CD5 Provides Viability Signals to B Cells from a Subset of B-CLL Patients by a Mechanism That Involves PKC

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Abstract

B-chronic lymphocytic leukaemia (B-CLL) is a heterogeneous disease characterized by an accumulation of B lymphocytes expressing CD5. To date, the biological significance of this molecule in B-CLL B cells remains to be elucidated. In this study, we have analysed the functional consequences of the binding of an anti-CD5 antibody on B-CLL B cells. To this purpose, we have measured the percentage of viability of B-CLL B cells in the presence or in the absence of anti-CD5 antibodies and also examined some of the biochemical events downstream the CD5 signalling. We demonstrate that anti-CD5 induces phosphorylation of protein tyrosine kinases and protein kinase C (PKC), while no activation of Akt/PKB and MAPKs is detected. This signalling cascade results in viability in a group of patients in which we observe an increase of Mcl-1 levels, whereas the levels of bcl-2, bcl-x_L and XIAP do not change. We also report that this pathway leads to IL-10 production, an immunoregulatory cytokine that might act as an autocrine growth factor for leukaemic B cells. Inhibition of PKC prevents the induction of Mcl-1 and IL-10, suggesting that the activation of PKC plays an important role in the CD5-mediated survival signals in B cells from a subset of B-CLL patients.

*Keywords***:** Signal Transduction; Kinases/Phosphatases; B-CLL; Apoptosis; B cells

1. Introduction

B-chronic lymphocytic leukaemia (B-CLL) is a lymphoproliferative disorder characterized by a monoclonal expansion of long-lived CD5+ B lymphocytes expressing low levels of surface immunoglobulin [1,2]. Despite their prolonged survival in vivo, B-CLL cells undergo spontaneous apoptosis when cultured in vitro. This points to the importance of the cellular microenvironment and the role of non-leukaemic cells in the biology of leukaemic cells [3-5]. Therefore, it is reasonable to assume that membrane-receptor signalling plays a critical role in maintaining the expansion and survival of the malignant cells. However, signalling pathways responsible for the prolonged survival are poorly understood. A full understanding of the pathways related to apoptosis resistance in these cells is critical in understanding the disease process.

CD5 (Ly-1, T1/Leu-1) is a monomeric type I transmembrane glycoprotein of 67 kDa that belongs to the scavenger receptor cysteine-rich (SRCR) family. This molecule is expressed on thymocytes and T cells, B1-a cells, and subpopulations of mature and activated B cells [6] . Proposed counter-receptors for CD5 include the B cell-specific CD72 [7], the gp40-80 [8,9], the IgV_H framework regions [10,11], and another broadly expressed cell surface protein [12]. The functional significance of these candidates in relation to CD5 activation has not been established.

Biochemical studies suggest that CD5 is associated with both the TCR complex [13] and the BCR complex [14]. CD5 possesses a large cytoplasmic domain containing multiple potential phosphorylation sites compatible with a function in signal transduction [15,16]. It has been proposed that CD5 plays an important role in transducing signals that are relevant to thymic development and immune response [17,18]. Although the precise role for CD5 in regulating immune responses remains uncertain, studies in CD5-deficient mice suggested a negative role of the molecule on

TCR [19] and BCR [20] signalling. TCR and BCR signal transduction occurs via activation of protein tyrosine kinases (PTKs), including members of the Src-family and Syk or ZAP-70 [21]. CD5 seems to negatively regulate antigen receptor-mediated signal transduction through recruitment of SHP-1, which dephosphorylates the PTKs activated after antigen receptor cross-linking [15, 22].

In addition to its role in inhibiting the antigen receptor-triggered signalling, it has been proposed that CD5 is an accessory molecule with its own signalling pathway. Gringhuis et al. [23] observed that the enhanced activation of the Ca^{2+}/calmodulin-dependent kinase type IV (CAM kinase IV) through CD5 costimulation was associated with an increase of the AP-1 activity at the interleukin (IL)-2 promoter in T lymphocytes, resulting in an elevated transcription and expression of the IL-2 gene. Subsequently, they presented evidence about the PI3K activation upon ligation of CD5 [24]. In another report, it was found that anti-CD5 mAbs induce the phosphatidylcholine-specific phospholipase C (PC-PLC)-dependent activation of acidic sphingomyelinase (A-SMase) in normal and lymphoblastoid T and B cells. Also, activation of protein kinase C-ζ (PKC- ζ) through diacylglycerol (DAG) and members of the MAPK cascade seem to be downstream events of the CD5 signalling pathway [25]. In B-CLL there are few studies to date which have addressed the possible role of CD5 molecule in activation or proliferation processes, the activation of A-SMase has been shown to occur after CD5 cross-linking [25], as well as the induction of IL-2 and a discrete proliferation of leukaemic B cells [26]. In contrast, the majority of works have reported CD5-induced apoptosis in these lymphoproliferative disease [27-29]. There are few data concerning CD5 signalling pathway in B-CLL, but recently Renaudineau et al. [30] have proposed that signals from CD5 could be transduced via CD79 in the vicinity of the BCR.

In the present study, we have investigated the effect of CD5 ligation on B-CLL B lymphocytes survival, and demonstrated a heterogeneous behaviour of leukaemic B cells in their susceptibility to CD5-mediated survival. We also set out to elucidate the signalling pathways induced by CD5 stimulation. We present evidence that CD5 ligation induces both Mcl-1 and IL-10 expression through a PKC-dependent pathway, which results in increased viability of B cells from a group of B-CLL patients.

2. Materials and methods

2.1. Patients and controls

After informed consent, heparinized peripheral blood samples were obtained from 50 patients with B-CLL diagnosed according to established morphologic and immunophenotypical criteria [31]. The diagnosis of B-CLL relied on characteristic cytological features of mature lymphocytes and a characteristic phenotype (CD5+, CD23+, FMC-7-, and low expression of surface immunoglobulin and CD79b). All patients were untreated or had not received chemotherapy for at least 6 months prior the study. These cases were collected from Haematology Units of the San Carlos and La Paz University Hospitals, Madrid, Spain. Tonsils were obtained from 7 routine tonsillectomy specimens from Otorhinolaryngology Unit of Puerta de Hierro University Hospital and were used as controls.

2.2. Cell isolation and culture

Tonsils were mechanically dispersed in PBS (Amresco, Solon, Ohio). PBMCs from heparinized peripheral blood B-CLL and from manually disaggregated tonsils were isolated by density gradient centrifugation (Comercial Rafer S.L., Zaragoza, Spain). CD19-positive cells of patients were isolated by negative selection using anti-CD2 and anti-CD14-conjugated immunomagnetic beads (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany). Tonsil T cells were depleted by rosetting with 2-aminoethylisothiourionium bromide (AET)-treated sheep erythrocytes (Sigma-Aldrich, St. Louis, MO and Oxoid S.A., Madrid, Spain, respectively). Percentages of CD19-positive cells were 97-100% in all cases, as determined by flow cytometry.

All studies were performed on freshly isolated leukaemic and normal B cells. After purification, B cells were cultured at 1 x 10^6 cells ml^{-1}, at 37ºC in a humidified 5%

CO_2 atmosphere in RMPI 1640 medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (all from Gibco, Life Technologies Inc., Rockville, MD). In the case of the tonsil cells, 15 μg ml^{-1} gentamycin (Schering-Plough, Kenilworth, NJ) were added. For stimulation experiments, the following reagents were added, as indicated: 1 μg ml^{-1} anti-CD5 monoclonal antibodies (mAbs) or 10 ng ml^{-1} PMA (Sigma-Aldrich). When the PKC inhibitor bisindolylmaleimide I (Bis I) (Calbiochem-Novabiochem Corporation, San Diego, CA) was used, cells were preincubated with Bis I in RPMI 1640 medium for 1 h prior to stimulation. Bis I was used at 1 μM dissolved in DMSO (final concentration of DMSO did not exceed 0.05%). In these experiments 0.05% DMSO was added to the control wells that contained the culture medium.

2.3. Antibodies

For immunophenotypic analyses mAbs against the following human molecules were used: CD3, CD5, CD16, CD56, CD14, CD45, HLA-DR (all from BD Biosciences, San Jose, CA), and CD19 (Caltag Laboratories, Burlingame, CA). For stimulation experiments, B cells were cultured with UCHT-2 anti-CD5 mAbs (BD Pharmingen, San Diego, CA) or with a control antibody (clone BZ-1; Diaclone Research, Besançon, France). The 4G10 FITC-anti-phosphotyrosine mAb (Upstate Cell Signaling Solutions, Lake Placid, NY) was used to determine the tyrosine-phosphorylated proteins by flow cytometry and Western blot. Antibodies against Mcl-1 and β-actin (both from Sigma-Aldrich), XIAP (MBL International Corporation, Woburn, MA), phospho-$PKC^{(Ser660)}$, phospho-$Akt^{(Ser473)}$, phospho-$SAPK/JKN^{(Thr183/Tyr185)}$, phospho-p44/42 (ERK) $MAPK^{(Thr202/Tyr204)}$, phospho-p38 $MAPK^{(Thr180/Tyr182)}$, Akt, SAPK/JNK, p44/42 (ERK) MAPK and p38 MAPK (all from New England Biolabs, Beverly, MA), and PKC (α, β,

γ; Upstate Cell Signaling Solutions) were used in Western blot analyses.

2.4. Flow cytometry

Immunophenotypic analysis was performed on cells immediately after separation or at different times in culture, as indicated. First, all antibodies were titrated to determine the amount that identified positive cells with the optimal mean fluorescence intensity (MFI). Surface staining was performed by direct immunofluorescence [32] and tyrosine-phosphorylated protein expression was determined by staining with a FITC-labelled anti-phosphotyrosine mAb. Flow cytometric analyses were performed on a FACSort using the CellQuest® research software (BD Immunocytometry Systems, San Jose, CA). Surface CD5 expression was calculated as the ratio of the MFI of antibody binding to the surface molecule relative to the MFI of non-specific binding of the corresponding isotype 'control' antibody.

2.5. Apoptosis assays

B cells were cultured in the presence or in the absence of anti-CD5 mAbs, and then stained with FITC-conjugated annexin-V (Roche Diagnostics Gmbh, Mannheim, Germany) and propidium iodide (PI) (Sigma-Aldrich). Following 15 min of incubation in the dark, cells were analysed by flow cytometry. Cell viability was measured as the percentage of annexin-V and PI-double negative cells (AV^-/IP^-). In selected experiments, data were expressed as the increase in anti-CD5-induced viability relative to spontaneous apoptosis: (percentage of AV^-/PI^- cells treated with anti-CD5 - percentage of AV^-/IP^- cells incubated in medium alone).

2.6. Determination of IgV_H mutational status

Total RNA was extracted from 3 to 5 x 10^6 B cells using guanidinium thiocyanate method (TRI_{ZOL}, Gibco). The obtained RNA was reverse transcribed into cDNA using 20 U AMV reverse transcriptase (Roche), following the manufacturer's instructions. The PCR reactions were set up in a GeneAmp PCR System 2400 cycler (Perkin Elmer Applied Biosystems, Foster City, CA) for each patient using one each six V_H1-6 or four J_H1-6 family-specific primers in combination with a J_H or V_H primer mix, respectively. These primers had been previously described [33]. The conditions of the PCR reactions were: one cycle of denaturation at 94ºC for 5 min, followed by 35 cycles of denaturing at 94ºC for 1 min, annealing at 56ºC for 1 min and extension at 72ºC for 1 min, with a final extension step at 72ºC for 10 min. The PCR products were then analysed by gel electrophoresis on a 2% agarose gel, excised and purified (MinElute Gel Extraction Kit, Qiagen, Hilden, Germany). Purified products were sequenced directly in a capillary sequencer (ABIPRISM 3730, Perkin Elmer Applied Biosystems). Nucleotide sequences were analysed by means of Chromas 1.45 software (Queensland, Australia) and compared with those available in the BLAST and IMGT databases. Sequences with more than 98 percent homology to the corresponding germ-line IgV_H sequence were considered unmutated.

2.7. Analysis of bcl-2 family and cytokine gene expression

bcl-2 family genes were PCR amplified as previously reported [29] from cDNA synthesized as described above. For the cytokine analysis the PCR mixture was identical to bcl-2 family genes and the primers were as follows: for the IL-2 gene forward 5'TGCAACTCCTGTCTTGCATT3' and reverse 5'ATGGTTGCTGTCTCATCAGC3', and for the IL-10 gene forward 5'GGCACCCAGTCTGAGAACAG3' and reverse

5’GCCTGAGGGTCTTCAGGTTC3’ (TIB MOLBIOL, Berlin, Germany). Amplifications were carried out, after the initial denaturation step for 5 min at 95ºC, for 28 cycles of 45 s at 95ºC, 45 s at 60ºC, and 2 min at 72ºC, with a final extension of 10 min at 72ºC. In total, 10 µl of each reaction was resolved on 2% agarose gels (Cambrex Bio Science, Rockland, ME), and bands were visualized by ethidium bromide staining (Sigma-Aldrich). In each case, 18S rRNA primers and competimers (QuantumRNA™ 18S, Ambion Huntingdon, UK) were added into the PCR reaction as standard for relative quantitation. Densitometric analyses were performed by using the Quantity One software system (Bio-Rad Laboratories, Hercules, CA).

2.8. Western blot analysis

B cells were lysed on ice for 20 min in lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% NP-40) containing 10 µg ml^{-1} aprotinin, 10 µg ml^{-1} leupeptin, 10 µg ml^{-1} pepstatin A, 2 mM PMSF, and 1 mM sodium orthovanadate (all from Sigma-Aldrich). The concentration of total proteins was determined using a kit from Pierce (Rockford, IL) and albumin as a standard. Cell lysates containing 10-15 µg of total proteins were resolved by 10% SDS-PAGE, transferred to nitrocellulose polyvinilidene difluoride (PVDF) membranes by using a semidry transfer system (both from Amersham Biosciences AB, Uppsala, Sweden), and blocked with 5% non-fat powdered milk in wash buffer (PBS, 1% Tween-20) for 2 h at room temperature. In the case of phosphoproteins, membranes were blocked with 5% BSA (Sigma-Aldrich). The PVDF membranes were then probed with primary antibodies and incubated overnight at 4ºC. After washing with 0.1% Tween-20 containing PBS, reactions were visualized with goat anti-mouse or anti-rabbit (both from Sigma-Aldrich) secondary antibodies conjugated with horseradish peroxidase (HRP) for 1 h. The binding of HRP was

detected by enhanced chemiluminiscence (ECL+Plus; Amersham) and exposure to film. For some experiments, blots were reprobed after exposure to stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris, pH 6.8) for 20 min at 55ºC. β-actin served as an internal loading control and rainbow markers (Amersham) as standard molecular weights.

2.9. Proliferation assays

Purified B lymphocytes (1 x 10^6 cells/ml) that were either non-treated or stimulated with anti-CD5 mAbs were cultured for 72 h at 37ºC in triplicate in flat-bottom 96-well plates (Costar, Cambridge, MA) under an atmosphere of 5% CO_2 in air. The cells were subsequently pulsed with BrdU (Sigma-Aldrich) and harvested 18 h later. The incorporation of BrdU was measured by staining with a FITC-conjugated anti-BrdU antibody (clone B44, BD Biosciences) and analysed by flow cytometry.

2.10. ELISA immunoassay

B cells were resuspended in culture medium at 1 x 10^6 ml^{-1} and distributed in flat-bottom 96-well plates, were incubated at 37ºC with anti-CD5 mAbs. After 48 h cell-free supernatants were harvested and assayed for IL-2 and IL-10 on an ELISA, following the manufacturer's instructions (Quantikine®; R&D Systems, Minneapolis, MN). The detection limits for IL-2 and IL-10 were 7 and 0.5 pg ml^{-1}, respectively.

2.11. Statistical analysis

Results were expressed as mean ± SD and SPSS v.10 software for Windows (SPSS, Chicago, IL) was used for data analysis. To assess the changes in apoptosis after CD5 stimulation, data was compared using the paired Student's *t* test when

normally distributed or the paired Wilcoxon´s test when not. Spearman rank correlation coefficients were used to identify relationships between quantitative variables. Differences were regarded as significant when $p < 0.05$.

3. Results

3.1. Surface CD5 engagement protects to B-CLL B cells from apoptosis in vitro in a group of patients

To determine the consequences of surface CD5 engagement in B-CLL, freshly isolated B lymphocytes from 7 B-CLL patients were cultured in medium or in the presence of increasing concentrations of anti-CD5. Subsequently, cell viability was measured every 24 h for up to 144 h by staining with annexin-V/PI and analysed by flow cytometry. We observed dose-dependent inhibition of apoptosis over the range of 0.25 to 2 μg ml^{-1} only in a group of patients (Fig. 1A). Taking into account these data, we used the 1 μg ml^{-1} dose of UCHT-2 anti-CD5 and 120 h of culture time to evaluate the changes in CD5-mediated response.

We next cultured B cells from 44 patients and 7 tonsils in medium or in the presence of 1 μg ml^{-1} anti-CD5 mAbs. Apoptosis was measured by the annexin-V/PI method and CD5-stimulated cell apoptosis was compared to spontaneous apoptosis. We considered that anti-CD5 protected from spontaneous apoptosis when the increment of viability at 120 h between both conditions was ≥ 8% (median value). Of the 44 patient samples, 19 showed a significant decrease in apoptosis in the CD5-stimulated B cells (58 ± 18 vs. 42 ± 18; $p < 0.0001$), 4 an increase (41 ± 28 vs. 54 ± 26; $p = 0.068$), and no change in the remaining 21 patients was observed (34 ± 26 vs. 32 ± 27) (Fig. 1B). Contrary to previously described [34), anti-CD5 did not modify the percentage of apoptosis in B cells from any tonsil sample (15 ± 13 vs. 17 ± 13). As the percentage of CD5 expression in tonsil B cells used as controls were 30-50%, we could not affirm completely that CD5 had no effect on the viability of this cells. Due to this it seemed to be better to compare the response to CD5 among the different patients classified as non responders, viability and apoptosis group. To ensure that our results were not a non-

specific effect of the antibody-Fc fragment, IgG1 control Ab was added to B cells from patients and controls cultured with medium alone. We observed no effect in apoptosis, for this reason, the culture condition with IgG1 control antibody was denominated as "medium". We also observed that anti-CD5-induced response was independent of extensive CD5 cross-linking (data not shown).

Since previously published data suggested that CD5 triggering could enhance the proliferation of B-CLL B cells [26], we next checked if the extended survival observed in some samples was due to proliferation. Cell proliferation in response to anti-CD5 was quantified by BrdU incorporation and staining with a FITC-conjugated anti-BrdU antibody. The results showed no proliferation in any of the 6 cases analysed (data not shown).

We then examined the relationship between CD5-mediated response and surface CD5 expression in 44 B-CLL patients. No correlation was observed, indicating that the effect observed in CD5-stimulated cells did not depend on the amount of CD5 expressed in these cells (r = -0.079, p = 0.611).

It is now well known that B-CLL is a very heterogeneous malignancy with a variable clinical course. B-CLL patients can be segregated into 2 subtypes based on the IgV_H gene mutational status, one group that expresses unmutated IgV_H and that it is strongly associated with a poor clinical course and another group with mutated IgV_H and a more favourable prognosis [35,36]. For this reason, we also analysed the mutational status of 19 B-CLL patients in relation to CD5-mediated response. Although we did not observe significant differences among the groups of response, the percentage of mutated cases was higher in the apoptosis and viability groups (3/3 and 9/10, respectively) than in non responders to CD5 (2/6). These findings suggested that mutated IgV_H B-CLL samples were more sensitive to CD5 stimulation.

3.2. PKC, but not Akt/PKB and MAPKs, is involved in the CD5-induced signalling pathway

Subsequently, we tried to verify whether ligation of CD5 in B-CLL was followed by some of the biological events previously described in other cell types [24,25,37]. The earliest recognizable event after receptor engagement by its ligand is the induction of PTK phosphorylation [38]. Besides this, signals mediated by phosphotyrosine proteins may have effects on B-CLL cell survival, because downregulation of these signals has been shown to be associated with a high frequency of apoptotic cells [39]. Then we examined whether CD5 ligation could induce tyrosine phosphorylation of cytosolic proteins in B-CLL samples. We investigated global tyrosine protein phosphorylation in purified B cells by flow cytometry and immunoblot analysis in 6 B-CLL patients. All leukaemic cells analysed displayed abnormally high tyrosine-phosphorylated proteins, as previously reported [40,41]. For this reason, to evaluate the relative increase in phosphotyrosines we considered the changes in MFI or in the density of bands identified on the immunoblot after CD5 stimulation (Fig. 2). The results showed that binding of anti-CD5 mAbs increased phosphorylation of PTKs in every case tested, with a maximum at 30 min ($p = 0.026$) (Fig. 2B).

These signalling events may lead to activation of kinases such as PI3K, PKC and several MAPKs, as reported in T lymphocytes [24,25]. Some of these kinases have been proposed to have an essential role in B-CLL B cell survival [29,42-45]. We aimed to asses whether some of such kinases were regulated by CD5 in B-CLL. For this purpose, we analysed the activation of PKC and the 3 MAPKs using antibodies against phosphorylated PKC, SAPK/JNK, p44/42 (ERK) MAPK and p38 MAPK, while activation of PI3K was studied using antibodies against phosphorylated Akt/PKB,

which has been identified as the direct downstream substrate of PI3K. This experiment was performed by Western blot in 6 B-CLL patients at 30 min and 48 h. Although we detected a basal PKC phosphorylation in all cases, as previously described [43], a significant increase in PKC phosphorylation was detected in CD5-stimulated B cells in 5 of 6 samples studied at 30 min as well as at 48 h ($p = 0.043$) (Fig. 3A), indicating the maintaining of these signal throughout the time. On the contrary, no appreciable effect on phosphorylation in the rest of molecules analysed was detected in any sample (Fig. 3B).

3.3. CD5 modulates Mcl-1 protein expression in B-CLL B lymphocytes

Members of the Bcl-2 protein family have been demonstrated to be directly related to apoptosis induction or resistance [29,46,47]. In addition, it has been reported that CD5 stimulation regulates the Bcl-2 protein family expression in B-CLL B cells [27,28]. We examined the levels of bax, bcl-2, bcl-x_L and mcl-1 gene expression in B-CLL after CD5 ligation. For these experiments, purified B cells from 5 B-CLL patients were cultured in medium or in the presence of anti-CD5 mAbs. After 48 h, B cells were harvested and mRNA levels were analysed by RT-PCR. We only found variation in the expression of mcl-1 after CD5 stimulation compared with cells cultured in medium alone (Fig. 4A).

We next studied the expression of Mcl-1 protein by Western blot. As seen in Fig. 4B, Mcl-1 expression was increased after CD5 stimulation in 5 of 6 B-CLL patients studied ($p = 0.043$). Densitometric analysis of Mcl-1 content in CD5-stimulated leukaemic B cells showed a 2.3-fold increase relative to non-stimulated B cells (range: 1.5- to 3.9-fold).

Many in vitro experiments have revealed other anti-apoptotic proteins that are thought to be relevant for the survival of these leukaemic cells, one of them is XIAP, a member of the inhibitor of apoptosis protein (IAP) family [29,44]. We examined the XIAP protein expression in 3 cases of B-CLL. The results showed that this protein was slightly induced in only 1 patient after CD5 stimulation, indicating that XIAP seems not to be a target of the CD5 signalling pathway in these leukaemic B cells.

We then wanted to determine if PKC activation was related to the induction of Mcl-1, for this B-CLL B cells from 3 patients were preincubated with 1 μM of the PKC inhibitor Bis I for 1 h and then stimulated with anti-CD5 mAbs. We used a PKC activator, PMA, as a positive control. Bis I at concentration of 1 μM had no cytotoxic effect on B-CLL cells, but a slight decrease in Mcl-1 expression was observed (data not shown). This could be reflecting because of the constitutive activation of PKC in B-CLL B cells, as previously described [43]. Densitometric analysis of RT-PCR products showed that both anti-CD5 and PMA induced a clear increase in mcl-1 expression, and this increase was completely arrested by Bis I in the case of anti-CD5 and partially in the case of PMA stimulation (data not shown). When we analysed the Mcl-1 protein induction by Western blot, the same results were observed, Mcl-1 augmented in 2 of the 3 samples studied and this increase was inhibited by the addition of Bis I (Fig 5).

3.4. CD5 modulates IL-10 expression

It has been shown that stimulation with anti-CD5 mAbs in B-1 and B-CLL B cells increase IL-2 secretion [26], cytokine that may be involved in the regulation of viability in an autocrine fashion [48]. Regarding this, we proposed to analyse the possible implication of IL-2 in CD5-mediated survival in B-CLL patients. For this, purified B cells from 6 B-CLL patients were stimulated with anti-CD5 mAbs. After 48

h cells were lysed to extract RNA and supernatants were harvested and frozen at –20ºC. IL-2 gene expression was determined by RT-PCR and cytokine secretion was measured by ELISA. Of the 6 samples studied only 1 expressed transcripts for IL-2. However, when we analysed the protein secretion of IL-2, we observed no production in any case (data not shown). To study the contribution of PKC in CD5-mediated IL-2 induction, we cultured the B cells in the presence or absence of the PKC inhibitor Bis I. In this case PKC was also implicated, as showed the complete inhibition of IL-2 mRNA expression when Bis I was used (Fig. 6B, top panel).

Subsequently, we wished to check the contribution of another cytokine, IL-10, because it has been described that CD5+ B cells produce more IL-10 in vitro after activation than the CD5- subset, and this production seems to depend on the constitutive expression of CD5 [49]. For this reason, we repeated the above experiment by measuring the mRNA levels and the protein secretion of IL-10. The results represented in Fig. 6 indicate that anti-CD5 up-modulated IL-10 mRNA expression in B-CLL cells. We observed higher levels of mRNA of this cytokine in 4 of 6 cases analysed (Fig. 6A) and an increase in protein production in 3 of these samples (data not shown). To further asses the implication of PKC in CD5-mediated IL-10 induction, we cultured leukaemic B cells in the presence or absence of Bis I. Selective inhibition of PKC resulted in suppression of IL-10 production. These data demonstrate the role of CD5 as an inducer of IL-10 mRNA and protein synthesis in B-CLL B cells and the implication of PKC in this pathway.

Interestingly, in a sample in which anti-CD5 induced a decrease in Mcl-1 expression, IL-10 expression was downmodulated. Furthermore, when we cultured with Bis I, this expression was recovered (data not shown), suggesting PKC implication in CD5-pathway when anti-CD5 provides both apoptotic and survival signals.

Based on these findings, we conclude that PKC activity seems to be necessary for Mcl-1 induction, IL-10 production and survival of B-CLL upon the CD5 stimulation.

4. Discussion

It is now clear that B-CLL is a heterogeneous disease at the clinical level as well as at molecular and cellular levels [35,36,50-52]. Probably, reflecting the clinical heterogeneity observed in patients, several signalling pathways may become affected during the initiation and course of this lymphoproliferative disorder. According to this, B-CLL cells have shown heterogeneous responses to stimulation via cell surface receptor including CD40 [53], BCR [40,54-57] and CD5 [28,26,27]. Here, we have demonstrated that CD5-signalling pathway is also heterogeneous but, in contrast with previously published data, we found that this pathway can induce viability in some patients. Previous works have reported that anti-CD5 treatment of B-CLL B cells results in apoptosis of these cells [27,28]. There are several significant methodological differences between these studies and ours. In their work, Pers et al. measured the apoptosis level after 18 or 36 h and Cioca and Kitano after 6, 12 or 24 h, whereas we extended the culture time to 120 h. Also, the concentrations used in the two published studies were higher than in our work (10, 15 and 1 $\mu g\ ml^{-1}$, respectively). If we analysed the first 24 h in our assays, the results were comparable to published series, we also observed apoptosis or no change in viability. We also detected an increase in apoptosis when anti-CD5 mAbs at 10 $\mu g\ ml^{-1}$ were used. Then, it seems that the differences in culture time and mAb concentration could explain the discrepancy between our results and those of Pers et al. and Cioca and Kitano. On the other hand, our findings are in agreement with a previous study in which is described that CD5 is capable of transducing positive signals [26]. In this work, Cerutti et al. showed that CD5 engagement delivers critical costimulatory signals in B-CLL B cells, as well as in B-1a and B-2 lymphocytes, although with different requirements and patterns. They also demonstrated that this costimulatory effect was associated with IL-2 production and a

slight proliferation. We only detected IL-2 mRNA in 1 of 6 patients analyzed and no proliferation in any case, probably reflecting again methodological differences between both studies. One possible explanation about CD5-induced viability observed by us is that low doses of anti-CD5 might indirectly stimulate the BCR through the previously demonstrated interaction [10,11] of the IgV_H framework regions with CD5, providing leukaemic B cells with activation signals and limited expansion in the absence of stimulation by environmental antigen [58]. These effects on survival might be relevant to B-CLL cells in vivo.

We have also analysed the signal transduction pathways triggered by anti-CD5 MoAbs. In the current study, we provide evidence supporting the implication of PKC in CD5-mediated response in B-CLL B cells. As previously reported [43], we found that the specific PKC inhibitor Bis I induces apoptosis in B-CLL cells, confirming a constitutive expression of PKC. Moreover, it is known that further activation of PKC plays an important role in the survival of B-CLL cells by enhancing the expression of anti-apoptotic proteins such as XIAP and Mcl-1 [59]. High levels of the anti-apoptotic protein Mcl-1 are commonly found in circulating B-CLL cells and might play an important role in the pathophysiology of this disease. In addition, previous studies have demonstrated that Mcl-1 expression is related to a failure to achieve a complete remission to chemotherapy [60]. Here, we have demonstrated that anti-CD5 increases Mcl-1 expression in most of the patients, whereas XIAP only in one case. We also found that the inhibition of PKC abrogates the augmentation of Mcl-1 and survival induced by anti-CD5, indicating the role of PKC in both processes. Since B cells express multiple PKC family members, including PKCα, βI/II, δ, ε, μ, ζ and λ [61] and to better understand the mechanism involved in CD5-mediated survival, it would be of

interest to elucidate the PKC isoform(s) involved in this pathway, which turn out to be a good target for therapy.

Cytokines are known to enhance not only the growth of the leukaemic B-CLL B cells in vitro but also promote the survival of these cells. It has been reported that IL-2 acts as a viability factor in B-CLL preventing the leukaemic cells from undergoing apoptosis in vitro [62], as well as enhancing and sustaining proliferation of B-CLL cells [63]. Several groups have also established the implication of IL-2 in CD5 signalling pathway in T cells [24] and B-1 and B-CLL B cells [26]. Contrary to what was expected, we have only found mRNA expression of this cytokine in one B-CLL patient and no protein expression in any case, indicating that IL-2 seems not to play a crucial role in CD5-mediated viability. However, our results suggested that IL-10 contribute to the enhancement of B-cell survival mediated by CD5 in B-CLL. Further IL-10 blocking experiments would be required in order to check whether Mcl-1 increase is a downstream event of IL-10-mediated response.

A number of studies have documented the role of IL-10 as a survival factor for human B-cells. Levy and Brouet [64] showed that IL-10 was able to rescue B cells of germinal centre from apoptosis by inducing Bcl-2 protein. IL-10 may act as an autocrine growth factor for B cells based on its stimulatory effects on in vitro B cell growth and differentiation [65,66]. Similar effects of IL-10 have been observed on CD40 activated B-CLL cells [67]. On the other hand, the development of CD5+ B cells appears to be regulated in part by IL-10 [68], as it has been described that CD5+ B cells produce more IL-10 in vitro after activation than the CD5- subset, and a constitutive expression of CD5 seems to be a requisite for this production [49]. However, available data regarding the role of IL-10 in the development and progression of B-cell malignancies are conflicting. Several in vitro studies have showed that IL-10 induces apoptosis in

unstimulated B-CLL B cells [69,70], since others observed that in some cases survival of B-CLL B cells was clearly higher in the presence of IL-10 [71].

In vitro data demonstrate that cytokines produced by B-CLL cells modulate the environment in which they accumulate [72,73]. In addition, these malignant cells have multiple points of contact with its neighbours and CD5 constitutes one of these points. Because there are several different ligands that appear to interact with CD5 [7,8, 10,12], regulation of signalling in B cells by CD5 probably differs depending on the origin of the cells, their differentiation stage and microenvironment [26]. It is possible that different signalling pathways could determine whether the CD5-ligand interaction results in viability rather than in apoptosis. Then, CD5 would be one of the survival factors contributing to the expansion of the malignant clone and perhaps to the reduced response to chemotherapy in vivo. These considerations raise important questions concerning the role of CD5 in B-CLL. Regarding this, it would be interesting to study the CD5-mediated response by using the natural ligands but, to date, none of the ligands for CD5 have been shown to be functional in vitro [74].

In conclusion, we have provided some amount of data that suggest the CD5 contribution to the survival of leukaemic B-CLL cells by a mechanism that involves PKC phosphorylation and Mcl-1 and IL-10 production. The recognition of intracellular targets that inhibit apoptosis in B-CLL B cells and cytokines that provide a favourable environment are of significance in order to develop more specific and definitive treatment strategies for this disease.

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Contributions: All the authors contributed significantly to this study. G. Perez-Chacon designed, executed and analysed the experiments, and wrote the paper including the final version. J.A. Vargas, S. Rosado, T. Martin-Donaire and I. Losada-Fernandez contributed in the analysis and interpretation of the data. J. Jorda and M Morado provided B-CLL samples. P. Perez-Aciego was involved in the project conception and design and gave the approval to the final version of the manuscript. G. Perez-Chacon takes primary responsibility for the paper. Authors reported no potential conflicts of interest.

References

[1] Keating MJ, Chiorazzi N, Messmer B, Damle RN, Allen SL, Rai KR, et al. Biology and treatment of chronic lymphocytic leukemia. Hematology (Am Soc Hematol Educ Program) 2003;:153-75.

[2] Chiorazzi N, Rai KR, Ferrarini M. Chronic lymphocytic leukemia. N Engl J Med 2005;352(8):804-15.

[3] Lagneaux L, Delforge A, Bron D, De BC, Stryckmans P. Chronic lymphocytic leukemic B cells but not normal B cells are rescued from apoptosis by contact with normal bone marrow stromal cells. Blood 1998;91(7):2387-96.

[4] Caligaris-Cappio F. Role of the microenvironment in chronic lymphocytic leukaemia. Br J Haematol 2003;123(3):380-8.

[5] Ghia P, Granziero L, Chilosi M, Caligaris-Cappio F. Chronic B cell malignancies and bone marrow microenvironment. Semin Cancer Biol 2002;12(2):149-55.

[6] Youinou P, Jamin C, Lydyard PM. CD5 expression in human B-cell populations. Immunol Today 1999;20(7):312-6.

[7] Van de Velde H, von Hoegen I, Luo W, Parnes JR, Thielemans K. The B-cell surface protein CD72/Lyb-2 is the ligand for CD5. Nature 1991;351(6328):662-5.

[8] Biancone L, Bowen MA, Lim A, Aruffo A, Andres G, Stamenkovic I. Identification of a novel inducible cell-surface ligand of CD5 on activated lymphocytes. J Exp Med 1996;184(3):811-9.

[9] Bikah G, Lynd FM, Aruffo AA, Ledbetter JA, Bondada S. A role for CD5 in cognate interactions between T cells and B cells, and identification of a novel ligand for CD5. Int Immunol 1998;10(8):1185-96.

[10] Pospisil R, Fitts MG, Mage RG. CD5 is a potential selecting ligand for B cell surface immunoglobulin framework region sequences. J Exp Med 1996;184(4):1279-84.

[11] Pospisil R, Silverman GJ, Marti GE, Aruffo A, Bowen MA, Mage RG. CD5 is A potential selecting ligand for B-cell surface immunoglobulin: a possible role in maintenance and selective expansion of normal and malignant B cells. Leuk Lymphoma 2000;36(3-4):353-65.

[12] Calvo J, Places L, Padilla O, Vila JM, Vives J, Bowen MA, et al. Interaction of recombinant and natural soluble CD5 forms with an alternative cell surface ligand. Eur J Immunol 1999;29(7):2119-29.

[13] Beyers AD, Spruyt LL, Williams AF. Molecular associations between the T-lymphocyte antigen receptor complex and the surface antigens CD2, CD4, or CD8 and CD5. Proc Natl Acad Sci U S A 1992;89(7):2945-9.

[14] Lankester AC, van Schijndel GM, Cordell JL, van Noesel CJ, van Lier RA. CD5 is associated with the human B cell antigen receptor complex. Eur J Immunol 1994;24(4):812-6.

[15] Perez-Villar JJ, Whitney GS, Bowen MA, Hewgill DH, Aruffo AA, Kanner SB. CD5 negatively regulates the T-cell antigen receptor signal transduction pathway: involvement of SH2-containing phosphotyrosine phosphatase SHP-1. Mol Cell Biol 1999;19(4):2903-12.

[16] Lozano F, Simarro M, Calvo J, Vila JM, Padilla O, Bowen MA, et al. CD5 signal transduction: positive or negative modulation of antigen receptor signaling. Crit Rev Immunol 2000;20(4):347-58.

[17] Azzam HS, DeJarnette JB, Huang K, Emmons R, Park CS, Sommers CL, et al. Fine tuning of TCR signaling by CD5. J Immunol 2001 May 1 ;166 (9):5464 - 72 2001;166(9):5464-72.

[18] Tarakhovsky A, Kanner SB, Hombach J, Ledbetter JA, Muller W, Killeen N, et al. A role for CD5 in TCR-mediated signal transduction and thymocyte selection. Science 1995;269(5223):535-7.

[19] Tarakhovsky A, Turner M, Schaal S, Mee PJ, Duddy LP, Rajewsky K, et al. Defective antigen receptor-mediated proliferation of B and T cells in the absence of Vav. Nature 1995;374(6521):467-70.

[20] Bikah G, Carey J, Ciallella JR, Tarakhovsky A, Bondada S. CD5-mediated negative regulation of antigen receptor-induced growth signals in B-1 B cells. Science 1996;274(5294):1906-9.

[21] Alberola-Ila J, Takaki S, Kerner JD, Perlmutter RM. Differential signaling by lymphocyte antigen receptors. Annu Rev Immunol 1997;15:125-54.

(22] Sen G, Bikah G, Venkataraman C, Bondada S. Negative regulation of antigen receptor-mediated signaling by constitutive association of CD5 with the SHP-1 protein tyrosine phosphatase in B-1 B cells. Eur J Immunol 1999;29(10):3319-28.

[23] Gringhuis SI, de Leij LF, Wayman GA, Tokumitsu H, Vellenga E. The Ca2+/calmodulin-dependent kinase type IV is involved in the CD5-mediated signaling pathway in human T lymphocytes. J Biol Chem 1997;272(50):31809-20.

[24] Gringhuis SI, de Leij LF, Coffer PJ, Vellenga E. Signaling through CD5 activates a pathway involving phosphatidylinositol 3-kinase, Vav, and Rac1 in human mature T lymphocytes. Mol Cell Biol 1998;18(3):1725-35.

[25] Simarro M, Calvo J, Vila JM, Places L, Padilla O, Alberola-Ila J, et al. Signaling through CD5 involves acidic sphingomyelinase, protein kinase C-zeta, mitogen-activated protein kinase kinase, and c-Jun NH2-terminal kinase. J Immunol 1999;162(9):5149-55.

[26] Cerutti A, Trentin L, Zambello R, Sancetta R, Milani A, Tassinari C, et al. The CD5/CD72 receptor system is coexpressed with several functionally relevant counterstructures on human B cells and delivers a critical signaling activity. J Immunol 1996;157(5):1854-62.

[27] Cioca DP, Kitano K. Apoptosis induction by hypercross-linking of the surface antigen CD5 with anti-CD5 monoclonal antibodies in B cell chronic lymphocytic leukemia. Leukemia 2002;16(3):335-43.

[28] Pers JO, Berthou C, Porakishvili N, Burdjanadze M, Le Calvez G, Abgrall JF, et al. CD5-induced apoptosis of B cells in some patients with chronic lymphocytic leukemia. Leukemia 2002;16(1):44-52.

[29] Cuni S, Perez-Aciego P, Perez-Chacon G, Vargas JA, Sanchez A, Martin-Saavedra FM, et al. A sustained activation of PI3K/NF-kappaB pathway is critical for the survival of chronic lymphocytic leukemia B cells. Leukemia 2004;18(8):1391-400.

[30] Renaudineau Y, Nedellec S, Berthou C, Lydyard PM, Youinou P, Pers JO. Role of B-cell antigen receptor-associated molecules and lipid rafts in CD5-induced apoptosis of B CLL cells. Leukemia 2005;19(2):223-9.

[31] Rozman C, Montserrat E. Chronic lymphocytic leukemia. N Engl J Med 1995;333(16):1052-7.

[32] Aman P, Gordon J, Lewin N, Nordstrom M, Ehlin-Henriksson B, Klein G, et al. Surface marker characterization of EBV target cells in normal blood and tonsil B lymphocyte populations. J Immunol 1985;135(4):2362-7.

[33] Hawkins RE, Zhu D, Ovecka M, Winter G, Hamblin TJ, Long A, et al. Idiotypic vaccination against human B-cell lymphoma. Rescue of variable region gene sequences from biopsy material for assembly as single-chain Fv personal vaccines. Blood 1994;83(11):3279-88.

[34] Pers JO, Jamin C, Le Corre R, Lydyard PM, Youinou P. Ligation of CD5 on resting B cells, but not on resting T cells, results in apoptosis. Eur J Immunol 1998;28(12):4170-6.

[35] Damle RN, Wasil T, Fais F, Ghiotto F, Valetto A, Allen SL, et al. Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. Blood 1999;94(6):1840-7.

[36] Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. Blood 1999;94(6):1848-54.

[37] Raman C, Kimberly RP. Differential CD5-dependent regulation of CD5-associated CK2 activity in mature and immature T cells: implication on TCR/CD3-mediated activation. J Immunol 1998;161(11):5817-20.

[38] Weiss A, Littman DR. Signal transduction by lymphocyte antigen receptors. Cell 1994;76(2):263-74.

[39] Laytragoon-Lewin N, Rossmann ED, Castro J, Mellstedt H. Significance of phosphotyrosine proteins, Bcl-2 and p53 for apoptosis in resting B-chronic lymphocytic leukemia (CLL) cells. Int J Cancer 2002;97(3):344-8.

[40] Lanham S, Hamblin T, Oscier D, Ibbotson R, Stevenson FK, Packham G. Differential signaling via surface IgM is associated with VH gene mutational status and CD38 expression in chronic lymphocytic leukemia. Blood 2002;101(3)1087-93.

(41] Contri A, Brunati AM, Trentin L, Cabrelle A, Miorin M, Cesaro L, et al. Chronic lymphocytic leukemia B cells contain anomalous Lyn tyrosine kinase, a putative contribution to defective apoptosis. J Clin Invest 2005;115(2):369-78.

[42] Carballo E, Colomer D, Vives Corrons JL, Blackshear PJ, Gil J. Phosphorylation of the MARCKS family of protein kinase C substrates in human B chronic lymphocytic leukemia cells. Leukemia 1995;9(5):834-9.

[43] Barragan M, Bellosillo B, Campas C, Colomer D, Pons G, Gil J. Involvement of protein kinase C and phosphatidylinositol 3-kinase pathways in the survival of B-cell chronic lymphocytic leukemia cells. Blood 2002;99(8):2969-76.

[44] Ringshausen I, Schneller F, Bogner C, Hipp S, Duyster J, Peschel C, et al. Constitutively activated phosphatidylinositol-3 kinase (PI-3K) is involved in the defect of apoptosis in B-CLL: association with protein kinase Cdelta. Blood 2002;100(10):3741-8.

[45] Petlickovski A, Laurenti L, Li X, Marietti S, Chiusolo P, Sica S, et al. Sustained signaling through the B-cell receptor induces Mcl-1 and promotes survival of chronic lymphocytic leukemia B-cells. Blood 2005;105(12):4820-7.

[46] Hanada M, Delia D, Aiello A, Stadtmauer E, Reed JC. bcl-2 gene hypomethylation and high-level expression in B-cell chronic lymphocytic leukemia. Blood 1993;82(6):1820-8.

[47] Kitada S, Andersen J, Akar S, Zapata JM, Takayama S, Krajewski S, et al. Expression of apoptosis-regulating proteins in chronic lymphocytic leukemia: correlations with In vitro and In vivo chemoresponses. Blood 1998;91(9):3379-89.

(48] Pistoia V. Production of cytokines by human B cells in health and disease. Immunol Today 1997;18(7):343-50.

[49] Gary-Gouy H, Harriague J, Bismuth G, Platzer C, Schmitt C, Dalloul AH. Human CD5 promotes B-cell survival through stimulation of autocrine IL-10 production. Blood 2002 Dec 15;100(13):4537-43.

[50] Schroeder HW, Jr., Dighiero G. The pathogenesis of chronic lymphocytic leukemia: analysis of the antibody repertoire. Immunol Today 1994;15(6):288-94.

[51] Damle RN, Batliwalla FM, Ghiotto F, Valetto A, Albesiano E, Sison C, et al. Telomere length and telomerase activity delineate distinctive replicative features

of the B-CLL subgroups defined by Ig V gene mutations. Blood 2004;103(2):375-82.

[52] Crespo M, Bosch F, Villamor N, Bellosillo B, Colomer D, Rozman M, et al. ZAP-70 expression as a surrogate for immunoglobulin-variable-region mutations in chronic lymphocytic leukemia. N Engl J Med 2003;348(18):1764-75.

[53] Granziero L, Ghia P, Circosta P, Gottardi D, Strola G, Geuna M, et al. Survivin is expressed on CD40 stimulation and interfaces proliferation and apoptosis in B-cell chronic lymphocytic leukemia. Blood 2001;97(9):2777-83.

[54] Lankester AC, van SG, van-der SC, van OM, van NC, van LR. Antigen receptor nonresponsiveness in chronic lymphocytic leukemia B cells. Blood 1995;86(3):1090-71.

[55] Bernal A, Pastore RD, Asgary Z, Keller SA, Cesarman E, Liou HC, et al. Survival of leukemic B cells promoted by engagement of the antigen receptor. Blood 2001;98(10):3050-7.

[56] Zupo S, Massara R, Dono M, Rossi E, Malavasi F, Cosulich ME, et al. Apoptosis or plasma cell differentiation of CD38-positive B-chronic lymphocytic leukemia cells induced by cross-linking of surface IgM or IgD. Blood 2000;95(4):1199-206.

[57] Chen L, Widhopf G, Huynh L, Rassenti L, Rai KR, Weiss A, et al. Expression of ZAP-70 is associated with increased B-cell receptor signaling in chronic lymphocytic leukemia. Blood 2002;100(13):4609-14.

[58] Pospisil R, Mage RG. CD5 and other superantigens as 'ticklers' of the B-cell receptor. Immunol Today 1998;19(3):106-8.

[59] Kitada S, Zapata JM, Andreeff M, Reed JC. Bryostatin and CD40-ligand enhance apoptosis resistance and induce expression of cell survival genes in B-cell chronic lymphocytic leukaemia. Br J Haematol 1999;106(4):995-1004.

[60] Kitada S, Andersen J, Akar S, Zapata JM, Takayama S, Krajewski S, et al. Expression of apoptosis-regulating proteins in chronic lymphocytic leukemia: correlations with In vitro and In vivo chemoresponses. Blood 1998;91(9):3379-89.

[61] Mischak H, Kolch W, Goodnight J, Davidson WF, Rapp U, Rose-John S, et al. Expression of protein kinase C genes in hemopoietic cells is cell-type- and B cell-differentiation stage specific. J Immunol 1991;147(11):3981-7.

[62] Huang RW, Tsuda H, Takatsuki K. Interleukin-2 prevents programmed cell death in chronic lymphocytic leukemia cells. Int J Hematol 1993;58(1-2):83-92.

[63] Mainou Fowler T, Copplestone JA, Prentice AG. Effect of interleukins on the proliferation and survival of B cell chronic lymphocytic leukaemia cells [see comments]. J Clin Pathol 1995;48(5):482-7.

[64] Levy Y, Brouet JC. Interleukin-10 prevents spontaneous death of germinal center B cells by induction of the bcl-2 protein. J Clin Invest 1994;93(1):424-8.

[65] Defrance T, Vanbervliet B, Briere F, Durand I, Rousset F, Banchereau J. Interleukin 10 and transforming growth factor beta cooperate to induce anti-CD40-activated naive human B cells to secrete immunoglobulin A. J Exp Med 1992;175(3):671-82.

[66] Rousset F, Garcia E, Defrance T, Peronne C, Vezzio N, Hsu DH, et al. Interleukin 10 is a potent growth and differentiation factor for activated human B lymphocytes. Proc Natl Acad Sci U S A 1992;89(5):1890-3.

[67] Fluckiger AC, Garrone P, Durand I, Galizzi JP, Banchereau J. Interleukin 10 (IL-10) upregulates functional high affinity IL-2 receptors on normal and leukemic B lymphocytes. J Exp Med 1993;178(5):1473-81.

[68] Ishida H, Hastings R, Kearney J, Howard M. Continuous anti-interleukin 10 antibody administration depletes mice of Ly-1 B cells but not conventional B cells. J Exp Med 1992;175(5):1213-20.

[69] Fluckiger AC, Durand I, Banchereau J. Interleukin 10 induces apoptotic cell death of B-chronic lymphocytic leukemia cells. J Exp Med 1994;179(1):91-9.

[70] Castejon R, Vargas JA, Romero Y, Briz M, Munoz RM, Durantez A. Modulation of apoptosis by cytokines in B-cell chronic lymphocytic leukemia. Cytometry 1999;38(5):224-30.

[71) Jurlander J, Lai CF, Tan J, Chou CC, Geisler CH, Schriber J, et al. Characterization of interleukin-10 receptor expression on B-cell chronic lymphocytic leukemia cells. Blood 1997;89(11):4146-52.

[72] Van Kooten C, Rensink I, Aarden L, van Oers R. Interleukin-4 inhibits both paracrine and autocrine tumor necrosis factor-alpha-induced proliferation of B chronic lymphocytic leukemia cells. Blood 1992;80(5):1299-306.

[73] Trentin L, Zambello R, Agostini C, Enthammer C, Cerutti A, Adami F, et al. Expression and regulation of tumor necrosis factor, interleukin- 2, and hematopoietic growth factor receptors in B-cell chronic lymphocytic leukemia. Blood 1994;84(12):4249-56.

[74] Berland R, Wortis HH. Origins and functions of B-1 cells with notes on the role of CD5. Annu Rev Immunol 2002;20:253-300.

Fig. 1**.** CD5-mediated response in B-CLL B cells. (A) Purified B lymphocytes from 7 B-CLL patients were cultured in triplicates in medium or in the presence of increasing doses of UCHT-2 anti-CD5 mAbs. The viability percentages were then measured every 24 h for up to 144 h by the annexin-V/PI method and analysed by flow cytometry. It is shown the dose-response curve of a representative case in which anti-CD5 induces viability and another case in which viability does not change at 120 h. (B) B cells from 44 B-CLL patients were then cultured in the presence or in the absence of 1 μg ml^{-1} anti-CD5 mAbs for 120 h. Figure represents the mean ± SD of the increment in viability induced by anti-CD5 mAbs (as compared with medium). (C) Representative cytometry plots showing the increment in viability after CD5 stimulation. Numbers indicate the percentages of viable cells (AV^-/PI^-).

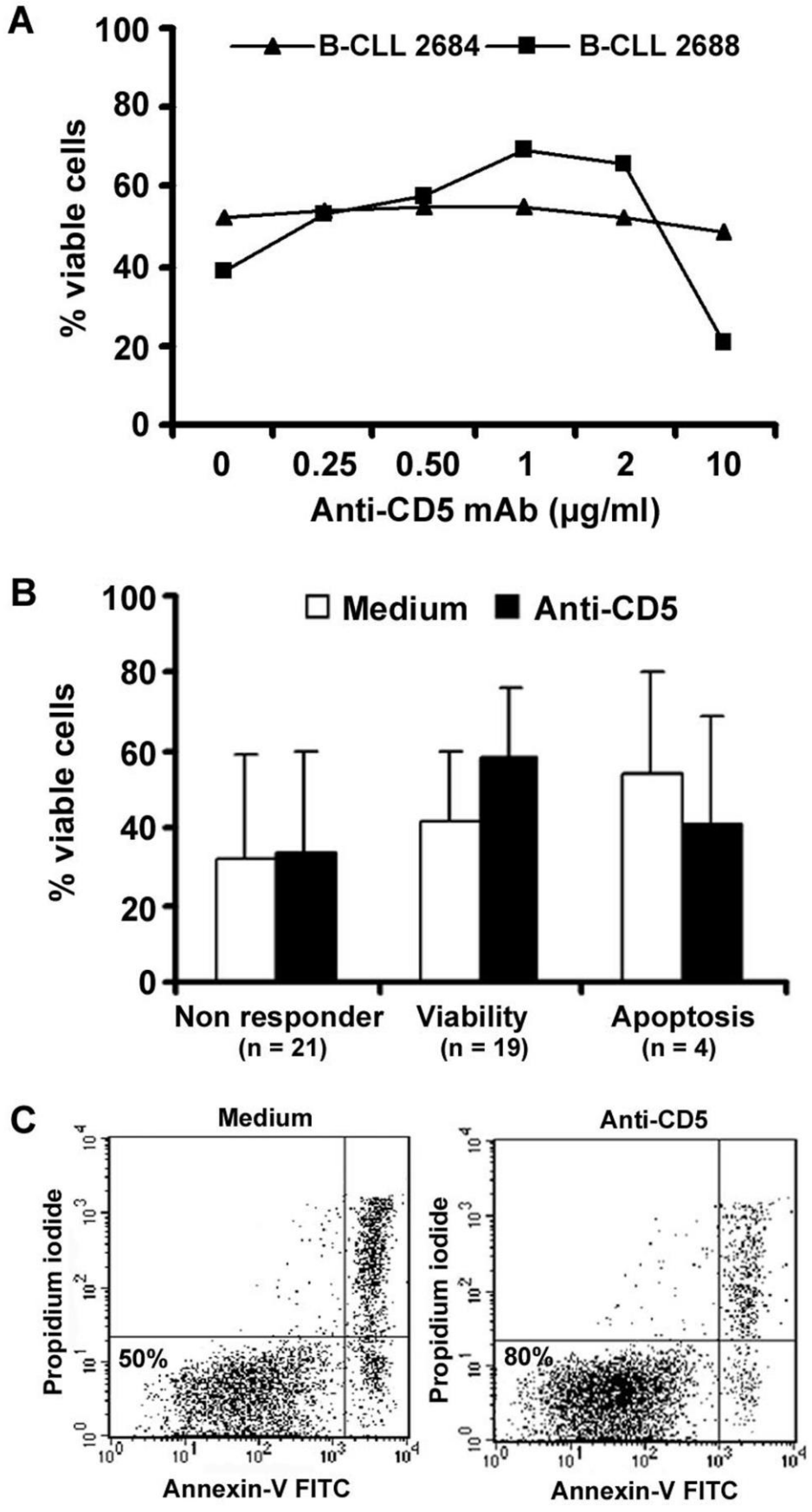
Fig. 2. Analysis of global tyrosine-phosphorylated proteins following anti-CD5 treatment. Purified B cells from 6 B-CLL patients were cultured in medium or in the presence of anti-CD5 mAbs and global tyrosine-phosphorylated proteins were analysed by flow cytometry and Western blot. (A) Histogram represents the mean fluorescence intensity (MFI) of anti-phosphotyrosine (anti-PTyr) mAb staining versus cell count of a representative sample at 30 minutes. (B) Mean ± SD of the increment in MFI of PTyr staining induced by anti-CD5 mAbs (as compared with medium) at the above indicated times (*p = 0.026). (C) A representative case of tyrosine-phosphorylated proteins analysed by Western blot. β-actin was used as loading control. Molecular weight (kDa) is indicated on the right.

Fig. 3. Anti-CD5 induces PKC phosphorylation in B-CLL B cells. B-CLL B cells were isolated from peripheral blood of 6 patients and then cultured with or without anti-CD5 mAbs. Cells were harvested after 30 min and 48 h and whole protein were extracted. 10-15 μg of protein were subjected to SDS-PAGE/immunoblot analysis by the use of specific antibodies as indicated. Membranes were stripped and reprobed with anti-β-actin as a loading control. (A) Top panel shows examples of patients in which anti-CD5 stimulation induces PKC phosphorylation at 48 h (1. Medium, 2. Anti-CD5). Bottom panel represents the densitometric analysis of Western blotting results at 48 h. Data are expressed as the mean ± SD of PKC fold increase in phosphorylation in CD5-stimulated B-CLL B cells relative to cells cultured in medium after normalization for total PKC (*p = 0.043). (B) Immunoblots from a representative case are shown; 5 additional patient samples gave similar results.

Fig. 4. Gene and protein expression of Bcl-2 family in response to CD5. Freshly isolated B cells from 5 B-CLL patients were cultured in medium or in the presence of anti-CD5 mAbs for 48 h. Total RNA and protein were extracted and then analysed by RT-PCR and Western blot, respectively. The level of product from the gene of interest was then normalized against the constant expression of the 18S rRNA. RT-PCR products were resolved on a 1% agarose gel and stained with ethidium bromide. (A) Top panel represents the mean ± SD of the relative expression of bcl-*2* family genes in B cells from 5 B-CLL patients. Bottom panel shows a representative B-CLL case in which anti-CD5 induces an increase of mcl-1 gene expression. (B) Top panel represents the mean ± SD of the expression of Mcl-1 relative to β-actin protein in B cells from 6 B-CLL patients studied. Bottom panel shows a representative B-CLL case in which anti-CD5 induces an increase of Mcl-1 protein expression.

Fig. 5. PKC inhibition abrogates Mcl-1 protein expression in B-CLL B cells. Purified B cells from 3 patients were cultured in medium (containing 0.02% DMSO) or in the presence of anti-CD5 or PMA without or upon preincubation with the PKC inhibitor Bis I. After 48 h, cells were recovered and lysed. A total of 10-15 μg of protein were subjected to 10% SDS/PAGE and immunoblotting with antibodies specific for Mcl-1, and for β-actin, used as loading control. Top panel represents the mean ± SD of the relative expression of Mcl-1 protein at the mentioned conditions. One representative experiment is shown (bottom panel).

Fig. 6. IL-10 gene expression induced by anti-CD5. Purified B cells from 6 B-CLL patients were cultured in medium or in the presence of anti-CD5 for 48 h. In order to study the implication of PKC in cytokine induction mediated by CD5, CD5-stimulated B cells were preincubated with or without the PKC inhibitor Bis I for 1 h. (A) Mean ± SD of the relative mRNA expression of IL-10 in B cells from 6 patients cultured at the above indicated conditions. (B) Top panel illustrates the case in which anti-CD5 induces both IL-2 and IL-10 gene expression. Bottom panel depicts a representative case of IL-10 induction, but not IL-2, triggered through CD5. In both cases, the expression of these cytokines was abrogated in the presence of Bis I.


A
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B-CLL 2688
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Anti-CD5 mAb (µg/ml)
B
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Anti-CD5
% viable cells
Non responder
(n = 21)
Viability
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Apoptosis
(n = 4)
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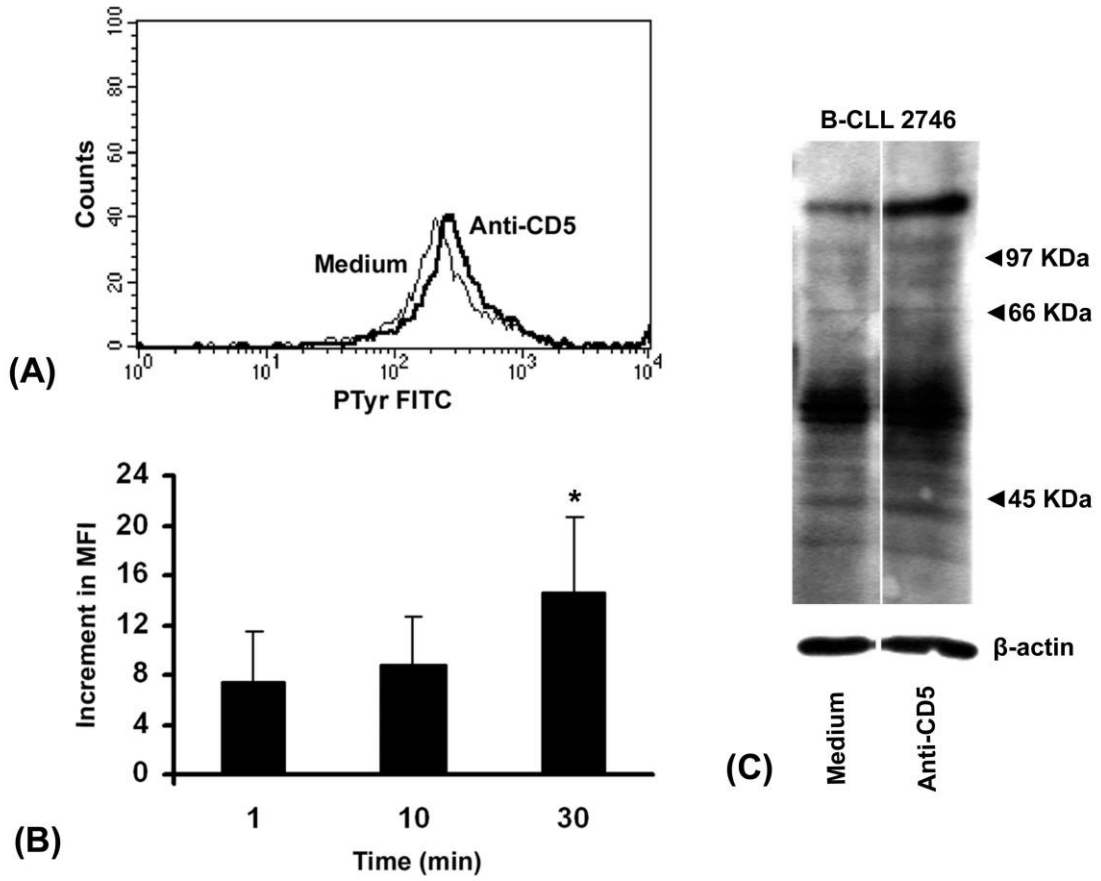
Anti-CD5
Propidium iodide
Annexin-V FITC
50%
80%


(A)
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Medium
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Time (min)
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B-CLL 2746
97 KDa
66 KDa
45 KDa
β-actin
Medium
Anti-CD5

Figure 2

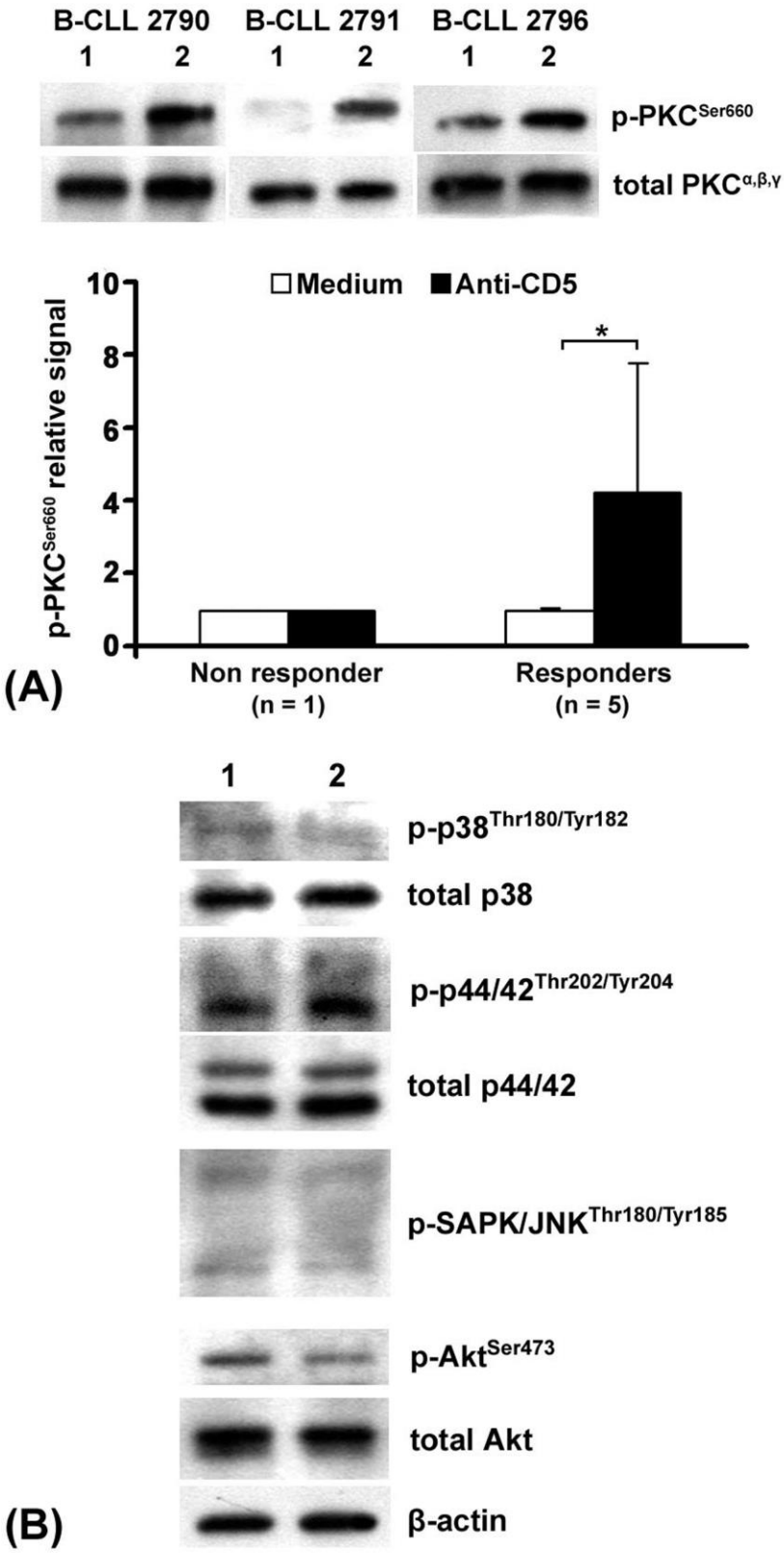
B-CLL 2790
B-CLL 2791
B-CLL 2796
1
2
1
2
1
2
p-PKC^{Ser660}
total PKC$^{\alpha,\beta,\gamma}$
□Medium ■Anti-CD5
p-PKC^{Ser660} relative signal
10
8
6
4
2
0
*
Non responder
(n = 1)
Responders
(n = 5)
(A)
1
2
p-p38$^{Thr180/Tyr182}$
total p38
p-p44/42$^{Thr202/Tyr204}$
total p44/42
p-SAPK/JNK$^{Thr180/Tyr185}$
p-Akt^{Ser473}
total Akt
β-actin
(B)

Figure 3

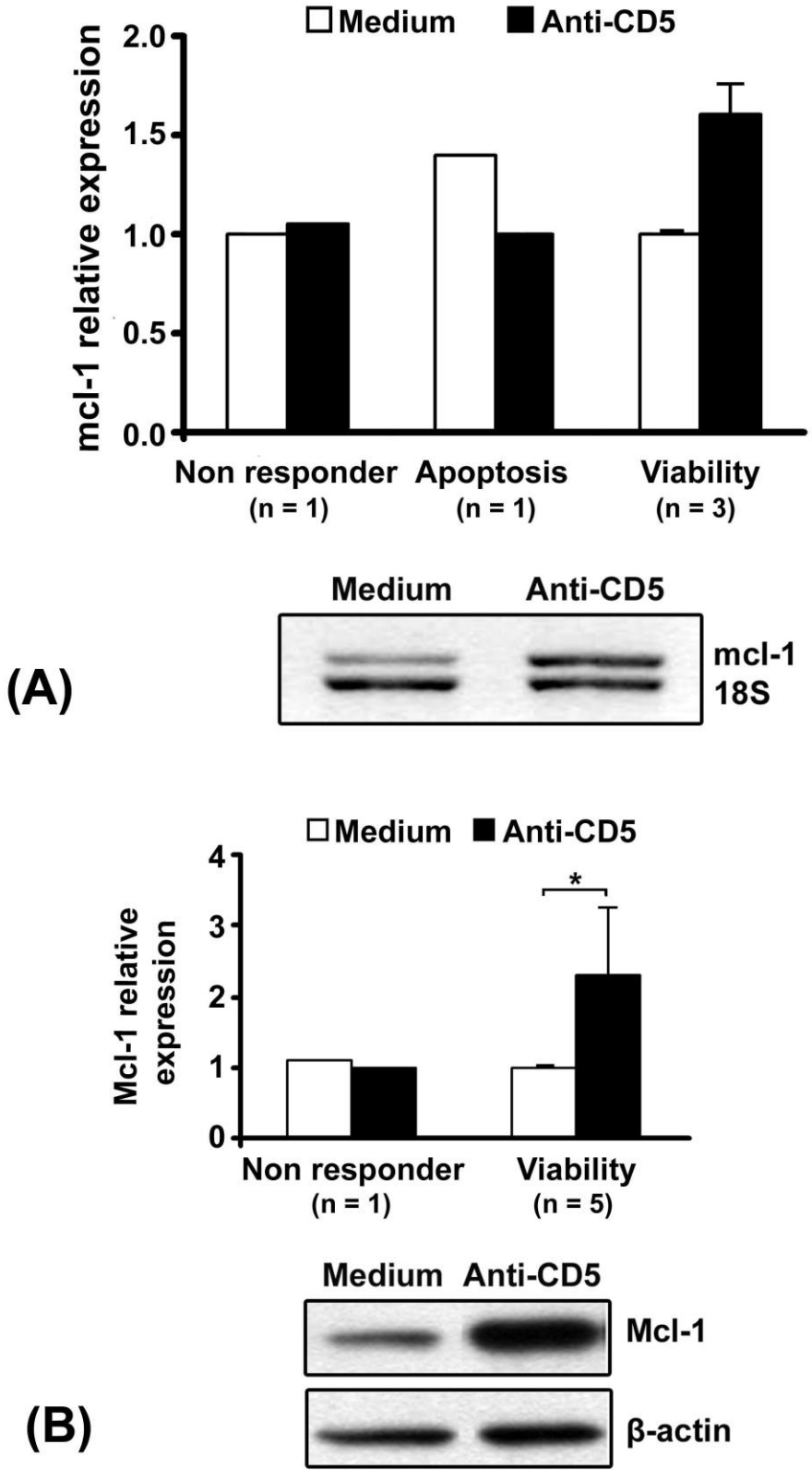
Medium
Anti-CD5
mcl-1 relative expression
2.0
1.5
1.0
0.5
0.0
Non responder
(n = 1)
Apoptosis
(n = 1)
Viability
(n = 3)
(A)
Medium
Anti-CD5
mcl-1
18S
Medium
Anti-CD5
Mcl-1 relative expression
4
3
2
1
0
*
Non responder
(n = 1)
Viability
(n = 5)
(B)
Medium
Anti-CD5
Mcl-1
β-actin

Figure 4

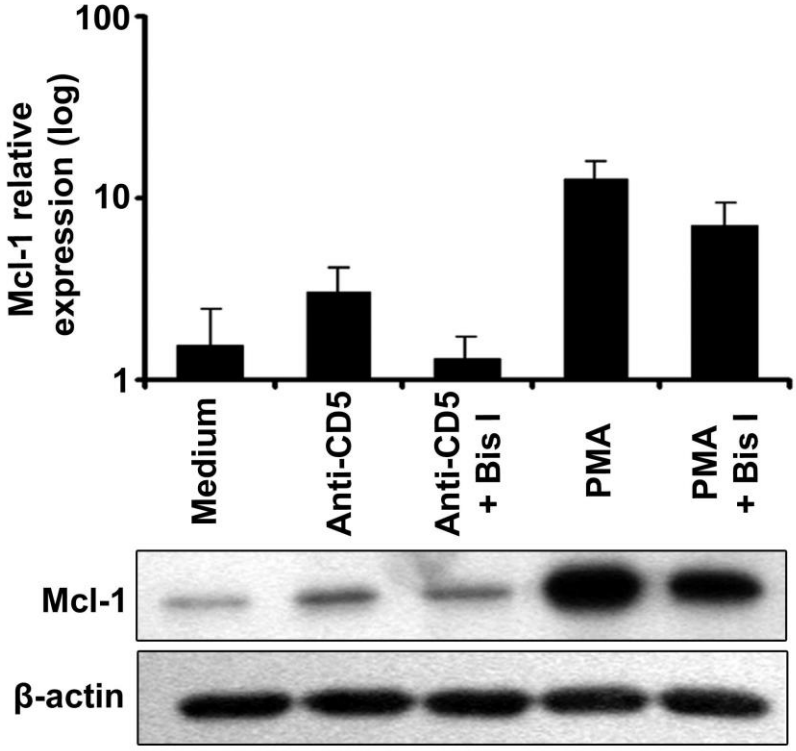
100
10
1
Mcl-1 relative expression (log)
Medium
Anti-CD5
Anti-CD5 + Bis I
PMA
PMA + Bis I
Mcl-1
β-actin

Figure 5

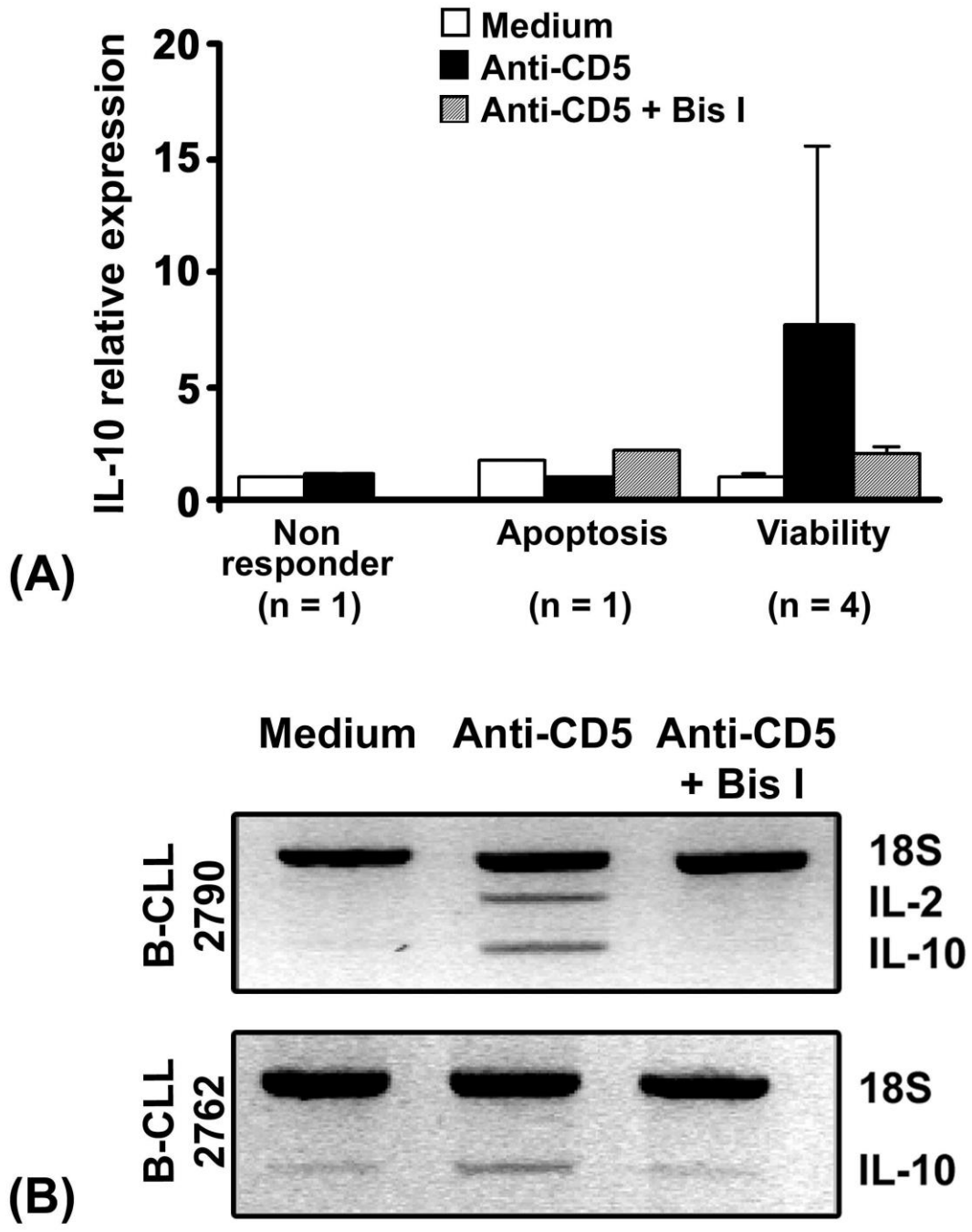
Medium
Anti-CD5
Anti-CD5 + Bis I
IL-10 relative expression
20
15
10
5
0
Non responder (n = 1)
Apoptosis (n = 1)
Viability (n = 4)
(A)
Medium
Anti-CD5
Anti-CD5 + Bis I
B-CLL 2790
18S
IL-2
IL-10
B-CLL 2762
18S
IL-10
(B)

Figure 6