cytotoxicity was mediated through this molecule. Concanamycin A has been shown to disrupt the granules in cytotoxic T cells and to inhibit PF-mediated cytotoxicity.[23,29] Our data show that, like CD8+ T-cell-mediated cytotoxicity, CD4+ T-mediated killing is blocked by concanamycin A in the majority of cases (Figure 6).

Since for three patients studied, blocking of apoptosis induced by CD4+ T cells was not completely inhibited by concanamycin A, we tested whether other PF-independent mechanisms could also be operating. Brefeldin A, a Fas/FasL blocker[24] failed to alter the cytotoxicity of either CD4+ or CD8+ T cells (Figure 6). This was not surprising since cross-linking of Fas expressed by B-CLL cells is not effective in inducing apoptosis[30] (*and unpublished observations*). Although unlikely to be playing a role in our experimental system, it has recently been shown that B-CLL cells become susceptible to Fas-induced apoptosis after 4-5 days of activation via CD40 suggesting that this mechanism could be operating in longer term cultures,[31] as compared to a *faster* PF-mediated killing. TNFα has been shown to be produced by cytotoxic T cells,[32] but in our experiments anti-TNFα also failed to block cytotoxicity. This is not surprising since TNFα has been shown to induce proliferation of B-CLL cells and even protect against *in vitro* apoptosis.[33] Another member of the TNFα family, TRAIL, which has been shown to be produced by cytotoxic T cells[34] failed to induce apoptosis when added *in vitro* to B-CLL cells

from 8 cases (*data not shown*). We cannot exclude that granulysin is contributing to the cytotoxicity since it is sensitive to CMA and is co-expressed with perforin.[35-37] This is currently under investigation.

The significance of the expansion of functionally potent cytotoxic $CD4^+ PF^+$ T cells in B-CLL is an important issue. Studies by others have shown that $CD4^+ PF^+$ are increased in several autoimmune disorders[19,20] and in acute and chronic viral infections.[26] Cytotoxic $CD4^+$ T cells kill virus-infected cells primarily through perforin.[26,38] Thus, the $CD4^+$ T cells could be specific for subclinical viral infections. In fact, recent studies have suggested that there is a high frequency of $CD4^+$ CMV-specific T cells in patients with B-CLL (*P. Moss, personal communication*). Experiments are currently underway to test this possibility. Alternatively, they might also have some specificity for tumour-associated antigens and could potentially play a role in killing B-CLL cells *in vivo*. That T cytotoxic precursors do exist *in vivo* is suggested by the fact that autologous T lymphocytes recognize B-CLL tumor-associated antigens such as IgVH CDR3 region[39] and that cytotoxic T cells have been generated, *in vitro*, against tumor-associated antigens from B-CLL.[11,13] Our data suggest the potential role of CD4 (and CD8) mediated cytotoxicity directed towards autologous leukemic cells as a future component of anti-tumor and anti-viral therapy.

Since $CD4^+$ cells that are $CD25^+$ and $CD45RB^{dim}$ have recently been shown to be terminally differentiated T regulatory cells[21] we determined whether the $PF^+ CD4^+$ population contained cells with immunoregulatory phenotype. Our finding that the intensity of CD45RB cells within the $CD4^+ PF^+$ population in B-CLL patients was higher than in their PF^- counterparts would argue that the PF^+ population contains fewer Treg cells than does the $CD4^+ PF^-$ subset (Figure 7A). However, there were significantly more CD25 expressing cells within the PF^+ $CD4^+$ from the B-CLL patients than from the controls (Figure 7B). This could also suggest that they were regulatory[41] or that they were simply responding to activation. Since IL-10 has also been shown to be an indicator cytokine present in T regulatory cells,[4,1] we examined PF^+ cells for intracellular IL-10 (Figure 7C). The $CD4^+ PF^+$ cells in B-CLL contained significantly more intracellular IL-10, than did their PF^- counterparts or PF^+ cells in normal controls indicating that cells within the $CD4^+ PF^+$ population could also have a regulatory role in B-CLL. This is currently under investigation. With regard to the differentiation status of the $CD4^+ PF^+$, the absence of CD28 expression previously shown by us[16] and more recently by others in control and virus infected individuals[26] suggests that this population of cells has been stimulated by antigen. However, the finding that they are not $CD45RB^{dim}$ would argue against their being mainly a chronically stimulated end-point differentiated population.[21]

In conclusion, we have provided evidence that the *ex vivo* $CD4^+ PF^+$ T cells are capable of killing autologous B-CLL tumor cells *in vitro* through a perforin-mediated mechanism. In addition, more of these cells in B-CLL contain IL-10 and express CD25 suggesting that they might have a regulatory role in B-CLL. Further experiments are in progress to determine the exact nature of these cells in relation to their origin, differentiation state, function and specificity in B-CLL patients.

NP helped to design the experiments, carry them out and interpreted results, produced the first draft of the paper and approved version to be published. LK carried out experiments and analyzed data, helped in drafting article, gave final approval for it to be published. AJ helped to design the experiments, helped with revising the first draft, approved final version to be published. KY clinician providing patients, helped with interpretation and revising manuscript, approved final version. MG provided bispecific antibodies, helped in design of experiments, revised article and approved final version. AA helped in the design and interpretation of experiments related to T regs, revised the paper, approved the final version. PM was involved in original concept and design, interpretation etc. Involved in first and subsequent drafts, approved final version and submitted it. The authors would like to acknowledge the help of Dr M Vukmanovic-Stejic in the study on T regulatory cells and Ms AL Tutt for supplying bispecific anti-CD3 × anti-CD19 mAb, Ysobel Howard for secretarial help with patients' records and Dr T. Kipps for helpful discussion. The authors reported no potential conflicts of interest.

This work was supported in part by a grant from the Special Trustees of the Middlesex Hospital and UCH and Royal Free & UCL Medical School and INTAS EU Grant 2239.

Manuscript received August 18, 2003. Accepted January 25, 2004.

References

1. Kipps TJ. Chronic lymphocytic leukemia and related diseases. In: Williams Hematology, Beutler E, Lichtman M, Coller B, Kipps TJ, eds. McGraw-Hill Inc, NY; USA. 1995. p. 1017-39.
2. Reed JC. Molecular biology of chronic lymphocytic leukemia. Semin Oncol 1998; 25:11-8.
3. Pers JO, Berthou C, Porakishvili N, Burdjanadze M, Le Calvez G, Abgrall JF, et al. CD5-induced apoptosis of B cells in some patients with chronic lymphocytic leukemia. Leukaemia 2002;16:44-52.
4. Zupo S, Isnardi L, Megna M, Massara R, Malavasi F, Dono M, et al. CD38 expression distinguishes two groups of B-cell chronic lymphocytic leukemias with different responses to anti-IgM antibodies and propensity to apoptosis. Blood 1996;88: 1365-74.
5. Pedersen IM, Buhl AM, Klausen P, Geisler CH, Jurlander J. The chimeric anti-CD20 antibody rituximab induces apoptosis in B-cell chronic lymphocytic leukemia cells through a p38 mitogen activated protein-kinase-dependent mechanism. Blood 2002; 99:1314-9.
6. Begleiter A, Mowat M, Israels LG, Johnston JB. Chlorambucil in chronic lymphocytic leukemia: mechanism of action. Leuk Lymphoma 1996;23:187-201.
7. Bellosillo B, Villamor N, Colomer D, Pons G, Montserrat E, Gil J. In vitro evaluation of fludarabine in combination with cyclophosphamide and/or mitoxantrone in B-cell chronic lymphocytic leukemia. Blood 1999;94:2836-43.
8. Wallgren A, Festin R, Gidlof C, Dohlsten M, Kalland T, Totterman TH. Efficient killing of chronic B-lymphocytic leukemia cells by superantigen-directed T cells. Blood 1993;82:1230-8.
9. Goddard RV, Prentice AG, Copplestone JA, Kaminski ER. Generation in vitro of B-cell chronic lymphocytic leukaemia-proliferative and specific HLA class-II-restricted cytotoxic T-cell responses using autologous dendritic cells pulsed with tumour

cell lysate. Clin Exp Immunol 2001;126: 16-28.
10. Bohlen H, Manzke O, Patel B, Moldenhauer G, Dorken B, von Fliedner V, et al. Cytolysis of leukemic B-cells by T-cells activated via two bispecific antibodies. Cancer Res 1993;53:4310-4.
11. Chu P, Wierda W, Kipps TJ. CD40 activation does not protect chronic lymphocytic leukemia B cells from apoptosis induced by cytotoxic T lymphocytes. Blood 2000;95:3853-8.
12. Kato K, Cantwell MJ, Sharma S, Kipps TJ. Gene transfer of CD40-ligand induces autologous immune recognition of chronic lymphocytic leukemia B cells. J Clin Invest 1998;101:1133-41.
13. Kipps TJ. Advances in classification and therapy of indolent B-cell malignancies. Semin Oncol 2002;29:98-104.
14. Henkart PA. Lymphocyte-mediated cytotoxicity: two pathways and multiple effector molecules. Immunity 1994; 1: 343-6.
15. Trapani JA, Davis J, Sutton VR, Smyth MJ. Pro-apoptotic functions of cytotoxic lymphocyte granule constituents in vitro and in vivo. Curr Opin Immunol 2000; 12:323-9.
16. Porakishvili N, Roschupkina T, Kalber T, Jewell AP, Yong K, Patterson K, et al. Expansion of $CD4^+$ T cells with a cytotoxic phenotype in patients with B-chronic lymphocytic leukaemia (B-CLL). Clin Exp Immunol 2001;126:29-36.
17. Shustov A, Luzina I, Nguen P, Papadimitriou JC, Handwerger B, Elkon KB, et al. Role of perforin in controlling B cell hyperactivity and humoral autoimmunity. J Clin Invest 2000;106:R39-47.
18. Caligaris-Cappio F. Biology of chronic lymphocytic leukemia. Rev Clin Exp Hematol 2000;4:5-21.
19. Xanthou G, Tapinos NI, Polihronis M, Nezis IP, Margaritis LH, Moutsopoulos HM. CD4 cytotoxic and dendritic cells in the immunopathologic lesion of Sjogren's syndrome. Clin Exp Immunol 1999;118:154-63.
20. Weyland CM, Bryl E, Goronzy JJ. The role of T cells in rheumatoid arthritis. Arch Immunol Ther Exp (Warsz) 2000;48:429-35.
21. Taams LS, Smith J, Rustin MH, Salmon M, Poulter LW, Akbar AN. Human anergic/suppressive $CD4^+CD15^+$ T cells: a highly differentiated and apoptosis-prone population. Eur J Immunol 2001;31:1122-31.
22. Glennie MJ, McBride HM, Worth AT, Stevenson GT. Preparation and performance of bispecific F(ab'γ)2 antibody containing thioether-linked Fab'γ fragments. J Immunol 1987;139:2367-75.
23. Kataoka T, Takaku K, Magae J, Shinohara N, Takayama H, Kondo S, et al. Acidification is essential for maintaining the structure and function of lytic granules of CTL. Effect of Concanamycin A, an inhibitor of vacuolar type H^+-ATPase, on CTL-mediated cytotoxicity. J Immunol 1994;153:3938-47.
24. Wen YJ, Min R, Tricot G, Barlogie B, Qing Y. Tumor lysate-specific cytotoxic T lymphocytes in multiple myeloma: promising effector cells for immunotherapy. Blood 2002;99:3280-5.
25. Krackhardt AM, Harig S, Witzens M, Broderick R, Barrett P, Gribben JG. T-cell responses against chronic lymphocytic leukemia cells: implications for immunotherapy. Blood 2002;100:167-73.
26. Appay V, Zaunders JJ, Papagno L, Sutton J, Jaramillo A, Waters A, et al. Characterisation of $CD4^+$ CTLs ex vivo. J Immunol 2002;168:5954-8.
27. Kabelitz D, Totterman TH, Nilsson K, Gidlund M. Phorbol ester treated chronic B lymphocytic leukaemia cells induce autologous T cell proliferation without generation of cytotoxic T cells. Clin Exp Immunol 1984;57:461-6.
28. El-Khatib M, Stanger BZ, Dogan H, Cui H, Ju ST. The molecular mechanism of FasL-mediated cytotoxicity by $CD4^+$ Th1 clones. Cell Immunol 1995;163:237-44.
29. Kataoka T, Shinohara N, Takayama H, Takaku K, Kondo S, Yonehara S, et al. Concanamycin A, a powerful tool for characterisation and estimation of contribution of perforin- and Fas-based lytic pathways in cell-mediated cytotoxicity. J Immunol 1996;156:3678-86.
30. Tinhofer I, Marschitz I, Kos M, Henn T, Egle A, Villunger A, et al. Differential sensitivity of $CD4^+$ and $CD8^+$ T lymphocytes to the killing efficacy of Fas (Apo-1/CD95) ligand + tumor cells in B chronic lymphocytic leukemia. Blood 1998; 91:4273-81.
31. Chu P, Deforce D, Pedersen I, Kim I, Kitada S, Reed JC, Kipps TJ. Latent sensitivity to Fas-mediated apoptosis after CD40 ligation may explain activity of CD154 gene therapy in chronic lymphocytic leukemia. Proc Natl Acad Sci USA 2002; 99:3854-9.
32. Gagnon SJ, Ennis FA, Rothman AL. Bystander target cell lysis and cytokine production by dengue virus-specific human $CD4(^+)$ cytotoxic T-lymphocyte clones. J Virol 1999;73:3623-9.
33. Nilsson J, Soderberg O, Nilsson K, Rosen A. Thioredoxin prolongs survival of B-type chronic lymphocytic leukemia cells. Blood 2000;95:1420-6.
34. Dorr J, Waiczies S, Wendling U, Seeger B, Zipp F. Induction of TRAIL-mediated glioma cell death by human T cells. J Neuroimmunol 2002;122:117-24
35. Pena SV, Krensky AM. Granulysin, a new human cytolytic granule-associated protein with possible involvement in cell-mediated cytotoxicity. Semin Immunol 1997;9:117-25.
36. Kaspar AA, Okada S, Kumar J, Poulain FR, Drouvalakis KA, Kelekar A et al. A distinct pathway of cell-mediated apoptosis initiated by granulysin. J Immunol 2001; 167:350-6.
37. Sun Q, Burton RL, Lucas KG. Cytokine production and cytolytic mechanisms of $CD4^+$ cytotoxic T lymphocytes in ex vivo expanded therapeutic Epstein-Barr virus-specific T-cell cultures. Blood 2002; 99: 3302-9.
38. Yanai F, Ishii E, Kojima K, Hasegawa A, Azuma T, Hirose S, et al. Essential roles of perforin in antigen-specific cytotoxicity mediated by human $CD4^+$ T lymphocytes: analysis using the combination of hereditary perforin-deficient effector cells and Fas-deficient target cells. J Immunol 2003;170:2205-13.
39. Rezvany MR, Jeddi-Tehrani M, Rabbani H, Lewin N, Avila-Carino J, Osterborg A, et al. Autologous T lymphocytes may specifically recognise leukaemic B cells in patients with chronic lymphocytic leukaemia. Br J Haematol 2000;111:608-17.
40. Shimizu J, Yamazaki S, Sakaguchi S. Induction of tumor immunity by removing $CD25^+CD4^+$ T cells: a common basis between tumor immunity and autoimmunity. J Immunol 1999;163:5211-8.
41. Roncarolo MG, Bacchetta R, Bordignon C, Narula S, Levings M. Type 1 T regulatory cells. Immunol Revs 2001;182:68-79.