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Selective killing of CD8$^+$ cells with a 'memory' phenotype (CD62L^{lo}) by the N-acetyl-D-galactosamine-specific lectin from *Viscum album* L

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Abstract

As reported previously by our group, among the toxic proteins from *Viscum album* L. only the mistletoe lectins (MLs) induce the apoptotic killing pathway in human lymphocytes. Although one may expect a homogenous distribution of carbohydrate domains on cell surface receptors for the carbohydrate binding B chains of the toxic protein, the sensitivity of cells to these B chains obviously differ. Here we report a selective killing of CD8$^+$ CD62L^{lo} cells from healthy individuals by the galNAc-specific ML III (and RCA$_{60}$, which binds to gal and galNAc), while the gal-specific ML I was less effective. This selective killing is not sufficiently explained by protein synthesis inhibition alone, since this subset was not affected by other ribosome inhibiting proteins such as the lectin from *Ricinus communis* (RCA$_{120}$), lectin from *Abrus precatorus* (APA), abrin A, and inhibitors of RNA, DNA and/or protein synthesis such as actinomycin D, mitomycin C, and cycloheximide. We conclude that CD8$^+$ cells with 'memory' phenotype (CD62L^{lo}) are more sensitive to the ML III-mediated killing than their CD8$^+$ CD62L^{hi} counterparts, CD4$^+$ T cells, and CD19$^+$ B cells. These cells probably express a distinct receptor with galNAc domains that is missing or not active on CD8$^+$ cells with a 'naive' phenotype.

Keywords: CD8$^+$ cells; cytotoxicity; apoptosis; cell surface molecules; mistletoe lectins; ricin; abrin; protein synthesis inhibition

Abbreviations: RIPs: ribosome-inactivating proteins; ML: mistletoe lectins; RCA: *Ricinus communis* agglutinin; APA: *Abrus precatorus* agglutinin; Mit C: mitomycin C; Act D: actinomycin D; CHX: cycloheximide; galNAc: N-acetyl-D-galactosamine; β-gal: β-galactose; WBCC: whole blood cell cultures; IL: interleukin; TNF-α: tumour necrosis factor alpha

Introduction

There is emerging evidence that lectins are dynamic contributors to tumour cell recognition, cell adhesion, signal transduction across membranes, mitogenic stimulation, and augmentation of host immune defence (reviewed by Mody *et al*, 1995). However, lectins were also recognised to induce apoptosis in several tumour cell lines, epithelial cells, macrophages, and human lymphocytes (Griffiths *et al*, 1987; Kim *et al*, 1993; Janssen *et al*, 1993; Khan and Waring, 1993; Kulkarni and McCulloch, 1995; Perillo *et al*, 1995; Büssing *et al*, 1996a).

A number of plant proteins such as the toxic lectins from *Ricinus communis, Abrus precatorus*, and *Viscum album* have been identified that catalytically damage eukaryotic ribosomes making them unable to bind the elongation factor 2, and consequently unable to perform the elongation step of protein synthesis (Stirpe *et al*, 1992). These 'ribosome-inactivating proteins' (RIPs) possess carbohydrate-binding B chains linked by hydrophobic bonds and disulphide bridges to the catalytic A chain (type 2 and type 4 RIPs). The lectin domains of these RIPs can bind to any appropriate carbohydrate domain on cell surface receptors, enabling the protein to enter the cell by receptor-mediated endocytosis (Stirpe *et al*, 1982, 1992; Endo *et al*, 1988). Subsequently, the catalytic A chain of the misletoe lectins (MLs) inhibit protein synthesis (Olsnes *et al*, 1989; Endo *et al*, 1988; Stirpe *et al*, 1992), and the cells undergo apoptosis (reviewed in Büssing, 1996b). It appears that both, glycoproteins and -lipids on the cell surface may act as binding sites for toxins.

The lectin chains of the various RIPs differ in their cellular interactions, as do the enzymic chains. This is suggested by the different lesions each toxin causes in animals (Stirpe *et al*, 1992), with ricin at high concentrations damaging primarily Kupffer and other macrophagic cells, whereas modeccin and volkensin affect both parenchymal and non-parenchymal liver cells. Since the type 1 RIPs are lacking a lectin subunit, these single chain RIPs are less toxic than type 2 RIPs; however, they are highly toxic to some cells, for instance macrophages and trophoblasts, possibly due to their high pinocytic activity (Stirpe *et al*, 1992). Thus, although one may expect a homogenous distribution of 'receptors' for the lectin B chains on the cell surface, the sensitivity of cells to the lectin-mediated cytotoxicity obviously differ. An unexpected finding reported recently by our group was related to the cytotoxicity mediated by the *N*-acetyl-D-galactosamine (galNAc)-specific ML III, which was more effective against CD8$^+$ T cells than CD19$^+$ B cells and CD4$^+$ T cells (Büssing *et al*, 1997). Here we report a predominant killing of CD8$^+$ CD62L^{lo} cells by the galNAc-specific ML III, while the β-

galactose (β-gal)-specific ML I was less effective. We conclude that $CD8^+$ cells with 'memory' phenotype ($CD62L^{lo}$) are more sensitive to the ML III-mediated killing than $CD4^+$ cells and $CD19^+$ cells and suggest that they may harbour 'receptors' with galNAc domains not expressed or less active on the surface of their $CD8^+$ $CD62L^{hi}$ counterparts.

Results

Killing of cultured human lymphocytes by ML III

The MLs induce an apoptotic cell death in cultured lymphocytes (Büssing *et al*, 1996a). Within 72 h, the number of Annexin-V^+ lymphocytes with low PI fluorescence (PI^{lo}) increased by the addition of ML III, however, even at a final concentration of 3 ng/ml (Table 1). The number of Annexin-V^+ PI^- apoptotic cells and Annexin-V^+ PI^{hi} necrotic cells did not significantly change by the addition of ML III, or protein and RNA synthesis inhibitors such as cycloheximide (CHX) and actinomycin D (Act D) that served as a positive control (Table 1). As shown in Figure 1, within 6 h of incubation with ML III at 10 ng/ml, no apoptotic cells were detected by flow cytometry as compared to controls, i.e. the cells did not bind Annexin-V and did not stain for mitochondrial membrane protein Apo2.7 which is expressed in cells undergoing apoptosis (Zhang *et al*, 1996), while the number of both, apoptotic and necrotic cells increased within 24 h. The cells undergoing apoptosis became permeable to PI within 48 h (Annexin-V^+ PI^{lo}) (Figure 1). However, within 48 h the number of lymphocytes that stain for mitochondrial membrane protein Apo2.7 went beyond the number of cells that bind Annexin-V, indicating that much more cells received a 'death signal' than actually change membrane configuration.

Killing of defined lymphocyte subsets by ML III

We wondered whether lymphocyte subsets may differ in regard of sensitivity to the ML III-mediated killing and analyzed the surviving lymphocytes by flow cytometry. Since ML III binds to granulocytes, monocytes and erythrocytes (but shows no blood group specificity and no hemolysis but agglutination), and thus the overall killing capacity towards the lymphocytes from whole blood cell cultures (WBCC) may decrease, we investigated both, Ficoll-isolated lymphocytes and lymphocytes from WBCC incubated with increasing concentrations of ML III.

In lymphocytes from WBCC, ML III at final concentrations of 50 and 100 ng/ml decreased the amount of $CD3^+$ T cells (Table 2), while $CD19^+$ B cells were less sensitive to the ML-mediated killing, as reported previously (Büssing *et al*, 1997). At a final concentration of 10 ng/mL ML III, $CD8^+$ cells declined, while the number of $CD4^+$ T cells remained almost unchanged. Predominantly the $CD28^-$ $CD8^+$ subset declined (Table 2). Within the $CD8^+$ cells, the $CD8^{lo}$ $CD38^+$ subset was eliminated, i.e. $CD3^+$ $CD8^{lo}$ T cells and $CD3^-$ $CD8^{lo}$ natural killer cells (data not shown). However, by increasing the concentration of ML III, also the $CD4^+$ T cells and the $CD28^+$ $CD8^+$ subset died.

Similar to lymphocytes from WBCC, in Ficoll-isolated lymphocytes the number of $CD8^+$ cells decreased by the addition of ML III, while the relative number of $CD4^+$ T cells and $CD19^+$ B cells increased at final concentrations of 3, 6 and 10 ng/ml ML III (Table 3). At 30 and 50 ng/ml ML III, also the number of $CD4^+$ cells declined, while the relative number of $CD19^+$ B increased. Addition of CHX at a final concentration of 10 μg/ml decreased the relative number of $CD19^+$ B cells, but did not affect the $CD8^+$ subset, while Act D killed $CD19^+$ B cells and $CD8^+$ cells at 1 μg/ml (Table 3). These results indicate that the $CD8^+$ cells are more sensitive to the ML III-mediated killing than the $CD4^+$ T cells and $CD19^+$ B cells.

Decrease of $CD8^+$ cells with 'memory' phenotype ($CD62L^{lo}$) by ML III

Using three-colour immunofluorescence staining, we observed that the $CD8^+$ cells from WBCC dying after application of 10 ng/ml ML III were predominantly of $CD28^-$ $CD62L^{lo}$ phenotype (Figure 2), while in Ficoll-isolated lymphocytes, also the $CD28^+$ $CD62L^{lo}$ subset decreased. However, after CD8 enrichment using magnetic beads, no significant numbers of $CD8^+$ $CD28^+$ $CD62L^{lo}$ cells were detected; thus, only the $CD28^-$ $CD62L^{lo}$ subset was affected by the toxic protein (Figure 2). Also in cultured WBCC from six HIV infected individuals, addition of ML III at 10 ng/ml resulted in a significant decline of $CD8^+$ $CD28^-$ $CD62L^{lo}$ cells, while the $CD8^+$ cells with $CD62L^{hi}$ phenotype were not affected by the toxic proteins (A Büssing, C Schnürer, U Pfüller, unpublished results). Thus, it is obvious that in both, $CD8^+$ cells from

Table 1 Flow cytometric analysis of lymphocytes undergoing cell death in response to ML III

	ML III (ng/ml)						CHX (μg/ml)	Act D (μg/ml)
	0	3	6	10	30	50	10	1
Annexin-V−/PI-	68.3±7.0	47.9±6.5	41.9±6.9	40.2±8.9	29.0±1.3	30.2±3.2	46.6±11.8	34.6±6.0
Annexin-V+/PI-	7.1±3.4	5.8±1.4	6.9±0.6	5.8±1.9	6.9±2.0	6.0±0.1	7.8±3.1	4.6±1.1
Annexin-V+/PI^{lo}	7.0±3.2	27.2±2.7	31.2±4.2	33.9±5.0	38.2±5.4	41.5±0.6	24.5±5.8	39.0±1.5
Annexin-V+/PI^{hi}	14.0±1.5	15.8±2.5	16.0±1.8	17.1±2.8	18.9±0.1	17.8±0.1	16.0±3.0	19.1±6.0

Ficoll-isolated lymphocytes from three healthy individuals were incubated for 72 h with the galNAc-specific toxic lectin from *Viscum album* L. (ML III) at various concentrations, and cycloheximide (CHX) or actinomycin D (Act D) as controls. Cells undergoing apoptosis bind Annexin-V but exclude the DNA-intercalating dye propidium iodide (PI), while necrotic cells (primary necrosis and late apoptosis) became permeable to PI. Viable cells are Annexin-V^- and PI^-. ML III at final concentrations of 0.5 and 1 ng/ml did not significantly increase the number of Annexin-V^+ PI^{lo} cells (data not shown)

WBCC and isolated lymphocytes, the $CD62L^{lo}$ subset decreased in response to ML III, while the $CD62L^{hi}$ subset remains almost unaffected.

In Ficoll-isolated lymphocytes, the $CD8^+$ $CD62L^{lo}$ cells declined at 3 and 6 ng/ml ML III, both, with and without CD28 molecules (Table 4), while the amount of $CD8^+$ $CD62L^{hi}$ cells increased. However, at higher concentrations such as 10 and 50 ng/ml ML III or treatment with Act D at 1 μg/ml, the relative amount of $CD8^+$ $CD28^-$ $CD62L^{lo}$ cells raised, while the $CD8^+$ $CD62L^{hi}$ cells declined (Table 4). CHX did not significantly affect the $CD8^+$ $CD28^-$ subsets but decreased the $CD62L^{hi}$ cells within the $CD8^+$ $CD28^+$

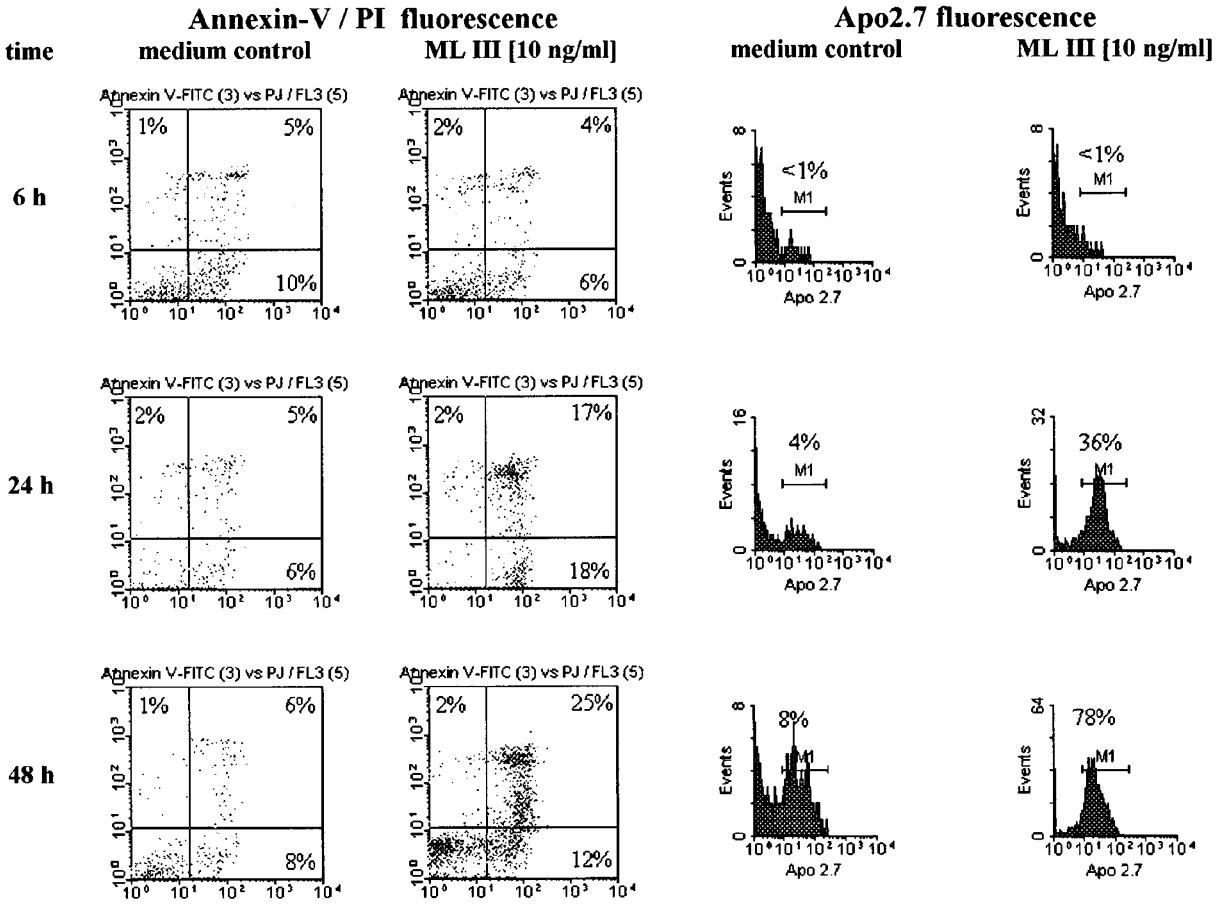

Figure 1 Flow cytometric analysis of lymphocytes undergoing cell death in response to ML III. Ficoll-isolated lymphocytes from healthy individuals were incubated for 6 h, 24 h and 48 h with ML III at a final concentration of 10 ng/ml. Annexin-V binds to membrane phospholipid phosphatidylserine translocated from the inner to the outer leaflet of the plasma membrane in cells undergoing apoptosis. Thus, cells undergoing apoptosis (lower right) bind Annexin-V but exclude the DNA-intercalating dye propidium iodide (PI), while necrotic cells (primary necrosis and late apoptosis) became permeable to PI (upper right). Viable cells are Annexin-V^- and PI^- (lower left). Fluorescence of mitochondrial membrane molecule Apo2.7 is given in the histograms, while the fluorescence of Annexin-V and PI is given in the dot plots

Table 2 Flow cytometric analysis of lymphocyte subsets from WBCC treated with ML III

	ML III [ng/ml]			
	0	**10**	**50**	**100**
Lymphocytes	4455±785	3354±1146	883±383	395±130
CD3+cells	73.2±3.2	71.4±4.7	52.7±7.16	44.7±5.7
CD19+cells[a]	14.2±1.9	22.8±5.7	28.5±4.5	37.7±4.6
CD4+cells	43.8±6.7	47.9±10.0	36.0±16.9	20.2±7.9
CD8+cells	29.3±6.5	18.7±6.1	14.4±3.9	14.8±4.9
CD8+CD28+cells	16.4±4.3	12.3±4.4	7.8±1.9	9.3±2.9
CD8+CD28−cells	12.8±2.2	6.4±2.4	6.6±3.2	5.5±2.0
CD4/CD8 ratio	1.60±0.60	2.82±1.27	2.48±1.44	1.55±1.04

WBCC from healthy individuals were incubated for 72 h at 37°C in the presence of ML III ($n=5$). The number of lymphocytes was given in arbitrary counts (cells in gate with lymphocytes of adequate cell size and granularity), while the relative amount of lymphocyte subsets were given in % of lymphocytes. [a]$n=3$

Table 3 Flow cytometric analysis of cultured lymphocytes treated with ML III

	ML III (ng/ml)						CHX (μg/ml)	Act D (μg/ml)
	0	3	6	10	30	50	10	1
Cells in gate (%)	77.7±8.3	68.4±8.0	59.1±11.7	49.1±13.8	21.0±21.4	16.6±18.4	68.2±11.3	11.3±7.8
CD19+cells	8.0±2.8	11.2±3.3	13.7±3.0	14.4±0.4	30.6±12.7	19.9±3.7	2.7±1.1	0.8±0.8
CD3+cells	80.0±7.9	84.0±3.1	79.5±4.9	77.2±1.2	50.0±6.6	61.1±34.9	91.6±5.5	61.9±21.9
CD4+cells	44.5±17.7	54.4±15.3	52.8±15.5	50.7±16.9	28.4±24.2	20.6±18.2	54.6±15.0	32.3±6.3
CD8+cells	29.8±5.4	22.3±3.8	20.6±5.2	19.3±4.8	10.1±0.3	6.9±2.4	29.9±8.2	13.5±5.2
CD4/CD8 ratio	1.60±0.84	2.55±1.01	2.78±1.24	2.87±1.35	2.78±2.32	2.62±1.79	2.01±0.88	2.71±1.26

Ficoll-isolated lymphocytes from three healthy individuals were incubated for 72 h at 37°C in the presence of ML III at various concentrations, and cycloheximide (CHX) or actinomycin D (Act D) that served as a control. Results of lymphocyte subsets are given in % of lymphocytes with adequate cell size and granularity. To calculate their relative number, the number of gated events is given in % of all cultured cells

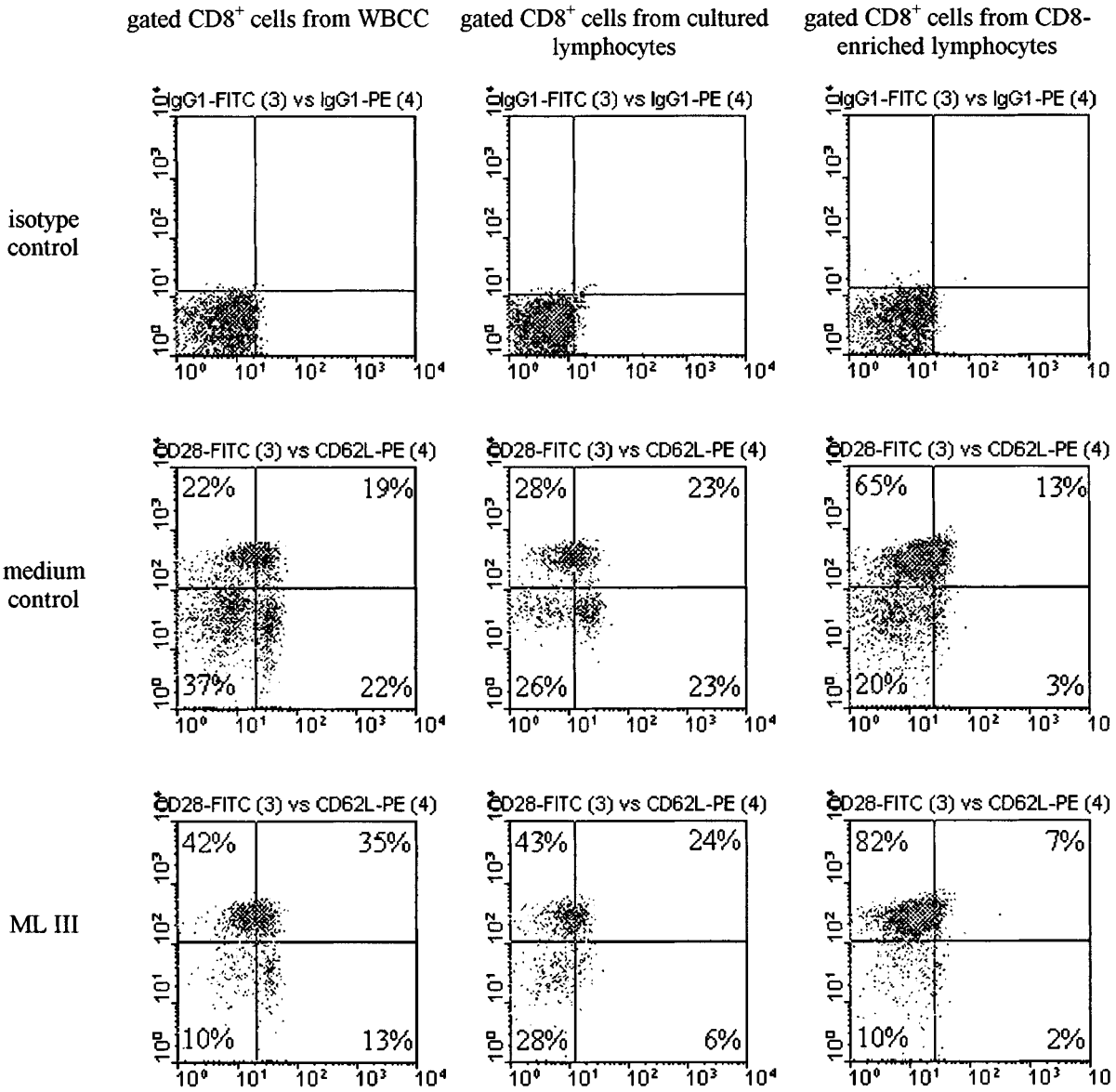

Figure 2 Representative three-colour immunofluorescence staining of CD8+ cells in 72 h cell cultures from WBCC, Ficoll-isolated lymphocytes, and lymphocytes after CD8 enrichment (99% CD3+ CD8+ cells) using magnetic beads (MACS, Miltenyi Biotec). ML III was added at a final concentration of 10 ng/ml. The gate was set on lymphocytes stained with ECD-conjugated anti-CD8 (Coulter). CD8+ cells were differentiated in subsets with high ($CD62L^{hi}$) and low ($CD62L^{lo}$) fluorescence intensity of L-selectin. L-selectin expression (γ-axis) was correlated with expression of CD28 molecules (χ-axis)

subset. The observed increase of $CD8^+$ $CD28^-$ $CD62L^{lo}$ cells in response to Act D and ML III at higher concentrations is probably due to the high cytotoxic potential of the drugs, resulting in a concomitant decrease of cell size and increased granularity of cultured lymphocytes (which have lost CD8 molecules and exhibited a $CD62L^{lo}$ phenotype), and a relative persistence of these late apoptotic affected cells (approved by Annexin-V binding and PI uptake) in the gate (Figure 3). However, this effect is not observed in WBCC. One may suggest that the ML III-mediated killing capacity in cultured lymphocytes is much higher as compared to WBCC, since granulocytes, monocytes, and erythrocytes do bind the toxic protein, and thus these differences may be due to a relatively lower concentration of active ML III in $CD8^+$ cells from WBCC.

Differences in the L-selectin expression on the surface of $CD4^+$ and $CD8^+$ cells treated with the MLs I-III

The expression of L-selectin, along with other markers, is suggested to distinguish naive T cells ($CD62L^{hi}$) from 'memory'/effector T cells ($CD62L^{lo}$ and $CD62L^-$, respectively) (Mobley *et al*, 1994; Beverley, 1996). Thus, the killing of $CD8^+$ $CD62L^{lo}$ and $CD8^+$ $CD62L^-$ cells by ML III raise several questions: Is this killing selective only for $CD8^+$ 'memory'/effector cells or also operative in $CD4^+$ $CD62L^{lo}$ cells, and are only 'receptors' with galNAc domains involved? To address these questions, we measured surface expression of CD62L on $CD4^+$ and $CD8^+$ cells from WBCC incubated with 10 ng/ml ML III. As shown in

Table 4 Flow cytometric analysis of $CD8^+$ cells from ML III-treated lymphocytes

	ML III (ng/ml)					CHX (μg/ml)	Act D (μg/ml)
	0	3	6	10	50	10	1
Cells in gate (%)	77.7±8.3	68.4±8.0	59.1±11.7	49.1±13.8	16.6±18.4	68.2±11.3	11.3±7.8
$CD^+CD28^-CD62L^{hi}$	16.6±6.4	26.1±11.1	31.9±14.9	18.2±5.4	13.4±4.8	17.8±13.0	1.3±1.9
$CD8^+CD28^+CD62L^{hi}$	36.4±13.9	49.8±1.0	40.0±4.5	31.2±6.4	14.1±8.4	24.6±1.8	2.8±2.7
$CD8^+CD28^-CD62L^{lo}$	23.6±13.6	10.2±3.0	14.5±9.8	31.8±5.3	58.6±12.1	22.1±7.5	53.7±13.3
$CD8^+CD28^+CD62L^{lo}$	23.4±6.4	14.9±8.5	12.1±8.6	18.8±8.0	19.3±1.1	35.5±20.9	64.1±24.1

Ficoll-isolated lymphocytes from three healthy individuals were incubated for 72 h at 37°C in the presence of ML III at various concentrations, and cycloheximide (CHX) or actinomycin D (Act D) that served as a control. The gate was set on lymphocytes stained with ECD-conjugated anti-CD8 (Coulter) and correlated expression of CD62L (γ-axis) *vs* CD28 (χ-axis). $CD8^+$ cells contain subsets with high ($CD62L^{hi}$) and low ($CD62^{lo}$) fluorescence intensity of L-selectin. Results are given in % of $CD8^+$ cells. To calculate their number, the relative amount of gated events is given in % of all cultured cells

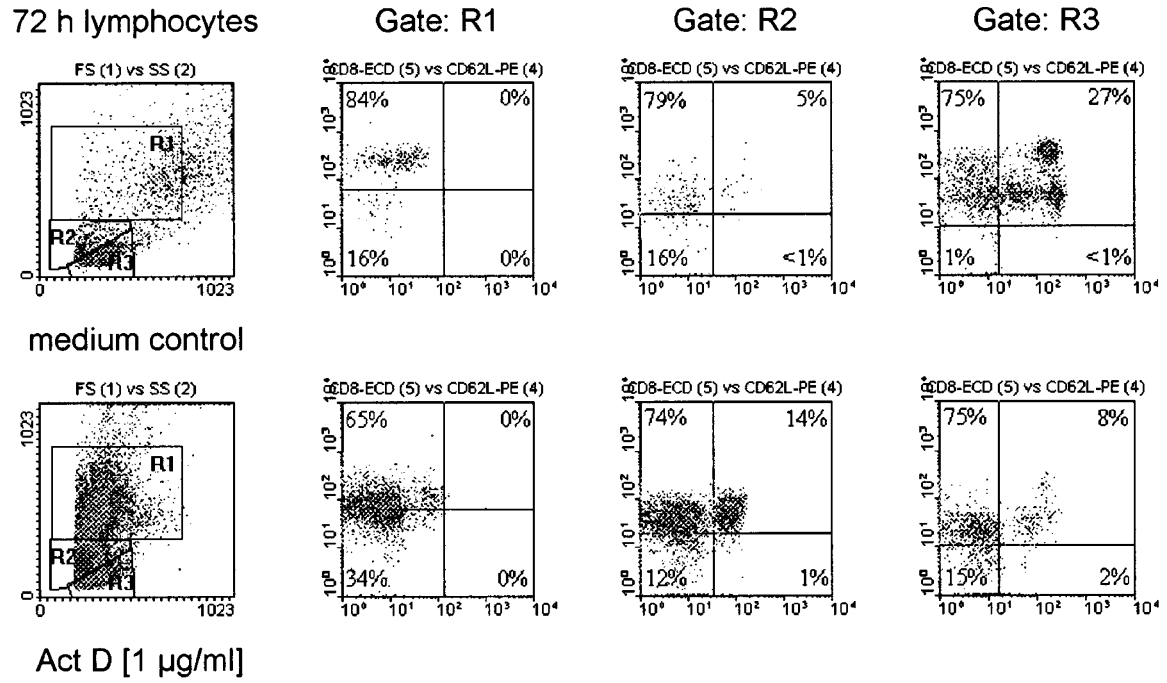

Figure 3 Representative immunofluorescence staining of $CD8^+$ cells from 72 h cultured Ficoll-isolated lymphocytes treated with Act D at a final concentration of 1 μg/ml. The gate was set on lymphocytes with adequate cell volume and granularity (R3), on cells with decreased cell size and increased granularity (R2), and on cells with severe increase of SSC signal (R1). All quadrants were strictly adjusted to the mouse anti-IgG1/IgG1 controls. L-selectin expression (γ-axis) was correlated with expression of CD8 molecules (χ-axis)

Table 5, ML III significantly decreased the amount of $CD8^+$ cells, thus, the proportion of $CD4^+$ cells increased. Within the $CD8^+$ cells, the amount of $CD62L^{lo}$ cells significantly declined, while, however, the decrease of $CD4^+$ $CD62L^{lo}$ cells was less severe. As shown in Table 6, the decline of $CD8^+$ cells was more pronounced by the addition of the galNAc-specific MLs II and III, while the β-gal-specific ML I was less effective (ML I $<$ ML II $<$ ML III). Predominantly the $CD28^-$ $CD8^+$ subset decreased (Table 6). The ML II and ML III-mediated decrease of the $CD8^+$ $CD62L^{lo}$ subset was substantially prevented by galNAc, while, however, the proportion of $CD4^+$ $CD62L^{lo}$ cells increased by the simultaneous addition of MLs and galNAc (Table 7). Several other sugars such as D-galactose, α-lactose, α-methyl-galactosid, lactitol, and melibiose were ineffective to inhibit the ML III-mediated killing (data not shown), while methyl-α-D-galactopyranosid resulted in a slight but insignificant inhibition.

We next examined whether the observed killing effects were specific for ML III or also observed in response to other drugs with properties of protein synthesis inhibition. Thus, we measured the L-selectin expression on the surface of $CD8^+$ cells from WBCC incubated for 72 h with the Ricinus communis agglutinin (RCA_{120}), ricin (RCA_{60}), Abrus precatorus agglutinin (APA), Abrin A, and ML III (each at a final concentration of 10 ng/ml), and with CHX (10 μg/ml). As shown in Figure 4, RCA_{120}, APA and CHX did not significantly alter the expression of CD62L and CD28 on the surface of $CD8^+$ cells as compared to the controls, while ML III and RCA_{60} eliminated the $CD8^+$ $CD62L^{lo}$ cells, however, with and without CD28 molecules. Further application of streptomyces toxins Mit C (0.5 μg/ml) and Act D (100 and 1000 ng/ml) did not result in significant changes of surface marker expression on $CD8^+$ cells as compared to CHX and medium control (data not shown).

Cytokines in the supernatants of lymphocyte fractions

In order to analyse whether the ML III-mediated killing of the $CD8^+$ $CD62L^{lo}$ cells may change the cytokine pattern in the culture supernatants, we determined the concentrations of type 1 and type 2-related cytokines, such as interferon-γ, interleukin (IL)-12, -4, -5, -6, -10, and also tumour necrosis factor (TNF)-α in the 72 h supernatants of CD8-enriched and CD8-depleted lymphocyte (n=6). As shown in Table 8, apart from large interindividual differences in the release of cytokines, application of ML III at 10 ng/ml did not significantly alter the supernatant concentrations of the investigated cytokines (a decrease of IL-6 was observed in three out of six experiments), while in the CD8-depleted cultures ML III raised the concentration of TNF-α. In Mit C-treated samples, a higher concentration of interferon-γ was observed in three out of six experiments. Thus, in $CD8^+$ cells no relevant changes of the cytokine pattern in the culture supernatants was observed in response to ML III. The TNF-α release in the supernatants of $CD4^+$ cells is probably due to a 'cytotoxic release' induced by ML III and Mit C.

Discussion

We wondered whether lymphocyte subsets may differ in their sensitivity to cell death induced by the toxic lectins from *Viscum album*, and observed a selective killing of $CD8^+$ cells with a 'memory' phenotype ($CD62L^{lo}$) by low concentrations of the galNAc-specific MLs. One may speculate that the $CD8^+$

Table 5 Flow cytometric analysis of $CD4^+$ and $CD8^+$ cells from WBCC after addition of ML III

ML III (ng/ml)	0	10
$CD4^+$ cells	40.3±8.6	48.5±10.1
$CD8^+$ cells	32.6±5.0	23.5±5.5
CD4/CD8 ratio	1.31±0.51	2.26±0.98
%$CD62L^{hi}$ in $CD4^+$	73.4±7.0	80.4±7.5
%$CD62L^{lo}$ in $CD4^+$	21.0±6.7	16.0±8.4
%$CD62L^{hi}$ in $CD8^+$	48.8±13.4	84.1±4.9
%$CD62L^{lo}$ in $CD8^+$	37.9±9.6	10.3±4.8

Results are means±S.D. of experiments with lymphocytes from 72 h cultured WBCC (n=16). ML III was added at a final concentration of 10 ng/ml. The surface expression of CD4 and CD8 molecules was given in % of lymphocytes, while the L-selectin expression is given in % $CD62L^{hi}$ and % $CD62L^{lo}$ within $CD4^+$ or $CD8^+$ cells, respectively. Results are significantly different compared to controls ($p \leqslant 0.001$; t-test)

Table 6 Flow cytometric anaysis of $CD4^+$ and $CD8^+$ cells from WBCC after addition of MLs I-III

	n	controls	ML I	ML II	ML III
CD4+	5	40.3±9.8	47.9±7.7	46.7±2.7	50.8±9.5
CD8+	5	33.7±3.5	28.9±4.2	27.3±3.8	25.2±5.0
CD28+CD8+	3	14.8±2.5	14.7±2.1	17.0±2.1	15.8±3.4
CD28−CD8+	3	19.4±4.2	15.6±4.1	14.3±3.5	11.4±4.3
CD4/CD8 ratio	5	1.23±0.46	1.71±0.47	1.75±0.32	2.11±0.68

WBCC were incubated for 72 h at 37°C in the presence of MLs I–III at a final concentration of 10 ng/ml. Results are means±S.D. of three to five different experiments

Table 7 Flow cytometric analysis of CD62L expression on the surface of $CD4^+$ and $CD8^+$ cells from WBCC incubated in the presence of MLs I–III and of galNAc

	galNAc	CD4+cells		CD8+cells	
		%$CD62L^{hi}$	%$CD62L^{lo}$	%$CD62L^{hi}$	%$CD62L^{lo}$
Controls	−	72.0±5.8	19.7±4.2	40.6±1.7	34.2±1.2
	+	70.7±7.4	22.7±6.0	40.3±1.8	36.3±0.3
ML I	−	76.3±6.9	16.2±4.3	72.0±15.1	21.6±1.9
	+	70.0±9.4	22.5±8.2	60.5±4.4	23.0±3.9
ML II	−	76.7±6.7	16.9±4.6	68.4±4.1	16.7±2.2
	+	66.0±12.3	27.0±9.8	46.2±4.8	31.8±3.5
ML III	−	80.4±7.7	14.3±5.6	83.6±6.6	9.3±3.9
	+	68.7±10.1	24.2±8.3	56.6±1.7	24.0±1.3

WBCC were incubated for 72 h at 37°C in the presence of mistletoe lectins (MLs) I–III. Results are means±S.D. of three different experiments. The MLs were added at final concentrations of 10 ng/ml, while the controls represent samples without added toxins. 15 mM galNAc were added to block the galNAc-specific MLs II and III. Results are given in %$CD62L^{hi}$ and %$CD62L^{lo}$ within CD4+ or CD8+ cells, respectively

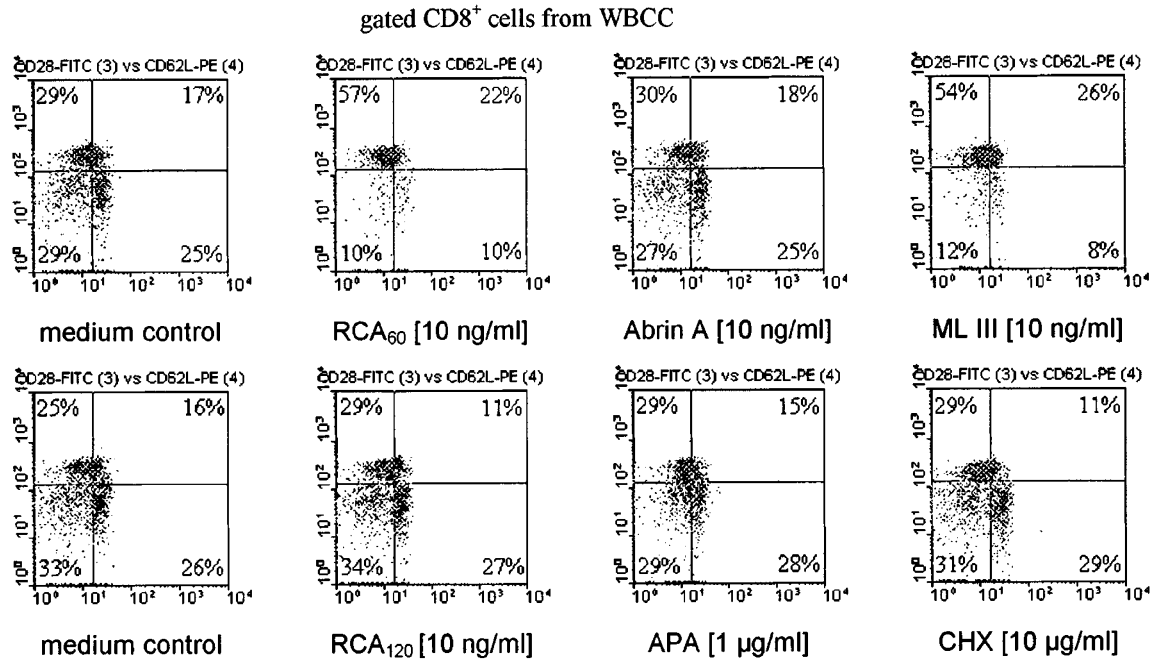

Figure 4 Flow cytometric analysis of $CD8^+$ cells from cultured WBCC incubated for 72 h in the presence of the plant lectins RCA_{120} (10 ng/ml), RCA_{60}, (10 ng/ml), Abrin A (10 ng/ml), APA (1 μg/ml), ML III (10 ng/ml), and the streptomyces toxin CHX (10 μg/ml). The gate was set on lymphocytes stained with ECD-conjugated anti-CD8 (Coulter). $CD8^+$ cells were differentiated in subsets with high ($CD62L^{hi}$) and low ($CD62L^{lo}$) fluorescence intensity of L-selectin. L-selectin expression (γ-axis) was correlated with expression of CD28 molecules (χ-axis). Results are representative for four independent experiments

Table 8 Cytokines in the supernatants of 72 h cultured lymphocyte fractions

			$CD8^+$ cells		$CD8^-$ cells (89% $CD4^+$ cells)		
	Cut off	Medium	ML III	MitC	Medium	ML III	MitC
(pg/ml)	(pg/ml)	control	(10 ng/ml)	(10 μg/ml)	control	(10 ng/ml)	(10 μg/ml)
IFN-γ	50	26±35	41±46	320±645*	117±148	155±163	155±163
IL-12	500	5720±6484	6205±6481	707±969	6372±7429	6229±7074	913±1326
IL-4	50	73±76	71±66	21±39	162±171	165±185	40±32
IL-5	50	79±96	76±84	113±234	105±112	95±97	18±11*
IL-10	20	35±15	57±77	61±84	728±689	243±261	427±458°
IL-6	50	468±891	300±558	339±380	15193±11775	14481±9462	22790±8727*
TNF-α	3	0.8±1.9	0.8±1.3	2.2±4.9	8±9	55±84°	183±178°

The cytokines in the supernatants of CD8-enriched lymphocytes (99% $CD3^+$ $CD8^+$ cells) and CD8-depleted lymphocytes (89% $CD4^+$ T cells) from six healthy individuals were determined by ELISA after 72 h incubation in the presence of ML III at a final concentration of 10 ng/ml. The alkylating drug mitomycin C (Mit C), that served as a positive control, was added at 10 μg/ml. Results are significantly different compared to control samples (*$P \leqslant 0.05$; °$P = 0.03$; Wilkoxon's sign rank test)

$CD62L^{lo}$ subset may differ from its $CD8^+$ $CD62L^{hi}$ counterpart by the expression of a yet undefined galNAc-containing receptor which mediates or facilitates endocytosis of the apoptosis-inducing proteins. This suggestion is corroborated by recent findings that interaction of ML B chains (but not the A chains) with appropriate receptors on lymphocytes increased intracellular Ca^{2+} (Büssing *et al*, 1996a) and inhibited the uptake of [^{3}H]thymidine (Metzner *et al*, 1987). However, this 'activation'-pathway probably does not involve T cell receptor triggering, since T cells did not upregulate CD25 or CD71 expression in response to the MLs (Büssing *et al*, 1996a). The reason why ML III selectively killed $CD8^+$ $CD62L^{lo}$ cells as compared to their $CD8^+$ $CD62L^{hi}$ counterparts, $CD4^+$ T cells and $CD19^+$ B cells is unclear. One may suggest different possibilities, such as (1) differences in ML-binding to the lymphocyte subsets, (2) hyperresponsiveness of $CD8^+$ $CD62L^{lo}$ cells to several cytokines, (3) higher susceptibility of these 'memory' cells to cell death triggered by the inhibition of protein synthesis, and (4) the presence of a specific 'receptor' preferentially expressed on $CD8^+$ $CD62L^{lo}$ cells which mediates subsequently cell death.

One explanation of the observed differences could be due to differences in ML-binding to the lymphocyte subsets. However, ML III exhibited similar cytotoxic activity to human lymphocytes as ML I (Göckeritz *et al*, 1994; Büssing *et al*, 1996a), although the binding of ML III was much lower as

compared to ML I (Göckeritz *et al*, 1994). Since ML I-mediated killing of $CD8^+$ $CD62L^{lo}$ cells was less effective as compared to ML III, one may suggest that binding of ML III to its 'receptor' is a more effective 'death signal'. But this does not explain the observed differences in the sensitivity between B cells and $CD4^+$ and $CD8^+$ T cell subsets to the toxic protein, since these subsets did not significantly differ in cell size. Alternatively, the binding sites may differ in $CD8^+$ $CD62L^{hi}$ 'naive' cells and $CD8^+$ $CD62L^{lo}$ 'memory' cells. As reported recently, the endogenous lectin galectin-1, which induced apoptosis in thymocytes and activated T cells (Baum *et al*, 1995; Perillo *et al*, 1995, 1997), preferentially binds to immature thymocytes as compared to mature cells (Baum *et al*, 1995). This was suggested to result from regulated expression of preferred oligosaccharide ligands on these cells. Thus, one may suggest similar differences in the expression of ML III ligands in 'naive' cells and 'memory' cells. However, this may be true for $CD8^+$ cells, but the ML III-mediated killing was less effective in the other subsets. An alternative view would be that cells exhibiting a 'memory' phenotype may be hyperresponsive to several cytokines released in response to ML III. Cytokines such as IL-1, -6 and TNF-α are recognised to be induced by the addition of ML I (Hajto *et al*, 1990). However, in CD8-enriched cultures treated with ML III, no significant changes in the release of type 1 and type 2-related cytokines, such as interferon-γ, IL-12, -4, -5, -10, and TNF-α, as compared to the control samples were observed.

Detailed studies suggest that for 'naive' phenotype cells ($CD44^{lo}$ $CD45RB^{hi}$ $CD62L^{hi}$), the rate of cell division is relatively slow, while $CD44^{hi}$ $CD45RB^{lo}$ $CD62L^{lo}$ cells divide at much higher rate (reviewed in Beverley, 1996). Such a rate of cell division obviously also implies a high rate of cell loss. Monteiro *et al* (1996) stated that the telomeric length was significantly shorter in $CD8^+$ $CD28^-$ cells, indicating an exhaustion of their replicative capacity. Indeed, the $CD8^+$ $CD28^-$ subset is reported not only to respond inadequately to a variety of stimuli (Lewis *et al*, 1994), but also to have reduced proliferative potential (Azuma *et al*, 1993). $CD8^+$ $CD28^-$ cells with an activated phenotype (CD38, CD45R0, HLA-DR) emerge during HIV infection (Levacher *et al*, 1992; Landay *et al*, 1993; Gougeon *et al*, 1996), and especially the $CD45R0^+$ ('memory') T cells from HIV infected persons were more susceptible to apoptosis as compared with the same subset in control subjects (Gougeon *et al*, 1996). Therefore, we suggest that the $CD8^+$ $CD28^-$ cells with 'memory' phenotype ($CD62L^{lo}$) are in a state of 'replicative senescence', and thus may be responsive to any death signal, or alternatively, are proned to undergo cell death, anyway. The $CD8^+$ $CD28^-$ subset was preferentially affected in WBCC, while, however, in isolated lymphocytes cultured in the presence of ML III, both the $CD8^+$ $CD62L^{lo}$ cells with and without CD28 molecules were affected. One may suggest that the less effective killing of $CD8^+$ $CD28^+$ $CD62L^{lo}$ subset in the WBCC might be due to distinct survival signals and/or growth factors present in the serum or released by monocytes or granulocytes. Monocytes may rescue this subset by an interaction of costimulatory signal with CD28 molecules on T cells, which provides important costimulatory signals for the development of T cell responses (reviewed by Boise *et al*, 1995).

It was reported previously that binding of the endogenous lectin galectin-1 to T cells involves recognition of carbohydrate epitopes on the T cell surface glycoproteins CD43 (leukosialin, sialophorin) and CD45 (tyrosine phosphatase involved in signal transduction) (Baum *et al*, 1995; Perillo *et al*, 1995). However, the presence of lectin-binding epitopes, while essential, may not be sufficient for cell lysis and probably some intracellular mechanisms are involved in the regulation of lectin-mediated cytotoxicity (Kim *et al*, 1993). Although the inhibitors of protein and RNA synthesis were potent toxicants for lymphocytes and induced apoptosis, the observed selective killing of $CD8^+$ $CD62L^{lo}$ cells by ML III is not sufficiently explained by protein synthesis inhibition alone. This subset was not affected by CHX, which acts at the level of the ribosome by preventing translation, or by the type 4 and 2 RIPs RCA_{120}, APA, and Abrin A. We suggest that ML III (and RCA_{60}) requires a high affinity surface receptor with galNAc domains to mediate the signal that ultimately leads to cell death within the $CD8^+$ $CD62L^{lo}$ subset, since (1) the ML III-mediated killing was substantially prevented by the addition of galNAc, and (2) the β-gal/galNAc-specific RCA_{60} but not the RIPs that binds preferentially to β-gal killed this defined subset. This suggestion is corroborated by preliminary results indicating that even a 2 h incubation of human lymphocytes with ML III, which was followed by an extensive washing of cells and continued incubation for 48 h in fresh medium, did kill the lymphocytes (Büssing, Wagner and Pfüller, unpublished observations).

In conclusion, this report describes for the first time that a plant toxin kills a defined subset of lymphocytes. Although the exact underlying mechanisms remain to be clarified, we observed a selective killing of $CD8^+$ cells with a 'memory' phenotype ($CD62L^{lo}$) by the type 2 RIPs ML III and RCA_{60} which both bind to terminal galNAc residues. It is unclear why $CD4^+$ T cells and $CD19^+$ B cells exhibiting this phenotype are less sensitive to the toxin as compared to the $CD8^+$ cells. This selectivity of cell targeted ML III makes it a candidate for the use in immunotoxin or ligand toxin synthesis, and immunomodulation, and also interposes several new aspects in the ongoing discussion (Matzinger, 1994; Ahmed and Gray, 1996) on the fate of 'memory' T cells.

Materials and Methods

Blood samples and culture conditions

Since in a previous investigation apparent differences in the cell function of T cells from WBCC and lymphocyte cultures treated with the streptococcal pyrogenic exotoxin A were observed (Büssing *et al*, 1995), for this investigation, the more '*in vivo*-like' WBCC was used. Heparinised (50 IU/ml) peripheral blood from healthy individuals was incubated 1 : 10 for 72 h at 37°C in chromosome medium A (Biochrom KG, Berlin, Germany).

In further experiments, Ficoll-isolated lymphocytes (1×10^6 cells/μl) were cultured in chromosome medium A before flow cytometric analysis. For enrichment of $CD8^+$ cells, isolated lymphocytes (3×10^6 cells/μl) were suspended in RPMI 1640 (Biochrom KG), supplemented with antibiotics and autologous plasma (10%), and separated with magnetic beads (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturers instructions. The $CD8^+$ cells were further incubated for 72 h at 37°C. Culture supernatants were carefully collected and kept frozen at −20°C.

Toxic proteins

The MLs differ due to their molecular weights (between 50 kDa and 63 kDa) and specificity. The ML I binds to β-gal, ML III to galNAc, whereas ML II binds to β-gal and galNAc (Franz *et al*, 1981). The MLs were extracted and purified as described previously (Eifler *et al*, 1993). For blocking experiments, 15 mM galNAc (Sigma Chemie) were added to block the galNAc-specific MLs II and III.

Lectin from *Ricinus communis* (RCA_{120}), lectin from *Abrus precatorus* (APA), and Abrin A from *Abrus precatorus*, which bind to galactose, and the DNA synthesis inhibitor mitomycin C (Mit C) from *Streptomyces caespitosus*, RNA synthesis inhibitor actinomycin D (Act D) from Streptomyces species, and the protein synthesis inhibitor cycloheximide (CHX) from *Streptomyces griseus* were obtained from Sigma Chemicals, Deisenhofen, Germany. Ricin (RCA_{60}), which binds to β-gal and galNAc, was a kind gift of Dr. Rudolf Eifler, Institute of Phytochemistry, University Witten/Herdecke.

Flow cytometric analysis of cultured cells

Flow cytometric analysis of surface molecules was performed on EPICS® XL-MCL flow cytometer (Coulter, Krefeld, Germany) using mAb against the following Ag: CD3, CD4, CD8, CD16, CD19, CD25, CD28, CD38, CD56, CD62L, CD95 (Coulter). For each sample, 10 μl of the mAb was added to 100 μl of cell suspension. Forward angle light scatter (FSC) and high perpendicular light scatter (SSC) were used to gate lymphocytes and to exclude cell debris. For surface molecule analysis, only lymphocytes with adequate cell volume (FSC signal) and granularity (SSC) were gated. These cells include both, viable cells and cells undergoing apoptosis (Annexin-V^+ PI^-), while cells with decreased FSC signal and increased SSC signal, which were excluded from analysis, contain late apoptotic cells (Annexin-V^+ PI^{lo}). All quadrants were adjusted to the anti-mouse isotype controls.

Viability analysis

To differentiate apoptotic and necrotic cells, Ficoll-isolated lymphocytes were analyzed for Annexin-V binding and propidium iodide (PI) uptake as described (Koopman *et al*, 1994; Vermes *et al*, 1995). Briefly, after washing of cultured cells (1×10^6/ml) with PBS and resuspension in binding buffer (10 nM Hepes/NaOH, pH 7.4, 140 nM NaCl, 2.5 nM $CaCl_2$), the cells were stained with 5 μl FITC-labeled Annexin-V (PharMingen, San Diego, CA, USA) and PI (5 μg/ml; Sigma). After 15 min of incubation at room temperature in the dark, again the binding buffer was added and cells were analyzed by flow cytometry. Additionally, the mitochondrial membrane protein Apo2.7, a 38 kD protein localised on cells undergoing apoptosis (Zhang *et al*, 1996), was analyzed. After digitonin-permeabilisation of cultured lymphocytes according to the method described by Zhang *et al* (1996), the cells were washed and resuspended in PBS, and subsequently stained with the PE-labeled mAb Apo2.7 (Immunotech, Hamburg, Germany).

Cytokine assays

The cytokines interferon-γ, IL-12, -4, -5, -10, -6, and TNF-α were determined in the culture supernatants as described previously (Stein *et al*, 1996). Briefly, microtiter plates were incubated overnight (4°C) with the monoclonal capture anti-cytokine Ab (PharMingen; IL-6 from R & D Systems, Wiesbaden, Germany), diluted in hydrogencarbonate buffer (0.1 M, pH 9.6), Recombinant or natural cytokines (standards) were added, and the supernatants of CD8-enriched and CD8-depleted lymphocytes. After 2 h at room temperature, plates were incubated with the biotinylated detecture Abs for another 2 h (room temperature), followed by peroxidase-conjugated avidine (Sigma) for 30 min at room temperature. Detection was performed by adding substrate (o-phenylenediamine) solved in citrate buffer (0.1 M, pH 5.0). The individual concentrations were measured with a SLT Spectra ELISA reader, using the SLT EasyFit software.

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