

The Significance of Low *bcl-2* Expression by CD45RO⁺ T Cells in Normal Individuals and Patients with Acute Viral Infections. The Role of Apoptosis in T Cell Memory

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Summary

The *bcl-2* gene product has been shown to prevent apoptotic cell death. We have now investigated the *bcl-2* protein expression by resting and activated mature T cell populations. Freshly isolated CD45RO⁺ T cells within CD4⁺ and CD8⁺ subsets expressed significantly less *bcl-2* than CD45RO⁻ (CD45RA⁺) T cells ($p < 0.001$). When CD45RA⁺ T cells within both CD4⁺ and CD8⁺ subsets were activated *in vitro*, the transition to CD45RO phenotype was associated with a decrease in *bcl-2* expression. Patients with acute viral infections such as infectious mononucleosis caused by Epstein-Barr virus infections or chickenpox, resulting from varicella zoster virus infection, had circulating populations of activated CD45RO⁺ T cells which also showed low *bcl-2* expression. In these patients, a significant correlation was seen between low *bcl-2* expression by activated T cells and their apoptosis in culture ($r = 0.94$, $p < 0.001$). These results suggest that the primary activation of T cells leads to the expansion of a population that is destined to perish unless rescued by some extrinsic event. Thus the suicide of CD45RO⁺ T cells could be prevented by the addition of interleukin 2 to the culture medium which resulted in a concomitant increase in the *bcl-2* expression of these cells. Alternatively, apoptosis was also prevented by coculturing the activated T lymphocytes with fibroblasts, which maintained the viability of lymphoid cells in a restinglike state but with low *bcl-2* expression. The paradox that the CD45RO⁺ population contains the primed/memory T cell pool yet expresses low *bcl-2* and is susceptible to apoptosis can be reconciled by the observations that maintenance of T cell memory may be dependent on the continuous restimulation of T cells, which increases their *bcl-2* expression. Furthermore, the propensity of CD45RO⁺ T cells to extravasate may facilitate encounter with fibroblast-like cells in tissue stroma and thus be an important additional factor which promotes the survival of selected primed/memory T cells *in vivo*.

The *bcl-2* proto-oncogene and its product have been shown to control the survival of both normal and malignant cells (1). The *bcl-2* gene was first identified in most follicular B cell lymphomas at the breakpoint of the translocation between chromosomes 14 and 18 (2, 3). It was subsequently shown that in lymphoid environments the low expression of the *bcl-2* gene product is associated with the selection or deletion of cells (4, 5). Thus, the expression of *bcl-2* by proliferating B cells in the germinal centers of LNs is low (5, 6). Similarly, immature cortical thymocytes undergoing selection are *bcl-2*⁻ (1). The product of the human *bcl-2* gene has been shown to block programmed cell death or apoptosis (1,

7, 8), and an induced increase in *bcl-2* expression rescues appropriate B cells or thymocytes from this suicide (5, 6, 9). In addition, in transgenic mice with upregulated *bcl-2* there is a prolongation of secondary immune responses (10, 11). These observations, taken together, suggest that factors that alter *bcl-2* expression are important in the development of both memory B cells and thymocytes (10, 12).

Although compelling data demonstrate that the *bcl-2* gene product may have a role in lymphoid selection, no data is available on the changes of the *bcl-2* protein during peripheral T cell activation and/or development. Clearly, the investigation of *bcl-2* expression by T cells before and after im-

immune stimulation *in vivo* is of considerable interest as a possible way in which activated T cells may be selected for survival. It has been shown that T lymphocytosis, especially within the CD8⁺ subset, is induced by acute viral infections and these cells are activated and enter the proliferative cycle *in vivo* (13–15). However, CD4⁺ and CD8⁺ cell numbers rapidly return to normal levels on disease resolution even though specific antiviral memory T cells persist *in vivo* (16). This suggests that mechanisms that determine if activated T cells survive or perish regulate the balance between the generation of a memory population and the reestablishment of cellular homeostasis after activation *in vivo*.

Recent studies have demonstrated that various phenotypic and functional changes parallel the differentiation of T cells from an unprimed to a primed/memory state (17–19). The most discriminating markers for unprimed and primed T cells in humans, the CD45RA and CD45RO antibodies, are directed to the high and low molecular weight isoforms of the leukocyte common antigen, respectively (19, 20). In this study, we have investigated the expression of bcl-2 protein by resting and activated subpopulations of T cells from normal individuals and patients with acute viral diseases caused by EBV and varicella zoster virus (VZV),¹ and related the bcl-2 expression to the LCA display on these cells. Our findings demonstrate that there is a decrease in bcl-2 protein expression in T cells after activation which leads to apoptosis unless they are rescued by appropriate factors, in analogy with the selection of B cells in germinal centers (5, 6). Thus, apoptosis can be prevented by the addition of exogenous IL-2, which maintains the activated T cells in cycle and induces the reexpression of bcl-2. In contrast, tissue stromal cells such as fibroblasts can also prevent the occurrence of apoptotic death in activated T cells, but enable them to attain a restinglike state with low bcl-2 expression. These data also provide clues into the interplay between mechanisms which may firstly lead to the persistence of T cell memory in humans, yet at the same time enable the homeostatic balance of total T cell numbers to be maintained after immune activation *in vivo*.

Materials and Methods

Patient and Control Samples. Heparinized venous peripheral blood was obtained from 10 patients with either acute infectious mononucleosis (seven males and three females; mean age 25 yr: range 19–45 yr) or with varicella zoster infections (six males and four females; mean age 31 yr: range 20–40 yr) who were admitted to the Infectious Diseases Unit at Coppett's Wood Hospital within 10 d after the onset of symptoms. Blood was also obtained from a male patient with acute HIV-1 infection who had p24 antigen in the serum before developing anti-p24 antibody. In addition, normal blood was obtained from 10 healthy laboratory personnel and medical students (five males and five females; mean age 28 yr: range 21–45 yr) and normal tonsils were obtained at elective surgery after antibiotic therapy. LN biopsies from HIV-1-infected individuals were frozen and analyzed in cryostat sections.

¹ Abbreviations used in this paper: MGG, May-Grunewald-Giemsa stain; PI, propidium iodide; VZV, varicella zoster virus.

Antibodies and Cytokines Used in the Study. The CD45RA (SN130; IgG1) and CD45RO antibodies (UCHL-1; IgG2a, generously provided by Professor P. C. L. Beverley, Imperial College Research Fund, London, UK) were previously shown to react with unprimed and primed T cells, respectively (20). CD4 (RFT4; IgG1) and CD8 (RFT8; either IgG1 or IgM) antibodies were used to identify and/or isolate helper and suppressor/cytotoxic subsets of T cells, respectively (21). A CD3 antibody (MEM-57; IgG1) was kindly provided by Dr. V. Horejski (Czechoslovak Academy of Sciences, Prague, Czechoslovakia) (22). The IgG1 antibody reacting with the bcl-2 gene product by recognizing a 26-kD protein (23) was obtained from Dr. D. Y. Mason (Nuffield Department of Pathology, Oxford, UK), and Dako Ltd. (High Wycombe, Bucks, UK). In this study, this protein will henceforth be referred to as bcl-2. Ig isotype-specific FITC or PE-conjugated affinity-purified goat anti-mouse second layer antibodies (Southern Biotechnology Associates, Birmingham, AL) were used at pretitrated optimal concentrations. Recombinant human IL-2 was kindly provided by Dr. Max Schreier (Sandoz Pharma Ltd., Basel, Switzerland) and recombinant GM-CSF was obtained from British Biotechnology Ltd. (Abingdon, Oxon, UK).

Preparation of Lymphocyte Subsets. CD2⁺ cells were prepared by E-rosetting from Ficoll-Hypaque (Nycomed, Oslo, Norway) separated PBMC as previously described (24). CD4, CD8, CD45RA, and CD45RO subsets of CD2⁺ cells were prepared by immunomagnetic bead depletion (DynaL Ltd., Wirral, UK) as described in detail elsewhere (25). Only negatively selected subsets were used in any of the assays. The subsets prepared in this way were regularly 90–95% positive for the CD45RA or CD45RO phenotype and 94–98% CD4 or CD8 positive.

Fibroblast/Lymphocyte Coculture. Human embryonic lung fibroblasts grown in 24-well plates (Falcon Labware, London, UK; Becton Dickinson, Ltd., Oxford, UK) in RPMI-1640 medium supplemented with L-glutamine, benzyl-penicillin, streptomycin (Gibco Ltd., Paisley, UK) and 10% of fetal bovine serum (FBS; Flow Laboratories Ltd., Rickmansworth, UK) were maintained in a humidified atmosphere containing 5% CO₂. The fibroblasts were used as confluent monolayers between passages 8–19. These cell lines were mycoplasma free as shown by Hoescht staining and an RNA probe (Laboratory Impex, Middlesex, UK). Every 3–4 d, half of the spent growth medium in fibroblast cocultures was replenished.

Lymphocyte Activation. T cell subsets were activated with 1 µg/ml PHA (Wellcome Ltd., High Wycombe, Bucks, UK) in the presence or absence of IL-2 (2 ng/ml; 26). Cells were harvested from replicate cultures at various times for phenotypic analyses. To establish IL-2-dependent cell lines, CD4⁺ or CD8⁺ T cells were first activated by PHA and IL-2 (2 ng/ml) for 6 d in bulk in the presence of 10% autologous adherent cells. The cells were then washed and resupplemented with IL-2 every 3–4 d. After 3–4 wk, the cells were reactivated with PHA in the presence of autologous adherent cells and recultured with IL-2 which was replenished every 3–4 d as before. To prevent overcrowding, the concentration of these cells was periodically readjusted. The acute withdrawal of IL-2 resulted in a rapid decrease in viability and increase in cell death by apoptosis as described (27).

Cell Staining. PBMC or T cell subsets were stained in suspension, as smears after cytocentrifuge preparation or in histological sections. First, membrane staining was performed and followed by membrane permeabilization to allow for cytoplasmic staining (28). The membrane markers used were CD3, CD4, CD8, CD45RA, and CD45RO antibodies and isotype-specific second layers conjugated to FITC (Southern Biotechnology Associates). The cells were fixed with 0.3–0.4% paraformaldehyde in PBS for 2 min,

washed with PBS containing 0.2% azide and 0.2% BSA, and permeabilized in 500 μ l of ice-cold 1:1 acetone methanol. This mixture was then incubated on ice for 15 min and washed twice with PBS plus azide before adding pretitrated optimal amounts of bcl-2 antibody. After washing, goat anti-mouse IgG conjugated to PE was added and incubated for 15 min at room temperature. The specificity of the method has been established by analyzing suspensions of tonsil cells containing surface(s) IgD⁺ B lymphocytes (bcl-2⁺) and CD38⁺ germinal center blast cells (bcl-2⁻). The cells were finally washed and fixed with 4% paraformaldehyde. The negative control antibodies for bcl-2 staining consisted of isotype-matched unreactive antibody followed by identical second layer labeling as above. The expression of bcl-2 on lymphocyte subsets was investigated by two-color immunofluorescence on a FACScan[®] (Becton Dickinson & Co.) and compared with similar staining performed in cyto centrifuge preparations. These methods have been described in detail elsewhere (29, 30). Double fluorescence staining was performed on acetone-fixed cryostat sections from tonsils and LN biopsies of HIV-1-infected individuals as described previously (31). After rehydration, CD8 and bcl-2 antibodies were added for 45 min, and after washing FITC or tetramethyl rhodamine isothiocyanate conjugated subclass-specific second layers were added in the presence of 10% normal human serum to inhibit nonspecific binding. After 45 min at room temperature, the sections were washed and then examined by fluorescence and confocal microscopy as above.

The Enumeration of the Absolute Number of Lymphocytes/Lymphoblasts. The Cytoron absolute (Ortho Diagnostic Systems, Ltd., High Wycombe, Bucks, UK) is a flow cytometer which allows the enumeration of absolute lymphocyte numbers identified by fluorochrome-labeled antibodies. Cells were fixed in 0.05% paraformaldehyde and gated on forward and 90° side scatter. The absolute number of fluorescent cells was then determined within the lymphoid gate. Dead cells, identified by their forward and side scatter profiles, were excluded from further analysis (32).

Detection of Apoptosis. Apoptosis was measured by four methods. First, the cleavage of DNA into oligonucleosomal fragments was tested as described previously (33). Briefly, 10⁶ cells from normal or virally infected individuals were snap frozen in liquid nitrogen and the pellets were resuspended in 20 μ l of 10 mM EDTA, 50 mM Tris/HCl buffer (pH 8) containing 0.5% sodium lauryl sarkosinate (BDH, Lutterworth, Leics, UK) and 0.5% mg/ml proteinase K (Pharmacia Biotechnology Ltd., Milton Keynes, UK).

After incubation at 50°C for 60 min, RNase A stock solution (10 μ l of 0.5 mg/ml; Sigma Chemical Co., Poole, Dorset, UK) was added, incubated for 1 h at 50°C, and the samples were then heated to 70°C. EDTA (10 μ l of 10 mM) containing 1% low melting point agarose (Sigma Chemical Co.), 0.25% bromophenol blue, and 40% sucrose was mixed with each sample. This mixture was loaded onto a 2% agarose gel containing 1 \times TBE buffer (90 mM Tris/borate, 1 mM EDTA) containing 250 ng/ml ethidium bromide (Sigma Chemical Co.) before electrophoresis (80 V, 1.5 h).

Second, the proportion of apoptotic cells present in cultures was also determined in cyto centrifuge preparations by their morphology, by chromatin condensation, nuclear fragmentation, and by a decrease of the nuclear/cytoplasmic ratio after May-Grunewald-Giemsa staining (MGG; see Fig. 3 F). Third, these cells were studied by electron microscopy. Fourth, the apoptotic cells were identified by double-color fluorescence technique using surface labeling in conjunction with nuclear labeling with propidium iodide (PI) after permeabilization with paraformaldehyde at a final concentration of 0.5% in PBS for 2 min (32). This technique identified a PI-reactive apoptotic population that was smaller in size than resting viable PI⁻ lymphocytes, but was distinct from debris and the non-viable cells exhibiting an increased 90° scatter. Similar proportions of apoptotic cells were detected by the three morphological methods.

Statistics. The results were analyzed by the Student's *t* test and by linear regression analysis.

Results

The Expression of bcl-2 by T Cell Subsets. PBMC isolated from 10 normal individuals and analyzed for bcl-2 expression within the CD3⁺ T cell cohort revealed that the majority (mean 80%) expressed bcl-2. A minor bcl-2⁻ population was, however, consistently found in each individual tested (Table 1) and these cells were observed mainly in the CD4 subset (Fig. 1 B). The CD45RO⁺ T cells expressed less bcl-2 than both the whole CD3⁺ ($p < 0.001$) and also the CD45RO⁻ T cell subsets ($p < 0.001$; Table 1, Fig. 1). Single cell analyses revealed that the difference between the bcl-2 expression between CD4⁺ and CD8⁺ subsets in normal individuals was due to the greater numbers of CD45RO⁺ cells within the former subset. The minor CD8⁺, CD45RO⁺ T cell population in normal individuals

Table 1. The Expression of bcl-2 by T Cell Subsets from Normal and Virally Infected Individuals

Groups	n	CD3	CD4	CD8	CD45RO ⁺	CD45RO ⁻
Normal	10	80 (73–84)*	82 (63–95)	83 (68–93)	63 (53–73) [§]	89 (78–96)
VZV	6	53 (40–67)**	70 (52–82) [‡]	65 (42–89) [†]	45 (28–48) [†]	63 (51–72)**
EBV	7	55 (38–67)**	71 (48–82) [‡]	49 (31–80)**	44 (16–68)	67 (46–89)**

* The proportion of bcl-2⁺ cells within PBMC T cell subsets analyzed by two-color immunofluorescence. The results represent the mean percentage (and range) of bcl-2⁺ cells in the different subsets as shown.

[‡] Not significantly different to normal as analyzed by the Student's *t* test.

[§] The proportion of bcl-2⁻ cells is significantly higher among normal CD45RO⁺ T cells than within the total CD3 population ($p < 0.001$).

^{||} Significant change from normal ($p < 0.02$).

[†] Significant change from normal ($p < 0.01$).

** Significant change from normal ($p < 0.001$).

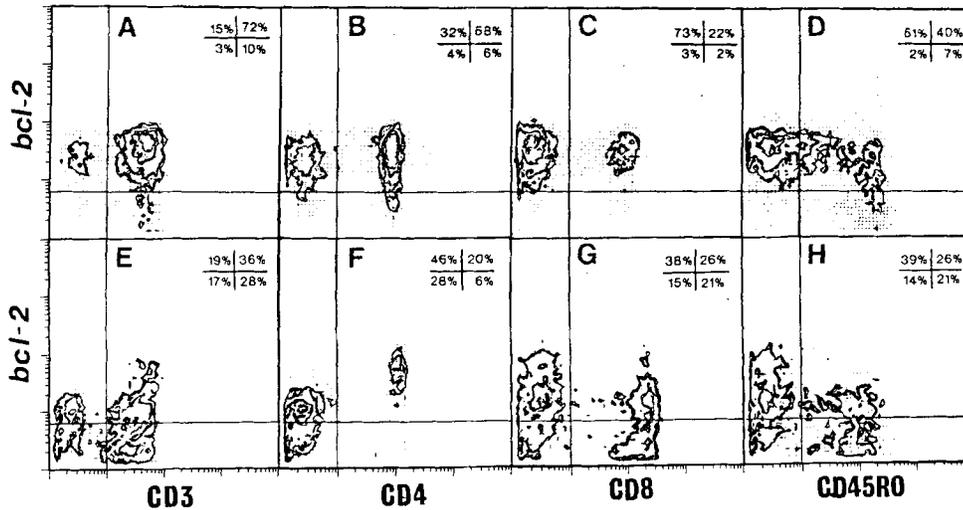


Figure 1. The expression of bcl-2 by T cell subpopulations from a normal individual (A-D) and a patient with acute EBV infection (E-H). PBMC were analyzed for coexpression of bcl-2 with other T cell markers by double-color immunofluorescence (5,000 cells in the lymphoid gate). The percentages shown represent the proportion of cells in each quadrant of the fluorescence gates, set using negative control antibodies. Within the CD8⁺ subset, only the brightly stained cells (CD3⁺) were analyzed whereas the CD8 dim (CD3⁻, C16⁺, CD56⁺) NK cells were excluded.

also included cells with low bcl-2 expression (data not shown). A bimodal distribution of bcl-2 on normal CD45RO⁺ T cells was consistently found. Cells expressing high levels of CD45RO showed low bcl-2 expression and vice versa (Fig. 1 D).

When the bcl-2 expression of T cells from normal individuals and patients with acute viral infections were compared, it was found that the bcl-2⁻ subsets within the CD3⁺ T cell population significantly expanded in both EBV and VZV infected individuals (*p* < 0.001; Table 1). Previous studies have already indicated that these patients have increased numbers of CD45RO⁺ T cells within the CD8⁺ subset (14), a finding which agrees with our own observations shown here. There was significantly decreased bcl-2 expression in both CD8⁺ and CD45RO⁺ subsets of virally infected as compared with normal patients, which largely accounted for the decrease of bcl-2 expression within the CD3⁺ popula-

tion (Table 1; see representative EBV patient in Fig. 1, E-H). It was noted that the CD3⁻ subset in both VZV and EBV patients also expressed lower levels of bcl-2 than normal individuals (representative experiment in Fig. 1 E). The CD3⁻, bcl-2⁻ cells are CD19⁺ and of the B-lineage (data not shown). When the bcl-2 reactivity of CD45RO⁺ (CD45RA⁻) PBMC from EBV and VZV patients was analyzed, it was found that they expressed significantly lower levels of bcl-2 than the CD45RO⁻ (CD45RA⁺) PBMC population, an observation which was also apparent in normal individuals (Table 1 and Fig. 1 H) and which was confirmed when these subsets were analyzed within purified T cells instead of PBMC populations (data not shown). The CD45RO⁻ PBMC cells from normal individuals however, express significantly higher levels of bcl-2 than those from EBV and VZV patients probably because of the presence of bcl-2⁻ B cells that are localized within the CD45RO⁻ subset in these

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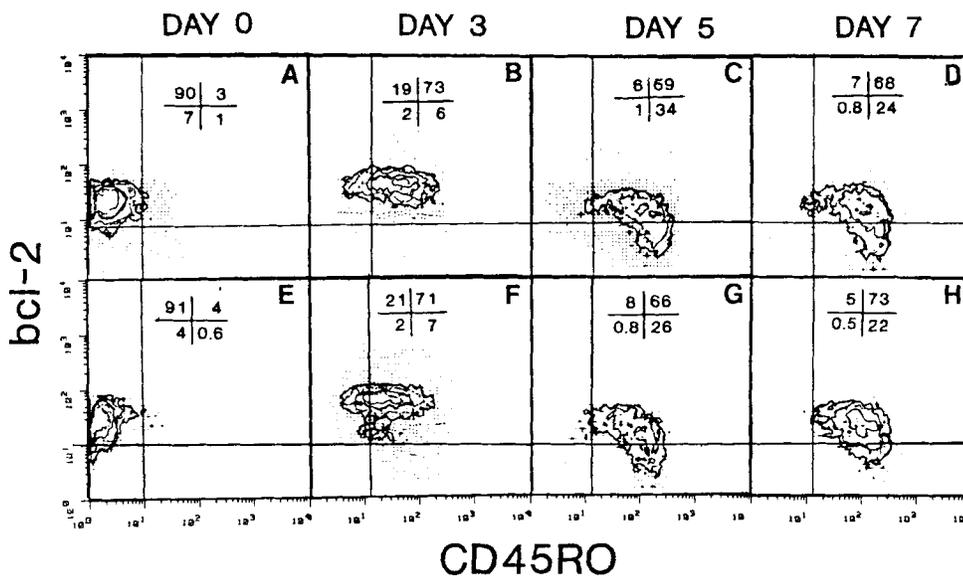


Figure 2. The changes in bcl-2 expression after PHA activation of CD4⁺, CD45RA⁺ (A-D) and CD8⁺, CD45RA⁺ cells (E-H) for various periods of time (day 0-7) in comparison with the appearance of CD45RO reactivity (5,000 cells in the lymphoid gate). For details see Fig. 1.

patients but not in the normal patients (Fig. 1, A and E). The low bcl-2 expression in the CD45RO⁻ PBMC population could also be due to activated T cells in transition from a CD45RA⁺ (CD45RO⁻) to a CD45RA⁻ (CD45RO⁺) phenotype that may have lost their bcl-2 expression before acquiring CD45RO reactivity. However, the kinetics of the loss of bcl-2 and gain of CD45RO reactivity by CD45RA⁺ T cells after activation render this latter possibility unlikely (see Fig. 2).

The Expression of bcl-2 by Normal T Cells during Activation In Vitro. The difference in bcl-2 expression between normal CD45RO⁺ and CD45RO⁻ T cell populations suggested that changes in the expression of this protein may occur as a result of T cell differentiation linked to recent priming. These observations were confirmed *in vitro* by activating purified T cells with mitogens such as PHA followed by the analysis of bcl-2 expression. CD4⁺, CD45RA⁺ (Fig. 2, A–D), and CD8⁺, CD45RA⁺ (Fig. 2, E–H) populations were isolated and activated with PHA in order to observe the changes in bcl-2 expression in parallel with the transition from CD45RA⁺ to CD45RO⁺ expression. Before activation, both the isolated CD4⁺ and CD8⁺ populations were <4% CD45RO⁺ (95% CD45RA⁺) and >95% bcl-2⁺ (Fig. 2, A and E). After 3 d of stimulation with PHA, 79% of the CD4⁺ cells and 75% of the CD8⁺ T cells started to express CD45RO (Fig. 2, B and F, respectively) but the majority of CD4⁺

(93%) and CD8⁺ (86%) T cells still remained bcl-2⁺. After 5 d of stimulation, 94% of the CD4⁺ and 93% of the CD8⁺ T cells expressed CD45RO (Fig. 2, C and G) and a bimodal distribution of bcl-2 developed in both CD4⁺ and CD8⁺ subsets (34 and 26% bcl-2⁻, respectively; Fig. 2, C and G). These results have also been confirmed in cytocentrifuge preparations stained for bcl-2 in conjunction with other lymphocyte markers (data not shown). The bcl-2⁻ cohort was still observed after 7 d of activation (Fig. 2, D and H).

The Association of Low bcl-2 Expression with Apoptosis in T Cells. In cytocentrifuge smears of normal tonsil cell suspensions stained for bcl-2 heterogeneity was observed (Fig. 3 a). The large blast cells (CD38⁺ germinal center B cell blasts) were bcl-2⁻ whereas the majority (>95%) of small lymphocytes were bcl-2⁺ (Fig. 3 a). In comparison, normal PBMC populations did not contain blast cells and were >80% bcl-2⁺ (Fig. 3 c). When the tonsil suspensions were activated for 48 h by PHA plus IL-2, T cell blasts developed and still showed strong expression of bcl-2⁺ (Fig. 3 b). bcl-2 was reduced in T cell blasts in longer term cultures (>84 h). Freshly isolated PBMC populations from patients with EBV and VZV infections included many activated T cell blasts that expressed low bcl-2 (Fig. 3 d) suggesting that in the patients *in vivo* these T cells were activated for longer periods (Fig. 2). We next investigated if the low expression of bcl-2 by T cells from EBV and VZV infected patients reflected their

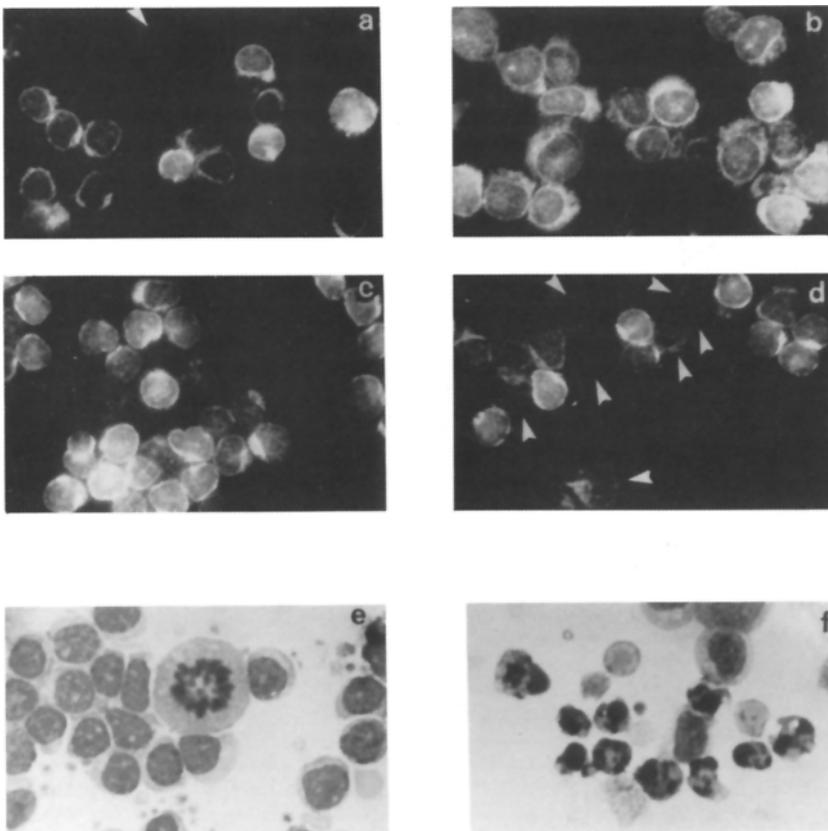


Figure 3. Bcl-2 expression of lymphocytes in cytocentrifuge smears were prepared from suspensions of tonsil before (a) and after activation with PHA and IL-2 (2 ng/ml) for 24 h (b), and from the fresh PBMC of a normal (c) and an EBV-infected donor (d). (White arrowheads) Blasts with low bcl-2 expression. The morphology of the lymphoid cells from the same EBV donor is also shown in MGG preparations that were freshly isolated (e) or cultured for 24 h in the absence of IL-2 (f).

susceptibility to suicide by an apoptotic process. To document these changes, adherent cells were removed from the PBMC populations before culture to prevent the clearance of apoptotic cells by phagocytosis (34). In many individuals, a proportion of lymphoid cells freshly isolated from patients with EBV and VZV infections showed characteristic hallmarks of apoptosis. The numbers of these cells were increased after 24 h of culture in the absence of any stimuli (Fig. 3 f). In normal individuals, apoptotic cells were rare either before or after 24 h of PBMC culture (data not shown). The presence of apoptotic cells in PBMC cultures of patients with viral infections was confirmed by DNA electrophoresis where oligonucleosomal DNA fragmentation of PBMC was observed in EBV and VZV infected but not normal individuals (Fig. 6). The apoptotic morphology of these cells was also formally confirmed by electron microscopy (data not shown).

Another direct confirmation of apoptosis at the single cell level was provided by double-color immunofluorescence using PI together with surface marker labeling (32). With this method we could confirm that the majority of PI-stained cells in patients with viral infections were amongst the CD8⁺ and CD45RO⁺ cells (Fig. 4, c and d). The proportion of PI-labeled cells correlated closely with the proportion of CD8⁺, CD45RO⁺ cells in apoptosis as determined by morphology. The PI-labeled cells were virtually unreactive with other markers such as CD4, CD16, and CD45RA.

The PI⁺ and PI⁻ populations were further investigated by their scatter characteristics. The PI⁻ population (Fig. 4 b, L) fell in the lymphocytic and blastic gate as expected (Fig. 4 a) and the PI⁺ population (Fig. 4 b, A) was smaller than normal lymphocytes (Fig. 4 a) and consisted of condensed apoptotic lymphocytes which, however, still showed intact membrane labeling. The fact that in the same samples healthy blast cells of CD8⁺, CD45RO⁺ phenotype and dividing cells were also present (Fig. 3 e) indicated that the activation/expansion and death by apoptosis were occurring simultaneously within the CD8⁺, CD45RO⁺ population of T cells in these patients. Normal individuals showed virtually no PI reactivity in T cell subpopulations (data not shown).

We next investigated if in EBV and VZV infection the numbers of T cells with low bcl-2 expression correlated with the cells undergoing apoptosis. The fresh cells were first investigated for bcl-2 expression by flow cytometry. The cells were cultured for 24 h and analyzed in cytocentrifuge smears for morphological signs of apoptosis developing during the incubation period. The correlation between the proportion of bcl-2⁺ cells in the fresh samples and the presence of apoptotic cells in the 24-h samples is shown in Fig. 5. There were significantly more bcl-2⁻ and apoptotic cells in VZV and EBV patients as compared with normal uninfected samples ($p < 0.001$ in both cases; Fig. 5). Furthermore, there was a strong positive correlation between the proportion of bcl-2⁻ cells and apoptosis in both the EBV ($r = 0.99$, $p < 0.001$) and VZV groups ($r = 0.73$, $p < 0.02$) analyzed separately or when the normal and patient groups were combined ($r = 0.94$, $p < 0.001$). A patient with acute HIV infection also showed low bcl-2 expression and increased apoptosis when compared with normal controls. This correlation between low bcl-2 expression and apoptosis was also found if the extent of DNA fragmentation seen after 24 h of culture was compared with the number of cells with low bcl-2 expression, before culture, as shown in a representative experiment (Fig. 6).

The Prevention of Apoptosis by IL-2 and Coculture on Fibroblasts. The previous observations suggested that low bcl-2 expression in CD45RO⁺ populations of EBV and VZV infected individuals was associated with the propensity for suicide. We investigated next the ability of IL-2 to prevent apoptosis in these cultures. The addition of IL-2 to PBMC from these patients at the initiation of cell culture for 24 h significantly reduced the proportion of apoptotic cells from 39 to 8% of the cultured populations ($p < 0.001$), whereas the addition of GM-CSF had no significant effect (Table 2). As the survival of activated T cells can also be promoted by fibroblasts (35), we investigated if the apoptosis of PBMC from patients with viral infections could be prevented by fibroblast coculture for 24 h. We found that fibroblast coculture significantly reduced the proportion of apoptotic cells

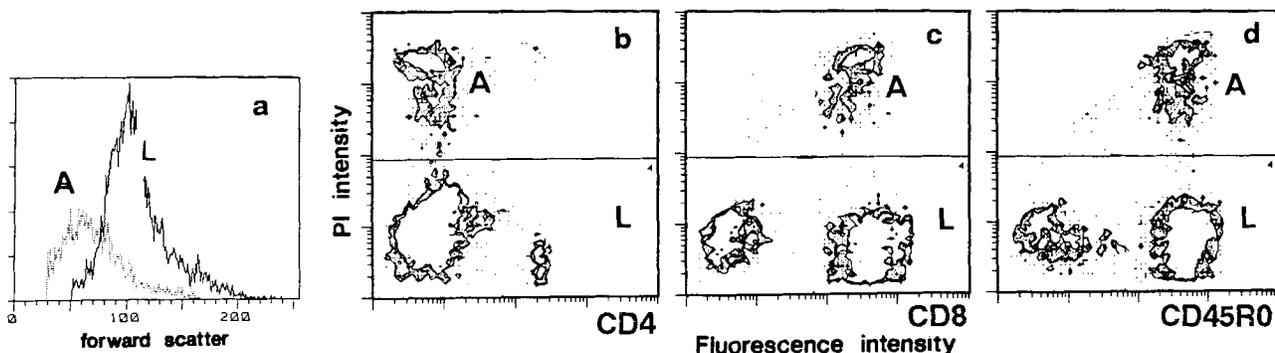


Figure 4. The identification of apoptotic cells by staining with PI in monocyte-depleted lymphocyte suspensions taken from an EBV donor and incubated for 24 h in the absence of IL-2. The PI⁺ apoptotic cells (A in b) are smaller than lymphocytes (L) when studied by forward scatter (a) and are CD4⁻ (b), CD8⁺ (c), and CD45RO⁺ (d). The percentage of PI-reactive cells showed a close agreement with the proportion of apoptotic cells identified by MGG morphology.

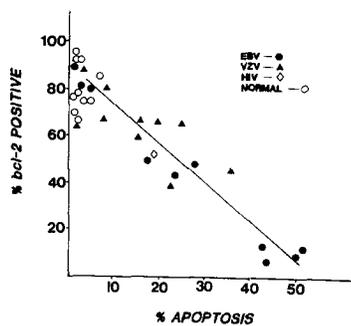


Figure 5. The association of low bcl-2 expression with apoptosis in EBV and VZV patients. PBMC were depleted of adherent cells and stained for bcl-2 and analyzed by flow cytometry (*y*-axis). Samples of the same populations were also cultured for 24 h in medium without any stimulus and the percentages of apoptotic cells among 1,000 cells were counted by two observers in smears stained with MGG (*x*-axis). Samples from normal blood (○) and from patients with acute EBV (●), VZV (△), and HIV-1 infections (◇) are shown.

from 39 to 16% ($p < 0.001$; Table 2) and substantially enhanced the number of viable cells recovered (data not shown).

To further investigate ways by which apoptosis may be prevented in activated T cells, we first established IL-2-dependent CD4⁺ and CD8⁺ T cell populations from normal individuals (see Materials and Methods). The use of these cell lines enabled the generation of large numbers of apoptotic T cells on IL-2 withdrawal, for study when required. These cells were CD45RO⁺. The CD8⁺ lines were highly cytotoxic in a lectin-mediated cytotoxic assay, and these blasts were phenotypically and functionally comparable to the T cells seen in the patients with acute viral disease. When IL-2 was removed from these cells, apoptosis was evident as soon as 24 h and greatly increased by 48–72 h (Fig. 7 *c*). The apoptotic changes were readily documented by both morphology and oligonucleosomal fragmentation (data not shown), indicating that the T cell line could be used to reproduce the apoptosis seen in cultured PBMC from EBV and VZV infected patients.

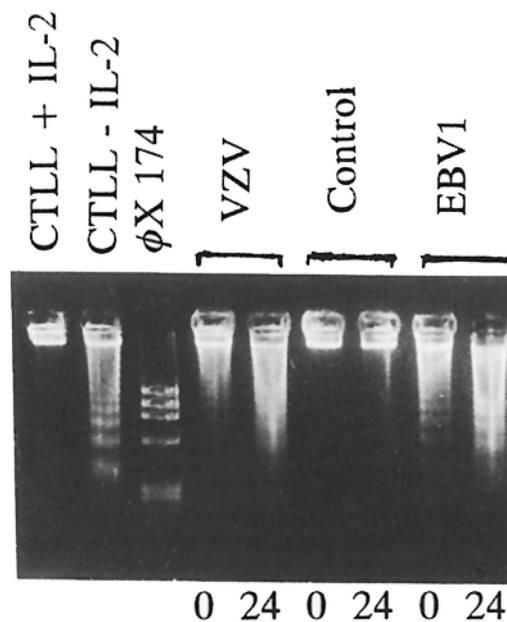


Figure 6. Fragmentation of DNA in lymphocytes from patients infected with EBV and VZV. PBMC samples from normal, VZV, and EBV patients were depleted of adherent cells and analyzed either before (0 h) or 24 h after incubation. EcoRI-treated ϕ X174 DNA were included as markers, and the controls included samples of an IL-2-dependent CTLL line cultured for 24 h in the presence or absence of IL-2.

We next investigated whether fibroblasts could also prevent apoptosis in these cell lines after IL-2 removal and also studied whether or not this increased survival was associated with changes in the bcl-2 expression of these cells. The cells in IL-2-supplemented cultures were large and blastlike on day 6 (Fig. 7 *g*) and the mean forward scatter (MFS), a measure of cell size, was 157 in this population. The CD8⁺ T cells cultured on fibroblasts for 6 d were smaller (Fig. 7 *e*; MFS 123). These cocultured T cells were, however, still marginally larger than the freshly isolated resting population (Fig. 7 *c*; MFS 123 vs MFS 110; data not shown). The results ob-

Table 2. The Prevention of Apoptosis in EBV-infected Patients by IL-2 and Fibroblasts

Expt.	Control*	IL-2	Fibroblasts	GM-CSF
1	61 [†]	15	23	ND
2	43	6.1	16.9	44
3	33.2	1.3	10.7	34.8
4	20.5	9.8	14.7	19.3
MEAN \pm SEM	39.4 \pm 8.5	8.1 \pm 2.9 [§]	16.3 \pm 2.6 [§]	32.7 \pm 6.9 [¶]

* Monocyte-depleted PBMC from EBV patients were cultured for 24 h alone, with fibroblast monolayers, or in the presence of 2 ng/ml of IL-2 or GM-CSF, respectively.

[†] Percentage of cells in apoptosis was determined by morphology in cytocentrifuge preparations.

[§] Significantly different from control as determined by the Student's *t* test ($p < 0.001$).

[¶] Not significantly different from normal.

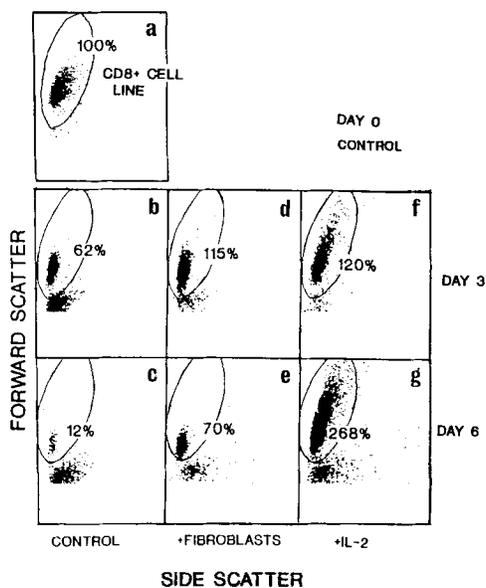


Figure 7. The prevention of apoptosis in an IL-2-dependent CD8⁺ T cell line by cocultivation on fibroblasts. IL-2-dependent lines (*a*: day 0 control) were cultured without IL-2 (*b* and *c*) on fibroblasts (*d* and *e*) or with IL-2 (2 ng/ml; *f* and *g*), and their absolute numbers were counted on the Cytoscan and shown as percent surviving cells. The representative results from one of five experiments are shown. Similar results were also obtained with IL-2-dependent CD4⁺ T cell lines.

tained for both CD4⁺ and CD8⁺ cell lines were similar. When cells were cultured for 6 d in the presence of IL-2, >91% of the population remained bcl-2⁺ (Fig. 8 *a*). In control samples cultured for 6 d without IL-2, only 12% of the original cellular input could be recovered in the viable gate (Fig. 7*c*) and these residual cells remained bcl-2⁺ (77%; data not shown). If the CD8⁺ T cells were cultured for 6 d on fibroblasts after IL-2 withdrawal, 70% of the original cells remained viable despite their lower bcl-2 expression in 50% of cells (compare Figs. 7 *e* and 8 *b*). These findings suggest that fibroblasts maintain the survival of T cells in a bcl-2 low state.

We then investigated whether in the absence of fibroblasts these cells may undergo apoptosis. After 6 d the CD8⁺ T cell line which was cocultured with fibroblasts was removed from the fibroblast monolayers and recultured in the presence or absence of IL-2. After a further 48 h in the presence of this cytokine, >90% of these cells regained bcl-2⁺ expression (Fig. 8 *c*) and the cell recovery remained high (>95%), excluding the possibility of the selective death of bcl-2⁻ cells. In the absence of IL-2, however, all cells perished within 96 h.

The Expression of bcl-2 by LNs of Normal and HIV-infected Individuals. The possibility that the bcl-2 expression by T cells from virally infected patients is an artefact in vitro has been excluded by documenting the presence of bcl-2⁻, CD8⁺ cells within LN populations from patients with viral infections. There was strong expression of bcl-2 in >90% of T cells in normal tonsil tissue. These bcl-2⁺ cells included CD4⁺ T cells localized inside the germinal centers (data not

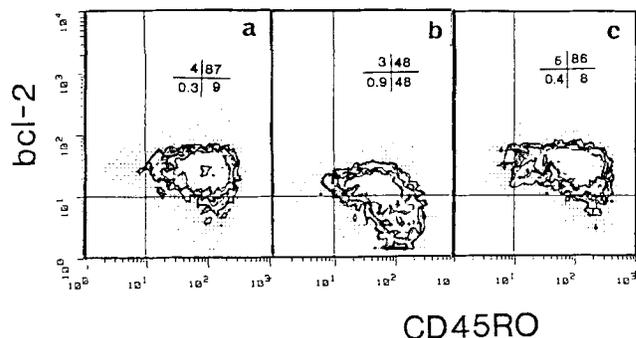


Figure 8. The changes of bcl-2 expression (*y*-axis) in correlation with CD45RO positivity (*x*-axis) in an IL-2-dependent CD8⁺ T cell line when these cells are cultured for 6 d in the presence of IL-2 (*a*) or fibroblasts (*b*). The bcl-2 expression of strongly CD45RO⁺ viable lymphocytes in the fibroblast cocultures dropped to low levels during the 6-d cultures (*b*). When these cells were recultured for 48 h in the presence of IL-2 (2 ng/ml; *c*) the bcl-2 expression returned to high values. Cells in similar cultures kept without IL-2 exhibit poor viability (data not shown, but see Fig. 7 *c*).

shown) as well as the CD8⁺ lymphocytes within the paracortical areas (Fig. 9, *a* and *b*). A different pattern was observed in the LNs of patients infected with HIV (Fig. 9, *c* and *d*). Extensive infiltrates of CD8⁺ cells were seen and the majority (60–80%) of these CD8⁺ T cells expressed CD45RO (31). A large proportion (30–60%) of these CD8⁺ T cells had reduced or negative bcl-2⁺ expression (Fig. 9, *c* and *d*). The pattern of bcl-2 reactivity in the B cells was, however, similar to that found in normal individuals revealing bcl-2 positivity in the B lymphocyte corona and low bcl-2 expression by B blasts inside the germinal center (data not shown).

Discussion

Previous studies have established the role of bcl-2 in the regulation of cell survival associated with the selection and/or deletion of germinal center B lymphoblasts (5, 6, 10) and cortical thymocytes (4, 9, 12). In these lymphoid organs, the loss of bcl-2 is linked with a short life span whereas the up-regulation of this protein results in the relative longevity of these recently generated cells. The bcl-2 protein has been shown to block apoptosis which enables the disposal of unwanted cells to occur (1, 7, 8). Nevertheless, the role of this proto-oncogene product has thus far remained uncharted in the regulation of life span and memory development in mature T cell populations despite the fact that, when stimulated by antigen, these cells also undergo differentiation (18–20).

In histological studies it has already been established that the majority of T cells in the paracortical areas are bcl-2⁺ (4, 29). We now show that not all circulating T cells are bcl-2⁺, but that a minor bcl-2⁻, CD3⁺ population is present in the blood. In normal individuals these cells are primarily of a CD4⁺, CD45RO⁺ primed phenotype, although the relatively small CD8⁺, CD45RO⁺ population in these individuals also shows low expression of this protein. As CD45RA⁺ T cells have been shown to develop CD45RO as a consequence of activation (17–20), these

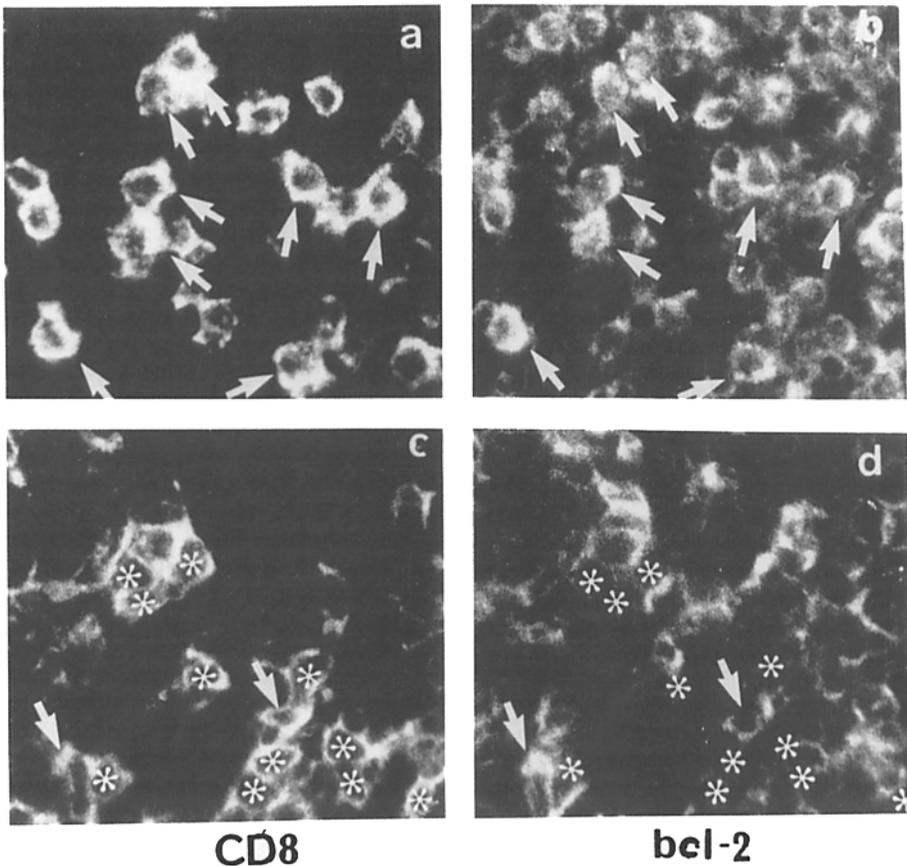


Figure 9. The CD8⁺ cells in normal tonsil (a) mostly express bcl-2 (arrows, b) whereas CD8⁺ cells in HIV-1-infected LN (c) frequently lack this protein (d, *). These sections were stained with double immunofluorescence for CD8 (a and c) and bcl-2 (b and d) and the same areas (a and b and c and d) were photographed with selective filters.

findings indicate that bcl-2 might be downregulated during the activation process. We investigated this possibility in two ways. First, patients with recent acute viral infections were studied to determine whether activated T cells, developing *in vivo*, lose their bcl-2. Such patients, particularly those who have EBV and VZV infections, have expanded circulating T cell populations expressing HLA-DR and CD45RO (14, 36). Second, we isolated CD45RA⁺ lymphocytes within both CD4 and CD8 subsets which were stimulated with PHA in 7-d cultures in order to investigate changes in bcl-2 expression in parallel with the transition from a CD45RA to a CD45RO phenotype *in vitro*. These studies clearly establish that the bcl-2 downregulation is associated with the development of CD45RO⁺ cells after activation and that these bcl-2⁻, CD45RO⁺ cells include a subset of both CD4 and CD8 lymphoblasts. Nevertheless, in viral infections, the CD45RO⁺, CD8⁺, bcl-2⁻ subset is predominant in the circulation, but low bcl-2 expression can also be demonstrated in a much smaller cohort of primed CD4⁺ T cells when sensitive single cell methods are used in these patients (14).

It has been well documented that the T cell lymphocytosis associated with both EBV and VZV infections is transient as the absolute number of circulating T lymphocytes and the relative proportions of CD4⁺ and CD8⁺ cells return to normal upon resolution of the disease (13-15, 37). This suggests a rapid clearance of the majority of activated T blasts *in vivo*. Indeed, the apoptotic death of these T cells

has been demonstrated by both morphology and DNA cleavage (36, 38). A balance must exist between cell death and survival, however, as immunological memory is retained after these infections and a higher cytotoxic precursor frequency of EBV and VZV specific T cells is found after primary infection (13, 16). We now propose that bcl-2 regulation may play a pivotal role in this balance, and that apoptosis is a major mechanism for the removal of unwanted T cells after resolution of viral disease.

Programed cell death or apoptosis, a suicide pathway resulting in endonuclease activation and the subsequent cleavage of DNA into nucleosomal fragments (39), is important in physiology, e.g., in metamorphosis, embryogenesis, and tissue atrophy where the restriction of cell numbers is essential (7). Apoptosis may also play a part in the life cycle of mature T cells because activated T cells and T cell lines perish by this process if IL-2 is removed (27). We have shown that the apoptosis of activated T cells from EBV and VZV patients is correlated with their reduced bcl-2 expression. The association of apoptosis with low bcl-2 expression is, however, not disease specific, as it was found in all acute viral infections studied including EBV and VZV, as well as the single case of acute HIV-1 infection studied. It is of interest that in normal individuals the Fas antigen, a marker associated with apoptosing cells, is elevated on CD45RO⁺ as compared with CD45RA⁺ T cells, and that there is a further increase of Fas on the CD45RO⁺ lymphoblasts in EBV-

infected individuals (36). Thus, the relationship between bcl-2 and Fas expression appears to be reciprocal on CD45RO⁺ T cells, and it would be of importance to determine if both molecules have roles in the same or different pathways leading to apoptosis.

Our data would suggest that after T cell activation *in vivo*, the expanded bcl-2⁻ population is destined to perish unless these cells are rescued from apoptosis. This situation is analogous to the rescue of bcl-2⁻ apoptosis-prone germinal center B cells by surface Ig and CD40 ligation and also by the presence of cytokines such as IL-4 (5, 6). The downregulation of bcl-2 as a result of both T and B cell activation provides a means by which an expanded lymphoid pool, arising as a result of immune stimulation, can be decreased, thus enabling the reestablishment of cellular homeostasis.

We have described two mechanisms that prevent the apoptosis of activated T cells. First, the continued presence of IL-2 maintains these cells in an activated state with elevated expression of bcl-2. Second, the interaction of T cells with a soluble fibroblast-derived factor enables these cells to return to a restinglike state but with low bcl-2 expression. In both these situations, the continued presence of IL-2 or the fibroblast factor is required and the T cells rapidly apoptose (at least *in vitro*) if either is removed.

Previous reports have already demonstrated that the coculture of apoptosis-prone leukocytes such as neutrophils (40), eosinophils (41), leukemic cells (30), and activated T cells (35) with monolayers of fibroblasts can prevent apoptosis and maintains the viability of these cells. The mechanism by which fibroblasts promote the survival of leukocytes, some of which are apparently bcl-2⁻, is unknown at present. Fibroblasts produce a wide array of cytokines, including TGF- β , GM-CSF, IL-1 β , IL-6 (42) and also extracellular matrix proteins such as collagen, vitronectin, and fibronectin (43), all of which may be potential candidates for promoting T cell survival. Other mechanisms, apart from IL-2 and fibroblasts, may also be required for the survival of activated T cells *in vivo* and enable these cells to return to a resting state. One such mechanism may be the engagement of the CD28 costimulatory pathway, in analogy with the prolongation of survival of germinal center B cells by the crosslinking of surface Ig together with CD40 ligation (6).

One of the crucial questions to be answered is how the proportion of activated T cells destined for either apoptotic death or for survival after an immune response is determined *in vivo*. One possibility is the competition for survival factors by activated T cells (7) and these factors fall into two broad categories. The first category of signals promote survival by maintaining the activated cells in cycle. In contrast, the second

group are those produced by stromal cells such as fibroblasts which enable the cells to return to rest. At the end of an immune response, limiting concentrations of the first set of factors such as antigen and/or cytokines would ensure that only the most efficient cells, i.e., those with the greatest affinity for antigen or the most efficient signal transduction pathways obtain sufficient stimuli to remain in cycle and survive (7). Some of the activated T cells, however, may be induced to return to rest by stromal factors but limiting amounts of these factors will, once again, only promote the viability of the most competitive cells. The competition between activated T cells will on the one hand enable homeostasis to take place after immune activation, as the cells that do not obtain these factors will perish, and on the other permit the retention of the most competent primed cells.

Many studies have shown that T cell memory resides within the CD45RO⁺ subset (17–20). It may therefore seem paradoxical that CD45RO⁺ T cells express low bcl-2 and are destined for an apoptotic death. It has been shown, however, that although memory to an antigenic encounter may persist for decades, the average life span of human T cells is less than 2 yr (44). The unexpectedly short T cell life span, together with the observations that recall responses to antigen reside within a cycling population of CD45RO⁺ cells (17, 44, 45) have suggested that T cell memory may persist as a consequence of the continual stimulation of a previously primed population (17). This hypothesis is supported by the demonstration that T cell memory responses *in vivo* are dependent on the presence of the original priming antigen (46). When placed in the context of our current data, this indicates that mechanisms that keep primed CD45RO⁺ T cells in cycle will elevate the bcl-2 in these cells, promote their survival, and thus allow for the persistence of a dynamically generated memory population. Alternatively, in the absence of reactivation, the propensity of CD45RO⁺ T cells to extravasate (45, 47) will facilitate their encounter with stromal fibroblasts and secure their viability until subsequent antigenic reencounter. This latter observation has implications for autoimmune disorders where fibroblast–lymphocyte interactions may become aberrant (35).

Collectively, our data suggest a scheme by which the changes in expression of the bcl-2 gene product by activated cells play a pivotal role in the balance between T cell death and survival after activation. The rescue of bcl-2⁻ primed T cells by various factors enables the generation of a dynamic primed/memory T cell population, yet also allows for the homeostatic maintenance of T cell numbers after immune stimulation *in vivo*.

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