

Intracellular tumor necrosis factor production by T- and B-cells in B-cell chronic lymphocytic leukemia

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Background and Objectives. The pathogenesis of B-cell chronic lymphocytic leukemia (B-CLL) has been linked with the production and activity of certain growth factors. Tumor necrosis factor (TNF- α) is important for the growth and survival of B-CLL cells. TNF- α promotes the proliferation of the malignant cell clone and is believed to play a role in the progression of B-CLL. The aim of our study was to examine the level of production and intracellular expression of TNF- α by T- and B-cells in B-CLL in correlation with stage of disease and clinical parameters.

Design and Methods. Using a three-color flow cytometry technique we analyzed intracellular TNF- α expression by B-cells (CD19⁺) and by T-cell subsets (CD3⁺/CD4⁺ and CD3⁺/CD8⁺) in peripheral blood (PB) and bone marrow (BM) from 40 patients with B-CLL and 24 healthy controls.

Results. A higher number of TNF- α -positive B-cells were found in PB and BM in B-CLL patients than in normal controls. In BM this difference was statistically significant ($p < 0.007$). Likewise, in PB and BM the percentage of T-cells expressing TNF- α was significantly higher in B-CLL patients than in normal controls (PB: $p < 0.00001$; BM: $p < 0.007$). CD3⁺/CD4⁺ cells from patients displayed a lower level of intracellular TNF- α expression than did CD3⁺/CD8⁺ cells (PB: $p < 0.0001$; BM: $p < 0.04$). The number of T-cells expressing TNF- α in B-CLL patients was higher in those with stages III-IV than in patients with early stage disease (PB: $p < 0.007$; BM: $p < 0.01$). Additionally, PB and BM T-cell subsets from patients in stages III-IV had a statistically significant higher level of cytoplasmic TNF- α expression than the corresponding cells from healthy controls (PB: $p < 0.02$; BM: $p < 0.05$). In PB the percentage of CD4⁺ and CD8⁺ T-cells expressing cytoplasmic TNF- α positively correlated with the stage of disease, total tumor mass (TTM) score and lymphocytosis. In BM only the percentage of CD8 T-cells positively correlated with TTM score and lymphocytosis. The expression of TNF- α in leukemic B-cells did not correlate with any progression parameters of disease.

Interpretation and Conclusions. The results obtained suggest that malignant B-cells are exposed to large numbers of T-cells able to synthesize and secrete TNF- α . Moreover, T-cells even though fewer than B-cells may be an important source of TNF- α in advanced stages of dis-

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ease. This indicates that the TNF- α can be associated with progression of B-CLL and may be implicated in some side-effects associated with this disease.

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Key words: intracellular TNF- α , B-CLL, T-cells, B-cells.

B-cell chronic lymphocytic leukemia (B-CLL) is characterized by an accumulation of mature CD5⁺ B-cells.¹ In addition to accumulation and clonal expansion of the malignant B-cell, several abnormalities have been demonstrated within the non-malignant T-cell population. These include morphologic and functional abnormalities of the T-cells, which can lead to defective T-cell/B-cell interactions.² The imbalance of immunoregulatory T-cells, mainly T-helper cells (CD4⁺) and T-suppressors (CD8⁺) may be the main cause of the immune deficiency observed in patients with B-CLL.^{3,4} The T-lymphocytes of B-CLL patients may be responsible, at least in part, for the defective B-cell maturation.⁵ Thus, phenotypic and functional defects within the T-lymphocyte population may play a role in the pathogenesis of B-CLL.⁶

Cytokines and other soluble factors, whether derived from the tumor (autocrine) or from the surrounding host cells (paracrine), may play an important role in the growth mechanism of the neoplastic clone.⁵ A number of studies assign some role to tumor necrosis factor (TNF- α) in activation and proliferation of B-cells.^{7,8,9} It has been shown that neoplastic B-lymphocytes from patients with B-CLL express TNF- α mRNA and release this cytokine spontaneously *in vitro*. Moreover, higher serum levels of TNF- α are detectable in patients with B-CLL compared to in normal controls, and there is an accompanying progressive increase in relation to the stage of disease.⁵

In this study the capability of CD3⁺ T-cells and CD19⁺ B-lymphocytes from B-CLL patients to produce TNF- α was examined and compared with that

of T- and B-cells from healthy controls. The intracellular concentration of TNF- α in peripheral blood and bone marrow T- and B-cells was investigated in relation to the stage of disease, total tumor mass (TTM) according to Jaksic and Vitale¹⁰ and lymphocytosis. There was a positive correlation between TNF- α production by T-cells, but not by B-cells (paracrine mechanism of growth), and stage of disease, lymphocytosis and total tumor mass. It seems that the growth of leukemic B-cells is controlled by endogenous TNF- α and a defect in this cytokine production could account for the progression of B-CLL.

Design and Methods

Patients

Peripheral blood (PB) and bone marrow (BM) were obtained from 40 newly diagnosed, untreated patients with B-CLL (26 men and 14 women, aged from 49 to 79 years). The diagnosis of B-CLL was made on the basis of clinical examination, morphologic and immunologic criteria. Patients were graded according to Rai's staging system¹¹ as follows: stage 0 (13 cases), stage I (10 cases), stage II (9 cases), stage III (1 case), stage IV (7 cases). The control samples consisted of peripheral blood from 24 healthy donors and bone marrow from 8 healthy donors. The Local Ethical Committee approved this study and all patients signed informed consent.

Reagents

Phorbol 12-myristate 13-acetate (PMA), ionomycin (calcium ionophore) and brefeldin A (BFA) were purchased from Sigma, the IntraPrep kit from Immunotech (Germany). The following fluorescein (FITC) or phycoerythrin (PE) conjugated monoclonal antibodies (MoAbs) used in this study were obtained from DAKO (Denmark): CD4 FITC (Clone MT310, IgG1), CD8 FITC (Clone DK25, IgG1), CD19 FITC (Clone HD37, IgG1), CD3 PerCP (Clone SK7, IgG1) and FastImmune anti-Hu-TNF- α PE (Clone 6401.1111, IgG1) were purchased from Becton Dickinson (USA). The IgG1 PE – conjugated mouse MoAb from Becton Dickinson was used as a negative control.

Cell preparation

Mononuclear cells were separated by density gradient centrifugation on Lymphoprep (Nycomed, Norway) for 25 minutes at 400g at room temperature. Interphase cells were removed and washed twice with phosphate-buffered saline (PBS).

Intracellular TNF- α staining

Cells (2×10^6 /mL) were cultured in RPMI 1640 supplemented with 2 mmol/L L-glutamine, 10%

fetal calf serum (FCS; Sigma), 100 U/mL penicillin (Sigma) and 100 μ g/mL streptomycin. (Sigma). In order to evaluate intracellular TNF- α expression, cells were incubated with 25 ng/mL of PMA and 1 μ g/mL of ionomycin in the presence 10 μ g/mL of brefeldin A (protein transport inhibitor) for 4 hours at 37°C in a 5% CO₂ atmosphere. Furthermore, this procedure was performed on non-activated lymphocytes using only BFA to assess the level of residual TNF- α synthesis from *in vivo* activation. Cultured cells were washed twice in PBS, divided into tubes at a concentration of 5×10^5 cells per tube and then stained with MoAbs to the following cell-surface markers: CD19, CD3, CD4 and CD8. Next, 10 μ L of each MoAb were added to appropriate tubes and incubated for 15 min at room temperature. Following membrane staining the cells were then fixed and permeabilized with the IntraPrep kit according to the manufacturer's instruction. Then the cells were incubated with 10 μ L of anti-TNF- α MoAb or IgG1 isotypic control. Finally the cells were washed and analyzed by flow cytometry directly after preparation.

Flow cytometric analysis

Samples were analyzed by two- and three-color flow cytometry using a Becton Dickinson FACS-Calibur instrument. Five data parameters were acquired and stored: linear forward and side scatter (FSC, SSC), log FL-1(FITC), log FL-2(PE) and log FL-3(PerCP). For each analysis, 20,000 events were acquired and analyzed using CellQuest software. An acquisition gate was established based on FSC and SSC that included mononuclear cells. A region, R1, was drawn around the lymphocytes (Figure 2a). Next, the R1 gated events were analyzed for CD3PerCP staining and positive cells (CD3⁺) were gated (region R2). We used dot plots of CD3PerCP versus SSC (Figure 2b). Two dot plots, CD4FITC versus SSC (Figure 2c) and CD8FITC versus SSC (Figure 2d), were established by combined gating of events using R1 and R2. As a result, CD4 and CD8 positive cells were identified as the cells in R3. The final dot plots CD4FITC versus mouse IgG1 PE (Figure 2e), CD8FITC versus mouse IgG1 PE (Figure 2f), CD4FITC versus TNF- α PE (Figure 2g) and CD8FITC versus TNF- α PE (Figure 2h) were established by combined gating of events using R1, R2 and R3. Isotype-matched antibody was used to verify the staining specificity and as a guide for setting the markers to delineate positive and negative populations.

The R1 gated events were also analyzed for CD19FITC staining and positive cells (CD19⁺) were

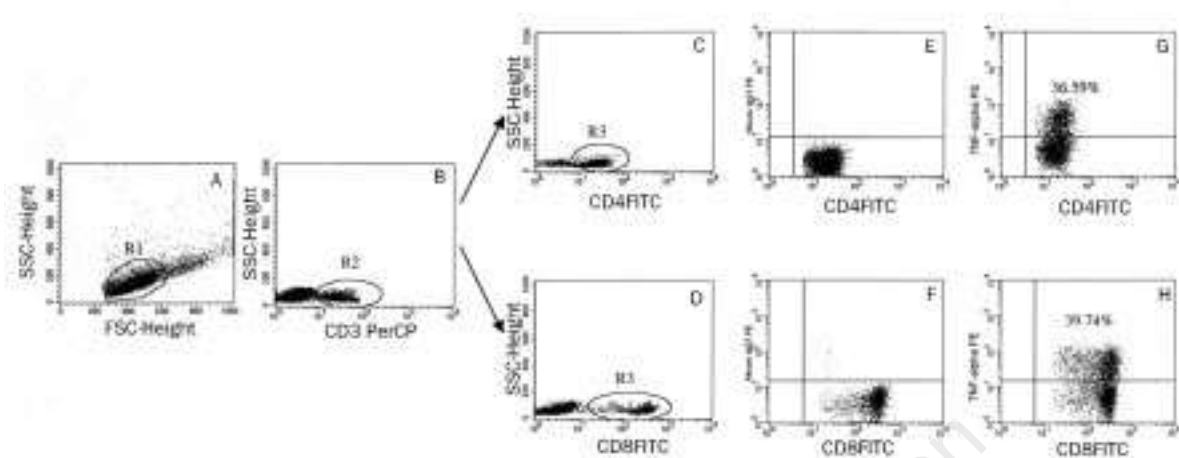


Figure 2. The dot plots show representative data from one B-CLL patient, illustrating the analysis method for identification of CD4⁺ T-cells and CD8⁺ T-cells expressing TNF- α following three-color staining. (a) The dot plot shows the forward scatter/side scatter (FSC/SSC) distribution and the gate (region R1) used to select lymphocytes for analysis. (b) The R1 gated events were then analyzed for CD3PerCP staining and positive cells (CD3⁺) were gated (region R2). The dot plot shows the SSC vs CD3PerCP distribution. (c,d). The two dot plots, CD4FITC vs SSC and CD8FITC vs SSC, were established by combined gating of events using R1 and R2. CD4 or CD8⁺ cells were thus identified as cells in R3. (e) The final dot plots, CD4FITC vs mouse IgG1 PE, (f) CD8FITC vs mouse IgG1 PE, (g) CD4FITC vs TNF- α PE and (h) CD8FITC vs TNF- α PE, were established by combined gating of events using R1, R2 and R3. The numbers in the upper right quadrant on the dot plots (g) and (h) represent the percentage of CD3⁺/CD4⁺ and CD3⁺/CD8⁺ cells (double-gated) expressing cytoplasmic TNF- α .

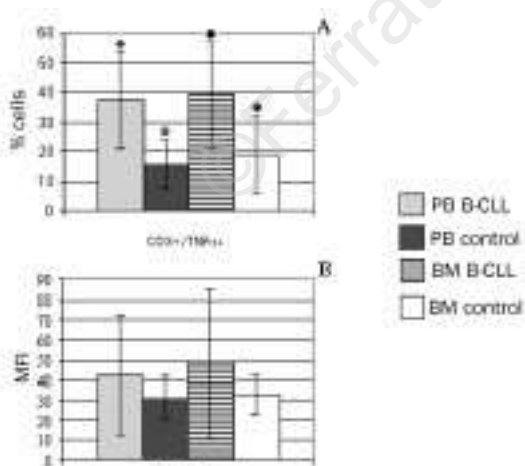


Figure 1. TNF- α expression by CD3⁺ T-cells from patients with B-CLL and from healthy controls. (A) Percentage of T-cells expressing intracellular TNF- α , (B) mean fluorescence intensity (MFI). * $p < 0.00001$ in PB, † $p < 0.007$ in BM.

gated (R2). The dot plots CD19FITC versus mouse IgG1 PE and CD19FITC versus TNF- α PE were established by combined gating of events using R1 and R2.

In this experiment, the mean percentage of cells secreting TNF- α and the level of TNF- α expression in cells, indicated by the mean fluorescence intensity (MFI), were analyzed. Expression of intracellular TNF- α was determined relative to the isotype control. For analysis, MFI cells were gated as described above and TNF- α expression in cells was plotted as a histogram (Figures 4A, 4B). The MFI values were expressed as linear values. Linear values refer to the dynamic range of signal intensities (10^0 – 10^4 or 1–10,000). Means were calculated using CellQuest software.

Statistical analysis

Wilcoxon's paired test was used to compare results in peripheral blood and bone marrow.

Mann-Whitney's U test was applied for the statistical comparison of results from patients with different stages of B-CLL. Mann-Whitney's U test was also used for the statistical comparison of

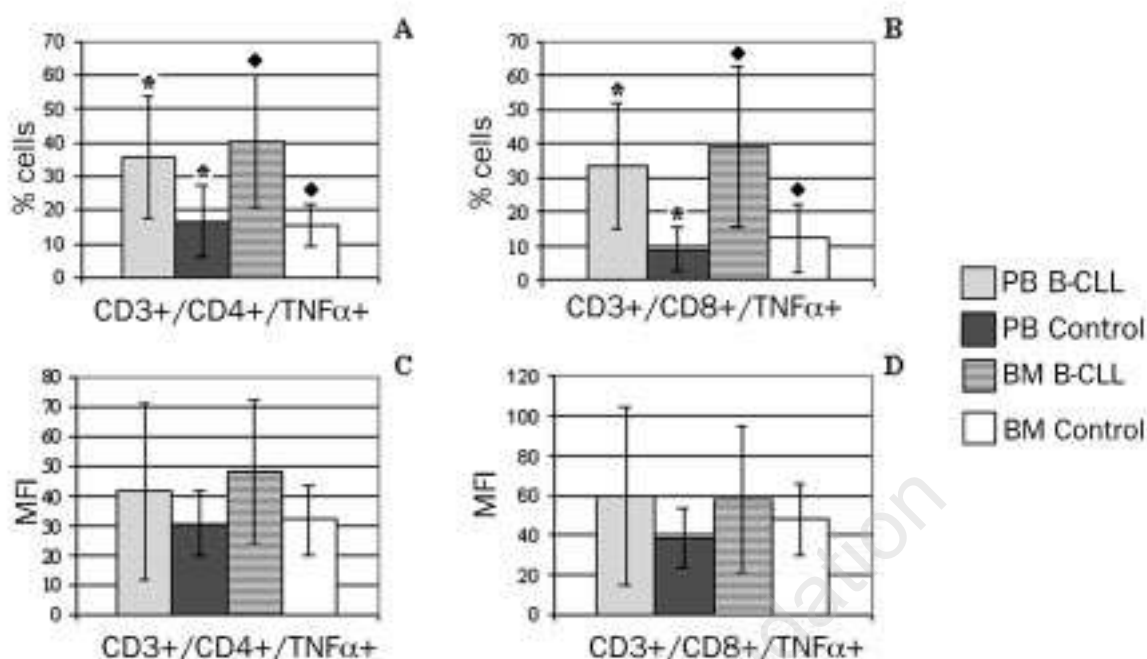


Figure 3. TNF- α expression by CD4⁺ and CD8⁺ T-cell subsets from patients with B-CLL and from healthy controls. (A,B) Percentage of T-cell subsets expressing intracellular TNF- α , (C,D) mean fluorescence intensity (MFI). (A) * $p < 0.0001$ in PB, † $p < 0.001$ in (B) * $p < 0.00001$ in PB, † $p < 0.002$ in BM.

results between patients with B-CLL and healthy donors. The relation between TNF- α expression in B-cell and T-cell subsets was analyzed by Spearman's rank test. Spearman's rank test was used to assess the relationship between intracellular TNF- α expression and other parameters of disease. R means the correlation coefficient of Spearman's rank. Differences were considered as statistically significant when the p -value was < 0.05 . Summary statistics are given as the mean \pm SD.

Results

Intracellular TNF- α expression was detected in both CD3⁺ and CD19⁺ cells from PMA- and ionomycin-activated cultures. In non-activation assays the percentage of cells with TNF- α expression was lower than 1%, comparable with the level of autofluorescence.

TNF- α expression by T cells

Figure 1A demonstrates that the percentage of T-cells from PB and BM expressing cytoplasmic TNF- α was significantly higher in patients with B-CLL (PB: 37.18 \pm 16.18%; BM: 39.22 \pm 17.84%) than in normal controls (PB: 15.70 \pm 8.12%; BM: 18.80 \pm

12.92%). This difference was statistically significant in PB and BM (respectively: $p < 0.00001$; $p < 0.007$).

It was found that the level of TNF- α expression in PB and BM, determined by the MFI, was higher in T-cells from patients than in T-cells from healthy controls, although these differences were not statistically significant (Figure 1B).

Analysis of T-cell subsets

Figure 2 shows the identification of CD4⁺ and CD8⁺ T-cells positively staining for intracellular TNF- α . In PB and BM the mean percentage of CD4⁺ T-cells expressing TNF- α was similar to the mean percentage of CD8⁺ T-cells expressing TNF- α .

As shown in Figure 3A, the mean percentage of CD3⁺/CD4⁺ T-cells in PB expressing cytoplasmic TNF- α was significantly higher in patients with B-CLL than in controls; 35.80 \pm 18.00% and 16.74 \pm 10.51%, respectively ($p < 0.0001$). Similarly, in BM the number of CD4⁺ T-cells expressing this cytokine was significantly higher in patients than in healthy controls (40.12 \pm 19.76% and 15.44 \pm 6.12%, respectively) ($p < 0.001$). Our results revealed that the percentage of CD3⁺/CD8⁺ T-lymphocytes from patients

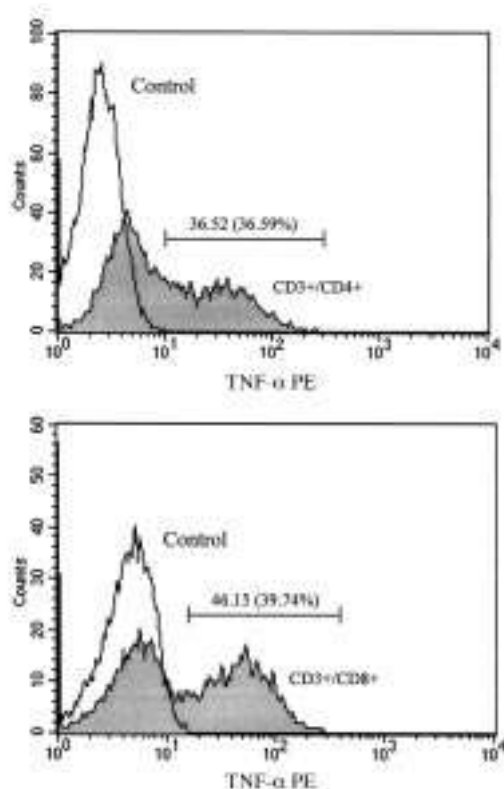


Figure 4. Intracellular TNF- α expression in CD3⁺CD4⁺ and CD3⁺CD8⁺ cells from one representative B-CLL patient. For analysis CD3⁺CD4⁺ and CD3⁺CD8⁺ cells were gated, as described in the *Design and Methods* section and as shown in Figure 2. TNF- α expression by T-cell subsets was plotted as a histogram. Expression of intracellular TNF- α (grey histogram) was determined relative to the isotype control (open histogram). Numbers in histograms are the mean fluorescence intensity (MFI) and percentage of TNF- α positive cells (between brackets). The x-axis corresponds to logarithmic fluorescence intensity and the y-axis to relative cell number.

with B-CLL expressing TNF- α was higher than that in healthy controls ($33.58 \pm 18.28\%$ vs. $9.12 \pm 6.27\%$) ($p < 0.00001$) (Figure 3B). Likewise, in BM the mean percentage of CD3⁺/CD8⁺ T-cells expressing intracellular TNF- α was significantly higher in patients ($39.01 \pm 23.48\%$) than in controls ($12.30 \pm 10.16\%$) ($p < 0.002$) (Figure 3B).

In PB the MFI indicative of TNF- α production per cell was significantly higher in CD3⁺/CD8⁺ lymphocytes (59.86 ± 44.71 MFI) than in CD3⁺/CD4⁺ lymphocytes (41.75 ± 29.82 MFI) ($p < 0.0001$). Likewise, in BM TNF- α production was significantly higher in CD3⁺/CD8⁺ T-cells (57.86 ± 36.81 MFI) than in CD3⁺/CD4⁺ T-cells (48.38 ± 24.12 MFI) ($p < 0.04$). Additionally, Figure 4 shows overlaid his-

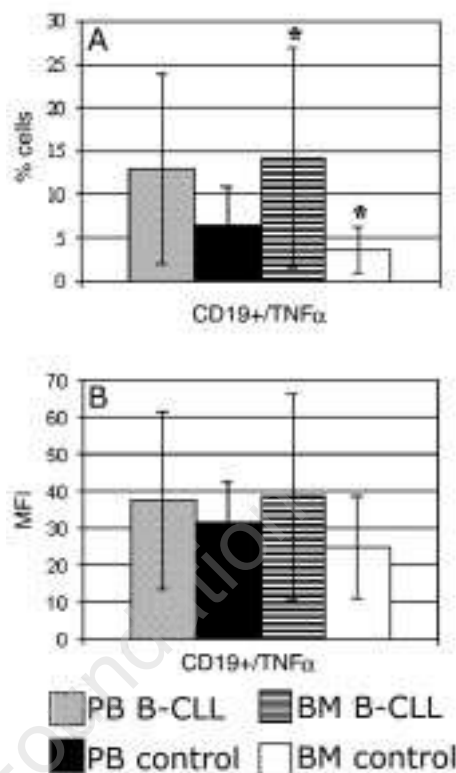


Figure 5. TNF- α expression by CD19⁺ B-cells from patients with B-CLL and from healthy controls. (A) Percentage of B-cells expressing intracellular TNF- α , (B) mean fluorescence intensity (MFI). * $p < 0.007$ in BM.

tograms of two T-cell subsets (CD3⁺/CD4⁺ and CD3⁺/CD8⁺) from PB tested with anti-TNF- α MoAb, and compared with negative controls. These representative histograms show a difference in MFI level between both T-cell subsets.

When we compared the expression of TNF- α in T-cell subsets from patients and healthy volunteers we found that in PB and BM there were not statistically significant differences in MFI between both groups (Figure 3C, 3D).

TNF- α expression by B-cells

Figure 5A illustrates the percentages of CD19⁺ B-cells expressing cytoplasmic TNF- α . Only in BM was the mean percentage of B-CLL CD19⁺/TNF- α ⁺ cells significantly higher than that of control

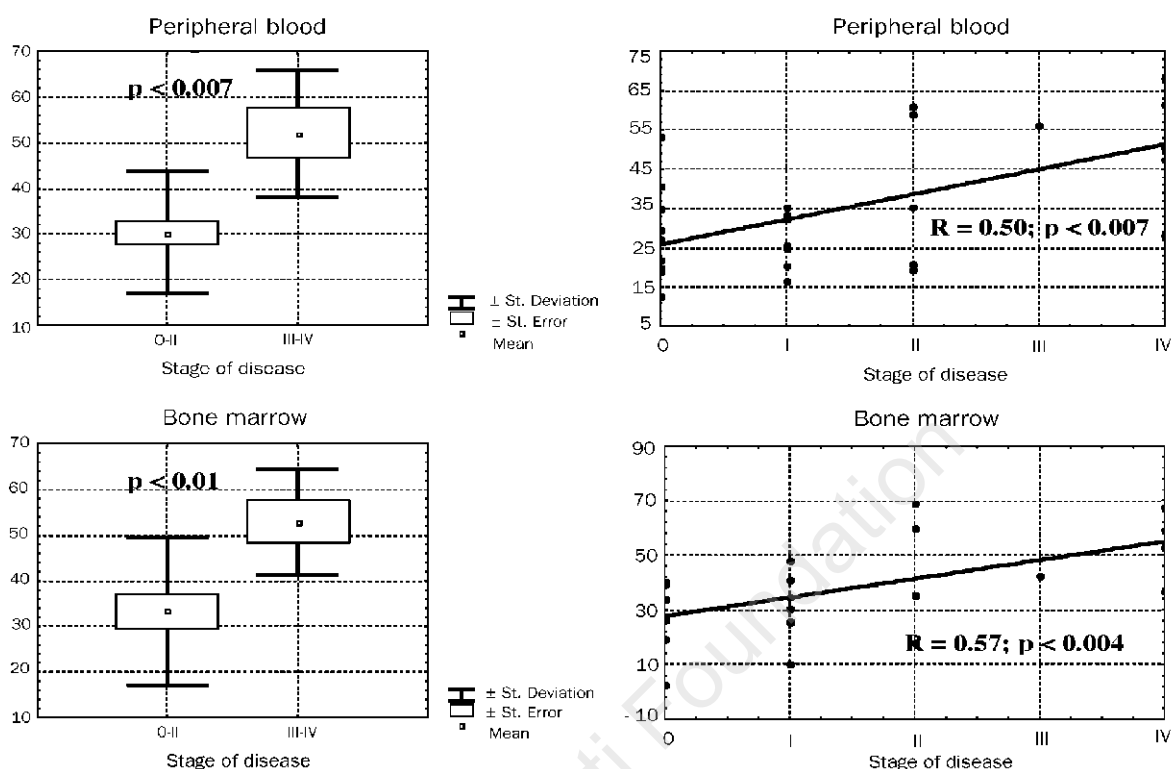


Figure 6. The box plots show statistically significant differences in percentages of CD3⁺ T-cells positive for TNF- α between patients in stage 0-II and patients in stage III-IV (left column). In PB and BM the percentage of CD3⁺ cells expressing cytoplasmic TNF- α positively correlated with the stage of disease. R – Spearman's rank correlation coefficient (right column).

CD19⁺/TNF- α ⁺ cells ($p < 0.007$). In PB and BM B-CLL CD19⁺ cells had a higher mean level of TNF- α expression in comparison with healthy controls. However this difference was not statistically significant (Figure 5B).

Relation between the expression of intracellular TNF- α and other clinical parameters

We looked for correlations between the B-CLL T-cells expressing TNF- α and clinical parameters. The first parameter assessed was clinical stage. As shown in Figure 6 the number of peripheral blood T-cells expressing TNF- α in B-CLL patients was higher in those with stages III-IV than in patients in early stages (PB: $p < 0.007$; BM: $p < 0.01$). In PB and BM the percentage of CD3⁺ cells expressing cytoplasmic TNF- α positively correlated with the stage of disease (respectively: $R = 0.50$; $p < 0.007$ and

$R = 0.57$; $p < 0.004$).

In PB the percentage of CD3⁺/CD8⁺ and CD3⁺/CD4⁺ cells producing TNF- α positively correlated not only with the stage of disease but also with lymphocytosis (respectively: $R = 0.40$; $p < 0.04$ and $R = 0.38$; $p < 0.05$). Furthermore, when the number of CD8⁺ and CD4⁺ T-cells positive for TNF- α were compared with total tumor mass (TTM) score the correlations were positive (respectively: $R = 0.49$; $p < 0.007$ and $R = 0.48$; $p < 0.009$). However, in BM only the percentage of CD8⁺ T-cells expressing cytoplasmic TNF- α correlated positively with TTM ($R = 0.46$, $p < 0.03$) and with lymphocytosis ($R = 0.40$; $p < 0.05$).

On the other hand, the MFI level did not correlate with the stage of disease or others parameters of disease. However, T-cell subsets from patients in stages III-IV had a statistically significant higher

level of TNF- α expression than corresponding cells from healthy control. Additionally, in our study the level of TNF- α expression in CD19⁺ cells positively correlated with its expression in CD8⁺ T-cells. The expression of TNF- α in leukemic B-cells did not correlate with parameters of disease.

Discussion

The results of the present study indicate that B- and T-cells from B-CLL patients produce TNF- α . Foa *et al.*¹² found that purified leukemic B-cells are capable of constitutional release of TNF- α . The observation that leukemic B-cells are capable of releasing TNF- α was further confirmed by the presence of the mRNA for this cytokine in primary or in pre-activated cells from B-CLL patients. This suggested that the presence of circulating high levels of TNF- α in the serum of B-CLL patients may derive from the neoplastic B-cell clone.¹² Rayes *et al.*,¹³ who assessed TNF- α production by PHA-stimulated CD2⁺ cells (which can be T- or NK-cells), detected higher TNF- α production in B-CLL patients than in healthy controls using an ELISA method. In our study the intracellular TNF- α was detected in both CD3⁺ T-cells and CD19⁺ B-cells. However, T-cells from PB and BM produced more cytoplasmic TNF- α than did the B-cells. Besides it was found that the percentage of T-cells expressing cytoplasmic TNF- α positively correlated with the stage of disease. The percentage of CD3⁺/TNF- α ⁺ cells was significantly higher in patients with more advanced disease (stage III and IV) than those in stage 0-II. The percentage of leukemic B-cells positive for TNF- α did not correlate with the stage of disease. In the study by Foa *et al.*¹² the cellular release of TNF- α by leukemic B-cells was significantly higher in patients with stage 0-I B-CLL than those with the stage II-III disease. These observations were not confirmed by all investigators, but the discrepancy may be due to technical differences. Adami *et al.*¹⁴ found that serum levels of TNF- α evaluated in the peripheral blood of B-CLL patients correlated with clinical stage (according to Rai's staging system) and relevant hematologic and immunologic data. Increased TNF- α serum levels were observed in all stages including stage 0 with a progressive increase in relation to the stage of disease.

Recently, Mainou-Fowler *et al.*¹⁵ detected higher expression of intracellular TNF- α by T-lymphocytes from patients than from controls. However, higher intracellular levels of TNF- α in T-cells was detected in some but not all cases of B-CLL. The per-

centage of T-cells that expressed cytoplasmic TNF- α was similar in patients and healthy controls, whereas, we found that the percentage of T-cells expressing intracellular TNF- α was significantly higher in patients with B-CLL than in normal controls. Likewise the percentage of CD19⁺ cells expressing intracellular TNF- α was higher in patients than in healthy controls.

In a study by Mainou-Fowler *et al.*¹⁵ cytoplasmic TNF- α was evaluated in purified blood T-cells from 17 patients (mostly in stages 0-III of disease). Intracellular TNF- α production was assayed in CD3⁺ T-cells. In our study we used mononuclear cells from peripheral blood and bone marrow of 40 patients in stages 0-IV of disease. We did not purify the T-cells. Furthermore, we used three-color flow cytometry to determine TNF- α expression by T-cell subsets – CD3⁺/CD4⁺ and CD3⁺/CD8⁺. We analyzed level of expression, indicated by the mean fluorescence intensity. Mainou-Fowler *et al.*¹⁵ used relative median fluorescence (RMF) to quantify cytokine expression. These differences are likely to account for discrepancies.

Our data show that B-CLL T-cells from PB and BM have higher percentages of cells positive for cytoplasmic TNF- α than do corresponding cells from the control population. When we compared the mean fluorescence intensity in B-CLL and control T-cell subsets only CD8⁺ and CD4⁺ T-cells from patients in stages III-IV had a statistically significant different MFI than the control T-cell subsets. T-cells from patients in stages 0-II also had higher MFI levels than T-cells from controls, but in this case the difference was not statistically significant.

Because of the overwhelming prevalence of the malignant B-cell clone, B-CLL patients show much lower percentages of circulating T-lymphocytes than those observed in normal peripheral blood samples.^{5,16} Although the relative percentage of T-cells in the peripheral blood is decreased due to the accumulation of abnormal B-cells, the absolute number of T-cells is frequently increased.^{5,17,18} Our data suggest that it is very likely that B-CLL cells, which are extremely frequent in the blood and can infiltrate lymphoid organs including the BM, are exposed to large numbers of T-cells directly able to synthesize and secrete TNF- α . What is more, T-cells may be an important source of this cytokine in advanced disease.

The method of analysis used in our study does not indicate that the B-CLL T-cells which expressed TNF- α also secreted this cytokine at an excessive rate. However, the mean fluorescence intensity

data indicated that T-cell subsets positive for intracellular TNF- α also produced more TNF- α per cell than the control T-cells. In our study CD3⁺/CD8⁺ cells from B-CLL patients displayed a significantly higher level of this cytokine expression in comparison with CD3⁺/CD4⁺ cells. Thus CD8⁺ T-cells and to a lesser extent CD4⁺ T-cells, are committed to produce TNF- α over the level seen in controls. Disease progression is associated with secondary immunodeficiency and with an increased risk of infections, which may be related to alterations in T-cell subsets and cytokine production.¹⁹ Numerous studies have shown a relative expansion of T CD8⁺ B-CLL cells with a frequently reduced percentage of CD4⁺ cells and a consequent overall significant reduction in the CD4:CD8 ratio.^{5,17,20} An inversion of the ratio of CD4⁺/CD8⁺ cell numbers and a deficiency in the function of T-helper cells were reported by several investigators.^{20,21,22}

Our assay system included the total peripheral blood and bone marrow lymphocytes. In addition to the abnormalities in the T-cell population, the presence of B-lymphocytes and cell-to-cell interactions may influence the behavior of T- and B-cells and their ability to produce intracellular TNF- α . In our study the level of TNF- α expression in CD19⁺ B-cells positively correlated with expression in CD3⁺/CD8⁺ and to a lesser degree with TNF- α expression in CD3⁺/CD4⁺ cells.

As the large tumor mass may be the main source of the TNF- α , even in the presence of a low secretory capacity on a per cell basis, we looked for some degree of correlation between expression of intracellular TNF- α and clinical parameters. In PB positive correlations were found between the number of CD3⁺/CD8⁺ and CD3⁺/CD4⁺ cells expressing TNF- α and the total tumor mass (TTM) score. What is more, in PB the number of both T-cell subsets positive for intracellular TNF- α was directly related to lymphocytosis. Jewell *et al.*²³ suggested that the correlation between changes in the lymphocyte count and levels of circulating cytokines may imply a role for autocrine cytokine production in the disease process in some patients with B-CLL. The B-CLL marrow is invariably infiltrated with leukemia cells. Furthermore, the extent of marrow infiltration correlates with clinical stage and prognosis.^{24,25} In our study the expression of TNF- α in leukemic B-cells did not correlate with parameters of disease. Moreover, in BM only number of CD3⁺/CD8⁺ cells expressing cytoplasmic TNF- α positively correlated with TTM and lymphocytosis. We can suppose

that different regulatory signals in the bone marrow and peripheral blood microenvironments may contribute to this difference.

TNF- α has an extensive array of activities towards both normal and malignant B-cells.⁹ *In vivo* leukemic B-cells from patients with B-CLL are arrested in the G₀ phase of the cell cycle,^{26,27} display enhanced survival and constitutively express high levels of the anti-apoptotic oncoprotein bcl-2.²⁶ Several observations indicate that *in vivo* signals from the microenvironment might influence the survival of B-CLL cells.^{28,29,30} In fact, when long-life leukemic cells are cultured *in vitro* they spontaneously die by apoptosis. A variety of cytokines have been shown to delay apoptosis of B-CLL cells when added *in vitro* to short-term cultures.^{31,32,33} However, the effects of TNF- α remain controversial.^{26,34} TNF- α production may be important for growth and survival of malignant B-cells because levels of TNF mRNA and protein are greatly increased when cells are cultured with rTNF itself.³⁴ Despite their developmental arrest *in vivo*, leukemic B-cells proliferate *in vitro* in the presence of TNF α .^{12,27,34,35} On the other hand, autologous serum containing high levels of soluble TNF receptors inhibits TNF- α -induced proliferation of CD19⁺ B-cells.³⁵

We can speculate that changes in the ability of T-cell subsets to produce cytoplasmic TNF- α , in addition to changes in tumor mass, are associated with progression of disease from an early to an advanced stage. However, further studies are required to explain this speculation.

In conclusion, in the present study we report that B-CLL B-cells and T-cell subsets produce higher amounts of cytoplasmic TNF- α than normal control cells. In addition T-cell subsets have higher expression of cytoplasmic TNF- α than CD19⁺ B-cells. Our results may confirm T-cell dysregulation. However further study is needed to elucidate what role the T-cell population and TNF- α production by T-cell subsets may play in the initiation and progression of B-CLL.

Contributions and Acknowledgments

AB designed the study and together with JR carried out the laboratory experiments, analyzed the samples, and prepared the manuscript. EW collected clinical data. AD and ZK were involved in interpretation of data, the statistical analyses and critically revising the intellectual content of the manuscript. They all approved the final version of the manuscript.

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Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

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PEER REVIEW OUTCOMES

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Dr. Smitha Sivaraman, who acted as an Associate Editor. The final decision to accept this paper for publication was taken jointly by Dr. Sivaraman and the Editors. Manuscript received November 8, 2001; accepted March 13, 2002.

What is already known on this topic

TNF α is purported to play a role in the pathogenesis of B-CLL, either in an autocrine or paracrine fashion. However an unequivocal delineation of the cells that produce the TNF α and its correlation to clinical pathogenesis of the disease has not been demonstrated.

What this study adds

This study demonstrates that T-cells rather than malignant B-cells produce higher levels of intracellular TNF α . The study further demonstrates the cellular subpopulations (CD3⁺/CD8⁺) that have higher intracellular levels of TNF α and show strong correlations between this population of cells and clinical parameters.

Potential implications for clinical practice

As clinicians today move increasingly in favor of targeted therapies, delineation of the precise role of TNF α in pathogenesis and the cellular location of abnormal cytokine production can help in designing therapies that can be directed towards rectifying such imbalances.

Smitha Sivaraman, Associate Editor