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Engagement of CD31 delivers an activating signal that contributes to the survival of chronic lymphocytic leukaemia cells.

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ABSTRACT

Herein we show that engagement of CD31 delivers a survival signal in chronic lymphocytic leukaemia (CLL) cells. We describe two groups of CLL, showing a different kinetics of apoptosis in vitro and distinct ratios between anti-apoptotic and pro-apoptotic proteins: CLL-I displayed low Bcl-x_L/Bax and Bcl-2/Bax ratio and underwent rapid apoptosis in vitro; CLL-II had high Bcl-x_L/Bax and Bcl-2/Bax ratio and were resistant to apoptosis for several days. Nurse-like cells, expressing vimentin, CD68 and CD31 were detected mainly in CLL-II cultures. Of note, CD31 cross-linking, obtained with a specific monoclonal antibody (mAb), induced PI-3K-dependent Akt phosphorylation and nuclear translocation of the NF-kBp65 and p52 subunits in both CLL groups, leading to up-regulation of Bcl-2 and Bcl-x_L transcription and increased cell survival. Binding to CD31⁺ stable transfectants, could also deliver an anti-apoptotic signal in B cells of both CLL-I and CLL-II, increasing the Bcl-2 and Bcl-x_L protein content, regardless the expression of CD38. On the other hand, the addition of the F(ab')₂ (that is unable to oligomerize the target molecule) of the anti-CD31 mAb prevented these effects. These data suggest that CD31 adhesion system may play a role also in vivo in maintaining CLL survival.

INTRODUCTION

Accumulation of long-lived, mature monoclonal B cells in peripheral blood, bone marrow and lymphoid tissues has long been recognised as the central feature of chronic lymphocytic leukaemia (CLL) (Rai and Patel, 1999; Meinhart et al, 1999; Danilov et al, 2006). Then, it has become evident that proliferation signals are crucial for the progression of the disease (Chiorazzi et al, 2005; Calissano et al, 2009). Nevertheless, CLL cells are resistant to both programmed cell death and to standard chemotherapy (Kitada et al, 1998; Rai and Patel, 1999; Meinhart et al, 1999); indeed, CLL show high levels of transcription of anti-apoptotic proteins belonging to the Bcl-2 family, while pro-apoptotic proteins as Bax are not increased (Schena et al, 1992; McConkey et al, 1996; Kitada et al, 1998). Despite this, CLL undergo spontaneous apoptosis in vitro (Collins et al, 1989; Sanz et al, 2004), thus implying that ex-vivo conditions lack important survival signals conceivably delivered from microenvironment (Meinhart et al, 1999; Caligaris-Cappio, 2003). Indeed, CLL can be rescued from apoptosis by co-culture with bone marrow stromal cells, due to increased expression of Bcl-2 and activation of cell cycle-associated kinases (Panayoditis et al, 1996; Lagneaux et al, 1998; Ringhausen et al, 2002; Longo et al, 2007). These observations indicate that positive signals occurring in the bone marrow may by-pass pro-apoptotic signals and sustain prolonged survival of CLL.

We and others have shown that the CD31 adhesion molecule can protect from starvation induced apoptosis both CD34⁺ hemopoietic precursors and endothelial cells, due to the activation of PI-3K-dependent phosphorylation of Akt and induction of Bcl-2 and Bcl-x_L antiapoptotic proteins, but not of pro-apoptotic proteins such as Bax (Ferrero et al, 2003; Gao et al, 2003). In CLL, CD38 engagement by CD31 has been reported to deliver a survival signal in leukemic B cells; in particular, CD38⁺CLL would receive such signal upon binding to CD31⁺ nurse like cells (NLC) in the bone marrow (Deaglio et al, 2006; Deaglio et al, 2010). Besides NLC, CD31 is expressed on multiple cell types, including endothelial cells, platelets and healthy peripheral blood mononuclear cells (PBMC) (DeLisser et al, 1994; Newman et al, 1997), thus making it possible that several types of interactions

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occur in the bone marrow between leukemic cells and other components of the microenvironment. However, there are reports showing that the CD38/CD31 couple does not deliver a survival signal in CLL (Tonino et al, 2008).

Herein we show that: 1) two groups of CLL can be defined, on the basis of Bcl-x_L/Bax and Bcl-2/Bax ratios; 2) oligomerization of CD31 at the surface of CLL induces PI-3K-dependent Akt phosphorylation, NF-κB nuclear translocation and transcription of Bcl-x_L and Bcl-2, but not that of Bax, thus increasing Bcl-x_L/Bax and Bcl-2/Bax protein ratios; 3) this signal leads to prolonged survival of CLL in vitro; 4) interactions between CD31 expressed by CLL and CD31-expressing transfectants leads to the same results, regardless the expression of CD38 by CLL.

MATERIALS AND METHODS

Patients. Thirty untreated patients with CLL were studied, provided IRB-approved informed consent, at the Clinical Haematology Division (Department of Haematology and Oncology, University of Genoa). The patients met the diagnostic criteria of the National Cancer Institute Working Group (NCI-WG) (Cheson et al, 1996) and were staged according to the Rai modified criteria (Rai and Han, 1990) as low risk (LR), intermediate risk (IR) and high risk (HR). Their clinical features and risk factors are summarized in table 1. Progressive disease is defined as a change in the clinical features (i.e. from LR to IR or from IR to HR or from LR to HR).

Isolation of B cells, cell culture and apoptotic assay. Peripheral blood mononuclear cells (PBMCs) were isolated from CLL patients by density gradient centrifugation as described (Poggi et al, 2004). Cells were then cultured (2x10⁶ cells/ml) in 24w flat bottomed plates (Corning Inc., Corning, NY 14831, USA) in culture RPMI medium with 10% FCS supplemented with penicillin, streptomycin and L-glutamine (all from Biochrom, Berlin, Germany) for different time periods (1, 3, 5, 7, 9, 14 days) at 37°C, recovered and used for the apoptotic assay. For CD31 cross-linking experiments, purified B cells were obtained from CLL patients, after depletion of monocytes by plastic adherence

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and addition of autologous red cells to PBMCs at 30:1 ratio, with the RosetteSep B cell enrichment kit (StemCell Technologies, Vancouver, Canada) and were >98% pure as assessed by CD19 staining (not shown). Cross-linking of CD31 (CD31-XL) was obtained by incubating B cells (2×10^6 /sample) with the specific M89D3 (IgG2a, 1µg/ml; now also achievable from BD Biosciences), that recognizes an activating epitope of the molecule (Poggi et al, 2003; Ferrero et al, 2003), purified from the producing hybridoma by affinity chromatography and prepared as described (Ferrero et al., 2003), for 20 min at 4°C. Cells were then added to 24 well flat-bottomed plates (Becton Dickinson, Oxnard, CA) coated with GAM (Sigma, 10µg/ml) and cultured for different time periods (1, 3, 5, 7, 9, 14 days) at 37°C; an unrelated murine mAb, UmAb-XL (1µg/ml, Sigma Chemicals Co. St. Louis MO), was used as a negative control. In some experiments B cells (10^6 /sample) were co-cultured on confluent monolayers of CD31 stable transfectants (D12P) or pcDNAIneo mock transfected (MOCK) or wild type NIH 3T3 cells, in the absence or presence of the F(ab')₂ of the M89D3 mAb (1µg/ml), fragment that does not oligomerise the molecule and do not deliver a signal (Poggi et al, 2003; Ferrero et al, 2003). In other samples, PBMC (instead of purified B cells) from CLL patients were cultured either in the absence or presence of the F(ab')₂ of the M89D3 mAb. Apoptotic events were evaluated by annexin-V labeling method, to show the exposure of phosphatidyl-serine at the external side of the plasma membrane, according to the manufacturer's protocol (Bender MedSystem GmbH, Vienna, Austria). B cells were identified by staining with an allophycocyanin (APC)-conjugated anti-CD19 mAb (BD Pharmingen). Apoptotic cells were differentiated from necrotic cells by flow cytometry (Cyan ADP cytofluorimeter, Beckman-Coulter S.p.A., Milan, Italy), after PI staining of non-permeabilized cells. At least 10^4 cells per sample were analysed and apoptotic cells were identified as annexin V⁺PI⁺ cells (Ferrero et al, 2003). In some experiments, 5×10^5 cells were washed and analysed on Cyan ADP after permeabilisation and PI staining to analyse DNA staining and to identify cells with DNA<2n as apoptotic cells (Ferrero et al, 2003). Viable, non apoptotic cells were evaluated by gating on CD19⁺ B cells, as annexinV⁻PI⁻ cells.

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Immunofluorescence and confocal microscopy. Samples from CLL cells were cultured for 10 days on glass slides (10^6 cells/mL); then, slides were washed to remove non-adherent cells and fixed in ice-cold 2% paraformaldehyde solution for 10 min. Surface staining was performed with the anti-CD14 (IgG1) monoclonal antibody (mAb) purchased from BD Pharmingen Europe (Milan, Italy), or the anti-CD68 mAb (Ki-M7, IgG1) from Serotec (Kidlington, OX5 1GE, UK) or the anti-CD31 mAb M89D3. Intracytoplasmic staining on permeabilised cells with 1% NP-40, was performed with the anti-prolyl-4-hydroxylase (P4H, IgG1) from Dako Italia (Milan, Italy) or the anti-vimentin (hVIN-1, IgG1) from Sigma followed by Alexa 647-conjugated anti-isotype goat anti-mouse immunoglobulins (GAM) (Invitrogen Molecular Probes Europe BV, Leiden, NL). Samples were then analysed by FV500 (Fluoview confocal Laser Scanning Microscope System, Olympus Europe GMBH, Hamburg, Germany) equipped with an He-Neon Red laser at 633nm to excite AlexaFluor 647 dye and associated to a IX81 motorized microscope (Olympus). Samples were observed with PlanApo 40x NA1,00 or 60x NA 1,40 oil objectives and data analysed with FluoView 4.3b computer program (Olympus). Surface staining for CD19 or CD38 and cytoplasmic staining for Zap70 were performed with the APC-conjugated anti-CD19 mAb or the phycoerythrin (PE)-conjugated anti-CD38 mAb (BD Pharmingen) or with the Alexafluor 488-conjugated anti-Zap70 (CALTAG Labs., Burlingame, CA, USA) as described and this method was validated with a semiquantitative western blotting (Scielzo et al, 2006) and samples were analysed on a Cyan ADP cytofluorimeter (Beckman-Coulter).

mRNA isolation, reverse transcription and Q-RT-PCR for SDF-1, Bcl-2, Bcl-x_L and Bax.

Adherent cells, or B cells, obtained from healthy donors (Hds) or CLL patients, were recovered; total RNA was extracted with TriPure (Roche Diagnostics, Milan, Italy) and reverse-transcribed with random primers. In some experiments B cells were also collected after CD31 cross-linking. Primers and probes for Bcl-2, Bcl-x_L and Bax were designed using the PrimerExpress software (Applied Biosystems, Foster City, CA, USA). The sequence (5' to 3') of the specific primers used are as

follows: Bcl-2 up-stream 5'-CgC CTT CgC CgA gAT gTC Cag Cca g, down-stream 5'-ACT TgT ggC CCA gAT Agg CAC CCA g (385-bp); Bcl-x_L up-stream 5'-TTg gAc AAT ggA CTg gTT gA, down-stream 5'-gTA gAg Tgg Atg gTC AgT g (780 bp); Bax up-stream 5'-TCC CCC CgA gAg gTC TTT T, down-stream 5'-Cgg CCC CAg TTg AAg TTg. Primers for SDF-1 amplifications and probes were purchased from Applied Biosystem (userid: Hs00171022_m1).

Q-RT-PCR was performed on the 7900HT FastRT-PCR system (Applied Biosystems) with the fluorescent Taqman method (Gabert et al, 2003). mRNAs were normalized to 18s as a control gene, and referred to a standard curve, i.e. serial dilutions of plasmids containing cloned sequences of 18s (Applied Biosystem). After subtracting the threshold cycle (C_T) value for 18s (Applied Biosystem) from the C_T values of the target genes, the ΔC_T values were converted with the formula $2^{-\Delta\Delta C_T}$ to show the fold relative increase in mRNA expression compared to levels prior to cross-linking of the different molecules which were given the arbitrary value of 1 (Fleige et al, 2006). Results are expressed as fold increase for SDF-1 or as Bcl-2/Bax or Bcl-x_L/Bax ratio.

ELISA for Bcl-2, Bcl-x_L and Bax and SDF-1 protein measurement.

Purified CLL B cells, or adherent cells obtained from CLL patients or healthy donors (Hds), before or after CD31 cross-linking, were lysed in 1mM EDTA, 0.5% Triton x-100, 10μg/mL aprotinin, 10μg/mL leupeptin, 3μg/mL pepstatin and 100μM PMSF (all from Sigma) in PBS; the lysates normalized for protein content and Bcl-2, Bcl-x_L or Bax proteins were measured by the commercial ELISA kit (DuoSet IC, R&D Systems Europe, Abingdon, UK), based on the use of capture and biotinylated detection antibodies, specific for each protein, followed by HRP-streptavidin. Following development with ABTS, plated were read at OD₄₀₅, referred to a standard curve as pg/mL and results expressed as Bcl-x_L/Bax or Bcl-2/Bax ratio. SDF-1 was measured by ELISA (Bender MedSystem) in cell lysates and in the SN of adherent cells, obtained from CLL patients, cultured for 24h in the absence or presence of CD31-XL and results, referred to a standard curve, are expressed as pg/mL.

Akt and NF- κ B activation assays. Phosphorylated Akt (pAkt) and total Akt (tAkt) were measured in the same samples by ELISA (BioSource Europe, Pantec, Turin, Italy) in CLL lysates prepared as above, before or 5min (optimal time, based on kinetics experiments) (Ferrero et al, 2003) after CD31-XL or UmAb-XL, with or without the PI-3K inhibitor LY294002 (5 μ M, Sigma). Data were referred to standard curves and results expressed as percentage of pAkt, normalized for tAkt.

NF- κ B activation was evaluated by the TransAM Assay Kit in nuclear extracts obtained using the Nuclear Extract Kit (Active Motif, Rixensart, Belgium) from CLL B lymphocytes before or 12h after CD31-XL. The time point was chosen on the basis of preliminary kinetics experiments in order to detect optimal NF- κ B activation. TransAM NF- κ B Kits is a 96-well plate with immobilized oligonucleotide containing the NF- κ B consensus site (5'-GGGACTTCC-3') that highly specifically binds to the active form of NF- κ B contained in cell extracts. Primary antibodies directed against an epitope of p65 or p52 that is accessible only when NF- κ B is activated and bound to its target DNA are then added, followed by HRP-conjugated secondary antibody and reaction developed by the specific substrate contained in the commercial kit. Plates were then read with and ELISA reader at OD_{450nm} and results expressed as p65 or p52 nuclear/cytoplasmic (N/C) ratio.

Determination of mutational status of IgV_H genes. The analysis of IgV_H genes mutational status was performed on cDNA as described (Poggi et al, 2004). Sequences were compared with those present in the V BASE sequence directory (<http://www.mrc-cpe.cam.ac.uk/imt-doc/vbase-home-page.html>) and in the IMGT/V-QUEST database (<http://imgt.cines.fr>). Results are expressed as percentages of germ line homology (Damle et al, 1999; Hamblin et al, 1999).

Statistical analysis. Data are presented as mean $\pm$ SD. Statistical analysis was performed using ANOVA, calculating the F ratio and, when corrected, applying the Bonferroni-Dunnett test with a

$\alpha=0.05$. Chi square analysis was also performed for some experiments to correlate of Bcl-2/Bax or Bcl-x_L/Bax with IgV_H genes mutational status.

RESULTS

Two groups of CLL can be identified on the basis of in vitro survival and of the ratio between anti-apoptotic and pro-apoptotic proteins.

Among the 30 patients analyzed, we have identified two groups of CLL, the former (from now on CLL-I) showing rapid in vitro apoptotic rate (14 out of 30 patients), the latter (CLL-II) more resistant to programmed cell death (16 out of 30 patients). In particular, upon in vitro culture, among CLL-I about 50% of B cells died by day 5 (cut off to assign each case to group I or group II) and almost no living cells were recovered after 10 days; conversely, only 10% of CLL-II cells underwent apoptosis by day 5 and about 50% of these cells were still alive on day 14 (Fig.1A and B). **Interestingly, the rate of apoptosis seems to be related to the stage of disease; indeed, the 14 patients belonging to CLL-I group, were all at the LR stage, whereas the 16 patients of CLL-II group were HR (12 out of 16) or IR (4 out of 16); moreover, the four patients at IR stage showed progressive disease (PD) over one year (table 1).**

To investigate the biochemical basis of such different survival rate, we addressed the question of whether transcription of the anti-apoptotic proteins Bcl-2 and Bcl-x_L, referred to a pro-apoptotic protein such as Bax, was different in the two different CLL groups. To this aim, mRNA was extracted from CLL cells immediately after isolation and at different days of culture and analyzed by Q-RT-PCR. First, we found that CLL-II show higher Bcl-x_L/Bax or Bcl-2/Bax ratio than CLL-I (1.3 vs. 0.6, Fig.1B left or 1.4 vs. 0.55 Fig.1B right). Second, on day 5 Bcl-x_L/Bax and Bcl-2/Bax ratio decreased in CLL-I (0.3 vs 0.6 Bcl-x_L/Bax, Fig.1B left and 0.3 vs. 0.5 for Bcl-2/Bax, Fig.1B right), while in CLL-II these ratios were still high, although decreased (1.1 on day 5 vs. 1.3 on day 0 for Bcl-x_L/Bax, Fig.1B left; 1.1 on day 5 vs. 1.4 on day 0 for Bcl-2/Bax, Fig.1B right). These results

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were confirmed also by measuring the content of each anti-apoptotic protein by ELISA referred to Bax as a ratio (Fig.1C). Of note, χ^2 analysis revealed a correlation between Bcl-xL/Bax or Bcl-2/Bax ratio and IgV_H mutational status ($p<0.05$) and Zap70 expression ($p<0.05$).

NLC are present in CLL-II cultures in higher number than in CLL-I cultures.

On the basis of these results, one may hypothesize that in CLL-II are operating survival mechanisms related to their resistance to spontaneous apoptosis and that they are reduced in CLL-I. Thus, we tried to identify such factors. It has been previously described that blood-derived, adherent NLC can protect CLL B cells from spontaneous apoptosis in vitro, through the production of SDF-1 (Burger et al, 2000). In keeping with this, in all CLL-II cultures we observed the presence of adherent cells, starting from day 3 and increasing in the following days (day 10 depicted in Fig.2), to which CLL B cells were attached (Fig.2Aa and 2Ab). On the other hand, a few adherent cells were found in cell cultures performed with cells isolated from peripheral blood of CLL-I donors (Table 1).

First, these adherent cells expressed CD31 (Fig.2Bb). Second, adherent cells were CD14⁻ (Fig.2Bc), CD68⁺ (Fig.2Bd), expressed the prolyl-4-hydroxylase (P4H, Fig.2Be), a marker of collagen-producing cells of mesenchymal origin, as fibroblasts or BMSC (Aggarwal et al, 2005; Anauta and Fibbe, 2007), and vimentin (Fig.2Bf) (Burger et al, 2000). Moreover, these cells were CD45⁺, CD33⁺, CD3⁻, CD19⁻ (not shown). Third, by Q-RT-PCR we found that adherent cells expressed transcripts for SDF-1 and the cytoplasmic protein (Fig.2C), in keeping with what described for NLC in previous reports (Burger et al, 2000); **nevertheless, the levels of transcription and expression of SDF-1 were significantly higher in CLL-II than in CLL-I NLC (Fig.2C), while the surface phenotype, including the intensity of CD31 expression, was superimposable in NLC from the 2 CLL groups (not shown). Of note, the number of NLC was significantly higher in CLL-II (mostly HR) than in CLL-I (LR) and related to the stage of disease (Table 1), IgV_H mutational status ($p<0.05$) and Zap70 expression ($p<0.05$) (χ^2 analysis).**

Signalling through CD31 protects CLL from apoptosis and induces anti-apoptotic proteins.

Based on previous observations on myeloid and endothelial cells (Ferrero et al, 2003; Gao et al, 2003), we planned a series of experiments to demonstrate that engagement of CD31 at the surface of CLL is able to improve their survival in vitro triggering the transcription of anti-apoptotic proteins. **First, we observed that CD31 was present at similar level of expression both on CLL-I and CLL-II: indeed, the mean fluorescence intensity (MFI) was 73 ± 9 arbitrary units (a.u.) on CLL-I vs 67 ± 6 a.u. on CLL-II (Table 1), compared to an intensity of 71 ± 8 a.u. in healthy B cells (Table 1).** Thus, CLL-I and CLL-II cells were subjected to CD31 cross-linking with the specific mAb and cultured up to 14 days, in the absence of any growth factor added. At different time points, cells were harvested and checked for apoptosis and for transcription of Bcl-2, Bcl-x_L and Bax. As shown in figure 3 (panel A), engagement of CD31 could delay the apoptotic rate of CLL-I, as more than 60% of alive cells were found on day 7 (<40% apoptotic cells, Fig.3A, left panel), while most of untreated cells or cells incubated with murine Ig died by the same day (about 70% apoptotic cells, Fig.3A, left panel). This effect was less evident on CLL-II (Fig.3A, right panel) possibly due to the fact that during the first days of culture CLL-II still have high levels of anti-apoptotic proteins, although decreased compared to day 0 (Fig.1B and C). Of note, on day 5 of culture after CD31 cross-linking, the ratio of Bcl-x_L/Bax significantly raised in both CLL-I (0.8 vs. 0.3) and CLL-II (1.1 vs. 0.7), compared to untreated cells or upon incubation of with an unrelated mAb (Fig.3B, left panel). Although to a lesser extent, an increase in Bcl-2/Bax ratio was also observed after CD31 oligomerisation in CLL-I (0.5 vs.0.2) and CLL-II (0.8 vs. 0.6), but not in untreated cells or incubated with unrelated mAb (Fig.3B, right panel). These data were confirmed by measuring the intracellular content of the mentioned proteins (Fig.3C), supporting that the engagement of CD31 can sustain the synthesis of anti-apoptotic proteins in CLL and delay their apoptosis rate.

Transcription of Bcl-2 and Bcl-x_L is regulated by nuclear translocation of the NF-κB subunits p65 and p52, respectively (Turco et al, 2004), moreover, the activation of PI-3K and subsequent Akt

phosphorylation is needed for NF- κ B activation (Cuni et al, 2004). Thus, we analysed whether CD31 cross-linking by specific mAb could trigger all these biochemical events. We found that basal levels of pAkt in freshly isolated B cells were higher in CLL-II than in CLL-I (Fig.4A, left vs. right). Interestingly, CD31 engagement was able to trigger phosphorylation of Akt, that was prevented by the specific inhibitor of PI-3K LY294002 (Fig.4A), in keeping with our previous data on CD34⁺ precursors (Ferrero et al, 2003). Of note, oligomerisation of CD31 induced the nuclear translocation (increase of the nuclear/cytoplasmic ratio) of NF- κ Bp65 (Fig.4B) and of NF- κ Bp52 (Fig.4C) in freshly isolated CLL-I; whereas no effect was observed in the presence of an unrelated mAb (Fig.4B and C). Activation of NF- κ B was not so evident in CLL-II that displayed, on day 0, a high N/C ratio of both subunits (Fig.4B and C); however, after 2 days of culture in the absence of stimuli, the N/C ratio of NF- κ B p65 and p52 decreased also in CLL-II that became responsive to the signal delivered via CD31, detectable 12h after cross-linking of the molecule, i.e. on day 3 (Fig.4C).

To obtain these survival and biochemical effects, cross-linking of the CD31 molecule was needed, since the F(ab')₂ fragment that binds to CD31 but is unable to be oligomerised by anti-isotype GAM (Ferrero et al, 2003), not only was not efficient, but could even exert an opposite effect on survival, possibly due to inhibition of cell-to-cell contacts (Fig.3A and B, not shown in Fig.4).

CD31 heterotypic homophilic interactions contribute to prevent CLL apoptosis.

To investigate whether the effects of CD31 engagement on CLL cells are mediated by heterotypic interactions, as may occur upon binding to NLC, we co-cultured the two groups of CLL onto monolayers of stable transfectants expressing CD31 (D12P) or CD31 negative mock pcDNAIneo transfectants (MOCK) or untransfected wild type cells and measured the transcription of anti-apoptotic proteins and cell apoptosis. Co-culture of either CLL-I (Fig.5A and C) and CLL-II (Fig.5B and C) with D12P transfectants induced Bcl-x_L (Fig.5A, upper panel for CLL-I, Bcl-x_L/Bax ratio 0.8 vs. 0.3 on day 5; Fig.5B, upper panel for CLL-II, Bcl-x_L/Bax ratio 1.4 vs. 0.6 on day 5), while Bcl-2 was enhanced to a lesser extent (Fig.5A, lower panel for CLL-I, Bcl-2/Bax ratio 0.7 vs. 0.3 on day 5;

Fig.5B, lower panel for CLL-II, Bcl-2/Bax ratio 1.2 vs. 0.8 on day 5). Of note, survival on day 7 was increased in both CLL-I (<30% apoptotic cells vs. >60%) and CLL-II (<20% apoptotic cells vs. >30%) by co-culture with D12P CD31⁺ transfectants (Fig.5C); moreover, addition of the anti-CD31 F(ab')₂ mAb significantly reduced the anti-apoptotic effect of co-incubation with D12P CD31⁺ transfectants (Fig.5C). CD31 negative mock pcDNAIneo transfectants (MOCK) (Fig.5C) or untransfected wild type (not shown) cells did not affect survival, Bcl-x_L/Bax nor Bcl-2/Bax ratios of CLL B cells.

To clarify whether CD31-CD31 interactions are implied in the delivery of biochemical signals in CLL leading to survival, CD38⁻CLL-I (n=10) were compared to CD38⁺CLL-II (n=11) in co-culture with CD31⁺D12P transfectants. **The MFI of CD38 expression on CD38⁻CLL-I was 9±2 vs 7±1 of the negative control, thus making it likely that these CD38 is really not expressed in these CLL.** Of note, survival signal delivered by CD31⁺ transfectants to CLL is present in CD38⁻CLL as well as in CD38⁺CLL (Fig.6A). Again, Bcl-x_L/Bax and Bcl-2/Bax protein ratio raised upon interaction with CD31⁺D12P transfectants both in CD38⁻ and in CD38⁺ CLL (Fig.6B). Both increased survival and enhancement of anti-apoptotic proteins were inhibited by covering CD31 with the specific F(ab')₂ mAb (Fig.6A and B). Taken together, these data seem in favour of an activating signal delivered via CD31 in CLL, possibly leading to increased survival.

Finally, as CD38 is a NF-κB target gene (Kang et al., 2006), we addressed the question of whether CD31-mediated signal could induce or up-regulate CD38 expression on CLL. To this aim, 10 CD38⁻ (MFI 9±2 a.u.) and 2CD38^{low} (30 or 35% positive cells, MFI 30 or 40 a.u.) were subjected to CD31 cross-linking (i.e. preincubated with the specific mAb, added to GAM-coated plates) or cultured onto CD31 positive transfectants (D12P) compared to CD31 negative mock transfectants, and cultured for 1, 3 or 5 days. No significant variations in the percentage of positive cells or intensity (MFI) of CD38 expression was observed at any of the time points analysed (not shown).

DISCUSSION

In this paper, we describe two groups of CLL with a different kinetics of apoptosis in vitro, and distinct ratios between anti-apoptotic and pro-apoptotic proteins. Of note, we show that engagement of CD31 induces PI-3K-dependent Akt phosphorylation and NF- κ B nuclear translocation in CLL, followed by the enhancement of anti-apoptotic protein transcription and increased survival.

In recent years, it has become evident that proliferation signals are crucial for CLL pathogenesis (Calissano et al, 2009). Nevertheless, enhanced survival of leukemic cells contributes to disease progression; moreover, CLL cells are resistant to standard chemotherapy that exploits apoptosis (Kitada et al, 1998; Meinhart et al, 1999; Danilov et al, 2006). Overexpression of anti-apoptotic genes, such as Bcl-2 and Bcl-x_L, deficiency of pro-apoptotic pathways and sustained activation of Akt and NF- κ B proteins have been implied in regulating the life and chemotherapy resistance of leukemic B cells (Munzert et al, 2002; Turco et al, 2004; Cuni et al, 2004). Thus, pharmacological inhibition of NF- κ B has been proposed to accelerate apoptosis and restore chemosensitivity in CLL (Pickering et al, 2007). We and other have described that, indeed, in a fraction of CLL Akt is constitutively phosphorylated and contributes not only to an increased nuclear translocation of NF- κ B, but also to a rise in the transcription of Bcl-x_L and Bcl-2 (Longo et al, 2007; Poggi et al, 2008). Herein, we confirm the existence of two groups of CLL, one with better survival rate and higher levels of anti-apoptotic proteins (CLL-II), the other with the opposite characteristics (CLL-I). **Of note CLL-I samples represent predominantly low risk CLL, whereas CLL-II samples represent predominantly high risk CLL, according to Ig V/H status, ZAP70 and CD38 expression and clinical stage.** In both groups, Bcl-x_L and Bcl-2 decreased during culture, while the apoptotic rate increased; supporting the notion that in vitro signals delivered by the microenvironment to leukemic cells (Lagneaux et al, 1998; Caligaris-Cappio et al, 2003) are missing. In the bone marrow, or in lymphoid tissues, intimate contact with stromal and endothelial cells or with NLC occur, and CLL can receive signals during these interactions. That CD38 engagement by CD31 leads to a survival signal in leukemic B cells, upon binding to CD31⁺ NLC,

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has already been demonstrated (Deaglio et al, 2006; Deaglio et al, 2010), and other adhesion molecules would contribute to this signal (Zucchetto et al, 2009).

In our study we provide evidence that also CD31 can deliver a positive signal in CLL, leading to the activation of those biochemical events needed for the enhancement of cell survival. Indeed, the engagement and oligomerization of the molecule by the specific mAb, induces PI-3K-dependent Akt phosphorylation, nuclear translocation of the NF- κ B subunits that contribute to up-regulate the transcription of Bcl-x_L and Bcl-2, with consequent increase in Bcl-x_L/Bax and Bcl-2/Bax protein ratio. Interestingly, a similar effect has been described by us and other authors in myeloid and endothelial cells (Ferrero et al, 2003; Gao et al, 2003). Second, upon CD31 cross-linking, in vitro survival of CLL is increased. Superimposable results were obtained by co-culturing both CD38⁻CLL and CD38⁺CLL with CD31-expressing transfectants; the finding that CD31⁺ transfectants, that do not express CD38, can trigger survival signals also in CD38⁻CLL strongly supports the hypothesis that CD31-CD31 homophylic heterotypic interactions may contribute to CLL resistance to apoptosis. These results are apparently not in agreement with other reports (Deaglio et al, 2006; Tonino et al, 2008); however, in the reported papers gene expression was evaluated, and not transcription or intracellular content of anti-apoptotic proteins. Moreover, subconfluent CD31⁺ transfectants were used, with a likely diffuse distribution of CD31 molecule at the cell surface. We and others have demonstrated that junctional distribution of CD31, as it occurs in vivo in vascular endothelium, is obtained only under confluent conditions and is important to achieve the so-called haptotactic gradient that is needed for initiating transendothelial migration of CD31⁺ leukocytes (Zocchi et al, 1996; Mamdouh et al, 2009). It is conceivable that this gradient is also needed to engage in a homophilic binding the molecule expressed by lymphocytes and deliver the activating signal. This explanation may also account for the missing evidence, so far, for a role of homophilic CD31-CD31 interactions in CLL.

In our culture experiments different types cell-to-cell adhesion were observed: indeed CLL cells could bind one to each other (homotypic binding) and to NLC grown in culture (heterotypic

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binding). During these events, both CD31/CD31 (homophilic) and CD31/CD38 (heterophilic) interactions, can occur, all contributing to the activation of pro-survival signal. We cannot exclude that soluble factors, such as SDF-1 produced and released by NLC, can also sustain CLL survival, although we could not detect SDF-1 in the supernatants of our CLL cultures (not shown). Thus, NLC might provide leukemic B cells with multiple survival signals, one mediated by SDF-1 as reported (Burger et al., 2000), the others delivered via CD38 and/or via CD31 through the different types of cell-to-cell interaction mentioned above. **It is of note that the number of NLC, significantly higher in CLL-II than in CLL-I, and the in vitro survival of leukemic B cells correlate not only with risk factors (CLL-II being predominantly high risk) and the stage of disease, but also with disease progression.** It might be hypothesized that in CLL that are going to show a worst clinical progression, NLC originally resident in the bone marrow, reach the bloodstream and contribute to leukemic cell survival, spreading and colonization of other tissues.

We also suggest that homophilic engagement of CD31 during transendothelial migration, which might be favoured in the areas of bone marrow enriched in blood vessels, or upon interaction with NLC, can sustain the transcription of anti-apoptotic proteins and activate both PI-3K and NF- κ B in CD38⁺CLL. This might be of interest as both these biochemical events and bone marrow angiogenesis, have been proposed as possible therapeutic targets in CLL (Cuni et al, 2004; Pickering et al, 2007; Mollica et al., 2007).

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Table 1. Clinical features of CLL patients.

Patients ¹	Sex	Age	Year ²	Stage ³	IgV/H% homol ⁴	WBC ⁵ (x10 ³ /μl ⁻¹)	L ⁵ (x10 ³ /μl ⁻¹)	Zap70 %pos ⁶	CD38 %pos ⁷	Apoptosis (%) ⁸	NLC ⁰ (n/field)	CD31 (MFI) ¹⁰
CLL-I												
1.	F	63	1997	IR	94.2	16.2	11.5	Neg	Neg	66	2	81
2.	M	58	2000	IR	94.0	18.1	13.1	Pos	Neg	72	4	73
3.	M	70	1999	LR	90.1	15.9	12.9	Neg	Neg	78	0	58
4.	M	65	2000	LR	91.9	23.3	18.0	Pos	Pos	69	0	64
5.	M	62	2002	LR	98.4	24.0	19.3	Pos	Neg	65	6	78
6.	F	58	2000	LR	97.2	15.7	10.9	Neg	Neg	73	4	79
7.	F	65	2000	LR	96.9	22.3	19.9	Pos	Neg	74	8	83
8.	F	64	1999	LR	96.3	16.7	11.5	Pos	Neg	78	6	80
9.	F	65	2002	LR	92.5	22.9	19.2	Neg	Neg	67	1	79
10.	F	56	1999	LR	89.7	28.8	26.8	Pos	Neg	76	8	66
11.	M	52	2002	LR	96.6	20.0	18.0	Neg	Neg	80	0	59
12.	M	62	1998	LR	96.9	12.6	10.6	Neg	Neg	72	9	86
13.	F	69	2003	LR	92.6	16.8	12.5	Neg	Neg	66	4	68
14.	M	71	2004	LR	91.9	18.8	12.9	Neg	Neg	76	6	67
CLL-II												
01.	M	68	2000	HR	91.3	18.6	11.6	Pos	Pos	18	50	68
02.	F	83	1994	HR	100	16.5	12.6	Pos	Neg	15	25	76
03.*	F	80	2002	HR	98.6	23.4	20.3	Pos	Pos	24	75	59
04.	M	48	2001	HR	99.7	16.2	12.3	Pos	Pos	30	48	75
05.	M	60	2002	HR	100	14.9	10.9	Pos	Pos	16	42	66
06.	F	55	2003	HR	98.7	18.9	15.3	Pos	Neg	26	48	71
07.*	M	71	2003	HR	98.2	26.0	21.5	Pos	Pos	25	29	68
08.	M	66	1990	HR	99.1	26.2	22.7	Pos	Pos	19	31	62
09.	M	62	2003	HR	97.6	16.3	12.7	Pos	Pos	20	42	58
10.*	M	53	2001	HR	98.0	23.2	18.0	Pos	Neg	30	49	64
11.	M	66	2001	IR	99.3	18.5	15.3	Pos	Pos	18	78	61
12.	M	65	2000	IR	100	19.1	15.7	Pos	Neg	16	62	76
13.	M	62	2002	IR	98.0	14.0	10.5	Pos	Neg	22	55	73
14.	M	61	2003	IR	98.6	21.5	18.3	Pos	Pos	28	60	67
15.	F	58	2004	HR	98.8	20.6	16.9	Pos	Pos	19	64	68
16.	F	62	2004	HR	99.8	23.2	19.9	Pos	Pos	18	65	63

¹Chemo-naïve patients who never had received chemotherapy unless indicated.* These patients underwent chemotherapy in 2005: fludarabine 25 mg/m²/day for 3 days; cyclophosphamide 250 mg/m²/day for 3 days.

²Year of diagnosis.

³Stage defined in 2004 (beginning of this study) according to the Rai classification.

⁴Ig mutations were evaluated as described. In most patients CLL were sIgM⁺ with the exception of patients 5,7,16,22 that were sIgD⁺. The sequences with a germ line homology 98% or higher were considered unmutated and those with a homology less than 98% mutated.

⁵WBC and among them lymphocyte count at the time of the study.

⁶Zap70 cytoplasmic expression was evaluated by immunofluorescence as described in materials and methods and was considered positive (Pos) when detected on >20% of B cells according to reported data.

⁷CD38 expression on leukemic B cells at the time of this study was evaluated by immunofluorescence and was considered positive (Pos) when detected on >28% of B cells according to reported data.

⁸Percentage of dead cells determined by Trypan blue exclusion on day 7 of culture.

⁹Number (n) of adherent cells/field counted at 200x magnification on day 7 of culture.

¹⁰Expression of CD31 on leukemic B cells evaluated by immunofluorescence as mean fluorescence intensity (MFI). CLL-I: MFI 73±9. CLL-II: 67±6. MFI on B cells from healthy donors: 72±8 (not shown in the table).

LEGEND TO FIGURES

Figure 1. In vitro survival of CLL on the basis of Bcl-x_L/Bax or Bcl-2/Bax ratio.

PBMC were isolated from CLL patients and cultured as described in the Materials and Methods section. At different time points (day 1, 3, 5, 7, 9 and 14), cells were recovered and the percentage of apoptotic or viable CD19⁺ cells (panel A, left vs right) or the Bcl-x_L/Bax ratio or the Bcl-2/Bax ratio, as mRNA (panel B) or protein ratio (panel C) were measured. Panel A: apoptotic cells were evaluated by FACS analysis on the basis of annexin V and PI staining; results are expressed as percentage of apoptotic (annexinV⁺PI⁺, left quadrant) or viable (annexinV⁻PI⁻, right quadrant) B cells, identified as CD19⁺ cells (gate on APC-CD19⁺ cells), and are the mean±SD from 14 CLL-I or 16 CLL-II. * p<0.01 vs. CLL-I. Panel B: mRNA was extracted from CLL-I or CLL-II cells immediately after isolation or at the indicated time points, reverse transcribed and analysed by Q-RT-PCR for Bcl-x_L, Bcl-2 or Bax expression. Panel C: the content of Bcl-x_L, Bcl-2 or Bax was measured in cell lysates from CLL-I or CLL-II, on day 1 and day 5, by ELISA and referred to a standard curve. Results are expressed as Bcl-x_L/Bax or Bcl-2/Bax ratio (B, left and right quadrants, for mRNA; C, upper and lower quadrants for proteins) and are the mean±SD from 14 CLL-I or 16 CLL-II. * p<0.01 vs. CLL-I; ** p<0.01 vs. day1.

Figure 2. Phenotype of NLC derived from CLL.

PBMC from CLL-I (n=14) or CLL-II patients (n=16) or Hds (n=10) were cultured for 10 days, then non adherent cells were removed, adherent cells fixed and stained with the indicated mAbs (panel B, one representative experiment) or recovered for evaluation of SDF-1 mRNA expression and protein content (panel C). Panel A: bright field of cultured cells from CLL-II patients (Aa, day 10) (Ab, day 10 after extensive washes to discard non-adherent CLL cells) at the indicated magnification, one representative experiment out of 10 performed with comparable results. Panel B: staining was performed on adherent cells from one representative CLL-II patient with an unrelated (Ba), or anti-

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CD31 (Bb), anti-CD14 (Bc), anti-CD68 (Bd), anti-P4H (Be), or anti-vimentin (Bf) mAbs followed by Alexafluor 647 conjugated anti-isotype reagent. Samples were analyzed on a confocal microscope at the indicated magnification. Panel C: RNA extracted from adherent cells of CLL-II or CLL-I patients or Hds was reverse transcribed and Q-RT-PCR was performed with probes for SDF-1 and results expressed as fold relative increase towards a housekeeping gene (18s) as described in Materials and Methods (panel Ca). Cells from CLL-II or CLL-I patients or Hds were also lysed and used for determination of SDF-1 protein content by ELISA (panel Cb). Mean±SD from CLL-II (n=16) or CLL-I (n= 14) or Hds (n=10). * p<0.01 vs. Hds. **p<0.01 vs. CLL-II.

Figure 3. CD31 oligomerization prevents CLL apoptosis and up-regulates Bcl-x_L and Bcl-2.

B cells from CLL patients were cultured under culture conditions that obtain cross-linking of CD31 (CD31-XL) upon incubation with the specific mAb (1µg/ml) and addition to GAM-coated plates, recovered at different days of culture (day 1, 3, 5, 7, 9 and 14), and the percentage of apoptotic CD19⁺ cells (panel A) or the Bcl-x_L/Bax and the Bcl-2/Bax mRNA (panel B) or protein (panel C) ratio were measured. None: CLL cells cultured without mAbs; aCD31F(ab')₂ and UmAb-XL: CLL incubated with the F(ab')₂ of the anti-CD31 mAb or with unrelated murine mAb, respectively, and cultured on GAM-coated plates. Panel A: apoptotic cells were evaluated in CLL by FACS analysis on the basis of annexin V and PI staining (AV⁺PI⁺) upon CD31-XL or UmAb-XL or a-CD31F(ab')₂ culture conditions at the indicated time points. Results are expressed as percentage of apoptotic B cells and are the mean±SD from 14 CLL-I (A, left quadrant) or 16 CLL-II (A, right quadrant). *p<0.01 vs. none or vs. UmAb-XL. Panels B and C: mRNA (panel B) or cell lysates (panel C) were obtained from CLL-I or CLL-II B cells on day 5 of culture under CD31-XL or UmAb-XL conditions, as indicated, and analyzed by Q-RT-PCR (panel B) or by ELISA (panel C) for Bcl-x_L, Bcl-2 or Bax expression. Results are expressed as Bcl-x_L/Bax (panel B, left quadrant for mRNA; panel C, upper quadrant for protein) or Bcl-2/Bax (panel B, right quadrant for mRNA; panel C,

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lower quadrant for protein) ratio and are the mean $\pm$ SD from 14 CLL-I or 16 CLL-II. * $p<0.01$ vs. none or UmAb-XL or aCD31F(ab')₂.

Figure 4. Akt activation and NF- κ B nuclear translocation of upon CD31 cross-linking in CLL.

Panel A: Phosphorylated Akt (pAkt) and total Akt (tAkt) were measured in the same samples by ELISA in CLL-I (n=14, left quadrant) or CLL-II (n=16, right quadrant) lysates, before or 5min after cross-linking of CD31 (CD31-XL) or an unrelated mAb (UmAb-XL), without (none) or with the PI-3K inhibitor LY294002 (5 μ M, Sigma). Ex-vivo: freshly isolated CLL. Data were referred to standard curves and results expressed as percentage of pAkt, normalized for tAkt. * $p<0.01$ vs. ex-vivo or UmAb-XL. ** $p<0.01$ vs. none.

Panels B and C: NF- κ B activation was evaluated by the TransAM Assay Kit in nuclear or cytoplasmic extracts obtained from B lymphocytes from CLL patients (n=14 CLL-I; n =16 CLL-II) before (ex-vivo) or 12h after cross-linking of CD31 (CD31-XL) or of an unrelated murine mAb (Ig-XL). In some experiments, CLL-II cells were seeded in culture medium alone for 2 days, recovered and CD31-XL, or UmAb-XL, was performed as above (panel D: 2days+12h after cross-linking). Antibodies directed against an epitope on p65 (panel B and D) or p52 (panel C and D) that is accessible only when NF- κ B is activated and bound to its target DNA were then added, followed by HRP-conjugated secondary antibody and reaction developed by the specific substrate contained in the commercial kit. Plates were then read with an ELISA reader at OD_{450nm}. Results are expressed as nuclear/cytoplasmic (N/C) ratio and are the mean $\pm$ SD from 14 CLL-I or 16 CLL-II. Panels B and C: * $p<0.01$ vs. ex-vivo or UmAb-XL. ** $p<0.01$ vs. CLL-I. Panel D: * $p<0.01$ vs. none or UmAb-XL.

Figure 5. Binding to CD31-expressing transfectants up-regulate Bcl-x_L and Bcl-2 preventing apoptosis in CLL. B cells from the two groups of CLL patients (n=10 CLL-I, panel A; n=10 CLL-II, B) were co-cultured on monolayers of CD31 expressing stable transfectants (D12P), in the absence or presence of the anti-CD31 M89D3 mAb F(ab')₂ (1 μ g/ml), or on mock pcDNAIneo-transfected

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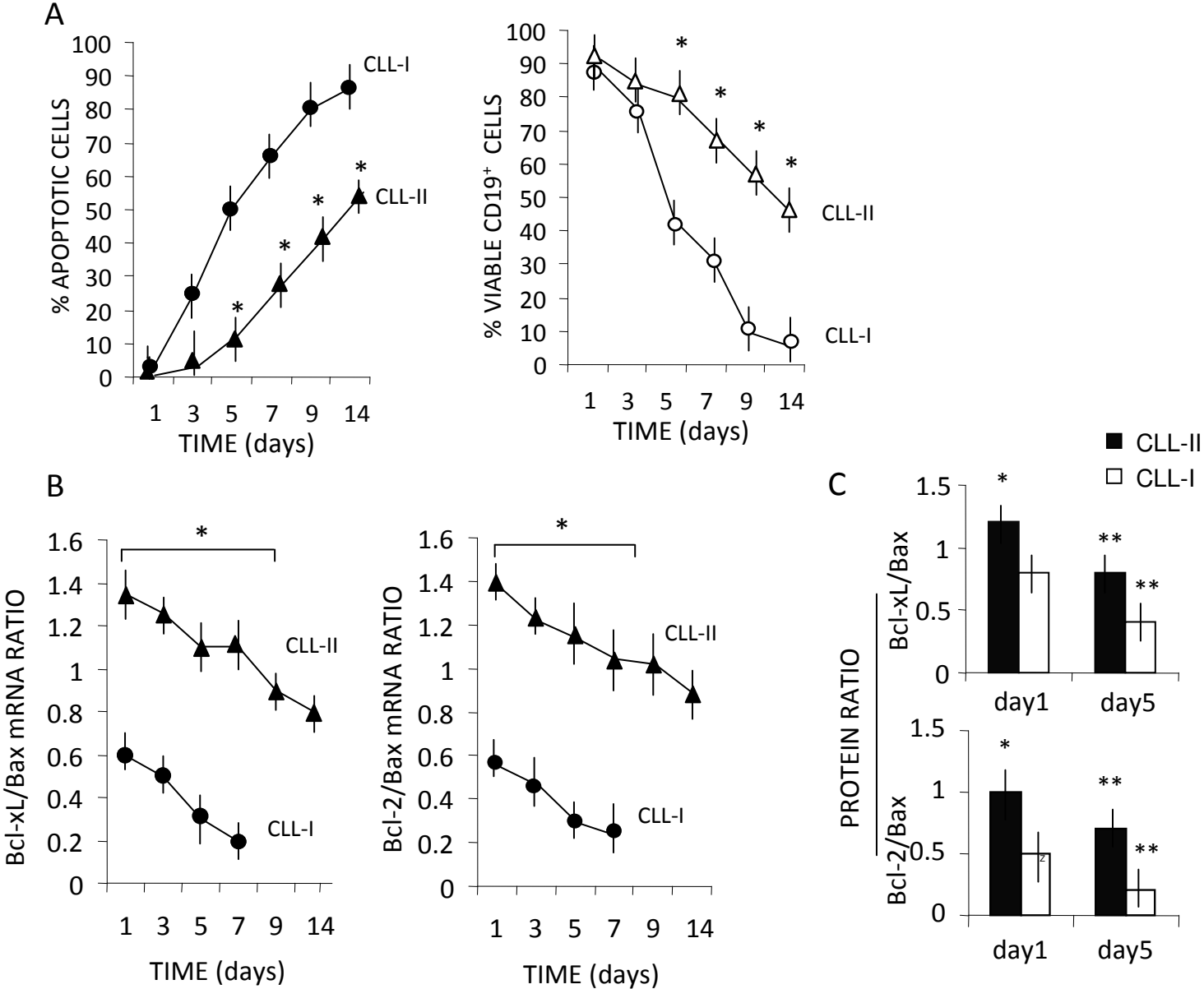
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CD31 negative NIH 3T3 cells (MOCK), recovered at different days of culture (day 1, 5, 7) and the Bcl-x_L/Bax or the Bcl-2/Bax ratio (panels A and B) or the percentage of apoptotic cells (panel C) were measured. Panels A and B: mRNA was extracted from B cells of CLL at the indicated time points, reverse transcribed and analyzed by Q-RT-PCR for Bcl-x_L, Bcl-2 or Bax expression. Results are expressed as Bcl-x_L/Bax (upper quadrants) or Bcl-2/Bax (lower quadrants) ratio and are the mean±SD from 10 CLL-I or 10 CLL-II. * p<0.01 vs. MOCK or vs. anti-CD31 F(ab')₂ mAb added. Panel C: apoptotic CD19⁺ cells were evaluated by FACS analysis (annexinV⁺PI⁺) on day 7 of co-culture of CLL-I or CLL-II cells on MOCK or D12P transfectants, in the absence or presence of the anti-CD31 F(ab')₂, as indicated. Results are expressed as percentage of apoptotic cells and are the mean±SD from n=10 CLL-I or n=10 CLL-II). * p<0.01 vs. MOCK. ** p<0.01 vs. D12P.

Figure 6. Effect of CD31 cross-linking on CD38⁺ and CD38⁻ CLL.

CD38⁻CLL-I (n=10) or CD38⁺CLL-II (n=11) were co-cultured with CD31⁺D12P transfectants, in the absence or presence of the anti-CD31 M89D3 mAb F(ab')₂ (1µg/ml). Panel A: cells were recovered on day 7 and viable cells were evaluated by FACS analysis on the basis of annexin V and PI staining; results are expressed as percentage of viable (annexinV⁻PI⁻) B cells, identified as CD19⁺ cells (gate on APC-CD19⁺ cells). * p<0.01 vs. MOCK. ** p<0.01 vs D12P.

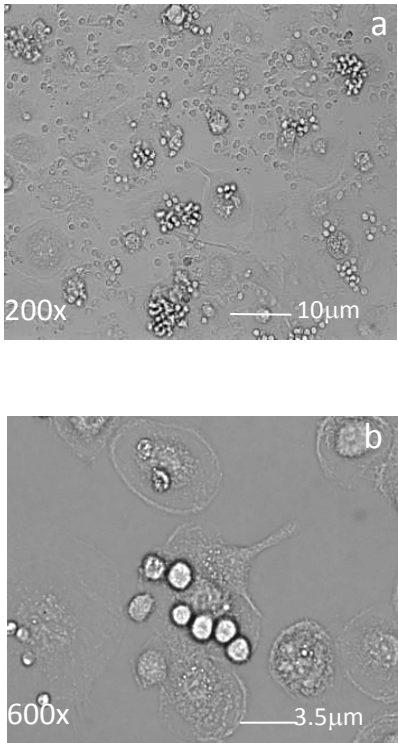
Panel B: parallel CLL samples were lysed and Bcl-x_L, Bcl-2 or Bax protein content ratio measured by ELISA. Results are expressed as Bcl-x_L/Bax (upper quadrant) or Bcl-2/Bax ratio (lower quadrant) and are the mean±SD from 11 CD38⁺CLL-II or 10 CD38⁻CLL-I. * p<0.01 vs. MOCK.



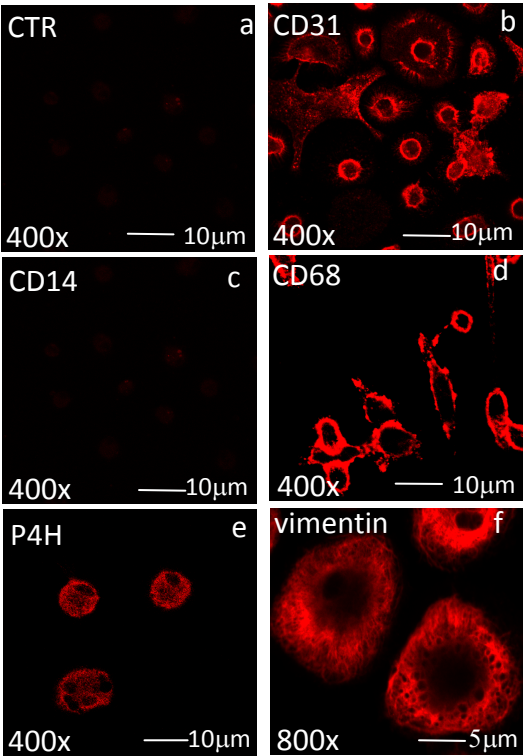
Poggi et al., Fig.1

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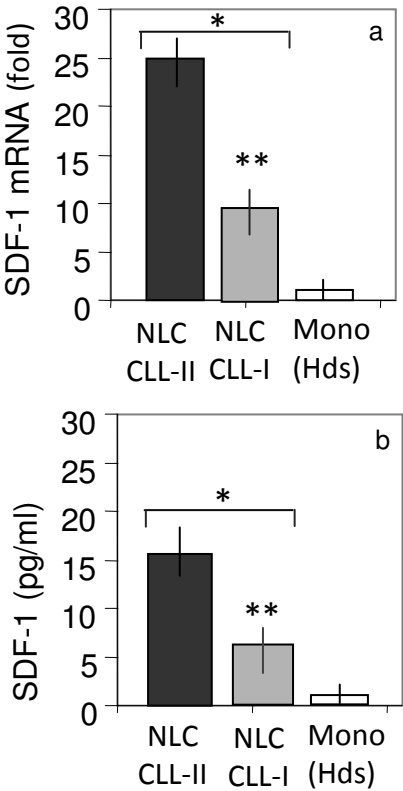
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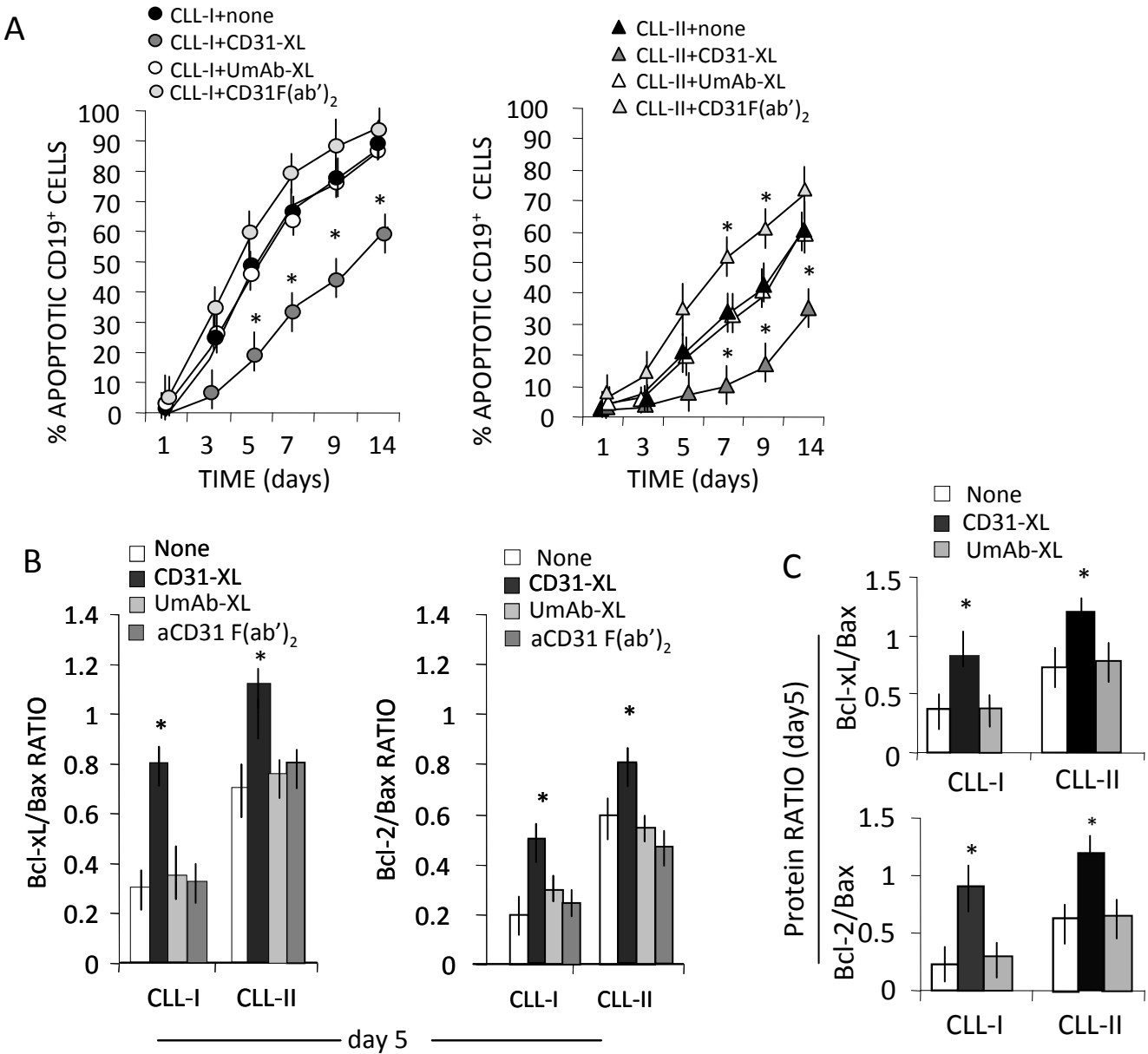


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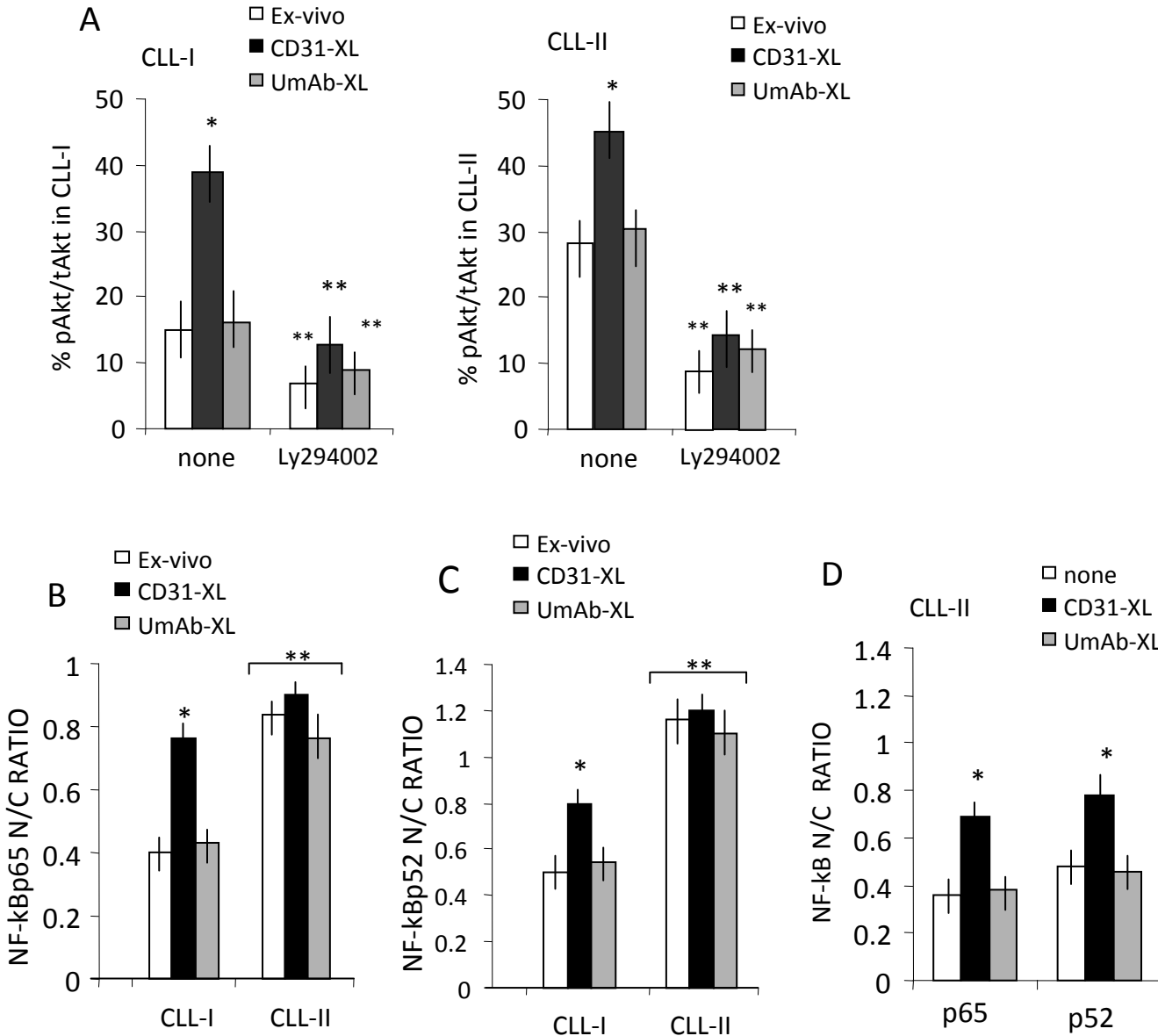


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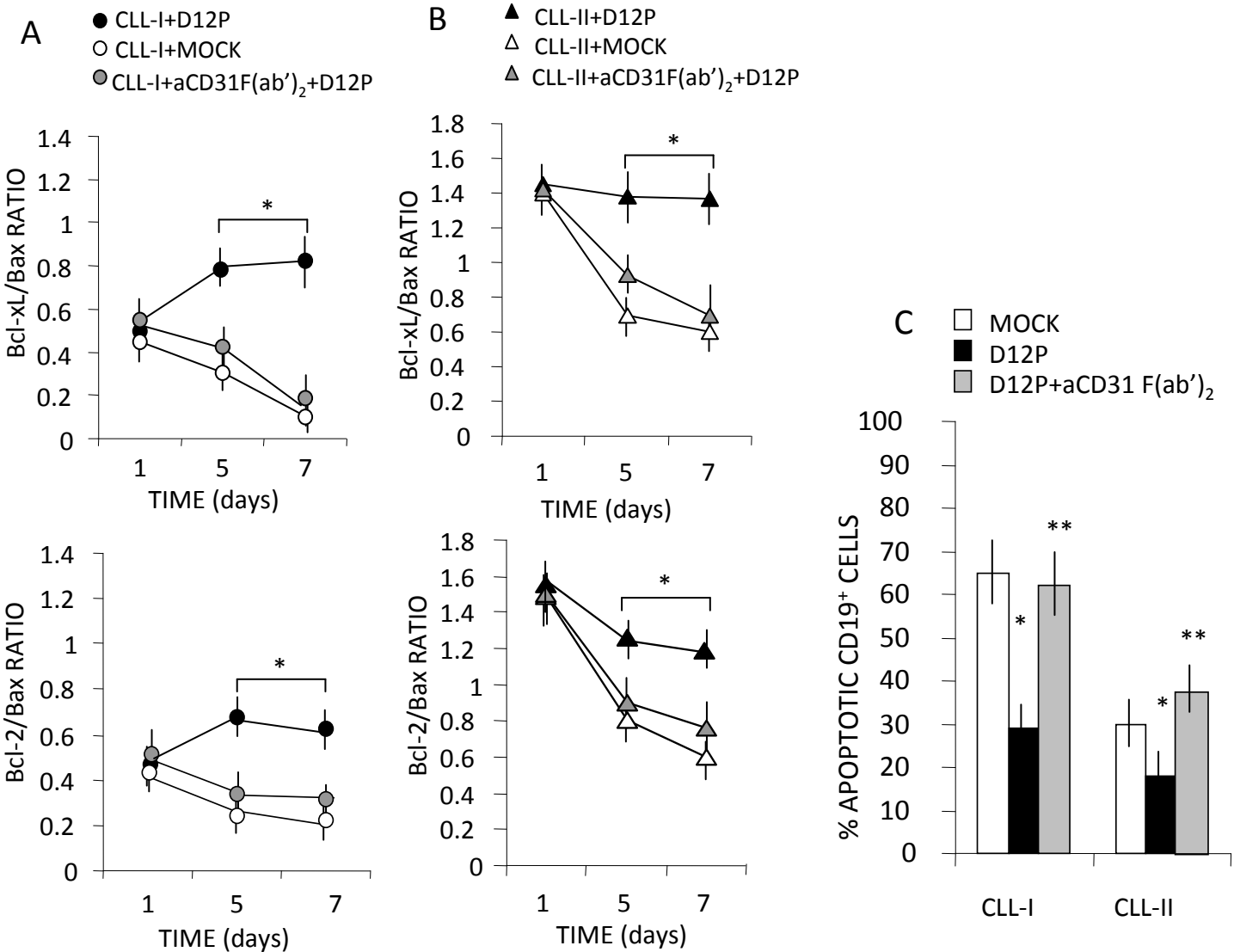




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Poggi et al., Fig.4



Poggi et al., Fig.5

