

## **CD3 hyporesponsiveness and in vitro apoptosis are features of T cells from both malignant and nonmalignant secondary lymphoid organs.**

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### Research Article

There is a dogma in tumor immunology that tumor-infiltrating lymphocytes (TIL) are defective based on their lack of antitumoral efficacy in vivo and on impaired response to in vitro functional tests. However, TIL have been compared usually with peripheral blood T lymphocytes, raising doubts on the conclusions drawn. Therefore, we compared TIL from B cell non-Hodgkin's lymphomas (NHL) with T cells from nonmalignant secondary lymphoid organs. NHL-TIL were unresponsive to activation by immobilized anti-CD3 mAb, although bypassing T cell receptor (TCR)/CD3 signaling led to proliferation. The poor proliferative responses of NHL-TIL could not be explained by quantitative defects in TCRzeta expression. NHL-TIL underwent marked spontaneous apoptosis in vitro with loss of approximately 50% of cells after 24 h of culture. This was associated with downregulation of the antiapoptotic Bcl-xL and Bcl-2 proteins, whereas viable NHL-TIL maintained their expression. IL-2, anti-CD3/IL-2, and manipulation of the Fas/Fas-ligand death pathway had no effect on NHL-TIL survival. Apoptosis was not due to increased cell cycling, as NHL-TIL were quiescent, nonproliferating cells. T cells from inflammatory, nonmalignant tissues gave similar functional results to NHL-TIL, suggesting the existence of factors common to the microenvironment of these diverse pathologies. Thus, the quiescent, anergic phenotype of NHL-TIL cannot be attributed solely to tumor factors, but rather is a feature of T cells from chronic inflammatory lesions.

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# CD3 Hyporesponsiveness and In Vitro Apoptosis Are Features of T Cells from Both Malignant and Nonmalignant Secondary Lymphoid Organs

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## Abstract

There is a dogma in tumor immunology that tumor-infiltrating lymphocytes (TIL) are defective based on their lack of anti-tumoral efficacy in vivo and on impaired response to in vitro functional tests. However, TIL have been compared usually with peripheral blood T lymphocytes, raising doubts on the conclusions drawn. Therefore, we compared TIL from B cell non-Hodgkin's lymphomas (NHL) with T cells from nonmalignant secondary lymphoid organs. NHL-TIL were unresponsive to activation by immobilized anti-CD3 mAb, although bypassing T cell receptor (TCR)/CD3 signaling led to proliferation. The poor proliferative responses of NHL-TIL could not be explained by quantitative defects in TCR $\zeta$  expression. NHL-TIL underwent marked spontaneous apoptosis in vitro with loss of  $\sim 50\%$  of cells after 24 h of culture. This was associated with downregulation of the antiapoptotic Bcl-x<sub>L</sub> and Bcl-2 proteins, whereas viable NHL-TIL maintained their expression. IL-2, anti-CD3/IL-2, and manipulation of the Fas/Fas-ligand death pathway had no effect on NHL-TIL survival. Apoptosis was not due to increased cell cycling, as NHL-TIL were quiescent, nonproliferating cells. T cells from inflammatory, nonmalignant tissues gave similar functional results to NHL-TIL, suggesting the existence of factors common to the microenvironment of these diverse pathologies. Thus, the quiescent, anergic phenotype of NHL-TIL cannot be attributed solely to tumor factors, but rather is a feature of T cells from chronic inflammatory lesions. (*J. Clin. Invest.* 1998. 102:1715–1723.)  
Key words: tumor infiltrating lymphocytes • non-Hodgkin's lymphoma • inflammatory tissue • Bcl-2 • anergy

## Introduction

A lymphocytic infiltrate is associated with almost all solid cancers and yet the presence of tumor-infiltrating T lymphocytes (TIL)<sup>1</sup> does not lead to an effective immune response in the majority of cases. The nonfunctionality of TIL has been attributed to various mechanisms, including a lack, or absence, of the costimulatory molecules CD80 and CD86 on tumor cells leading

to anergy (1–3), and the production of suppressive factors by the tumor (4, 5).

Many studies have documented poor proliferative responses of TIL from different types of cancers to mitogenic stimuli (6–10). In B cell non-Hodgkin's lymphoma (NHL), TIL have been shown to proliferate poorly to IL-2, immobilized anti-CD3 mAb, and to a combination of the two, although occasionally NHL-TIL proliferate normally (11, 12). Most studies have used peripheral blood T lymphocytes (PBL-T) as controls for TIL. However, data from T cells derived from tissues not involved by a malignant process, such as the lamina propria (13), synovium (14), and intestine (15), show a similar pattern of impaired proliferation.

A variety of alterations in signal transduction molecules has been reported in both mouse tumor models (16, 17) and in TIL from human cancers (9, 18). In view of the poor proliferative responses of TIL to T cell receptor (TCR)/CD3 stimulation, much attention has focused on the TCR $\zeta$  component of the complex (19). Decreased levels of TCR $\zeta$  have been found in TIL from human renal cell (9) and colorectal (20) carcinoma and in splenic T cells from tumor bearing mice (16). However, some workers have suggested that reduced TCR $\zeta$  levels may be artifactual and due to digestion by granulocytic proteases (17, 19, 21). In NHL-TIL, Wang et al. have reported normal levels of TCR $\zeta$ , CD3 $\epsilon$ , and of the protein tyrosine kinases, p56<sup>lck</sup>, p59<sup>lyn</sup>, and ZAP-70 (22), although they did find qualitative changes in p56<sup>lck</sup>. Once again, however, few studies have used tissue T cells as controls for TIL and where this has been done tonsillar tissue was found to express the same levels of TCR $\zeta$  as lymphomas (22). These findings, combined with the data from proliferation assays in vitro, suggest that tissue T cells are more appropriate controls than PBL-T for assessing the functionality of NHL-TIL.

In this study, we have directly compared NHL-TIL with T cells from inflammatory, nonmalignant secondary lymphoid organs (referred to as reactive T cells) and PBL-T. NHL-TIL and reactive T cells were both found to show hyporesponsiveness to TCR/CD3 activation but express similar levels of TCR $\zeta$  as PBL-T. One factor in the poor proliferation of TIL and reactive T cells may be an increased propensity to undergo apoptosis in vitro as compared with PBL-T. Indeed, Salmon et al. have shown a marked susceptibility of synovial T cells in rheumatoid arthritis to undergo apoptosis in vitro (23). We found that NHL-TIL and reactive T cells were remarkably

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1. *Abbreviations used in this paper:* AV, annexin V; FasL, Fas-ligand; MFI, mean fluorescence intensity; NHL, non-Hodgkin's lymphoma; PBL-T, peripheral blood T lymphocytes; PI, propidium iodide; reactive T cells, T cells from chronic inflammatory, nonmalignant secondary lymphoid organs; TCR, T cell receptor; TIL, tumor-infiltrating T lymphocytes.

similar in terms of their poor *in vitro* survival and that the Fas/Fas-ligand (FasL) pathway was not involved in this phenomenon. In addition, our results show that the CD3 hyporesponsiveness of these cell populations is not principally attributable to their increased degree of spontaneous apoptosis. Thus, T cells from different chronic inflammatory lesions are functionally similar and the phenotype of NHL-TIL cannot be attributed solely to tumor-specific factors.

## Methods

**Samples and cell preparation.** 13 cases of NHL were studied, consisting of 10 lymph node biopsies (9 follicular lymphomas and 1 chronic lymphocytic leukemia) and 3 splenectomy samples (splenic lymphoma with villous lymphocytes, mantle cell lymphoma, and lymphoplasmacytic lymphoma). Control tissues were whole tonsils from children undergoing tonsillectomy for recurrent infections, lymph node biopsies showing benign follicular hyperplasia, and splenectomies performed for autoimmune hemolytic anemia and trauma. All biopsies were received fresh, in a sterile container without any medium, and were processed immediately. Cell suspensions were obtained either by repeated perfusion with complete medium using a 19G needle or by mincing over a wire mesh. The cell suspensions were then filtered through a 100- $\mu$ m filter to remove aggregates and viability was assessed by trypan blue exclusion. Peripheral blood mononuclear cells were isolated by Ficoll-Paque centrifugation of peripheral blood from patients with NHL and healthy donors. All cells were cryopreserved in 10% DMSO with 20% FCS and stored in liquid nitrogen until testing.

Highly purified T cell populations were obtained by negative selection using a cocktail of hapten-modified Abs (CD11b, CD16, CD19, CD36, and CD56) followed by antihapten magnetic microbeads (pan-T cell isolation kit; Miltenyi Biotec, Bergisch Gladbach, Germany). For the purification of some tissue samples, anti-CD19 magnetic microbeads were also added to take account of the greater proportion of B cells. All purified T cell populations were CD19<sup>-</sup>, CD56<sup>-</sup>, and > 96% (and most were > 98%) CD3<sup>+</sup>. Cell cultures were performed with complete medium: RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 1 mM sodium pyruvate.

In this paper, T cells from NHL biopsies will be referred to as NHL-TIL; T cells from benign lesions as reactive T cells; and T cells from peripheral blood as PBL-T.

**Surface and intracellular immunofluorescence analysis.** The following FITC-conjugated mAbs were used for detection of surface molecules: CD4 (S3.5), CD8 (3B5), CD25 (CD25-3G10), CD45RA (MEM56), CD45RO (UCHL1), HLA-DR (TU 36) (all from Caltag Laboratories, Burlingame, CA), CD62L (Dreg 56; Immunotech, Marseille, France), and CD86 (FUN-1; PharMingen, San Diego, CA). Other directly conjugated mAbs used were anti-CD3-TRICOLOR (S4.1), CD19-TRICOLOR (SJ25-C1; Caltag), CD45RO-PE (Immunotech), and CD86-PE (FUN-1; PharMingen). CH11 (Immunotech), an IgM anti-Fas (CD95) mAb, was used unconjugated and revealed by a FITC-labeled goat anti-mouse Ig (Caltag). Pretitrated quantities of mAbs were added for 15 min at 4°C and cells were then washed in staining buffer (PBS, 5% FCS, and 0.02% sodium azide) and fixed in 1% paraformaldehyde. All tests were analyzed on a EPICS XL flow cytometer (Coulter Corp., Hialeah, FL). Isotype control Abs were used to establish the PMT settings and singly stained samples allowed compensation for double- and triple-color immunofluorescence tests.

For intracellular labeling, cells were fixed in PBS 1% paraformaldehyde for 20 min at 4°C, washed, and then permeabilized with staining buffer containing 0.1% saponin and the specific Ab for 20 min at 4°C. Cells were washed in staining buffer and, if required, the secondary Ab was added, also diluted in staining buffer containing 0.1% saponin. Anti-human Bcl-2 mAb (124; DAKO, Glostrup, Denmark) was FITC-conjugated and compared with an isotype control. Bcl-x<sub>L</sub>

was detected by a polyclonal anti-Bcl-x<sub>L/S</sub> Ab<sup>2</sup> (Santa Cruz Biotechnologies, Santa Cruz, CA) and a FITC-labeled goat anti-rabbit Ig (Caltag) as the secondary Ab. Anti-TCR $\zeta$  (TIA-2; Coulter) Ab was detected with a PE-labeled goat anti-mouse Ig (Caltag). Staining with the secondary Ab alone was used as the negative control for TCR $\zeta$  and Bcl-x<sub>L/S</sub>. We confirmed the positivity of the anti-Bcl-2 and anti-Bcl-x<sub>L/S</sub> Abs by staining the t(14;18) containing lymphoma cell line, RL, and PHA blasts, respectively (data not shown).

**Cell survival, apoptosis, and cell cycle analysis.** All experiments were performed on pure T cell populations isolated from rapidly thawed cryopreserved samples. Viability was assessed by trypan blue exclusion and 10<sup>5</sup> trypan blue negative cells per well were planted in 96-well round bottom plates (Costar) in 0.2 ml of complete medium. Apoptosis was detected by the appearance of phosphatidylserine residues at the cell surface by labeling with annexin V (AV) (Annexin V-FITC kit; Bender MedSystems, Vienna, Austria) for 10 min at room temperature, in the dark. Propidium iodide (PI) (20  $\mu$ g/ml) was added to exclude necrotic cells from analysis. The population of live lymphocytes was gated on using the morphological criteria of forward and side scatter. For cell cycle analysis, cells were washed in PBS and then fixed and permeabilized with 1 ml of ice-cold 70% ethanol for 1 h at 4°C. After centrifugation and washing, 50  $\mu$ g/ml PI (Sigma Chemical Co., St. Louis, MO) and 75  $\mu$ g/ml RNase A (Sigma) were added for 45 min at 37°C. Samples were analyzed immediately on an EPICS XL.

The time course of cell survival was evaluated by daily harvesting of independent wells and assessing viability by trypan blue exclusion (all measurements made in duplicate) and apoptosis and cell cycle analysis as above. The effect of various agents on cell survival was tested by addition at the start of cultures of: IL-2 (50 U/ml; Eurocetus, Amsterdam, The Netherlands); immobilized anti-CD3 mAb (CD3ana3, see below); immobilized anti-CD3 plus IL-2; 10  $\mu$ g/ml of the anti-Fas (CD95) blocking Ab, ZB4 (MBL, Nagoya, Japan); or an isotype control. Induction of apoptosis by activation of Fas (CD95) was performed as previously described (24) with minor modifications. In brief, cells were incubated with the agonistic anti-Fas Ab, APO-1 (APO-1.3; Alexis, San Diego, CA) for 1 h at 37°C, then cross-linked with a rat anti-mouse Ig (10  $\mu$ g/ml; Caltag) and examined for AV staining. PHA blasts (24) were used as positive controls.

**T cell proliferation assays.** 5  $\times$  10<sup>4</sup> T cells per well were planted in 96-well round-bottomed plates in a final volume of 0.2 ml of complete medium. Cells were cultured in triplicate wells with medium alone or with the following stimuli: anti-CD3 mAb (CD3ana3, produced in our laboratory), which was used in cross-linking conditions, i.e., pre-immobilized onto wells at a 1:5,000 dilution of ascites; 10 ng/ml PMA (Sigma); 10<sup>-6</sup> M ionomycin (Sigma); IL-2 (50 U/ml); and 1  $\mu$ g/ml anti-CD28 (CD28.2; kindly donated by D. Olive, Marseille, France). Combinations of these stimuli were also used, as detailed in the text. Cultures were incubated in a humidified incubator with 5% CO<sub>2</sub> in air at 37°C for 96 h. During the last 8 h of the culture, 1  $\mu$ Ci of [<sup>3</sup>H]thymidine (ICN, Irvine, CA) was added per well and then cells were harvested on a Packard Harvester (Packard, Meridian, CT). Incorporation of radioactivity was determined using a microplate scintillation counter (Top Count; Packard).

**Statistical analysis.** All data were analyzed using the nonparametric Wilcoxon test.

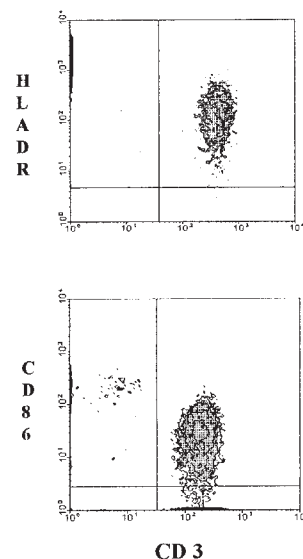
## Results

**NHL-TIL and reactive T cells express a predominantly activated, memory immunophenotype.** First, we established whether the

2. No Bcl-x<sub>L</sub>-specific mAb was commercially available at the time of this study. In all tissues and cell lines studied, Bcl-x<sub>L</sub> is dominant (mRNA 100 times greater than all other isoforms of Bcl-x) and Bcl-x<sub>S</sub> has not been detected in mature human lymphocytes (34, 71). Thus, it is possible to use an anti-Bcl-x<sub>L/S</sub> Ab to specifically detect the Bcl-x<sub>L</sub> protein in mature human T cells by flow cytometry.

Table I. Immunophenotyping of NHL-TIL

	CD3+	%OF CD3+ T CELLS POSITIVE FOR:								RATIO CD4:CD8
		CD62L	CD45RA	CD45RO	HLA-DR	CD25	CD86	CD4	CD8	
FL1	22	12	17	81	100	36	75	91	9	10.1
FL2	49	11	19	76	100	21	16	78	22	3.5
FL3	60	16	5	95	99	19	79	81	19	4.2
FL4	14	18	28	79	100	18	60	44	56	0.8
FL5	-	18	17	89	100	15	4	40	57	0.7
FL6	-	18	31	79	56	5	5	77	21	3.7
FL7	54	30	15	95	86	15	8	60	40	1.5
CLL	12	64	20	93	94	8	14	63	37	1.7
MCL	5	17	9	92	72	21	23	60	40	1.5
SLVL	5	20	50	81	100	30	46	44	56	0.8
MEAN	28	22	21	86	91	19	33	64	36	2.8
MEDIAN	18	18	17	89	100	19	20	62	39	2.6
RANGE	5-60	11-64	5-50	76-95	72-100	8-36	4-79	40-91	9-57	0.7-10.1



Percentages were determined by flow cytometry of mononuclear cell or purified T cell suspensions. Double staining confirmed the presence of a population of CD45RA<sup>+</sup>CD45RO<sup>+</sup> NHL-TIL in the cases where the total for the individual markers was > 100% (data not shown). (Inset) Flow cytometric images of HLA-DR and CD86 expression on CD3<sup>+</sup> cells from FL3. FL, Follicular lymphoma; CLL, chronic lymphocytic leukemia; MCL, mantle cell lymphoma; SLVL, splenic lymphoma with villous lymphocytes.

distributions of T cell subsets in reactive T cells and NHL-TIL were comparable by immunophenotyping the mononuclear cell suspensions of NHL and control tissues using double and triple immunofluorescence flow cytometry. Table I shows that the majority of NHL-TIL expressed the memory/effector marker CD45RO along with the activation marker HLA-DR. The median CD4/CD8 ratio was 2.6. It is of note that the median HLA-DR positivity was 100% (Table I and inset), whereas that of CD25 was only 19%. CD86 (B7.2) is well char-

acterized as a costimulatory molecule on antigen presenting cells; however, its role on T cells remains unclear. CD86 expression is very low on resting PBL-T but is upregulated during T cell activation (25, 26), being maximal at 24–48 h and then decreasing (i.e., similar to CD25). In this context, it is interesting to note the elevated expression of CD86 in some NHL-TIL (Table I and inset). The same profile of markers was shown by control reactive T cells (Table II), with a median CD4/CD8 ratio of 2.7, although the range of activation marker

Table II. Immunophenotyping of Reactive T Cells

	CD3+	% of CD3 <sup>+</sup> T cells positive for:								Ratio CD4/CD8
		CD62L	CD45RA	CD45RO	HLA-DR	CD25	CD86	CD4	CD8	
LN 1	70	37	73	24	21	9	9	69	31	2.2
LN 2	61	10	32	64	10	9	6	80	20	4.0
SPL 1	73	2	16	75	100	1	ND	64	36	1.8
SPL 2	55	5	37	64	100	5	6	55	45	1.2
SPL 3	50	5	18	82	12	2	13	75	25	3.0
SPL 4	60	5	28	61	19	3	4	57	43	1.3
TON 1	21	5	17	81	90	5	22	74	26	2.9
TON 2	20	6	17	73	21	7	15	55	45	1.2
TON 3	20	6	20	70	100	5	30	73	27	2.7
TON 4	24	2	9	80	90	4	21	76	24	3.2
TON 5	—	20	36	65	28	8	0	69	23	3.0
Mean	45	9	28	67	54	5	13	68	31	2.4
Median	52.5	5	20	70	28	5	11	69	27	2.7
Range	20–73	2–37	9–73	24–82	12–100	1–9	0–30	55–80	20–45	1.2–4.0

Percentages were determined by flow cytometry of mononuclear cell or purified T cell suspensions. TON, Tonsil; LN, lymph node; SPL, spleen; ND, not done.

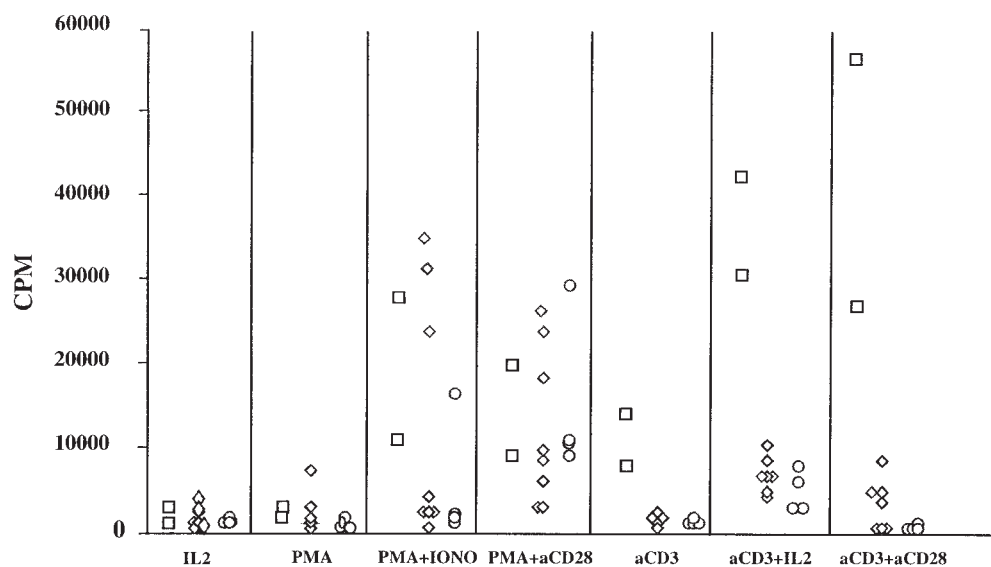


Figure 1. Poor proliferation of NHL-TIL and reactive T cells to TCR/CD3 activation.  $5 \times 10^4$  T cells were planted in 96-well round-bottomed plates in complete medium and activated with IL-2 (50 U/ml), PMA (10 ng/ml)/ionomycin ( $10^{-6}$  M), immobilized anti-CD3 (aCD3), aCD3/IL-2, and aCD3/aCD28 (1  $\mu$ g/ml). Results are expressed as cpm and are the mean of triplicate wells representing incorporation of [ $^3$ H]thymidine during the last 8 h of 96-h cultures. Normal PBL-T ( $n = 2$ ; squares), reactive T cells ( $n = 7$ ; diamonds), and NHL-TIL ( $n = 4$ ; circles).

expression was broader. Of the activation markers, CD25 ( $P = 0.001$ ), but not HLA-DR ( $P = 0.06$ ) or CD86 ( $P = 0.15$ ), was significantly higher in NHL-TIL. Overall, reactive T cells and NHL-TIL had a similar CD4/CD8 ratio and predominantly expressed an activated, memory phenotype which is clearly distinct from that of PBL-T (27). The immunophenotype of PBL-T from NHL patients did not differ from that of normal PBL-T (data not shown).

**Impaired TCR/CD3 activation in NHL-TIL and reactive T cells as compared with PBL-T cannot be attributed to decreased TCR $\zeta$  expression.** Using a variety of activation stimuli (IL-2, PMA, PMA/ionomycin, and PMA/anti-CD28), we found that NHL-TIL and reactive T cells showed a heterogeneous pattern of responses, with samples showing a lower, similar, or greater level of proliferation than normal PBL-T (Fig. 1). However, the most striking observation was the nonproliferation of all NHL-TIL and reactive T cell samples with immobilized anti-CD3 (Fig. 1). The combination of anti-CD3/CD28 was ineffective in NHL-TIL and anti-CD3/IL-2 only produced weak proliferation in NHL-TIL and reactive T cells, whereas both combinations were strongly synergistic in PBL-T. All conditions involving anti-CD3 in NHL-TIL and reactive T cells gave statistically significant differences as compared with PBL-T (anti-CD3,  $P = 0.0379$ ; anti-CD3/IL-2 and anti-CD3/anti-CD28,  $P = 0.0382$ ). In NHL-TIL, the best proliferative response was always seen with PMA/anti-CD28, indicating that these cells can be activated by bypassing the TCR/CD3 signaling pathway and that the CD28 pathway is functional. These results cannot be explained simply by the subset differences between NHL-TIL and PBL-T, with a marked CD45RO $^+$  predominance among NHL-TIL (mean CD45RO $^+$  = 86%, see Table I), as activation of accessory cell-depleted PBL-T with immobilized anti-CD3 mAb leads to proliferation of the CD45RO $^+$  and not the CD45RA $^+$  subset (28, 29).

To further investigate the impaired signaling via the TCR/CD3 complex, the expression of TCR $\zeta$  was studied. At day 0, all the populations tested had > 90% of T cells positive for TCR $\zeta$ , with a similar mean fluorescence intensity (MFI) (Fig. 2, dotted lines). After 24 h of culture in complete medium, gated cells in all populations showed spontaneous upregulation of TCR $\zeta$  expression (Fig. 2, solid lines). Notably, NHL-

TIL did not show decreased TCR $\zeta$  expression as compared with reactive T cells or normal PBL-T.

**Increased spontaneous apoptosis and poor survival kinetics of NHL-TIL and reactive T cells as compared with PBL-T is not due to increased cell cycling.** We studied the survival kinetics of these three populations cultured in complete medium without any stimuli. AV binding was used to detect the loss of asymmetry of the cell membrane and the appearance of phosphatidylserine residues at the cell surface, which is a relatively early

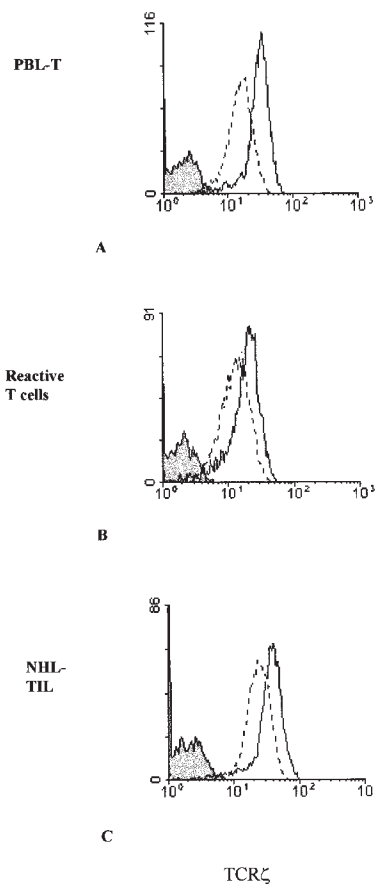
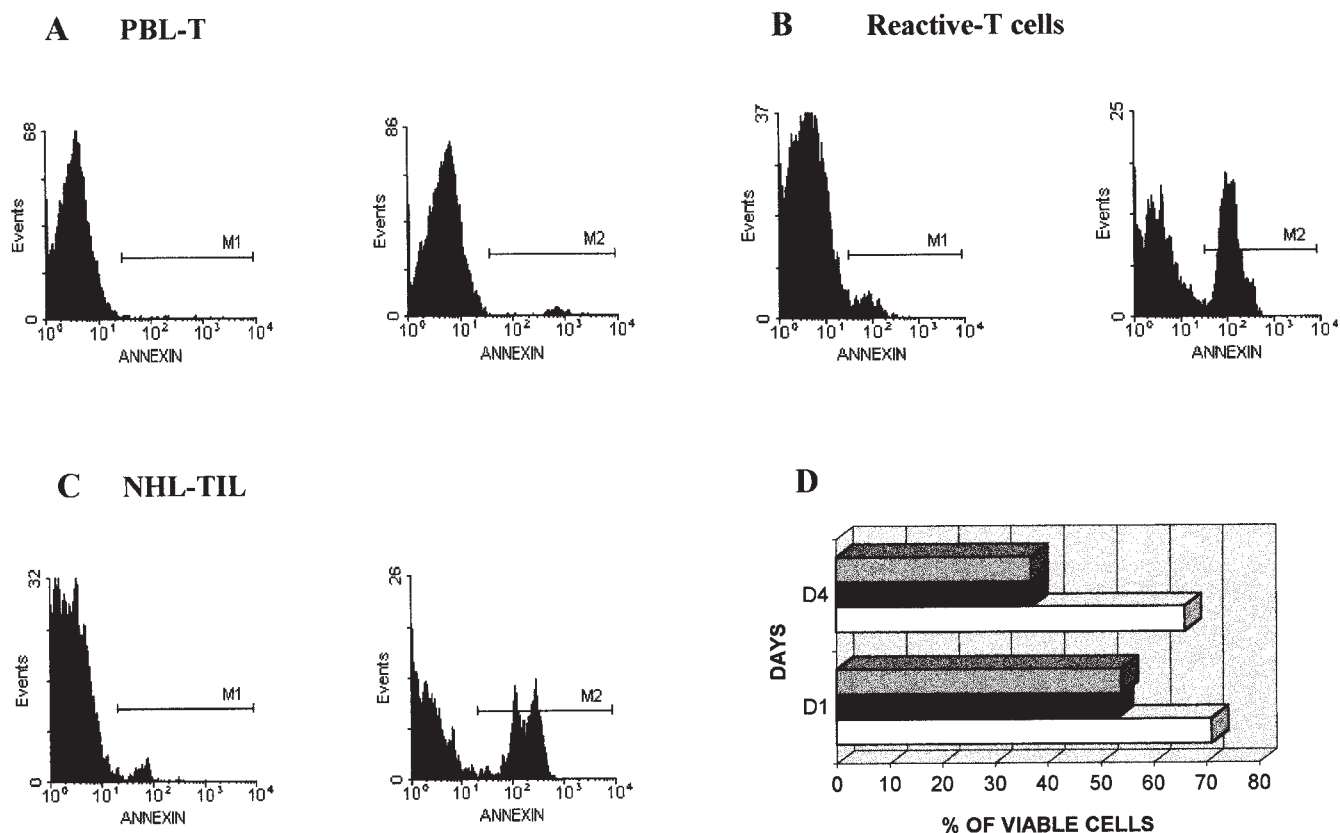


Figure 2. Similar expression and modulation of TCR $\zeta$  by NHL-TIL and reactive T cells as compared with PBL-T. Intracellular staining for TCR $\zeta$  was performed immediately after T cell purification (dotted lines) and after 24 h in culture in complete medium, without additional stimuli (solid lines). Controls are represented by filled curves.



**Figure 3.** Poor viability of NHL-TIL and reactive T cells in culture. T cells were cultured in 96-well round-bottomed plates at  $10^5$  trypan blue–negative cells/well. Apoptosis was measured as the percentage of AV<sup>+</sup> cells immediately after T cell purification and after 24 h in complete medium. Forward and side scatter characteristics were used to gate the population of live T cells. Representative examples of AV staining at the initiation of cultures (left-hand panels) and after 24 h (right-hand panels) are shown for (A) normal PBL-T: M1 = 1.5%, M2 = 3%; (B) reactive T cells: M1 = 3.3%, M2 = 21.2%; (C) NHL-TIL: M1 = 2.3%, M2 = 20.4%. (D) Absolute numbers of viable cells were calculated by subtracting the percentage of AV<sup>+</sup> cells in the live gate from the number of trypan blue–negative cells. Results are expressed as a percentage of the number of viable cells planted at day 0. Normal ( $n = 2$ ) and NHL ( $n = 7$ ) PBL-T data were identical and therefore combined (white bars). Reactive T cells,  $n = 9$  (gray bars). NHL-TIL,  $n = 3$  (black bars).

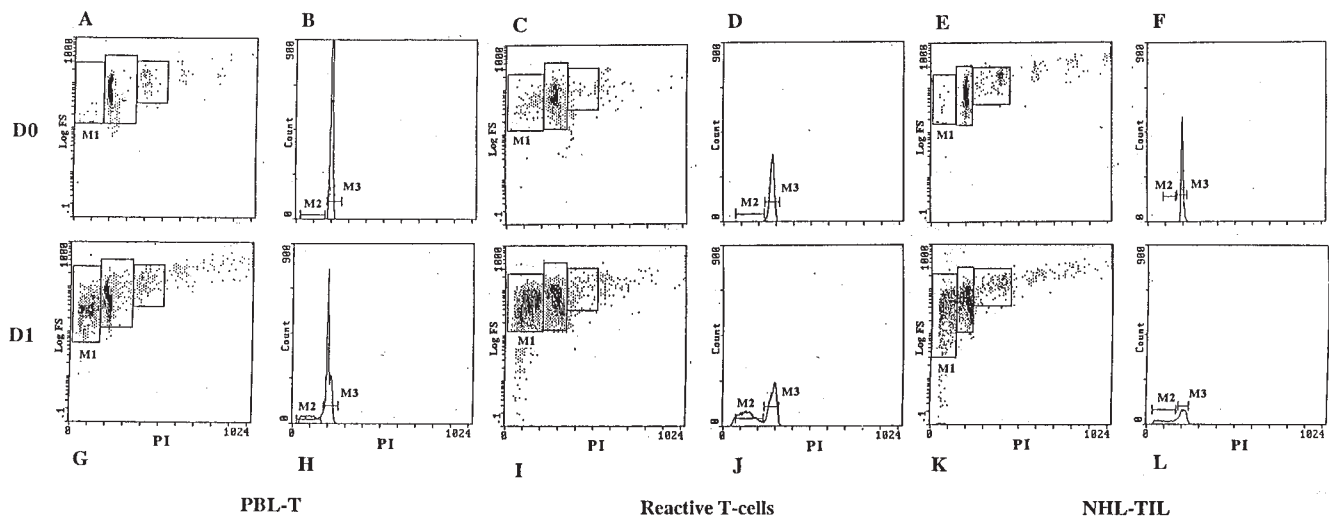
cellular event during apoptosis (30). Using forward and side scatter to gate the population of live T cells, we found that this gate contained < 1% of necrotic cells (PI<sup>+</sup> cells) but a significant proportion of cells were AV<sup>+</sup>. Reactive T cells and NHL-TIL showed greater spontaneous apoptosis than PBL-T (Fig. 3, A–C, shows representative examples of AV staining). Since early apoptotic cells can be AV<sup>+</sup> but can still exclude vital dyes (31), the number of surviving cells for the kinetic studies was calculated by deducting the percentage of AV<sup>+</sup> cells in the live gate by flow cytometry from the number of trypan blue–negative cells (both performed on independent but identical wells). Fig. 3 D shows that the cell survival kinetics of NHL-TIL was significantly poorer than PBL-T, with only 54% of planted cells still viable at day 1 and 37% at day 4 (the differences were statistically significant at day 1:  $P = 0.025$ ; and day 4:  $P = 0.0037$ ). The kinetics of reactive T cell viability (53% at day 1 and 37% at day 4) was completely superimposable with that of NHL-TIL.

Apoptosis in activated T cells has been correlated to cell cycle progression (32). As NHL-TIL and reactive T cells were immunophenotypically activated cells, cell cycle analysis was performed to see if the increased apoptosis was attributable to a greater proportion of cycling cells. As shown in Fig. 4, at day 0 virtually all cells were quiescent in G0/1 phase with no DNA synthesis or mitotic cells, regardless of the type of sample: 99%

for normal PBL-T, 96% for reactive T cells, and 98% for NHL-TIL (see M3 in Fig. 4, B, D, and F, respectively). After 24 h of culture in complete medium, cycling cells could still not be detected (Fig. 4, H, J, and L). However, apoptosis was more marked in NHL-TIL (Fig. 4 L, M2 = 37%) and reactive T cells (Fig. 4 J, M2 = 44%) than normal PBL-T (Fig. 4 H, M2 = 22%), as shown both morphologically by the appearance of a population of smaller cells (Fig. 4: compare M1 between A and G, C and I, and E and K) and in terms of a reduced DNA content, < 2n (Fig. 4: compare M2 between B and H, D and J, and F and L), confirming the results with AV. Thus, the increased apoptosis of NHL-TIL and reactive T cells was not associated with an increased proportion of cycling cells.

*Viable NHL-TIL and reactive T cells modulate Bcl-2 and Bcl-x<sub>L</sub> expression in culture.* Changes in the levels of expression of Bcl-2 and Bcl-x<sub>L</sub> have been shown to be correlated with protection from, or the induction of, apoptosis in various T cell populations (33). Recently, the levels of Bcl family proteins as detected by flow cytometry have been correlated with the findings on immunoprecipitation (34). Using flow cytometry to analyze the expression of these two proteins in single cells in the live gate, we found that all populations studied (NHL-TIL, reactive T cells, and PBL-T) gave the same profile immediately after T cell purification, with 100% of cells positive for both

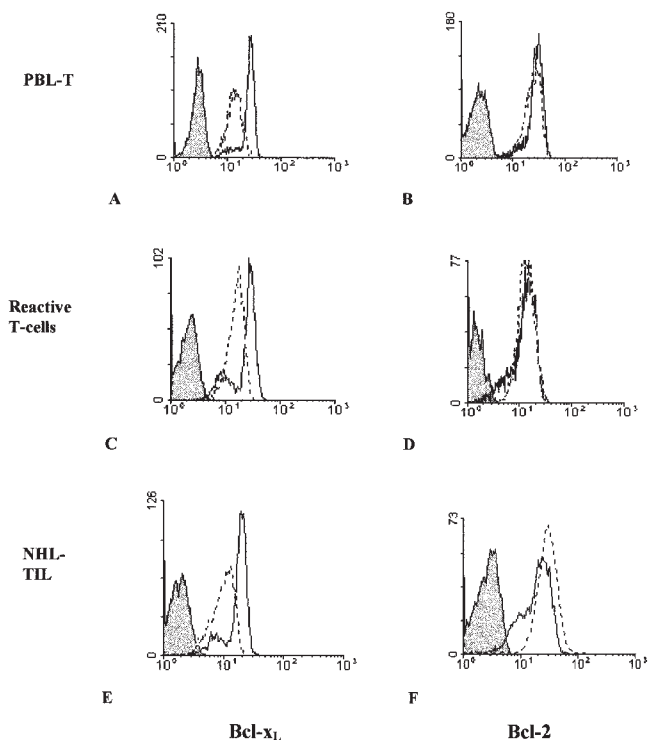




**Figure 4.** Cell cycle analysis: NHL-TIL are quiescent but show marked apoptosis during in vitro culture. Purified T cell populations were stained with PI for cell cycle analysis immediately after magnetic sorting (D0) and after 24 h of culture in complete medium, without additional stimuli (D1). Morphology (size) is shown on a logarithmic scale (Log FS) against PI content (A, C, E, G, I, and K). The rectangular gates are placed as an aid to visualize the population, with M1 highlighting small, apoptotic cells. DNA content of the entire population, after exclusion of doublets, is represented by PI staining (B, D, F, H, J, and L). The marker M2 delineates apoptotic cells (DNA content < 2n) and M3 represents cells in G0/1 phase (DNA content = 2n).

molecules and at a similar MFI (Fig. 5, dotted lines). After 24 h of culture, Bcl-2 and Bcl-x<sub>L</sub> expression in viable cells consistently showed two peaks in all populations: a major peak with a similar MFI as on day 0, and a minor peak with a lower MFI (Fig. 5). Although there were individual variations within each sample group, the Bcl-2<sup>low</sup> and Bcl-x<sub>L</sub><sup>low</sup> peaks were most marked in NHL-TIL and reactive T cells. Outside the live gate, Bcl-x<sub>L</sub> and Bcl-2 expression was low (data not shown) and all cells were AV<sup>+</sup>. Thus, PBL-T, reactive T cells, and NHL-TIL all showed similar patterns of expression of Bcl-2 and Bcl-x<sub>L</sub> and viable cells in culture maintained Bcl-2 and Bcl-x<sub>L</sub> expression, whereas apoptotic cells had low levels of both molecules.

**CD3 hyporesponsiveness of NHL-TIL and reactive T cells is not directly related to apoptosis.** Our results raised the question of whether the poor proliferative response of NHL-TIL and reactive T cells to anti-CD3 mAb was related to their marked in vitro apoptosis. First, we excluded the possibility that TCR/CD3 stimulation during the proliferative assays was leading to activation-induced cell death. The effect of IL-2, anti-CD3 mAb, and anti-CD3/IL-2 on NHL-TIL and reactive T cell survival was determined by trypan blue exclusion and AV staining (as described above). None of these conditions had any significant effect as compared with medium alone after 24 h of culture: 11.8, 9.6, 9.5, and 8% AV<sup>+</sup> cells for medium, IL-2, anti-CD3, and anti-CD3/IL-2, respectively. As NHL-TIL and reactive T cells are Fas (CD95) positive (Table III and data not shown), the role of Fas was studied using the Fas agonist Ab, APO-1, and the blocking Ab, ZB4.<sup>3</sup> Table III shows that while



**Figure 5.** Viable T cells modulate expression of Bcl-x<sub>L</sub> and Bcl-2. Intracellular staining for Bcl-x<sub>L</sub> (A, C, and E) and Bcl-2 (B, D, and F) was performed immediately after T cell purification (dotted lines) and after 24 h in culture in complete medium, without additional stimuli (solid lines). Forward and side scatter characteristics were used to gate the population of live T cells. Negative controls are represented by filled curves.

3. To control for the ability of ZB4 to inhibit Fas-mediated apoptosis by FasL, PHA blasts were incubated with recombinant FasL, both in the presence and absence of ZB4 or an isotype control antibody. 90% of PHA blasts entered apoptosis after a 24-h incubation with FasL. The control antibody had no effect, whereas ZB4 reduced the percentage of apoptotic cells to 15%. (Experiments were kindly performed by Françoise Le Deist.)

**Table III. T Cells from Chronic Inflammatory Lesions Are Resistant to Fas-induced Apoptosis**

	% of Fas <sup>+</sup> cells (MFI)	Apoptosis (% of AV <sup>+</sup> PI <sup>-</sup> cells)						
		Medium	APO-1 (ng/ml)				CH11 100 ng/ml	Isotype control 100 ng/ml
			2.5	25	100	1000		
Reactive T cells	100 (7.1)	22.7	17.5	18.5	19.6	19.1	15.5	
PHA blasts	100 (49.4)	6.4	9.7	21	26.9	7.5	9.8	

Fas (CD95) expression was determined with the anti-Fas mAb, CH11. For apoptosis induction, two anti-Fas mAbs were used: a Fas-activating Ab to induce apoptosis, APO-1, and CH11 as a control as it did not provoke apoptosis in our system. An irrelevant (IgG3) mAb was also used as an isotype control for APO-1. Cells were incubated in medium alone or with APO-1, CH11, or the isotype control mAb, followed by the cross-linking Ig and cultured for 4 or 24 h at 37°C. PHA blasts were used after 7 d of activation (i.e., 2 d of PHA/IL-2 followed by 5 d of IL-2). The results at 4 h shown as AV<sup>+</sup>PI<sup>-</sup> cells were maximal at this time point.

APO-1 induced increased apoptosis in PHA blasts, reactive T cells were completely resistant to Fas signaling by APO-1. ZB4, a blocking anti-Fas Ab, had no effect on survival kinetics (up to 4 d of culture). Thus, the Fas/FasL pathway does not appear to be involved in the marked spontaneous in vitro apoptosis of NHL-TIL and reactive T cells. As the vast majority of cell death occurred during the first day of culture (see Fig. 3 D), we investigated the proliferative capacity of the remaining viable T cells after 24 h in medium alone by replanting them in wells coated with anti-CD3 mAb. Once again, no proliferation was seen (data not shown). Thus, on the one hand, the conditions used to activate NHL-TIL and reactive T cells do not augment apoptosis and, on the other hand, the cells resistant to apoptosis remain nonresponsive to TCR/CD3 stimulation. These results show that the nonproliferative and apoptotic phenotypes of T cells from malignant and nonmalignant chronic inflammatory lymphoid organs can be dissociated.

## Discussion

The profound in vitro hyporesponsiveness of NHL-TIL and reactive T cells to TCR/CD3 activation raises the question of their in vivo functional capacity and whether they are in an anergic state. While NHL-TIL have normal levels of the signaling molecules TCR $\zeta$ , CD3 $\epsilon$ , p56<sup>lck</sup>, p59<sup>lyn</sup>, and ZAP-70, which are involved in the early events after TCR/CD3 activation, p56<sup>lck</sup> is constitutively phosphorylated and shows low activity (22). Also, NHL-TIL do not increase the nuclear binding activity of NF- $\kappa$ B and there is no induction of Rel A into the nucleus after activation by anti-CD3 mAb or IL-2 (18). Similar changes have been described in T cells in in vitro mouse models of anergy and in T cells from tumor-bearing mice (16, 17, 35–38). Thus, based on the hyporesponsiveness to TCR/CD3 stimuli and signaling alterations described above, NHL-TIL can be regarded as anergic T cells. Given that the whole population of NHL-TIL appears to be involved and that the pattern of TCR V $\alpha$  and V $\beta$  usage of NHL-TIL is polyclonal (39), the in vivo induction of anergy is a polyclonal phenomenon, not restricted to a few antigen- or tumor-specific T cell clones. The similarity between NHL-TIL and reactive T cells presented

in this study suggests that there are common mechanisms responsible for their state of anergy, namely, the inflammatory microenvironment. In reactive and B-NHL lymph nodes, a wide variety of cytokines has been identified (40–43). IL-10 is produced by T cells, monocytes/macrophages, activated B cells, and malignant B-NHL cells (43, 44). It has a direct inhibitory effect on T cell proliferation and IL-2 production and an indirect effect via downregulation of accessory cell function (44–47). Also, chronic activation of T cells in the presence of IL-10 promotes the development of poorly proliferative CD4<sup>+</sup> T cells which can downregulate immune responses (48). Another candidate cytokine is TGF- $\beta$ , produced by lymphocytes and monocytes/macrophages. It inhibits T cell activation, cytotoxic T lymphocyte development, and TNF- $\alpha$  and IFN- $\gamma$  production (49, 50). Whereas tumor cell secretion of TGF- $\beta$  has been demonstrated in human carcinomas (51), glioblastomas (5), and Hodgkin's disease (52), little data exist on TGF- $\beta$  in B-NHL (53). In addition to soluble mediators, further complexity arises from possible interactions between NHL-TIL themselves. The strong expression of CD86 in some NHL-TIL (see Table I and inset) and their expression of cytotoxic T lymphocyte A-4 (54) provide another mechanism to downregulate the immune responses of NHL-TIL via these two molecules (55–59).

NHL-TIL and reactive T cells showed poorer in vitro survival and greater spontaneous apoptosis than PBL-T. Surprisingly, IL-2 did not prevent this spontaneous apoptosis. This may be due to the fact that these populations were not actively cycling, as the IL-2R $\gamma$  chain-common cytokines have been shown to inhibit activated T cell apoptosis in a variety of situations (60–62), but this is associated with increased Bcl-x<sub>L</sub>, Bcl-2, and cell cycling (23, 60). Autologous fibroblasts prevented the rapid in vitro apoptosis of T cells from rheumatoid synovium and this was mediated, at least in part, by integrins with the upregulation of Bcl-x<sub>L</sub> but no increased cell cycling (23). These findings suggest that the in vivo survival of T cells at sites of inflammation is critically dependent on stromal factors, both cell–cell contact and soluble factors, with an important role for integrin interactions and type I interferons (23; and Pilling, D., A.N. Akbar, J. Girdlestone, N.J. Borthwick, D. Scheel-Toellner, C.D. Buckley, and M. Salmon, manuscript submitted for publication). Thus, NHL-TIL and reactive T cells, once denied the multiple signals and the anchorage of the lymph node microenvironment, are subject to anoikis (homelessness) and show an increased propensity for apoptosis in vitro, as described for adherent cells (63). Anoikis may also explain the noncycling nature of NHL-TIL and reactive T cells, as loss of anchorage-dependent signals is associated with a block of cell cycle progression in the G1 phase (64).

Our results show that the CD3 hyporesponsive and apoptotic phenotypes of NHL-TIL and reactive T cells can be dissociated. Interestingly, in a variety of models, apoptosis can be dissociated from proliferation (65), cell growth (66), and T cell anergy (67). The activated immunophenotype of NHL-TIL and reactive T cells and their resistance to Fas-mediated apoptosis are also features of a murine in vivo model of tolerance (68) and anergized human T cell clones (69). Induction of anergy was associated with only small increases in Bcl-x<sub>L</sub>, Bcl-2, and Bax expression as compared with the high levels in productively stimulated clones (69). The expression of these molecules in resting T cells is upregulated during activation (70). Thus, the similar levels of expression of Bcl-x<sub>L</sub> and Bcl-2



among NHL-TIL, reactive T cells, and normal PBL-T further support the hypothesis that NHL-TIL and reactive T cells have not been primed efficiently *in vivo* and are anergic populations.

In conclusion, our results demonstrate that NHL-TIL and T cells from chronic inflammatory, nonmalignant conditions show hyporesponsiveness to TCR/CD3 activation and poor *in vitro* survival kinetics, with similar modulation of the antiapoptotic molecules Bcl-2 and Bcl-x<sub>L</sub>. Thus, the quiescent, nonproliferating, anergic phenotype of NHL-TIL cannot be attributed solely to tumor-related factors, rather there are factors common to inflammatory microenvironments leading to downregulation of the recruited T cells in an antigen-independent manner. Therapeutic strategies in B-NHL based on manipulation of the immune system must take into account the phenomena of anergy and spontaneous *in vitro* apoptosis described here.

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