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Overexpression of the *bcl-2* oncogene in the mouse pre-B cell line SPGM-1 protects from apoptosis, but does not affect blocked B-lineage differentiation and lineage switch towards macrophage like cells

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Abstract

The CD5$^+$ mouse pre-B cell line SPGM-1 is able to undergo lineage conversion towards a macrophage like cell type. Although one of the κ-light chain alleles is rearranged in V-Jκ4 configuration, no protein is expressed nor could the expression be induced. Infection with and expression of the human *bcl-2* gene protected SPGM-1 cells from apoptosis, allowing the rearrangement of their second κ-allele in V-Jκ5 configuration. mRNA transcripts of the V-Jκ regions were detected only in SPGM-1 $\times$ *bcl-2* cells, but not in SPGM-1, while κ-light chain protein was not found in any of the cell lines. The myeloid differentiation potential of SPGM-1 cells was not affected by overexpression of the human bcl-2 gene. Upon appropriate stimulation, SPGM-1x*bcl-2* cells became enlarged, adhered to the plastic surfaces and lost their immunoglobulin μ heavy chain expression.

Keywords: pre-B cells; lineage switch; immunoglobulin light chain rearrangement; lymphocyte differentiation; *bcl-2*

Abbreviations: BCR, B cell receptor; PMA, phorbolester; LPS, lipopolysaccharide; IL, interleukin; PBS, phosphate buffered saline; NEA, non-essential amino acid; 2-ME, 2-mercaptoethanol

Introduction

The expression of the CD5 (Ly-1) antigen distinguishes two B-cell subpopulations, the CD5$^-$ 'B2'-, and the CD5$^+$ 'B1'-cells (Hardy *et al*, 1994). Additionally to the expression of CD5, B1 cells differ from B2 cells by several other features, e.g. their main localisation in the peritoneal cavity, their ability to self-replenish, their altered IgM:IgD ratio on the cell surface and their independence of T-cell help. B1 cells develop in fetal liver and predominate in early ontogeny. In the adult mouse they are replenished by a self renewing, peritoneum-located, precursor B-cell of unknown differentiation status. B1 cells produce and react to another set of cytokines as B2 cells do. Optimal induction and progression of B1 cell differentiation is dependent on peritoneal stromal cells, while bone marrow stromal cells do not work equally well in this regard (Hardin *et al*, 1995).

B1 cells follow the same differentiation-pathway as is known for conventional B2 cells. Precursor cells develop via proB and preB stages to virgin B cells and then mature B-cells, which become antibody forming plasma cells upon antigenic stimulation. These differentiation stages are identified by phenotypic and genotypic markers. While cells within the proB stage have both their heavy and light chain loci in germline configuration, preB cells express cμ heavy chains in association with surrogate light chains. Finally, on the surface of virgin B cells a mature B cell receptor (BCR) of the IgM isotype, consisting of cμ heavy chain and κ or λ light chains, is found (Rolink and Melchers, 1991).

At the pre-B cell stage light chain loci are recombined. The germline κ-light chain locus consists of about 350 Vκ (variable) elements, 5 Jκ (joining) elements (Jκ3 being a pseudogene) and 1 Cκ (constant) element. Within the recombination process, a particular Vκ element is connected directly to one of the Jκ elements. The DNA stretch between these elements is excised and thereby lost. This new V-Jκ element is associated later to the Cκ segment by RNA splicing. In the case of an out-of-frame recombination the newly produced V-Jκ elements are said to be non-productive. Cells which bear non-productively rearranged immunoglobulin-chains are negatively selected, they die by apoptosis. The proto-oncogene *bcl-2* is an inhibitor of apoptosis in differentiating B-cells. Its expression is strictly regulated during B-cell differentiation, being low in stages at which cells are lost by apoptosis and high at stages at which cells are long-lived (Merino *et al*, 1994). Overexpression of *bcl-2* in various cell lines and in lymphocytes of transgenic mice inhibits apoptosis induced by different cytotoxic conditions, revealing that distinct pathways to apoptosis all converge upon a common effective mechanism that is blocked by Bcl-2 (McDonnell *et al*, 1988; Sentman *et al*, 1991; Strasser *et al*, 1991a,b, 1994).

For a long time it was postulated that cells cannot switch their differentiation program from one particular hemopoietic lineage to another lineage. But in the last 15 years several

cases have been reported, showing that some B cell precursors, mostly from the $CD5^+$ ($Ly\text{-}1^+$) B1 type, could differentiate to mature macrophages. These lineage-switches occurred spontaneously (Palacios and Steinmetz, 1985) or were induced by cytokines (IL-3; Bauer *et al*, 1986), by drug treatment (5-azacytidine; Boyd *et al*, 1982) or by retroviral transformation (Klinken *et al*, 1988).

Another model of switching from pre-B to macrophage-like cells is the mouse cell line SPGM-1. SPGM-1 cells represent immortalized pre-B cells, as shown by phenotypic and genotypic characterization. Upon treatment with either IL-3 (Martin *et al*, 1993) or a combination of phorbolester (PMA) and calcium ionophore (ionomycin) (Spencker *et al*, 1995) SPGM-1 cells lose their pre-B characteristics and acquire macrophage-like features. This differentiation pathway is documented in detail, however, it is not known whether SPGM-1 cells are also able to develop to a more advanced B-cell stage. In order to address this question, we defined the exact differentiation stage of SPGM-1 and tried to induce these pre-B-cells to differentiate to cells expressing a mature B-cell antigen receptor.

Here we demonstrate that SPGM-1 cells (1) have rearranged one allele in V-Jκ4 configuration, but do not produce measurable amounts of mRNA, detectable by RT–PCR, and (2) upon protection from apoptosis by *bcl-2* overexpression rearrange their second κ-allele and express V-Jκ specific mRNA in amounts detectable by RT–PCR. This mRNA is not translated to light chain protein measurable by any technique and the translation cannot be induced. (3) The potential of SPGM-1 cells to undergo lineage conversion into macrophages is not affected by the overexpression of *bcl-2*. These data indicate that SPGM-1 cells have a block in differentiation that prevents their transition from the pre-B to the virgin and mature B-cell stage.

Results

Functional expression of the human *bcl-2* gene in SPGM-1 cells

Apoptosis is a physiological process involved in normal development, differentiation and homeostasis (Wyllie *et al*, 1980). We hypothesized that SPGM-1 cells, which are induced to differentiate to the mature B cell stage might die by apoptosis and thus escape our analysis. To overcome this problem, we infected SPGM-1 cells with a retrovirus containing the human *bcl-2* gene. The transfected pool was cloned and four clones further analysed. The expression of the hu-Bcl-2 protein in the resulting cells was verified by FACS analysis (Figure 1, lower panels, show the data for one representative clone in comparison to the vector-only infected cells (Figure 1, upper panels) and Western blotting (data not shown).

The antiapoptotic function of the Bcl-2 protein was tested using several inducers of apoptosis. In Figure 2 the results for a glucocorticoid (Figure 2B), for ionomycin (Figure 2C), and for irradiation (Figure 2D) are depicted. All of the stimuli lead to cell death of the whole culture of vector-only infected cells within 1 to 4 days, depending on the stimulus.

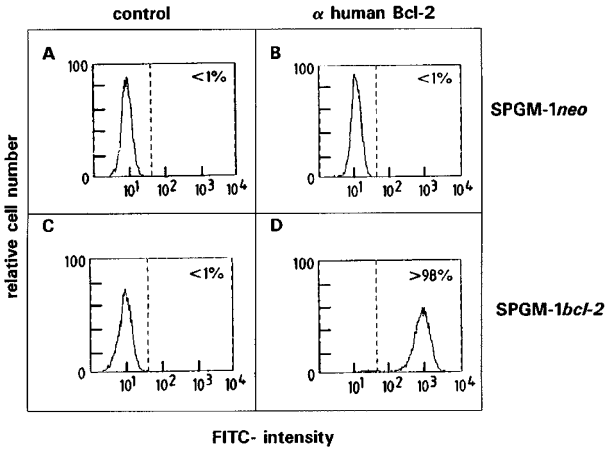

Figure 1 FACS analysis of hu-Bcl-2 expression in infected SPGM-1 cells. Vector-only (SPGM1*neo*; **A+B**) or *hu-bcl-2* infected (SPGM*1bcl-2/neo*; **C+D**) SPGM-1 cells were either stained with an irrelevant antibody (**A+C**) or with an antibody recognizing the hu-Bcl-2 protein (**B+D**) and analysed in the FACScan. Only SPGM-1xbcl-2 cells showed an increased fluorescence by staining with an antibody specific for human Bcl-2, while the vector-only infected cells were negative. (control: irrelevant isotype matched antibody).

In contrast, the *bcl-2* expressing cells survived well during the 7 day observation period (Figure 2 A–D).

These data demonstrate that after infection the hu-Bcl-2 protein is functionally expressed in SPGM-1 cells, hereafter designated 'SPGM-1*xbcl-2*'.

Recombination of light chain loci in SPGM-1

Cells of the mouse pre-B cell-line SPGM-1 express the immunoglobulin μ heavy chain (cμ) in association with the surrogate light chain (vpre-B, λ5) (Martin *et al*, 1993). The next step in B-cell maturation is the downregulation of surrogate light chain expression followed by the appearance of κ or λ light chains. The light chain genes have to be recombined prior to their expression; these recombination events were detected by a PCR-based method. As an initial step samples were standardized for equal DNA content by amplifying a specific sequence of the house keeping gene aldolase (Figure 3, lanes 9–12). The analysis of SPGM-1 light chain loci showed a Vk-Jk4 recombination of at least one κ-light chain allele, detected by the appearance of a 140 bp PCR product with the Vκ-Jκ4 primers and a 500 bp PCR product using the Vκ-Jκ5 primers (Figure 3, lanes 3 and 7). Genomic DNA extracted from control WEHI231 cells, which contain a Vκ-Jκ1 recombination, resulted in the expected 1100 bp and 1500 bp PCR products, using the Vκ-Jκ4 and Vκ-Jκ5 primer-pairs, respectively (Figure 3, lanes 1 and 5). No PCR product was obtained using DNA from the T cell line EL-4, whose immunoglobulin light chain alleles are in germline configuration, resulting in a DNA-stretch between their Vκ and Jκ elements too long to be amplified by PCR under the conditions used (Figure 3, lanes 2 and 6). These results, in combination with restriction analysis of the PCR products (not shown) proved the specificity of the selected primers.

In the *bcl-2* expressing SPGM-1 cells a second recombination of Vκ-Jκ5 could be detected indicated by

an additional PCR product of 140 bp using the Vκ-Jκ primers (in Figure 3, lane 8, the result for one clone is documented, being representative for all four SPGM-1*xbcl-2* clones tested). We then asked, if these two recombination events are due to two individual subpopulations within the cell culture, or due to the recombination of the second allele in SPGM-1*xbcl-2* cells. Subclones of SPGM-1*xbcl-2* cells were generated by limiting dilution technique and seven of these further analyzed. All of them showed signals for both recombination events, Vκ-Jκ4 and Vκ-Jκ5, indicating that in SPGM-1*xbcl-2* cells both alleles are rearranged in the same fashion (data not shown).

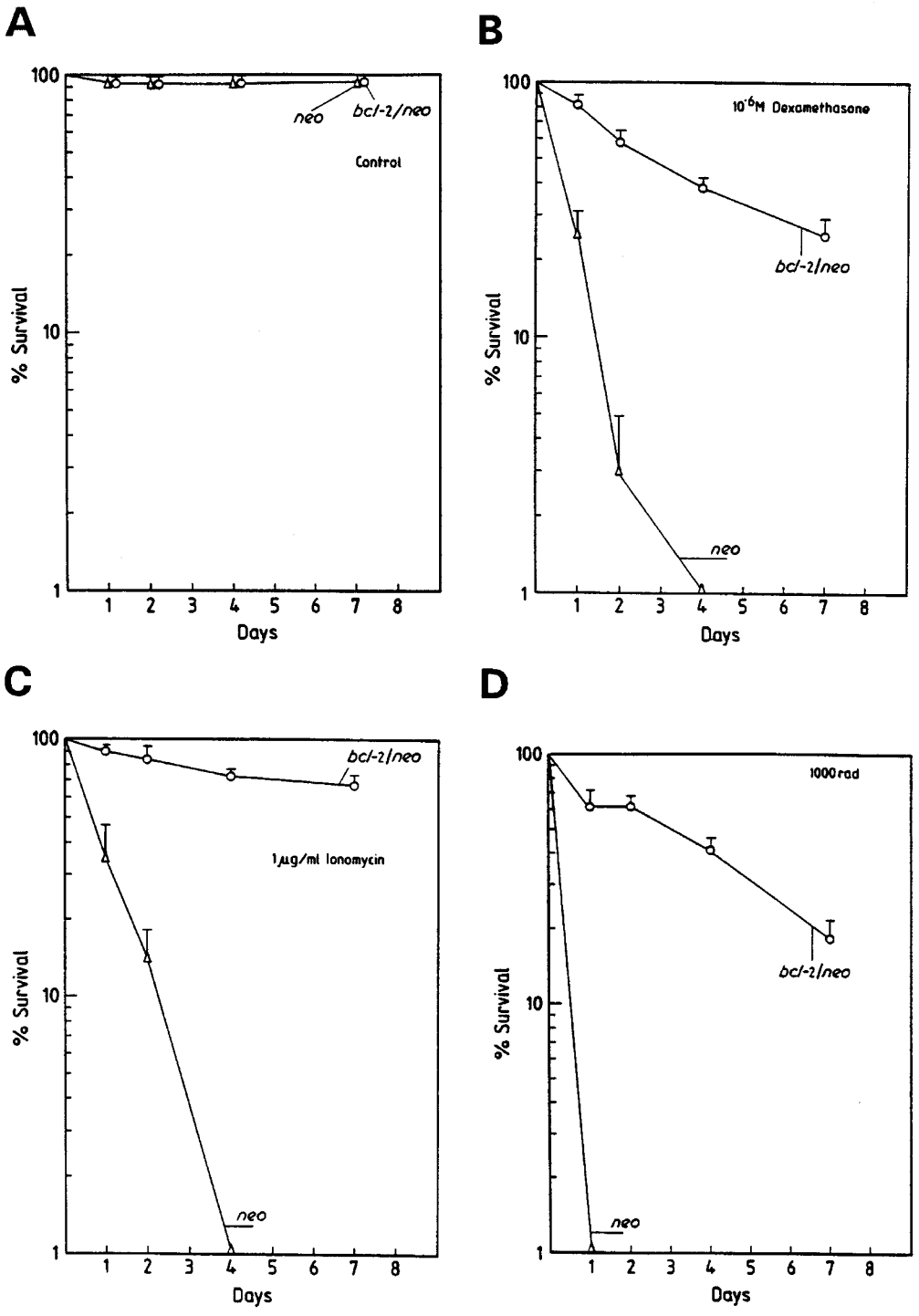

Figure 2 Expression of hu-Bcl-2 protects SPGM-1 cells from induced cell death. Vector-only (Δ-neo) and *hu-bcl-2* infected SPGM-1 cells (o -bcl-2/neo) were either left untreated (**A**) or treated with 10^{-6} M dexamethasone (**B**), 1 μg/ml ionomycin (**C**), 1000 rad γ-irradiation (**D**). At the time points indicated cell survival was determined by trypan-blue exclusion. (Results are means ± s.d. of one representative experiment out of three identical ones).

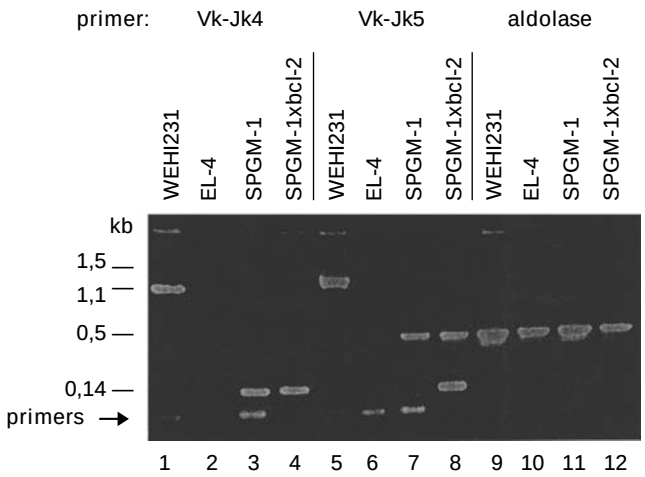

Figure 3 PCR-analysis of the Vκ-Jκ rearrangement in SPGM-1 cells. Genomic DNA from different lymphocyte cell lines was extracted and V-Jκ specific sequences were amplified using specific primers (see Table 2) in a PCR. Aldolase specific sequences were amplified as positive controls. Products were separated by agarose gel electrophoresis and visualised by ethidium bromide staining. The products of different length are due to individual V-Jκ recombination events.

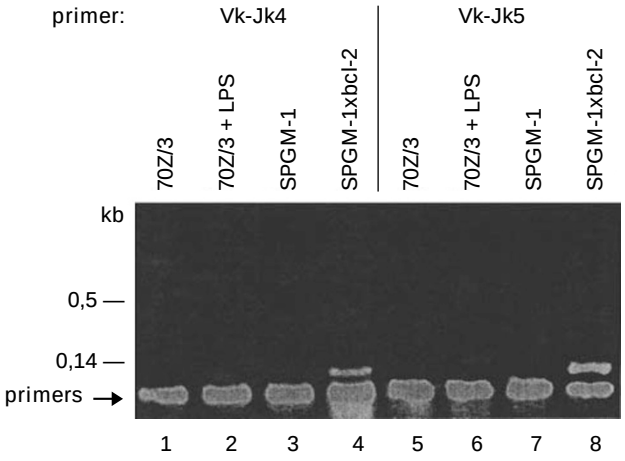

Figure 4 RT-PCR analysis of the transcription of mRNA for Igκ-light chains. RNA of untreated or LPS (50 μg/ml, 48 h) treated 70Z/3 cells, as well as SPGM-1 and SPGM-1*xbcl-2* cells was isolated, reverse transcribed and amplified using V-Jκ specific primer pairs as indicated. The products were separated by agarose gel electrophoresis and visualised by ethidium bromide staining. Light chain mRNA could neither be detected in untreated 70Z/3 (lanes 1 and 5), in LPS-treated 70Z/3 (lanes 2 and 6), nor in SPGM-1 cells (lanes 3 and 7), whereas specific products were obtained in SPGM-1*xbcl-2* with V-Jκ4 (lane 4) and V-Jκ5 (lane 8) specific primer pairs.

Transcription of the Igκ light chain gene

We used the highly sensitive RT – PCR method to examine the transcription of the κ light chain genes in SPGM-1 or SPGM-1*xbcl-2*. From both cell lines mRNA was prepared and used as a template for reverse transcription. Amplification using a Vκ-Jκ4 primer pair yielded no product for SPGM-1 cells (Figure 4, lane 3), but a signal of the expected size of 0.14 kb in SPGM-1*xbcl-2* cells (Figure 4, lane 4), demonstrating that only the Bcl-2 expressing cells transcribe their κ-light chain gene. SPGM-1*xbcl-2* cells also synthesized mRNA from the second rearranged allele resulting in a signal with the Vκ-Jκ5 primers (Figure 4, lane 8), whereas SPGM-1 (Figure 4, lane 7) or the 70Z/3 control cells showed no signal (Figure 4, lanes 5 – 7).

No PCR products were obtained in control amplifications, where the reverse transcription step was carried out without enzyme, proving that the RNA preparations were free of DNA contaminations. RNA from 70Z/3 cells served as controls. In 70Z/3 cells, which can be induced to express κ-light chains by LPS, no V-Jκ4 or V-Jκ5 mRNA could be detected, because their κ-locus is recombined in V-Jκ1 configuration.

These results indicate that B-lineage differentiation in SPGM-1 is blocked at the level of κ-light chain synthesis. This transcriptional block in B cell differentiation can be shifted to a certain extent by overexpressing the *bcl-2* gene in SPGM-1 cells, resulting in the synthesis of κ-light chain mRNA.

Translation of κ light chain mRNA

Expression of κ-light chain protein was investigated in SPGM-1 and SPGM-1*xbcl-2* cells by FACS analysis in intact cells for surface expression (Figure 5A-D) and in permeated, pepsin-treated cells for intracellular expression (Figure 5E-H). Protein for κ-light chains could neither be detected on the surface of SPGM-1 (Figure 5A) or in SPGM-1*xbcl-2* (Figure 5B) cells nor intracellulary (Figure 5E and F, respectively). The anti-Igκ antibody detected κ-light chains on the surface of 70Z/3 cells which were pretreated with LPS (Figure 5D), while no κ-light chain protein was observed on the surface of untreated 70Z/3 cells (Figure 5C). In LPS treated 70Z/3 cells a signal for κ-light chain protein was found in the permeated cells (Figure 5H), proving that the permeabilization of the cells allowed the antibody to detect κ-light chain protein within the cells. In untreated 70Z/3 cells κ-light chain protein could not be detected intracellulary (Figure 5G), proving that this antibody did not recognize other proteins unspecifically. In addition, κ-light chain surface expression could not be induced by any reagent known to promote B-cell differentiation, such as interleukins and chemicals (summarized in Table 1). The negative FACS data were supported by Western blotting, as well as by biosynthetic labelling and subsequent immunoprecipitation, which did not detect κ-light chain protein in SPGM-1 or SPGM-1*xbcl-2* cells (data not shown). These results demonstrate that Igκ light chain translation does not take place in SPGM-1 cells. And, in contrast to the transcriptional block, this translational block cannot be abolished by overexpression of the *bcl-2* gene.

Overexpression of hu-Bcl-2 in SPGM-1 does not affect lineage conversion

Having shown that Bcl-2 overexpression does not completely overcome the block in B lymphoid differentiation in SPGM-1, we studied possible effects of Bcl-2 expression on the myeloid differentiation potential. The most prominent feature of SPGM-1 cells undergoing lineage switch is their altered morphology. SPGM-1 cells were treated with a combination of phorbolester

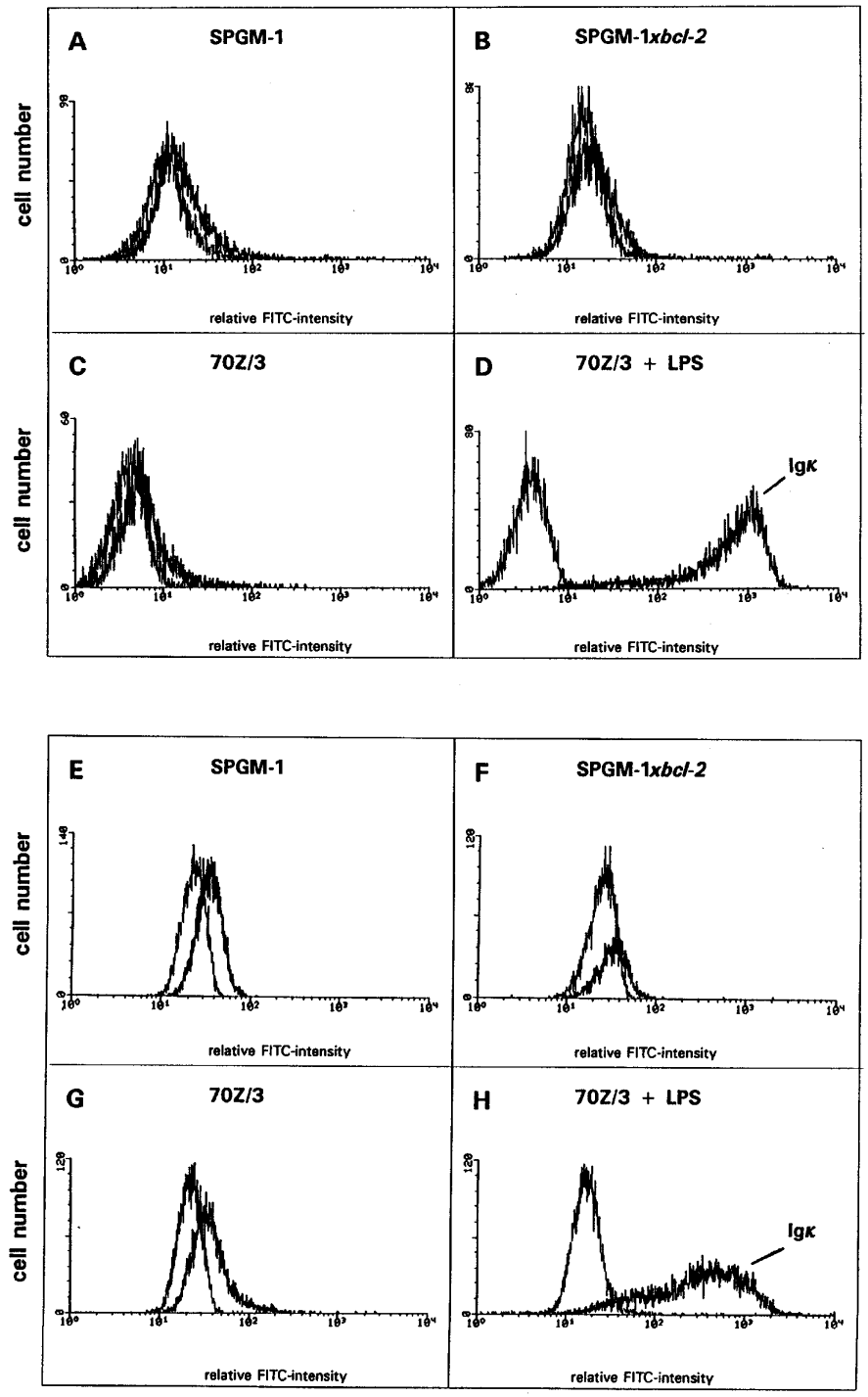

Figure 5 Analysis of Igκ light chain protein expression by FACS. 5A-D: Surface expression of Igκ light chain protein: Intact SPGM-1 cells (**A**), SPGM-1*xbcl-2* cells (**B**), as well as untreated 70Z/3 cells (**C**) and 70Z/3 cells treated with LPS (50 μg/ml, 48 h; **D**) were stained with a FITC labeled Igκ specific monoclonal antibody and analysed in the FACScan. Only the positive control of LPS treated 70Z/3 cells showed an enhanced fluroescence compared to the autofluorescence of the cells, demonstrating that Igκ light chains were detectable under these conditions, whereas SPGM-1 and SPGM-1*xbcl-2* lacked surface Igκ light chains. 5E-H: Intracellular detection of Igκ light chain protein. The same cells as above were treated with pepsin (1 mg/ml, 10 min, 37°C) in order to remove surface light chains and permeabilized with 0.5% saponin. Igκ light chain proteins were detected by a FITC labeled specific monoclonal antibody. Only in the positive control of LPS-treated 70Z/3 cells Igκ light chain protein could be detected under these conditions (**H**), whereas none could be found in untreated 70Z/3 (**G**), SPGM-1 (**E**), or SPGM-1*xbcl-2* (**F**), indicating the absence of Igκ light chain protein in these cells.

(PMA) and calcium ionophore (ionomycin). The suspension cells (Figure 6 A) adhered to plastic surfaces, became enlarged, and formed extensions (Figure 6 B). SPGM-1*xbcl-2* cells, also normally blastoid and non-adherent (Figure 6 C), differentiated exactly in the same manner showing the typical appearance of macrophage-like cells (Figure 6 D). Another striking event, high-lighting the loss of B-lineage characteristics, is the downregulation of the immunoglobulin μ heavy chain (cμ). Both, SPGM-1 and SPGM-1*xbcl-2*, showed cμ suface expression (Figure 7 A and C) which was lost after 2 days of treatment with the combination of PMA and ionomycin (Figure 7 B and D). Acquisition of macrophage-like features and concomitant loss of B-lymphoid markers, suggest that overexpression of the *hu-bcl-2* gene, and hence protection from apoptosis, does not affect the potential of SPGM-1 cells to undergo lineage conversion.

Table 1 Summary of substances tested to induce B-lymphoid differentiation in SPGM-1

20% WEHI 3BD conditioned medium (interleukin-3)
20% AG8-IL5 conditioned medium (interleukin 5)
20% ST-2 conditioned medium (interleukin 7)
20%P388D1 conditioned medium (interleukin 6 and 1)
20% EL-4 conditioned medium (interleukin 2)
20% interleukin 10 containing conditioned medium
2ng/ml recombinant human interleukin 1β
50 μg/ml lipopolysaccharide (LPS) from E.coli (Sigma)
1 μg/ml anti cμ antibody from hybridoma E4.2
50 nM 12-O-tetradecanoylphorbol-13-acetate (Sigma)
1 μg/ml ionomycin (Sigma)
20 μM all-trans retinoic acid (RA, Sigma)
1.5% dimethylsulfoxide (DMSO, Sigma)

Discussion

The bipotent mouse pre-B-1 cell-line SPGM-1 switches its lineage commitment towards macrophage differentiation

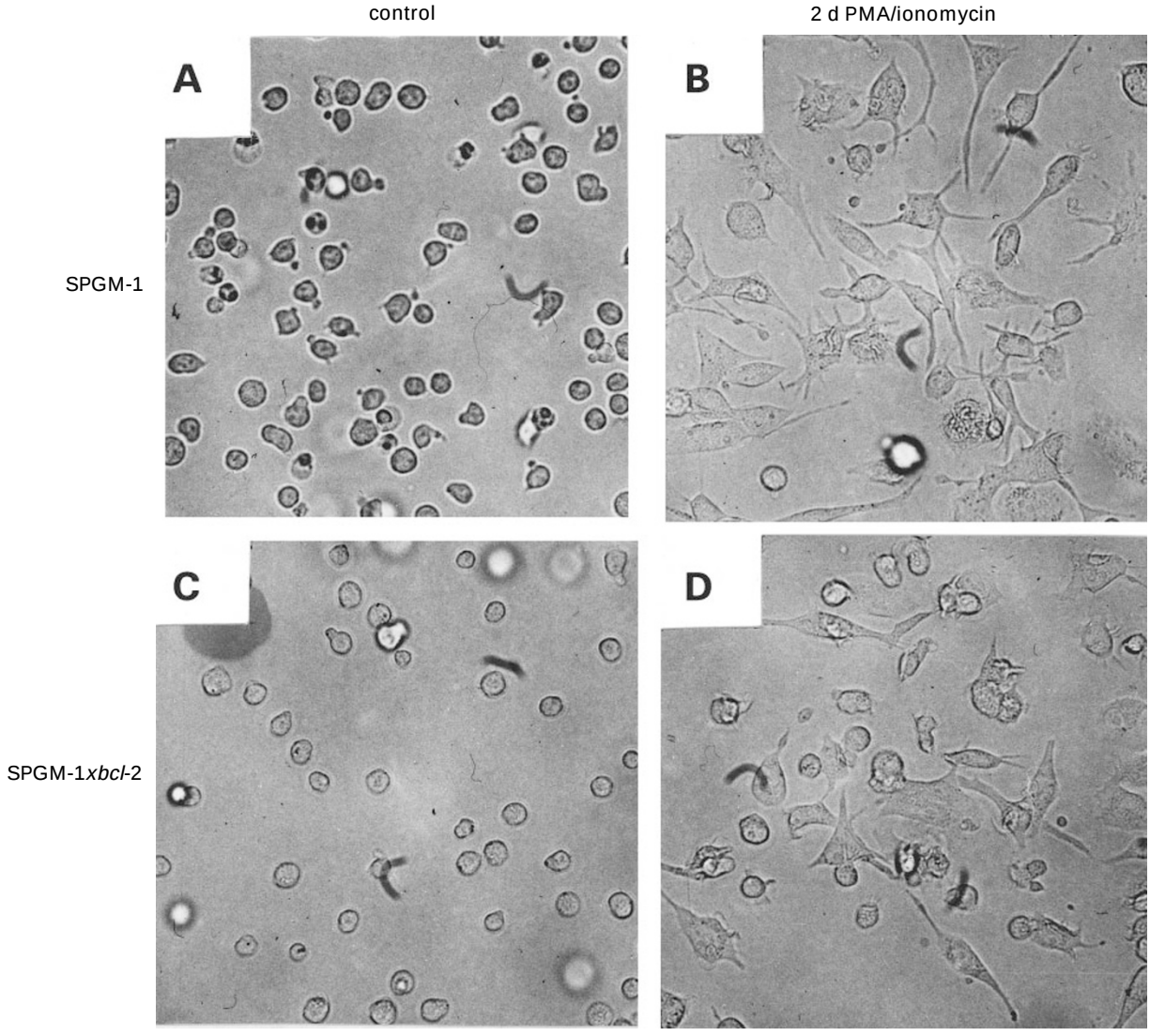

Figure 6 Morphological changes in SPGM-1 and SPGM-1*xbcl-2* cells after PMA/ionomycin-induced lineage switch. SPGM-1 and SPGM-1*xbcl-2* cells were treated with PMA (50 nM) plus ionomycin (0.5 μg/ml) for 2 days. Cells are depicted before treatment (SPGM-1: **A**; SPGM-1xbcl-2: **C**) and at the end of the incubation period. Both differentiated SPGM-1 (**B**) and differentiated SPGM-1*xbcl-2* (**D**) showed the typical morphology of adherent, macrophage-like cells (magnification 100 $\times$).

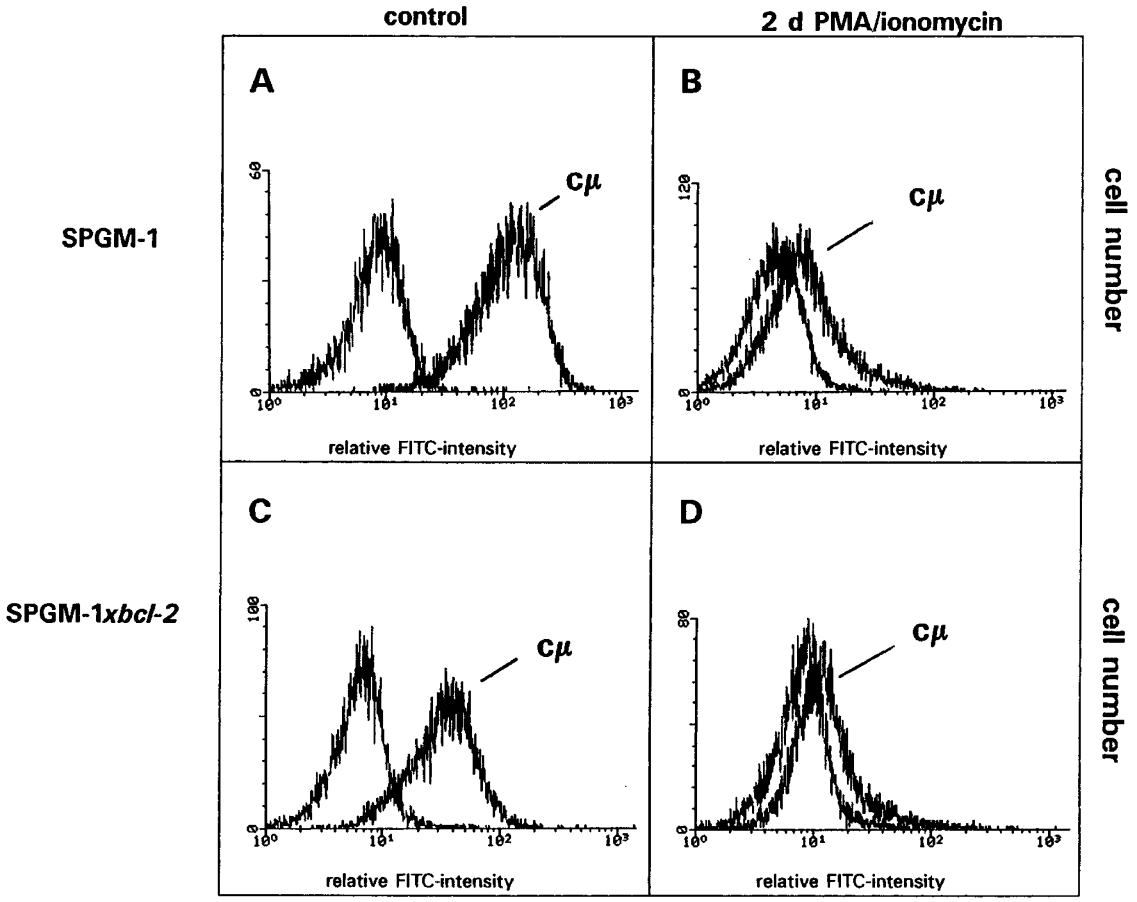

Figure 7 Loss of cμ heavy chain surface expression after PMA/ionomycin-induced lineage switch is not affected by Bcl-2. SPGM-1 (**A** and **B**) and SPGM-1*xbcl-2* (**C** and **D**) cells were either left untreated (**A** and **C**) or incubated with PMA (50 nMol/l) plus ionomycin (0.5 μg/ml) for 2 days (**B** and **D**). Thereafter the cells were stained with a FITC labeled monoclonal antibody recognizing the murine cμ heavy chain and analysed in the FACScan. The untreated cells show a shift in fluorescence with the FITC-labeled antibody compared to their autofluorescence (**A** and **C**). After the lineage switch cμ heavy chain surface expression is lost in both SPGM-1 (**B**) and SPGM-1*xbcl-2* (**D**) cells.

upon treatment with IL-3 (Martin *et al*, 1993) or a combination of calcium ionophore and phorbolester (Spencker *et al*, 1995). The aim of this study was to investigate the ability of this cell line to differentiate to a mature B-cell expressing an intact immunoglobulin antigen receptor.

SPGM-1 cells express a pre-B immunoglobulin receptor, consisting of the μ heavy chain (cμ) and the surrogate light chain, encoded by the λ5 and v-pre-B genes. Prior to the expression of conventional immunoglobulin light chains, the germline loci have to be recombined (Rolink and Melchers, 1991). This was analysed in SPGM-1 using a modified PCR technique (Schlissel and Baltimore, 1989). The results showed that although SPGM-1 cells had recombined at least one allele in V-Jκ4 configuration (Figure 3) they did not express mRNA for κ light chains (Figure 4). Concerning their immunoglobulin light chain gene, SPGM-1 cells thus exhibit the same differentiation status as the pre-B cell line 70Z/3. This cell line bears a V-Jκ1 recombined light chain gene, which is not transcribed to κ mRNA. In 70Z/3 cells κ gene transcription can be induced by either IL-1 or LPS (Sakaguchi *et al*, 1980). Attempts to induce synthesis of κ light chain mRNA in SPGM-1 by a series of reagents published to be efficient in similar systems (summarized in Table 1) failed. We reasoned that this may be due to the loss of differentiating SPGM-1 cells by apoptosis.

In order to rescue such differentiating SPGM-1 cells for analysis, we took advantage of the antiapoptotic *bcl-2* gene (Vaux and Strasser, 1996). An expression construct encoding *human-bcl-2* (Vaux *et al*, 1988) was introduced into SPGM-1 cells and permanent clonal lines established, designated SPGM-1*xbcl-2*. Bcl-2 protein expression was verified by FACS-analysis (Figure 1) and by Western-blotting (data not shown). The hu-Bcl-2 protein conveyed antiapoptotic properties to SPGM-1*xbcl-2* cells, which survived treatment with reagents known to induce apoptosis for a prolonged period of time, whereas normal SPGM-1 cells quickly died under such conditions (Figure 2). In these SPGM-1*xbcl-2* clones we readdressed the question of a possible progression to mature B-cells. A V-Jκ4 recombination was found in SPGM-1, demonstrating that at least one light chain allele was rearranged. Analysis of genomic DNA from SPGM-1*xbcl-2* clones revealed an

additional V-Jκ5 recombination (Figure 3), suggesting that either both alleles were rearranged in one individual cell or, alternatively, two cell lines were accidentally co-cultured, one with a V-Jκ4 recombination, the other with a V-Jκ5 recombination. Generation of subclones from all four original SPGM-1*xbcl-2* clones proved that this event was due to a sequential rearrangement of both alleles in a single cell, as all seven subclones of SPGM-1*xbcl-2* tested showed both recombinations (data not shown). In contrast to SPGM-1 cells, which were negative for mRNA for κ-light chains, SPGM-1*xbcl-2* cells expressed mRNA of both alleles (Figure 4). Thus, Bcl-2 expression allowed the rearrangement of the second allele and enabled the transcription of the respective genes into mRNA in SPGM-1*xbcl-2* cells.

The fact that prevention of apoptosis by overexpression of the *bcl-2* gene may lead to an ongoing recombination of the light chain loci was already observed by Rolink *et al*. (1993) who found a continued recombination of the light chain loci in primary pre-B-cells from Eμ-*bcl-2* transgenic mice after removal of IL-7 from cell cultures. We could neither detect κ-light chain protein (Figure 5) on the cell surface or intracellulary in SPGM-1*xbcl-2* cells, nor could we induce κ-light chain protein expression, supporting the notion that the transcripts identified by RT-PCR were sterile. Thus, although protection from apoptosis allowed us to detect a further attempt of SPGM-1 cells to progress towards mature B cells, i.e. the rearrangement of the second κ-light chain allele and mRNA synthesis, hu-Bcl-2 overexpression did not abolish the block in B-cell differentiation.

The potential of SPGM-1 cells to switch their lineage commitment towards macrophages was not affected by overexpression of hu-Bcl-2. The parental SPGM-1 cells as well as SPGM-1*xbcl-2* cells could be induced to switch from the pre-B cell differentiation state to macrophage-like cells, high-lighted by the acquisition of macrophage morphology (Figure 6) and the loss of the B-lymphoid marker μ-heavy chain (Figure 7).

In summary, we show that protecting SPGM-1 cells from apoptosis by overexpressing human Bcl-2 does not allow to overcome the block in B-lymphoid differentition which denies SPGM-1 to produce a mature immunoglobulin antigen receptor, consisting of μ-heavy and κ-light chains, whereas it does not affect the potential of SPGM-1 cells to switch lineages and differentiate towards macrophage-like cells. In this respect SPGM-1 cells differ from 70Z/3 cells, which can be induced by LPS to express κ-light chains and surface IgM. 70Z/3 may also switch out of their pre-B cell state to differentiate towards macrophages, however, in contrast to SPGM-1 cells, this event occurs spontaneously and with a low frequency (Tanaka *et al*, 1994).

The reason for the block in B cell differentition of SPGM-1 is unclear. Preliminary sequence analysis showed, that the V-Jκ4 junction is recombined in frame (data not shown), but other defects within the κ mRNA may be responsible for its sterility. The detailed characterisation of the differentiation block in SPGM-1 may yield new insights into the molecular mechanisms governing B-cell maturation at the crucial time point of conventional light chain expression, possibly not only important for SPGM-1 cells but also for normal B1-cell development.

Materials and Methods

Cell culture

SPGM-1 cells were grown in Iscoves-modified-Dulbeccos-medium containing 10% fetal calf serum (FCS), 2 mM L-glutamin (Glu), 1 mM pyruvate (Pyr), 1 × non-essential aminoacids (NEA) and 50 μM 2-mercaptoethanol (2-ME) at 37°C and 10% CO_2 in a humidified atmosphere. Cultures were passaged three times a week to a density of $2-2.5\times10^5$ cells/ml. To maintain the *bcl-2* expressing SPGM-1 cells ('SPGM-1*xbcl-2*'; engineered in the lab of S Cory, WEHI, Melbourne, Australia) the above medium was further supplemented with 1 mg/ml geneticin (G418; Sigma, Taufenkirchen, Germany).

Cell survival was determined by trypan blue exclusion at 1, 2, 4 and 7 days after treating *bcl-2* or vector-only infected cells with either 1 μg/ml ionomycin, 10^{-6} M dexamethasone or 1000 rad γ-irradiation.

For induction of differentiation, cells were pelleted and resuspended in fresh medium containing stimuli, alone or in combination, as listed in Table 1. Cells were either incubated alone or cocultured with irradiated ST-2 stromal cells (kindly provided by Dr S-I Nishikawa, Medical School Kumamoto, Japan) for three days under normal cell culture conditions. All other cell lines (WEHI231, 70Z/3, EL-4, ST-2) were grown in RPMI1640 medium supplemented with FCS, Glu, Pyr, NEA and 2-ME.

DNA and RNA isolation

To isolate nucleic acids 1×10^7 cells were washed in icecold PBS and the cell pellets were either immediately processed or frozen at −80°C. Genomic DNA was isolated using the QIAamp Blood Kit and RNA using the RNeasy Total RNA Isolation Kit (Qiagen, Hilden, Germany) according to the manufactuer's instructions.

First strand cDNA synthesis

1 μg of total RNA was reverse transcribed in a total volume of 30 μl using StrataScript reverse transcriptase (Stratagene, LaJolla, USA) by the protocol described by the manufacturer.

Polymerase chain reaction (PCR)

0.5 μg of genomic DNA or 5 μl of the first strand cDNA solution were used as PCR template. Amplification was carried out in a total volume of 50 μl containing 1 × PCR buffer (Gibco, Eggenstein, Germany), 2 mM $MgCl_2$ (Gibco), 0.2 mM dNTPs (Pharmacia, Uppsala, Sweden), 2 μM of each primer (custom made by Pharmacia) and 1 U Taq polymerase (Gibco). After an initial denaturation step (5 min, 94°C) 35 amplification cycles were run. Cycles consisted of one minute steps of 68°C annealing, 72°C elongation and 94°C denaturation. Resulting PCR products were separated in a 2% agarose gel and visualised by ethidium bromide staining. Specific amplification of Igκ light chain sequence was performed using a degenerated Vκ-primer, which recognises about 80% of all known Vκ elements (Schlissel and Baltimore, 1989) in combintion with a primer specific for a particular Jκ segment. Primers specific for the Jκ4 and Jκ5 elements were created based on the sequence published by Lebecque and Gerhart (1990). The κ-light chain primer sequences used are listed in Table 2a, the aldolase primers are published elsewhere (Wesche *et al*, 1996).

Table 2a Sequences of primers used to detect Ig-κ by PCR

Primer	Sequence 5′→3′
Vκ sense	GGC TGC AGS TTC AGT GGC AGT GGR TCW GGR AC
(degenerate primer)	S=G or C; R=A or G; W=T or A
Jκ 4 antisense	TAT TTC CAA CTT TGT CCC CGA GC
Jκ 5 antisense	CTT GGT CCC AGC ACC GAA CGT GAG C

Table 2b Expected length of PCR products

Recombination: primer-pair	Vκ-Jκ1	Vκ-Jκ4	Vκ-Jκ5
Vκ+Jκ4	1100 bp	140 bp	
Vκ+Jκ5	1500 bp	500 bp	140 bp

Flow cytometry

Immunoglobulin κ light chain and μ heavy chain molecules were analyzed by direct immunofluorescence. Cells were washed with PBS and then labelled by a standard procedure using FITC-conjugated monoclonal antibodies (Dianova, Hamburg, Germany) and analyzed in a FACScan II flow cytometer (Becton Dickinson, Heidelberg, Germany). Intracellular proteins were stained according to a previously described method by Assenmacher *et al*. (1994). In short: PBS washed cells were treated with pepsin (1 mg/ml, 10 min, 37°C), washed and then fixed with 2% formaldehyde and then incubated with 20 μg/ml of anti κ light chain antibody in 0.5% bovine serum albumin, 0.02% NaN_3, and 0.5% saponin. The human Bcl-2 protein was identified using a specific antibody, Bcl-2-100 (Pezzella *et al*, 1990) and a FITC labeled goat anti mouse IgG (Southern Biotechnology) as second reagent.

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