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# *In vitro* susceptibility of $CD4^+$ and $CD8^+$ T cell subsets to fludarabine

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**Abstract**

Administration of the adenosine analogue fludarabine (FLU) *in vivo* induces a profound and prolonged T lymphopenia which mainly affects $CD4^+$ cells. To better understand the mechanistic basis underlying this preferential depletion, we analyzed the *in vitro* susceptibility of T cell subsets to FLU-induced apoptosis. Contrasting with observations *in vivo*, our results showed that treatment of peripheral blood mononuclear cells with FLU induced a higher level of apoptosis in $CD8^+$ than in $CD4^+$ T lymphocytes. This increased sensitivity of $CD8^+$ T cells to FLU was observed in samples from both, healthy donors and B cell chronic lymphocytic leukemia patients, and resulted in higher CD4:CD8 ratios in FLU-treated than in untreated cultures ($P < 0.01$). Expression of factors involved in FLU transport and metabolism was then evaluated by quantitative real time-PCR in normal T cell subsets. It was found that mRNA levels of human equilibrative nucleoside transporter-1 nucleoside transporter were higher whereas deoxycytidine kinase and IMP/GMP selective 5′-nucleotidase mRNA levels were lower in $CD4^+$ cells. However the dCK/cN-II ratio was 2-fold greater in $CD8^+$ than in $CD4^+$ T lymphocytes, which could account for the higher apoptosis levels observed in the $CD8^+$ subset. These results favor the view that decreased CD4:CD8 ratios in FLU-treated patients should be attributed to differences in cell recovery and/or homing between T cell subsets.
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*Keywords:* Fludarabine; Purine nucleosides; T lymphocyte subsets; Apoptosis; Leukemia

## 1. Introduction

FLU is an analogue of adenosine resistant to deamination which is widely used for the treatment of B cell chronic lymphocytic leukemia (B-CLL) and other low-grade hematological malignancies [1–4]. More recently, FLU has successfully been used in nonmyeloablative preparative regimens for stem cell transplantation [5–7].

Before entering cells, FLU is rapidly dephosphorylated by membrane ectonucleotidases (CD73) and transported inside the cell *via* nucleoside-specific membrane carriers. Once inside, it requires phosphorylation by deoxycytidine kinase (dCk) for cytotoxic activity [8,9]. 5′-Nucleotidases may have an effect opposite to that of dCK, decreasing the amount of activated FLU forms inside the cells [10]. FLU is cytotoxic both against dividing and resting cells [8]. In dividing cells, FLU inhibits DNA synthesis and ribonucleotide reductase enzyme [8,11], while in quiescent cells its main mechanism of cytotoxicity appears to be the inhibition of cellular DNA repair processes [12,13].

One of the major complications related to FLU therapy is the high incidence of severe infections, that has been attributed to the profound and prolonged T lymphopenia induced by this drug [14–16]. Different studies, mainly performed in BCLL patients, showed that $CD4^+$ and $CD8^+$ T lymphocyte subsets are rapidly depleted after initiation of FLU therapy, with values remaining below normal for many months [15,17]. *In vivo* data indicate that $CD4^+$ subset is preferentially affected by FLU with median CD4

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*Abbreviations:* FLU, fludarabine (9-β-D-arabinosyl-2-fluoroadenine-monophosphate); B-CLL, B cell chronic lymphocytic leukemia; hENT1, human equilibrative nucleoside transporter-1; dCK, deoxycytidine kinase; cN-II, IMP/GMP selective 5′-nucleotidase; PBMC, peripheral blood mononuclear cells; mAb, monoclonal antibodies; 18S, 18S ribosomal RNA.

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counts ranging from 150 to 200 cells/μL after the first three courses of treatment [17]. A lesser effect is observed on the $CD8^+$ subset, resulting in a decreased CD4:CD8 ratio. It has been suggested that this could be related to a higher sensitivity of $CD4^+$ T lymphocytes to the cytotoxic effect of FLU compared to that of $CD8^+$ T cells. Differences in T cell recovery following FLU treatment might also be responsible for prolonged CD4 lymphophenia [18–20]. However, the mechanistic basis underlying the preferential depletion of $CD4^+$ T cells by FLU has not yet been defined.

Here we evaluate the *in vitro* susceptibility of T lymphocyte subsets to FLU by analyzing CD4:CD8 ratios in cultures of peripheral blood mononuclear cells (PBMC) from both healthy donors and BCLL patients. In addition, we studied the expression of factors involved in FLU transport and metabolism. Our results show that $CD8^+$ T lymphocytes were more sensitive to FLU-induced apoptosis than $CD4^+$ T cells *in vitro* and that this higher sensitivity might be due to a higher degree of activation of FLU in $CD8^+$ cells.

## 2. Materials and methods

### 2.1. Reagents

Hystopaque, acridine orange, ethidium bromide and propidium iodide were purchased from Sigma Chemical Co. Fetal calf serum, RPMI 1640, streptomycin and penicillin were obtained from Life Technologies. Fluorescein isothiocyanate (FITC) and phycoerythrin (PE)-conjugated monoclonal antibodies (mAb) specific for CD3, CD4, CD8 and control antibodies with irrelevant specificities, as well as Annexin V-FITC were purchased from Immunotech. Fludarabine (9-β-D-arabinosyl-2-fluoroadenine-monophosphate) was obtained from Schering Argentina.

### 2.2. Cell isolation and culture

Studies were performed in peripheral blood samples obtained from 17 healthy donors and from 17 patients diagnosed with B-CLL according to standard clinical and laboratory criteria [21]. Patients and age-matched healthy donors were informed about the objectives of the study and gave their consent. B-CLL patients were either untreated (15 samples) or had not received cytoreductive chemotherapy for at least 3 months before investigation (two samples). At the time of the analysis, all patients were free from clinically relevant infectious complications, including HIV infection. Clinical features of B-CLL patients are depicted in Table 1.

PBMC were isolated by centrifugation over a Ficoll-Hypaque layer (Hystopaque), washed twice with saline and resuspended in RPMI 1640 supplemented with 10% fetal calf serum, 100 U/mL penicillin and 100 μg/mL streptomycin. Then, aliquots of 0.15 mL of these cells ($2 \times 10^6$ $mL^{-1}$) were placed in 96-well flat-bottom microplates and cultured at 37° in an atmosphere of 5% $CO_2$, with or without FLU for 72 hr.

Table 1
RAI staging and percentages of circulating T lymphocytes ($CD3^+$ cells) in B-CLL patients

| Patient | RAI | Age | Sex | Previously treated | T lymphocytes (%) |
|---|---|---|---|---|---|
| 1 | 0 | 63 | M | No | 6 |
| 2 | 0 | 69 | F | No | 18 |
| 3 | 0 | 75 | F | No | 14 |
| 4 | 0 | 86 | F | No | 5 |
| 5 | 0 | 88 | F | No | 3 |
| 6 | 0 | 85 | F | No | 7 |
| 7 | 0 | 64 | M | No | 18 |
| 8 | 0 | 67 | F | No | 28 |
| 9 | 0 | 62 | M | No | 31 |
| 10 | 0 | 72 | M | No | 9 |
| 11 | 0 | 66 | M | No | 19 |
| 12 | 0 | 71 | M | No | 16 |
| 13 | I | 72 | M | No | 17 |
| 14 | I | 72 | M | No | 25 |
| 15 | I | 72 | F | Yes | 27 |
| 16 | I | 71 | F | Yes | 4 |
| 17 | I | 70 | F | No | 9 |

### 2.3. Immunophenotypic analysis

$CD4^+$ and $CD8^+$ T lymphocyte subsets were analyzed by double immunofluorescence staining using PE-anti-CD3 and FITC-anti-CD4 or FITC-anti-CD8 mAbs and flow cytometry. Briefly, aliquots of $3 \times 10^5$ PBMC were incubated with saturating concentrations of conjugated mAbs for 30 min at 4°. Then, cells were washed twice in saline supplemented with 1% FCS, resuspended in Isoton II (Coulter Diagnostics) and analyzed immediately using a FACScan cytometer (Becton Dickinson) with CellQuest software.

### 2.4. Quantitation of cell death by Annexin V staining and flow cytometry

The percentage of overall cell death (apoptosis and necrosis) in $CD4^+$ and $CD8^+$ lymphocytes was measured by flow cytometry using Annexin V-FITC. Briefly, $3 \times 10^5$ PBMC were stained with PE-conjugated anti-CD4 or anti-CD8 mAb, washed twice and incubated with saturating concentrations of Annexin V-FITC according to manufacturer's instructions. Negative controls were performed simultaneously using irrelevant PE-conjugated MoAb of the corresponding Ig isotype.

### 2.5. Quantitative PCR

The levels of mRNA expression of the human equilibrative nucleoside transporter-1 (hENT1) nucleoside transporter, dCK and cN-II were assessed by quantitative real

time PCR in $CD4^+$ and $CD8^+$ T lymphocytes from healthy donors. To this aim, T cell subsets were purified from PBMC using magnetic beads coated with CD4 or CD8 mAbs (Dynal) according to the manufacturer's instructions. The purity of each population was checked by cytometric analysis and was found to be >95%. Cellular RNA extraction and cDNA synthesis were performed as previously described [22]. Quantitative real-time PCR was carried out in a Lightcycler detection system (Roche) as previously described [22]. Briefly, cDNA (5 μL) was mixed with primers (300 nM each), LightCycler-FastStart DNA Master SYBR Green I (Roche) (hENT1) or LightCycler-FastStart DNA master hybridization probes (Roche) (18S, dCK and cN-II), and probes (130 nM; if necessary) in a total volume of 20 μL for 40 cycles. Primer and probe sequences are published elsewhere [22].

The data were expressed as $C_t$, which is the PCR cycle number at which the accumulated fluorescent signal in each reaction crosses a threshold above background. Mean $C_t$ values were then normalized to the expression level in reference to a 18S ribosomal RNA: $\Delta C_t$ = sample mean $C_t$ − control mean $C_t$. The results were then expressed as $2^{-(\Delta C_t)}$. For each sample, a ratio between the studied gene $2^{-(\Delta C_t)}$ values and 18S ribosomal $2^{-(\Delta C_t)}$ values were calculated and considered as final amount of mRNA. All samples were analyzed in three separate experiments.

### 2.6. Statistical analysis

The Wilcoxon nonparametric test was used to analyze the statistical significance of the experimental results.

## 3. Results

### 3.1. Differential in vitro susceptibility of normal T lymphocyte subsets to FLU

PBMC from 17 healthy donors were incubated with different concentrations of FLU (1–25 μM) for 72 hr. Apoptosis was determined by fluorescence microscopy using acridine orange and ethidium bromide as previously described [23]. T cell subsets evaluation was determined in those cultures in which the percentage of apoptosis induced by FLU was at least 2-fold higher than spontaneous apoptosis. For most samples, this difference between spontaneous and FLU-induced apoptosis was observed with 10 μM FLU. T cell subsets in untreated and FLU-treated cultures were evaluated by flow cytometry using double staining with mAbs directed to CD3, CD4 and CD8. Results from a representative experiment are depicted in Fig. 1. Apoptotic cells (Fig. 1A) could be easily distinguished from viable cells because of their lower forward light scatter, consistent with reduction of cell size and cytoplasmic volume occurring during apoptosis [24]. T lymphocyte subsets within the region of viable cells were then depicted as shown in Fig. 1B. In this example, it can be seen that the $CD8^+$ subset represented an average of 28% of viable T cells in untreated cultures, but only 10% after incubation with FLU.

Using this methodology, we found that treatment with FLU affected the survival of $CD8^+$ T cells to a greater extent compared to $CD4^+$ T lymphocytes. Figure 2A shows the percentages of viable $CD4^+$ and $CD8^+$ T cells after 72 hr incubation with or without FLU for the 17 samples assessed. In all but two samples, there was an

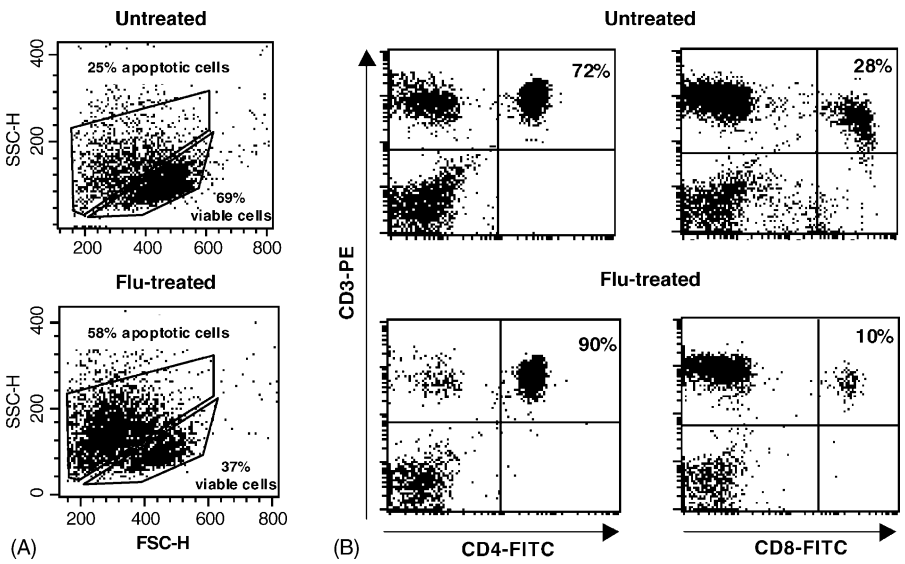


Fig. 1. *In vitro* treatment with FLU differently affects the survival of $CD4^+$ and $CD8^+$ T cells. PBMC ($2 \times 10^6$ $mL^{-1}$) isolated from healthy donors were incubated in the presence or absence of FLU (1–25 μM) for 72 hr at 37°. (A) Viable and apoptotic cells in untreated and FLU-treated cultures can be distinguished according to forward/side scatter profiles. (B) T cell subsets within the region of viable cells were analyzed by double immunofluorescence staining and flow cytometry using anti-CD3 mAb labeled with PE and anti-CD4 or anti-CD8 mAbs labeled with FITC. The numbers in the top right quadrant represent the percentage of viable T CD4 or CD8 cells in untreated and FLU-treated cultures. Results from a representative sample (N = 17) are shown.

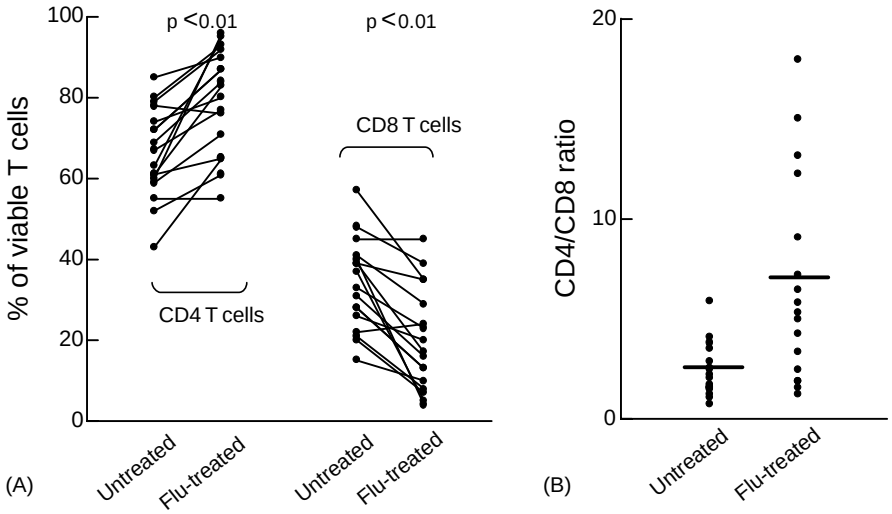


Fig. 2. Differential *in vitro* susceptibility of $CD4^+$ and $CD8^+$ T cells to FLU in 17 healthy donors evaluated. T cell subsets were analyzed as described in Fig. 1. (A) Percentages of viable $CD4^+$ and $CD8^+$ T cells for each sample incubated for 72 hr in the absence (untreated) or presence (FLU-treated) of FLU (1–25 μM). (B) Higher CD4:CD8 ratios in FLU-treated compared to untreated cultures ($P < 0.01$). Each dot represents an individual sample. Mean values are indicated by a line.

increase in the proportion of viable $CD4^+$ T cells and a concomitant decrease in that of $CD8^+$ T cells as a consequence of FLU treatment. These differences in FLU-induced apoptosis resulted in higher CD4:CD8 ratios (Fig. 2B). In all samples, levels of apoptosis determined by flow cytometric alterations of light-scattering properties were similar to those obtained by fluorescence microscopy using acridine orange and ethidium bromide.

Increased susceptibility of $CD8^+$ T cells to FLU was further confirmed by measuring the binding of Annexin V in antigen-defined populations. Results in Fig. 3 show that, under our experimental conditions, there were no differences in spontaneous cell death (apoptosis and necrosis) between $CD4^+$ and $CD8^+$ compartments. As expected, incubation of PBMC with 10 μM FLU for 72 hr increased the percentage of Annexin V-positive cells within both T cell subsets. However, the proportion of Annexin V-positive cells was significantly ($P < 0.02$) higher in the $CD8^+$ compared to the $CD4^+$ subset indicating that $CD8^+$ T cells were more susceptible to FLU-induced apoptosis.

### 3.2. Expression of factors involved in FLU metabolism in normal T cell subsets

To test whether the different susceptibility of $CD4^+$ and $CD8^+$ T lymphocytes to FLU-induced apoptosis was due to different mechanisms associated with FLU metabolism, we analyzed by quantitative real time-PCR the mRNA levels of hENT1, dCK and cN-II at baseline. As shown in Fig. 4, mRNA levels of hENT1 transporter were higher whereas dCK and cN-II mRNA levels were lower in $CD4^+$ cells than in $CD8^+$ T lymphocytes. More importantly, the dCK/cN-II ratio, which provides information regarding the global degree of activation of FLU in cells, was higher in $CD8^+$ cells (1.75) than in $CD4^+$ cells (0.94).

### 3.3. Differential susceptibility to FLU of T lymphocyte subsets from B-CLL patients

To determine whether FLU might exert different *in vitro* effects on T cell subsets from leukemic patients suffering of

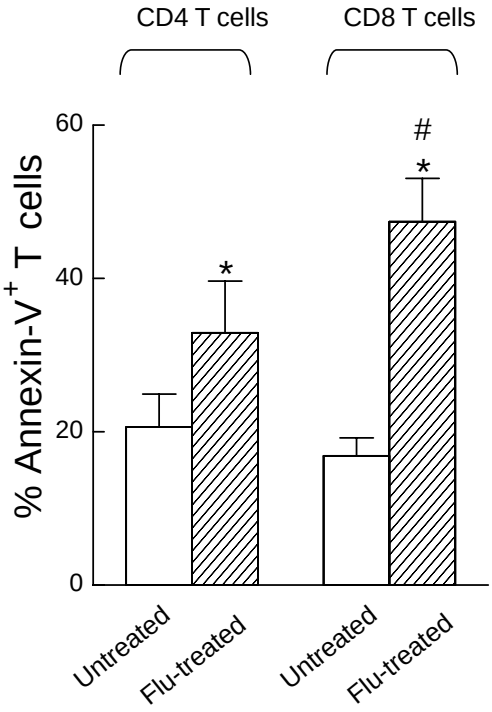


Fig. 3. Cell death in T cell subsets evaluated by Annexin V. PBMC were cultured *in vitro* for 72 hr in the absence or presence of FLU (1–25 μM). Then, the percentage of Annexin V-positive cells in T cell subsets was measured by two-colour flow cytometry after staining with PE-conjugated anti-CD4 or anti-CD8 and Annexin V-FITC. Results are expressed as the mean ± SEM of eight samples evaluated. (*) Statistical significance ($P < 0.01$) compared to untreated cells. (#) Statistical significance ($P < 0.05$) compared to $CD4^+$ T cells treated with FLU.

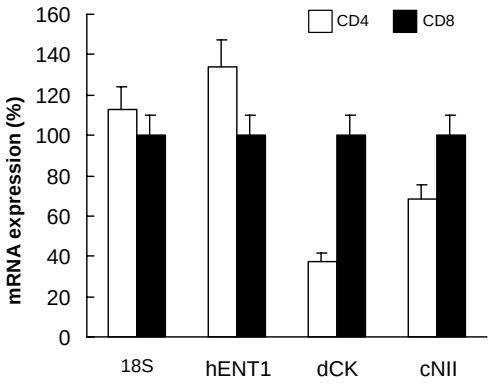


Fig. 4. Quantitative real time-PCR analysis of factors implicated in the activation or degradation of FLU in normal T cell subsets. RT-PCR of 18S; hENT1; dCK and cN-II were performed as described in Section 2 in $CD4^+$ and $CD8^+$ T cells purified from PBMC using magnetic beads coated with CD4 or CD8 mAbs. Results are expressed as the mean ± SEM of eight samples analyzed.

B-CLL, we next evaluated $CD4^+$ and $CD8^+$ T lymphocytes in control and FLU-treated PBMC from 17 B-CLL patients by cytometric analysis as described for healthy donors. Results from a representative patient are depicted in Fig. 5.

As in normal PBMC, we found that treatment with FLU affected the survival of $CD8^+$ T cells to a greater extent compared to $CD4^+$ T lymphocytes. In all patients evaluated, increased susceptibility of $CD8^+$ cells to FLU-induced apoptosis resulted in higher CD4:CD8 ratios (Fig. 6).

## 4. Discussion

Our data show that *in vitro* FLU induced a higher degree of apoptosis on $CD8^+$ than on $CD4^+$ T lymphocytes, both in healthy donors and in B-CLL patients. A possible explanation for these results are differences between $CD4^+$ and $CD8^+$ T lymphocytes in the expression of transporters or enzymes involved in FLU transport or metabolism. Previous studies determined that cell sensitivity to FLU may depend both on dCK and 5′-nucleotidase levels of expression or activity [10,25]. Hapke *et al.* [26] demonstrated that transfection of a retroviral vector containing the dCK gene in tumor cells increased dCK expression and activity resulting in an increased sensitivity to FLU. It was also shown that T cells with increased 5′-nucleotidase accumulated less nucleotides from exogenously added FLU and were more resistant to this drug than did parental T lymphocytes [27]. Thus, higher sensitivity of $CD8^+$ cells to FLU may be ascribed to the higher expression of dCK observed in these cells compared to $CD4^+$ T lymphocytes. Moreover, when analyzing dCK/cN-II ratios, $CD8^+$ cells expressed 2-fold more dCK than cN-II mRNA levels. This may result in a higher intracellular accumulation of FLU-triphosphate nucleotides explaining the higher apoptosis levels observed in the $CD8^+$ lymphocyte subset. It should be pointed out that a limitation of our study is that we only measured dCK and cN-II mRNA expression and not protein expression or enzyme activities. For dCK, downregulation of enzyme activity has been shown to correlate with decreased levels of mRNA expression [28,29]; however, at present time, whether cN-II mRNA expression correlates with protein levels or enzyme activity is not known.

Induction of apoptosis by FLU in lymphoid subpopulations has been previously evaluated by Consoli *et al.* [30] without finding any differences between $CD4^+$ and $CD8^+$ T cells from B-CLL patients with high-risk disease.

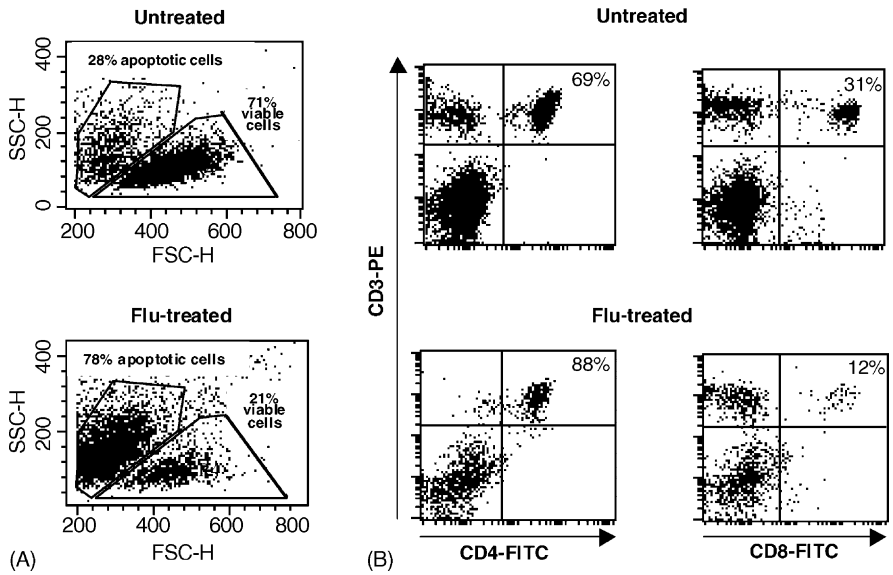


Fig. 5. *In vitro* exposure to FLU differently affects the survival of $CD4^+$ and $CD8^+$ T cells from B-CLL patients. PBMC ($2 \times 10^6$ $mL^{-1}$) isolated from B-CLL patients were incubated in the presence or absence of FLU (1–25 μM) for 72 hr at 37°. (A) Viable and apoptotic cells in untreated and FLU-treated cultures. (B) T cell subsets within the region of viable cells were analyzed as described in Fig. 1. The numbers in the top right quadrant represent the percentage of viable CD4 or CD8 T lymphocytes in untreated and FLU-treated cultures. Results from a representative sample (N = 17) are shown.

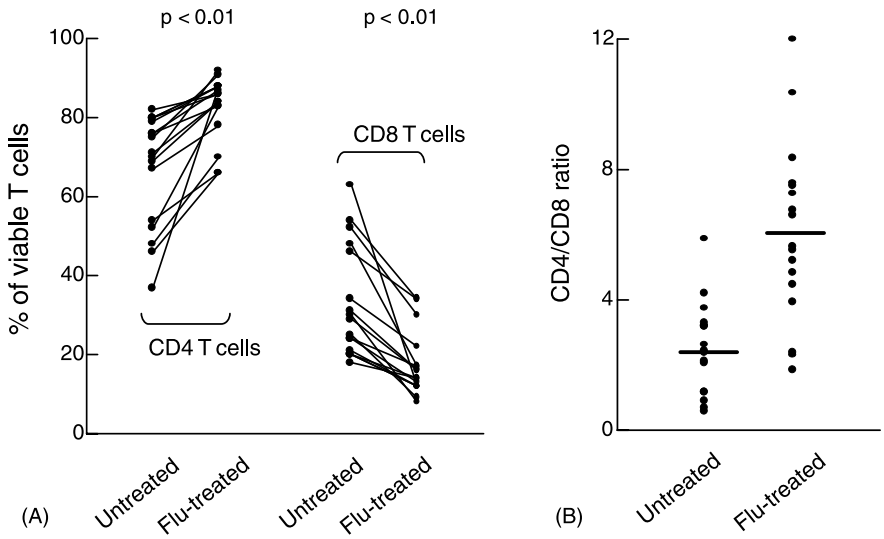


Fig. 6. Differences in *in vitro* sensitivity of $CD4^+$ and $CD8^+$ T cells to FLU in 17 B-CLL patients evaluated. T cell subsets were analyzed as described in Fig. 1. (A) Percentages of viable $CD4^+$ and $CD8^+$ T cells for each sample incubated for 72 hr in the absence (untreated) or presence (FLU-treated) of FLU (1–25 μM). (B) Higher CD4:CD8 ratios in FLU-treated compared to untreated cultures ($P < 0.01$). Each dot represents an individual sample. Mean values are indicated by a line.

Although we do not have an explanation for these contrasting results, they might be attributed, among other factors, to differences in clinical staging and pretreatment status of the patients evaluated in both reports. In fact, all of our patients were at low RAI stages and none but two had received cytoreductive chemotherapy before this study (see Table 1). It is conceivable that prior cytotoxic treatment may cause resistance to FLU, not only in leukemic cells but also in non-malignant lymphocytes. Further studies will be required to determine whether $CD8^+$ T cells from B-CLL patients at high risk and/or heavily pretreated lose their increased sensitivity to FLU-induced apoptosis.

Increased *in vitro* susceptibility of $CD8^+$ T cells to FLU contrasted with findings in B-CLL patients treated *in vivo* with FLU, which showed a profound $CD4^+$ T lymphopenia [14,17,31]. Assuming that our observations *in vitro* reflect what happens *in vivo* upon administration of FLU, the most likely explanation for prolonged $CD4^+$ lymphopenia in B-CLL-treated patients may be the slow rate of recovery of this population compared to $CD8^+$ T cells. In fact, it was shown that after intensive chemotherapy, $CD8^+$ T cells had a faster doubling time than $CD4^+$ T cells, returning to pretherapy values 3 months later, while $CD4^+$ recovery remained incomplete up to 12 months post-therapy [18–20]. On the other hand, changes in CD4:CD8 ratios in FLU-treated patients could also result from differences in redistribution of T cell compartments among lymphoid tissues, bone marrow and blood. In this regard, the alterations in T cell compartments induced by steroid treatment have been attributed to differences in homing between $CD4^+$ and $CD8^+$ T cells [32,33].

In conclusion, we show that $CD8^+$ T lymphocytes are more sensitive to *in vitro* FLU exposure than $CD4^+$ lymphocytes. This may be due to a higher degree of activation of FLU in $CD8^+$ T cells, which could be confirmed by dosages of intracellular phosphorylated FLU. Our results can help to understand the basis for T lymphocyte depletion after FLU therapy. Thus, since $CD4^+$ T cells are less sensitive to FLU-induced apoptosis than $CD8^+$ lymphocytes, decreased CD4:CD8 ratios in FLU-treated patients should be attributed to differences in cell recovery and/or homing between T cell subsets.

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