Cytokine regulation of apoptosis and Bcl-2 expression in lymphocytes of patients with systemic lupus erythematosus

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Abstract

Both faulty regulation of apoptosis and the inappropriate expression of several interleukins have been considered important defects of lymphocytes in the human autoimmune disease systemic lupus erythematosus (SLE). We therefore tested the *in vitro* effect of recombinant interleukin (IL)-2, 4, 7, and 15 on peripheral blood mononuclear cells from patients with SLE and from healthy volunteers. Intracellular Bcl-2 and Bax expression was measured by fluorocytometry and the rate of apoptosis was determined by the TUNEL technique and propidium iodide staining. IL-2, IL-4, IL-7 and IL-15 led to a significant increase in Bcl-2 and a reduction in cell death rates, which was even more pronounced in SLE. Bax levels remained unchanged. Interestingly, the high *ex vivo* Bcl-2 content of lymphocytes from some SLE patients was maintained after growth factor withdrawal. Anti-apoptotic cytokine signaling may significantly influence the deregulation of cell death in SLE lymphocytes. *Cell Death and Differentiation* (2000) 7, 966–972.

Keywords: systemic lupus erythematosus; Interleukin-2; Interleukin-7; Interleukin-15; Bcl-2; Bax

Abbreviations: SLE, systemic lupus erythematosus; IL-2, Interleukin-2; IL-7, Interleukin-7; IL-15, Interleukin-15

Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by a broad array of variable clinical features, autoantibodies, and deranged cytokines. Because of findings in animal models leading to autoimmunity¹ and in spontaneous experimental autoimmunity,^{2,3} apoptosis rates and apoptosis-related proteins in SLE have been investigated by several groups. Two findings have emerged from these studies: (1) Bcl-2 protein is elevated in bone marrow cells and peripheral blood T lymphocytes of patients with SLE;^{4–7} and (2) In SLE, *in vitro* apoptosis rates

are elevated, and presumably there are more apoptotic cells in the periphery.^{4,8–10} From these findings, two interrelated questions arise: How do the contradictory findings of an elevated antiapoptotic protein, Bcl-2, and an elevated programmed cell death rate match? And which factors influence either of these features in active lupus? Theoretically, the finding of elevated Bcl-2 could be due to technical problems. In discordance with this argument (and in spite of some contradictory observations,^{11–13} however, the wealth of well-controlled data from our and other laboratories find a consistent elevation in active SLE as well as a significant correlation of the Bcl-2 content with measures of disease activity and therefore strongly speak against such an idea.^{4–7,14} One could also hypothesize that, in addition to the upregulation of Bcl-2, its counterpart Bax might be upregulated, too, leaving the Bcl-2/Bax ratio unchanged. Since Bax has indeed not been investigated thoroughly in SLE it was therefore studied as part of the present work.

Alternatively, additional factors, such as cytokines, could be responsible for the elevation of Bcl-2. Finally, one can also hypothesize that the *in vitro* apoptosis rate is mostly artefactual in the sense that it is due to withdrawal of survival factor *in vitro*. A number of cytokines are likely to influence the rate of programmed cell death.^{15–19} Thus, survival factors such as cytokines would elevate Bcl-2 *in vivo*, but cells dependent on these cytokines would die at an increased rate when subjected to an environment without these survival factors.

We therefore decided to investigate the influence of human cytokines on the lymphocytes of SLE patients and compare them to those of healthy control individuals. In the present study, we will show that the elevated Bcl-2 is not balanced by an elevation of Bax and that the γ -chain cytokines IL-2, IL-4, IL-7, and IL-15 upregulate Bcl-2 in healthy controls to levels seen in active SLE. In lupus cells, however, these cytokines lead to further Bcl-2 upregulation, in some patients to an even higher degree than in the controls. Moreover, these cytokines also prevent the increased apoptosis occurring *in vivo*.

Results

IL-2 and IL-4 induce Bcl-2 in healthy and lupus lymphocytes

The addition of either recombinant human interleukin 2 (IL-2) or IL-4 for 72 h led to a significant rise of Bcl-2 in healthy control PBMC (Table 1). Indeed, control lymphocytes cultured in the presence of IL-2 reached Bcl-2 levels similar to those seen in SLE cells *ex vivo* (Figure 1). Within SLE cells, however, the already increased Bcl-2 expression was even

Table 1 Mean fluorescence intensities of Bcl-2 antibody in lymphocytes

	Healthy controls (n=19)		SLE (n=25)		
	<i>P</i> vs RPMI		<i>P</i> vs RPMI		<i>P</i> vs HC
<i>Ex vivo</i>	333 ± 64	0.0001	473 ± 141	0.05	0.0001
72 h RPMI	271 ± 55		427 ± 133		0.0001
72 h IL-2	484 ± 12	0.00001	550 ± 29	0.0001	0.05
72 h IL-4	421 ± 82	0.0001	591 ± 109	0.0001	0.001

Figure 1 FACS analysis of intracellular Bcl-2 in lymphocytes from SLE patients and healthy controls (HC) after incubation for 72 h in serum free unsupplemented medium (RPMI) without or with interleukin-2 (IL-2) or interleukin-4 (IL-4). Results are expressed as mean fluorescence intensity (mfi)

further enhanced by IL-2 (Figure 1). Thus, in analogy to the Bcl-2 levels *ex vivo*,⁷ SLE cells expressed significantly higher levels of Bcl-2 than controls after culture in medium supplemented with IL-2 or IL-4.

IL-2 and IL-4 decrease lymphocyte programmed cell death *in vitro*

At the same time, the addition of IL-2 or IL-4 significantly decreased the frequency of *in vitro* programmed cell death of SLE lymphocytes cultured in unsupplemented medium (Table 2). Under IL-2 stimulation, the lupus apoptosis rates were almost down to the rates of programmed cell death in control lymphocytes. However, some further reduction was also seen among control cells after interleukin-2 or -4 supplementation. Nevertheless, neither in controls nor in SLE could IL-2 or IL-4 fully abrogate apoptosis under the culture conditions employed.

IL-7 and IL-15, but not TNF- α exert effects similar to IL-2 on Bcl-2 expression and apoptosis

In nine SLE patients, the effect of adding IL-2 or IL-7 or IL-15 to the culture medium for 24 h only was compared to resting culture conditions in unsupplemented medium alone. IL-7 and IL-15 had analogous effects on Bcl-2 expression and on the rate of apoptotic cells to those already described above for IL-2 in healthy volunteers as well as in SLE patients (Tables 3 and 4). Similar to IL-2, IL-7 and IL-15 also significantly reduced the frequency of apoptosis in lupus

PBMC of SLE (Table 4). Among cells from healthy controls, this reduction did not reach significance. Figure 3 shows the time kinetics of apoptosis in one representative SLE patient and in a control individual. The addition of TNF- α did not have any impact on *in vitro* apoptosis in this and five analogous experiments (data not shown). Interestingly, in this set of patients as well as in the total cohort studied here, there was an excellent correlation between clinical disease activity as measured by the ECLAM score and *ex vivo* Bcl-2 (not shown).

Bcl-2 decreases and apoptosis increases under resting conditions

As shown in Figure 2, when PBMC were maintained in serum-free culture medium for 72 h ('resting culture'), the Bcl-2 content of viable lymphocytes decreased significantly both in SLE patients and in healthy controls. Group comparison (SLE vs controls) showed that SLE lymphocytes had a significantly higher Bcl-2 content also after culture in serum free conditions (Table 1). This was in part due to a small subgroup of SLE patients whose lymphocyte Bcl-2 content even increased after incubation in plain medium without any growth factors added (Figure 2). We could, however, not find any significant differences of these patients to the rest of the cohort with regard to their disease activity, organ manifestations, or autoantibodies. The lymphocytic Bcl-2 content was also elevated in freshly drawn blood of a small number of patients with septicemia ($n=5$), but decreased swiftly after incubation in unsupplemented medium (not shown).

Intracellular Bax signal intensity was not different between patients and controls. Bax levels also did not change after *in vivo* culture with or without cytokines (not shown).

Discussion

SLE T cells are characterized by increased expression of the anti-apoptotic protein Bcl-2 as well as increased propensity to undergo apoptosis. These observations are made persistently⁵⁻⁹ (and the present study), although they appear contradictory. These two phenomena could be perceived as a consequence of preexisting T cell activation, which occurs *in vivo* in SLE.²⁰⁻²³ Moreover, increased lymphocyte apoptosis occurs in diseases like viral infection or even in patients with severe burns. Elevated Bcl-2 is likewise found in the lymphocytes of patients with bacterial and viral infection and is thus by no means specific for autoimmune disease.

On the other hand, Bcl-2 is not elevated in rheumatoid arthritis PBMC,⁷ although rheumatoid arthritis

Table 2 Per cent apoptotic cells (propidium iodide)

	Healthy controls (n=17)		SLE (n=30)		
	<i>P</i> vs RPMI		<i>P</i> vs RPMI		<i>P</i> vs HC
<i>Ex vivo</i>	0.4±0.2	0.0001	1.7±1.6	0.05	0.0001
72 h RPMI	6.7±2.9		12.3±8.5		0.01
72 h IL-2	3.1±1.2	0.0005	8.2±5.0	0.0001	0.5
72 h IL-4	5.1±3.4	0.03	8.7±7.3	0.0001	0.03

Table 3 Mean fluorescence intensities of Bcl-2 antibody in lymphocytes

	Healthy controls (n=7)		SLE (n=9)		
	<i>P</i> vs RPMI		<i>P</i> vs RPMI		<i>P</i> vs HC
<i>Ex vivo</i>	294 ± 52	0.63	391 ± 121	0.14	0.11
24 h RPMI	277 ± 31		333 ± 63		0.08
24 h IL-2	326 ± 19	0.043	379 ± 87	0.011	0.2
24 h IL-7	361 ± 33	0.015	439 ± 98	0.0004	0.11
24 h IL-15	324 ± 32	0.06	393 ± 85	0.0008	0.10

Table 4 Per cent apoptotic cells (TUNEL)

	Healthy controls (n=19)		SLE (n=25)		
	<i>P</i> vs RPMI		<i>P</i> vs RPMI		<i>P</i> vs HC
<i>Ex vivo</i>	2.1±3.0	0.02	1.6±1.1	0.002	0.65
24 h RPMI	11.3±10.5		19.3±12.1		0.18
24 h IL-2	10.3±9.3	0.33	11.0±7.8	0.009	0.86
24 h IL-7	9.2±8.4	0.09	12.2±9.8	0.02	0.52
24 h IL-15	10.1±9.0	0.36	10.6±11.5	0.004	0.93

also represents an autoimmune disease with considerable lymphocyte activation. In addition, double staining experiments with conventional activation markers such as CD25 or HLA-DR have repeatedly failed to allocate increased Bcl-2 content or increased apoptosis to activated lymphocytes, although the data clearly showed that T lymphocytes are responsible for these phenomena⁷ (and unpublished data). Therefore mechanisms other than conventional T-cell activation might be responsible for deregulating cell death in SLE. The deregulation of the cytokine network in SLE^{24,25} might well be operative in determining the observed deregulation of *in vitro* cell survival in this disease.¹⁰

In this study we show that SLE lymphocytes respond to the *in vitro* exposure to exogenous IL-2, 4, 7 and 15, all of which belong to a group of cytokines sharing a receptor element, the common γ -chain. Although the Bcl-2 content of these cells is already significantly enhanced without cytokine addition, as compared to healthy controls, the increase in Bcl-2 is even more pronounced after incubation with these cytokines. This overreaction to γ -chain interleukins may be of considerable importance. After all, these cytokines are known to allow for T cell activation and proliferation; they also prevent apoptosis induced by activation or deprivation of growth factors.^{26–28} Lymphocytes with a tuned up survival state (as indicated by a high Bcl-2 content) which even respond more strongly to anti-apoptotic cytokine signals could contribute a disease-perpetuating factor as indicated by

experimental animal systems.^{29–33} Thus, defects of cell death signal transduction pathways in SLE might lead to the survival of autoreactive cells and to cell activation even without the expression of conventional activation markers.^{34,35} Since the anti-apoptotic cytokines used here share a common receptor element, the gamma chain which uses denominated intracellular signaling molecules,³⁶ the study of cell death signal transduction and of Janus kinases or signal transducers (STATs)^{37,38} and their inhibitors^{39,40} is warranted in SLE.

At this point, we do not have a conclusive explanation for our finding, that among the SLE patients studied, the 'abnormal' increase in Bcl-2 persisted (and was sometimes even augmented) during 72 h of culture in medium devoid of exogenous growth factors (Figure 2). Our experimental conditions did, however, include the presence of other mononuclear cells during the 'resting' culture, so that ongoing cellular interactions or autocrine cytokine circuits cannot be excluded.

The observations made here, including the correlation of Bcl-2 expression and cytokine-induced enhancement of Bcl-2 expression, are compatible with a role of cytokine-induced anti-apoptotic events in the pathogenesis of SLE. Since synergistic effects of Bcl-2 genotypes and cytokine abnormalities have been reported to predispose to disease expression,^{41,42} further dissection of the mechanisms and regulation of proapoptotic and cytokine-induced cell death may lead to a better understanding of the pathogenesis of SLE.

Figure 2 Cytofluorographic determination of intracellular Bcl-2 in lymphocytes from SLE patients and healthy controls (HC) *ex vivo* or after incubation for 72 h in serum free unsupplemented medium (RPMI). Results are expressed as mean fluorescence intensity (mfi)

Materials and Methods

Patients

Forty consecutive outpatients (37 female, age 34 ± 16.3 years (mean $\pm$ SD) fulfilling at least four ACR criteria for the classification of SLE gave informed consent to the use of their blood for this study. Disease duration was 6.5 ± 6.2 years, disease activity as measured by the ECLAM score⁴³ was moderate to low (2.8 ± 1.6). Thirty-one patients were on low dose steroids (range 5 to 10 mg/day), 17 were taking azathioprine in doses between 50 and 150 mg/day, 14 patients had been prescribed 200 mg of daily chloroquine and 15 were on steroids only without further immuno-modulatory medication. Statistical comparison of Bcl-2 signal and levels of apoptosis did not show any differences when the patient cohort was stratified according to different treatments. A subgroup of nine female patients and seven age-matched female controls without any medication was used for the experiments using IL-2, IL-7 and IL-15. Patients who had received intravenous cyclophosphamide within 6 weeks prior to this investigation or patients with clinical or laboratory signs of concurrent infectious disease were not included in this study. A convenience group of 39 healthy laboratory personnel (15 female, age 27 ± 4.2) served as a control population. While there was no equivalence in the age- and sex-matching of the controls and patients, large series of observations on Bcl-2 levels or *in vitro* apoptosis did not show any influence of age or sex on these parameters (Stummvoll *et al*, submitted).

Figure 3 Representative examples for the time kinetics of apoptosis as determined by the TUNEL-reaction in a SLE patient and a healthy control. Cytokines added to the cultures are denoted in the insert, RPMI denotes medium devoid of any growth factors

Blood cell purification and culture

PBMC were isolated from heparinized blood using Ficoll-Paque density gradient centrifugation. Blood from at least one healthy volunteer was always processed in parallel with the patient samples. Isolation and staining of the cells was performed within 30–60 min after drawing the blood. Culture of cells was performed for 24 or 72 h under standard conditions in 96-well plates in RPMI 1640 without human or bovine calf serum in order to avoid any growth or death signals generated by exogenous biological sources ('resting medium'). Recombinant human (rh)-IL-2 (Biosource International, Camarillo, CA, USA), IL-4, IL-7, IL-15 (Pharma Biotechnologie, Hannover, Germany) or TNF- α (PBH, Hannover, Germany) was added to cell cultures in concentrations of 10 to 100 u/ml.

After incubation in plastic wells, non-adherent cells were recovered by rinsing with medium, cell recovery varied from 58 to 77%. The addition of the various cytokines did not lead to significant variances of recovery rates. Viability of all cells cultured for 24 and 72 h in RPMI with or without cytokine supplementation always exceeded 90 and 85%, respectively, when tested by trypan blue exclusion and there was no significant effect on viability observed after addition of the different cytokines.

Measurement of intracellular Bcl-2 and Bax protein

PBMC were analyzed immediately after venipuncture and isolation or culture. Cells were fixed and permeabilized using the Fix&Perm cell permeabilization kit (An der Grub Inc., Kaumberg, Austria). Intracellular staining⁷ was performed simultaneously during the

Figure 4 Intracellular staining of peripheral lymphocytes with a monoclonal Bcl-2 antibody. (a) Representative result from a SLE patient. The dark line shows the fluorescence intensity after 24 h of incubation in unsupplemented medium, the grey line depicts the result after incubation in identical medium plus human recombinant IL-2. (b) Result from a healthy control individual. Insert graph shows the gates set: the region R2 containing largely non-lymphocytic mononuclear cells is excluded from analysis

permeabilization step with the addition of fluorescein-(FITC)-conjugated mouse-anti-Bcl-2 monoclonal antibody (DAKO A/S, Glostrup, Denmark) or Ig-isotype-matched control-antibody as well as with a polyclonal rabbit anti-bax antibody (Santa Cruz, CA, USA) and phycoerythrin (PE)-conjugated donkey anti-rabbit Fab2 fragment (Accurate Chemicals, New York, NY, USA). Cytofluorographic analyses were performed on a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA, USA) with the acquisition gate excluding all cells but lymphocytes (e.g. see insert, Figure 4). Bcl-2-fluorescence was recorded using linear signal-amplification. The instrument's settings were kept constant throughout the series of experiments, day-to-day variation of instrument parameters was tested by comparing the healthy controls analyzed in parallel with the patients' samples each day and was found to amount to a coefficient of variation of less than 10%.

Previous double and triple staining experiments⁷ had shown that the abnormalities in Bcl-2 expression as well as in apoptosis were confined to the T lymphocytes, but there was no significant difference

in Bcl-2 expression or apoptosis between the different T lymphocyte subsets analyzed (CD4, CD8, CD4/8 double negative). Cells staining with CD25, HLA-DR, or CD45RO had shown decreased Bcl-2 signals. Since this was again seen in this study for unstimulated as well as for stimulated cells of SLE patients and controls, data of such subset analyses are not shown here.

Detection of apoptosis

The percentage of cells undergoing programmed cell death was measured in all cases by staining with propidium iodide in hypotonic citrate-buffer and analyzing the percentage of viable cells with a hypodiploid DNA content. In addition, 33 samples were tested also by the terminal deoxynucleotidyl-transferase (TdT) mediated dUTP nick end labeling technique (TUNEL) using the cytofluorimetric In-Situ Cell Death Detection Kit with fluorescein (Boehringer Mannheim, Mannheim, Germany). The percentage of FITC-fluorescing cells – reflecting apoptotic DNA fragmentation – was recorded using label mix

without TdT as negative controls and PBMC incubated for 24 h with prednisolone as positive controls. For both methods, the analysis was performed by cytofluorometry as above, including both dying and viable lymphocytes into the acquisition gate, but excluding all cells of larger size and/or higher granularity. By determining the frequency of apoptotic cells with different methods, we saw an excellent correlation ($r=0.89$, $P=0.0001$) between hypotonic propidium iodide staining and the TUNEL technique.

Statistical analysis

This included group comparison by the Mann-Whitney and Wilcoxon tests as well as Student's *t*-test (with Tukey-Kramer correction for multiple comparisons, when appropriate). Values are given as mean $\pm$ SD. *P*-values <0.05 were considered significant.

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