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ORIGINAL ARTICLE

Michael Stanglmaier · Simone Reis · Michael Hallek

Rituximab and alemtuzumab induce a nonclassic, caspase-independent apoptotic pathway in B-lymphoid cell lines and in chronic lymphocytic leukemia cells

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Abstract The monoclonal antibodies (MoAbs) alemtuzumab (anti-CD52) and rituximab (anti-CD20) produce objective clinical responses in patients with chronic lymphocytic leukemia (CLL). However, their mechanisms of action are not fully understood. Therefore, we investigated the mechanisms of lymphoma and CLL cell killing by two anti-CD20 antibodies (rituximab, B1) and by alemtuzumab. All antibodies induced complement-independent cell death in B-lymphoid cell lines Raji, Ramos, and Mec-1. The efficiency of cell killing was increased by the addition of human complement in Raji but not Ramos cells. Both alemtuzumab and rituximab also killed freshly isolated CLL cells, with a much stronger response for alemtuzumab (from eight of eight patients) compared to rituximab (from two of six patients). Cell morphology and Western blot analyses revealed that the antibody-induced cell death lacked some typical features of apoptosis such as chromatin condensation or poly-ADP-ribose polymerase (PARP) cleavage. Taken together, the results suggest that the tumor killing activity of these MoAbs is not only mediated by complement-mediated cytotoxicity (CDC) or antibody-dependent cytotoxicity (ADCC), but also by a nonclassic, caspase-independent apoptotic pathway.

Keywords Chronic lymphocytic leukemia · Monoclonal antibody therapy · CD20 · CD52 · Caspase-independent apoptosis

M. Stanglmaier (✉) · S. Reis · M. Hallek
Clinical Cooperation Group for Gene Therapy, GSF—National Institute of Health and Environment,
Marchioninistr. 25,
81377 Munich, Germany
e-mail: m.stanglmaier@gmx.de
Tel.: +49-89-70076622
Fax: +49-89-70076611

Present address:
M. Stanglmaier
Trion Research,
Am Klopferspitz 19,
82152 Planegg-Martinsried, Germany

Present address:
S. Reis
Institut für Pathologie und Rechtsmedizin,
Universitätsklinikum Ulm,
Prittwitzstrasse. 6,
89075 Ulm, Germany

M. Hallek
Klinikum Großhadern, Medical Clinic III,
Marchioninistr. 15,
81377 Munich, Germany

M. Hallek
Genzentrum, Universität München,
Feodor-Lynen-Strasse 25,
81377 Munich, Germany

Introduction

Chronic lymphocytic leukemia (CLL) is the most common leukemia in the Western world and is characterized by the accumulation of long-lived B lymphocytes. Despite much progress during the past decade, the disease still remains incurable. Therefore, new therapeutic strategies are highly warranted. Recent results suggest that the monoclonal antibodies (MoAbs) against CD20 (rituximab) and CD52 (alemtuzumab) have good therapeutic potential in CLL.

Rituximab monotherapy shows some activity in the therapy of relapsed CLL [1–3]. The activity was dose dependent: higher doses or more frequent application yielded response rates of up to 75% [3]. However, the full potential of rituximab seems to unfold when given simultaneously with chemotherapy, in particular fludarabine (plus cyclophosphamide). The concomitant application of fludarabine (with or without cyclophosphamide) and rituximab in first-line treatment of CLL allows achieving more than 90% overall response and more than 50% complete remissions [4–6].

The antibody alemtuzumab has shown remarkable activity in fludarabine-refractory patients. It yields response rates up to 40% in this poor prognostic group [7, 8]. It seems to be particularly active in clearing blood and marrow from leukemic cells [9]. An overall response rate

of 87% with alemtuzumab as first-line therapy was achieved in a phase II study with 41 CLL patients: 19% of the patients showed a complete response [10]. In two studies with pretreated CLL patients, including patients who failed to respond to fludarabine therapy, an overall response rate of 33% was induced by alemtuzumab administration.

The target antigens for these two therapeutic antibodies are quite different, and their functional role in the immune system is only partially understood. CD20 is a 33- to 37-kDa transmembrane protein that is expressed on all mature B cells, except early pre-B cells and terminally differentiated B cells [11]. The biological function of CD20 on B cells is not entirely clear. Structural data and ectopic expression of CD20 suggest that it might act as a calcium channel and might be involved in B-cell activation and regulation of B-cell growth [12, 13]. However, mice carrying a CD20 gene disruption did not show any major effect on the differentiation and function of B lymphocytes [14]. CD52 is a 21- to 28-kDa 12 amino acid glycopeptide belonging to the class of glycosylphosphatidyl inositol (GPI)-anchored proteins. CD52 is expressed on most circulating B and T lymphocytes as well as on a variety of B- and T-cell lymphomas, but not on hematopoietic stem cells [15, 16] The function of CD52 on B and T cells is poorly understood.

At least three different mechanisms of leukemia cell lysis induced by anti-CD20 and anti-CD52 antibodies were proposed so far, including antibody-dependent cellular cytoxicity (ADCC), complement-dependent cytoxicity (CDC), and the induction of programmed cell death [17]. These mechanisms may act synergistically. It was shown recently that anti-CD20 MoAbs might induce apoptosis in vitro [18, 19]. Rituximab was shown to induce complement-mediated cell lysis of some B-cell lines in vitro [20]. On the other hand, rituximab showed substantial decrease in its tumor cell killing activity in Fcγ receptor-deficient mice. This suggests that ADCC acts as a major tumor cell killing mechanism in vivo [21] It was shown that ADCC and complement are involved in CD52-mediated cell lysis [22]. In the B-cell line Wien 133 alemtuzumab induced apoptosis [23].

In the present study we show that both rituximab and alemtuzumab can induce cell death in B-cell lines and B lymphocytes purified from CLL patients in vitro. Cell death induction was independent of complement and ADCC. Although cell death induction showed apoptotic characteristics as assessed by annexin V/propidium iodide and DiOC(6) measurements, we did not observe classic, caspase-dependent apoptotic steps such as cleavage of poly-ADP-ribose polymerase (PARP) [24]. This indicates that a caspase-independent form of programmed cell death might be induced by both MoAbs, rituximab and alemtuzumab.

Materials and methods

Cell culture

The Burkitt's lymphoma (BL) cell line Raji and Ramos were a kind gift of Dr. Hammerschmidt (GSF, Munich, Germany), and the CLL cell line Mec1 was a kind gift of Dr. Caligaris-Cappio (Milano, Italy). Cells were maintained in RPMI-1640 media (Gibco, Karlsruhe, Germany) supplemented with 10% fetal calf serum (FCS) (Gibco, Karlsruhe, Germany).

CLL cells

Peripheral blood samples were obtained from CLL patients after informed consent. The diagnosis of CLL was based on standard clinical and laboratory criteria. CLL cells were purified by Ficoll (Gibco, Karlsruhe, Germany) gradient centrifugation, washed with phosphate-buffered saline (PBS) (Gibco, Karlsruhe, Germany) and resuspended in RPMI or IMDM, supplemented with 10% FCS and cultured at 37°C and 5% CO_2.

Antibodies and reagents

Rituximab and alemtuzumab were kind gifts from Roche (Eppstein-Bremthal, Germany) and Medac Schering Onkologie (Munich, Germany), respectively. The monoclonal anti-CD20 antibody B1 was purchased from Coulter (Krefeld, Germany). The secondary antibodies goat antihuman IgG (Cg) and goat antimouse (GAM) were purchased from DAKO (Hamburg, Germany). The polyclonal antibodies against PARP and protein kinase C-δ (PKC-δ) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, Heidelberg, Germany). Secondary antibodies (coupled with horseradish peroxidase, ECL detection system) were purchased from Amersham (Freiburg, Germany). Primary antibodies were used at 1:1000 dilution, secondary antibodies were used in dilution from 1:2500 to 1:5000.

Reagents for cell lysis were purchased from Sigma Chemicals (Deisenhofen, Germany). sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed with chemicals provided by Bio-Rad (München, Germany). Acrylamide/bisacrylamide was purchased from Boehringer Bioproducts (Ingelheim, Germany).

Human serum served as a source of complement. It was prepared from the blood of ten healthy donors and stored in aliquots until use at −20°C. When appropriate, serum was heat inactivated at 56°C for 1 h.

The annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit was purchased from Bender Med Systems (Eching, Germany). DiOC(6) and propidium iodide were obtained from Sigma Chemicals (Deisenhofen, Germany). Hoechst 33342 was purchased from Hoechst (Frankfurt, Germany).

Cell viability

Cells were cultured at 1×10^6 ml^{-1} in 96 well plates for 1 h in RPMI without FCS, added with 10 μg/ml mAb and where indicated with 50 μg/ml secondary goat antimouse and goat antihuman antibody, respectively, incubated for 1 h at 37°C and 5% CO_2 and diluted to a final concentration of 5×10^5 ml^{-1} in RPMI and 5% FCS. At the indicated time points, cells were collected and the number of viable cells was counted by trypan blue exclusion. All experiments were done in triplicates. For detection of complement-mediated cell lysis, cells were supplemented with 5% active and heat-inactivated human serum pooled from ten healthy donors and sterile filtered, 15 min prior to adding the antibodies.

Annexin V/propidium iodide measurement

A total of 2×10^5 cells were collected at the indicated points of time, washed with 2 ml PBS, and resuspended in 100 μl binding buffer. Then 2 μl of annexin V and 2 μl of propidium iodide solution were added and cells were incubated for 20 min in the dark at room temperature (RT). Cells were washed with PBS, resuspended in 400 μl of binding buffer, and annexin V and propidium iodide staining was detected by flow cytometry (Epics, Coulter, Krefeld, Germany).

DiOC(6) measurement

A total of 2×10^5 cells were collected at the indicated time points, washed with 2 ml PBS, resuspended in 500 μl PBS, and supplemented with 10 μl of 40 nm DiOC(6) solution, incubated for 30 min at RT in the dark. Cells were washed again with 2 ml PBS, resuspended in 1 ml PBS, and DiOC(6) staining was detected by flow cytometry.

Hoechst 33342/propidium iodide staining

Cells were stained as described elsewhere [25]. Briefly, 2×10^6 cells were harvested at the indicated time points, washed with PBS, and resuspended in 1 ml PBS. Thereafter 2 μl of Hoechst 33342 solution (1 μg/μl) were added and cells incubated for 30 min at 37°C and 5% CO_2. Then 10 μl PI solution (1 μg/μl) were added and cells incubated for 2 min at RT. Cells were pelleted and resuspended in 100 μl PBS. Hoechst 33342/PI staining was detected using a fluorescence microscope (Leitz Dialux EB with Orthomat-W, Leitz, Wetzlar, Germany).

Western blot

A total of 2×10^6 cells were incubated as described (cell viability) with antibodies, etoposide (VP-16), and dexamethasone (CLL cells). After 24 h, cells were counted and lysed in 50 μl lysis buffer/1×10^6 cells. For this step, cells were collected, precipitated by centrifugation (10 min, 1000 rpm, 4°C), and washed with PBS. Cells were incubated for 30 min at 4°C with lysis buffer. Lysates were cleared from debris by centrifugation (10 min, 1000 rpm, 4°C).

Protein extracts were mixed with 5× gel loading buffer, denaturated by heating for 5 min at 95°C, and loaded on 10–12% SDS-PAGE gels. Gel electrophoresis and immunoblotting were performed using standard methods. After electrophoresis, proteins were transferred to a polyvinylidene difluoride (PVDF) (Millipore, Eschborn, Germany) or a nitrocellulose (Schleicher and Schüll, Dassel, Germany) membrane. Thereafter transfer membranes were blocked with 5% skin milk, incubated with a primary antibody for at least 1 h, washed, and incubated for 40 min with a horseradish peroxidase-conjugated secondary antibody. Proteins were visualized using the ECL detection system (Amersham, Freiburg, Germany) according to the instructions of the manufacturer.

Results

Different anti-CD20 antibodies reduce the cell viability of neoplastic B cells

To study the effect of CD20 MoAbs we incubated the human B-cell lymphoma line Raji (Burkitt's lymphoma) with two anti-CD20 MoAbs, rituximab and B1, both at a concentration of 10 μg/ml. Because it had been shown previously that crosslinking of the antibody B1 with a secondary antibody was necessary to induce cell death in the Burkitt's lymphoma cell line Ramos [26], Raji cells were incubated with anti-CD20 MoAbs in the absence and presence of crosslinking MoAbs (50 μg/ml) [(Fig. 1a)]. To test whether the crosslinking MoAbs had any effect, cells were also incubated with these MoAbs alone. To exclude effects of the complement system due to the FCS, an artificial supplement (BMS) was used (Fig. 1b). Cell viability was determined after 24 h of incubation by trypan blue exclusion. As shown in Fig. 1a, rituximab alone had no effect on cell viability. Crosslinking of rituximab with a goat antihuman IgG antibody (Cg) reduced the number of viable cells to about 55% compared to untreated cells, while the number of living cells was not affected by the crosslinking antibody alone. In contrast to rituximab, B1 alone reduced the number of viable cells to about 47% after 24 h. Crosslinking of the B1 antibody with the GAM antibody further reduced the number of viable cells to about 38% compared with untreated cells. Again, incubation with the crosslinking antibody GAM alone had no effect. As a positive control, cells were also incubated with 68 μM VP-16, a topoisomerase II inhibitor known to induce apoptosis [27]. As seen in Fig. 1, VP-16 reduced the number of viable cells to about 40% compared to controls. The experiments were also repeated by using BMS instead of heat-inactivated FCS. The results obtained

with BMS were comparable to the experiments using heat-inactivated FCS (Fig. 1b). Similar results were also obtained with Mec1 and Ramos cells (data not shown), but the overall reduction of viable cells seen in Ramos cells was somewhat lower compared to Raji cells.

Rituximab- but not B1-mediated cytoxicity is complement dependent.

Complement-dependent cytoxicity (CDC) is an important mechanism for the elimination of normal and malignant B cells in vivo and in vitro. Rituximab was reported to induce CDC in some human cell lines [20]. Therefore, we wished to examine the capacity of rituximab to induce CDC in our system. Human serum (concentration 5%) was used as a source of human complement. In a control experiment we used 5% heat-inactivated human serum (data not shown). Raji cells were starved for 1 h in the absence of serum and then preincubated with 5% human serum for 15 min before adding the MoAbs. In the presence of rituximab, the number of viable Raji cells was reduced to 10% after 24 h (Fig. 1c). The addition of the crosslinking antibody to rituximab significantly increased the number of viable cells to 34% compared to untreated cells, while the crosslinking antibody alone had no effect. This reduced effect of rituximab in the presence of crosslinking MoAbs might be explained by an impaired steric access of C1q to the Fc part of rituximab, caused by the secondary antibody. Active human complement had no effect on B1 antibody alone or in combination with the crosslinking antibody GAM.

When heat-inactivated human serum was used, similar results as with BMS and FCS were obtained (data not shown). Moreover, cell death induced by VP-16 was not affected by the presence of complement system. We also tested the complement-dependent cell lysis of Ramos cells by rituximab (data not shown). This cell line showed no complement-dependent lysis. We therefore compared the expression of CD55 and CD59, two known complement inhibitors on the two cell lines by fluorescence-activated cell sorter (FACS) analysis of the mean fluorescence intensity (MFI). The expression of CD55 was equally high in both cell lines, while the expression level of CD59 was very low in Raji cells (MFI 37) compared to Ramos cells

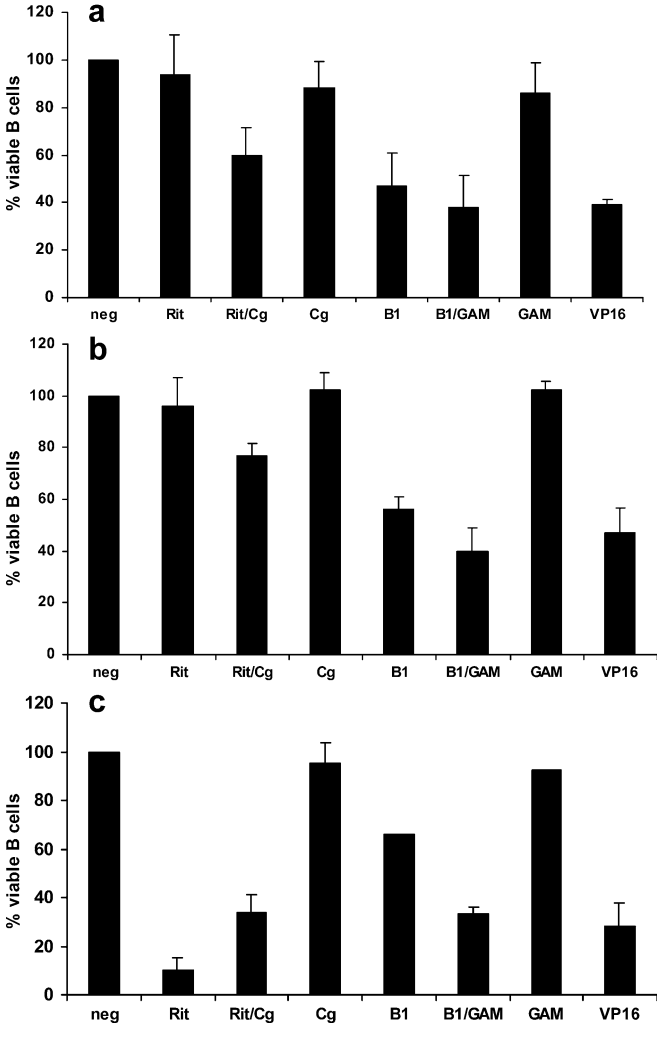

Fig. 1 Reduction of cell viability by anti-CD20 antibodies. Raji cells were incubated with 10 μg/ml rituximab and B1, respectively, with or without 50 μg/ml crosslinking antibody goat antihuman (*Cg*) or goat antimouse (*GAM*), respectively, or crosslinking antibody alone. As positive control 68 μM VP-16 was used. Cell viability was determined after 24 h of incubation at 37°C by trypan blue exclusion. **a** Heat-inactivated FCS (10%) was used as supplement. **b** The artificial supplement BMS was used. **c** Complement-mediated reduction of viability of Raji cells. Cells were incubated with 10 μg/ml rituximab or B1 with or without 50 μg/ml crosslinking MoAbs in the presence of 5% active human serum

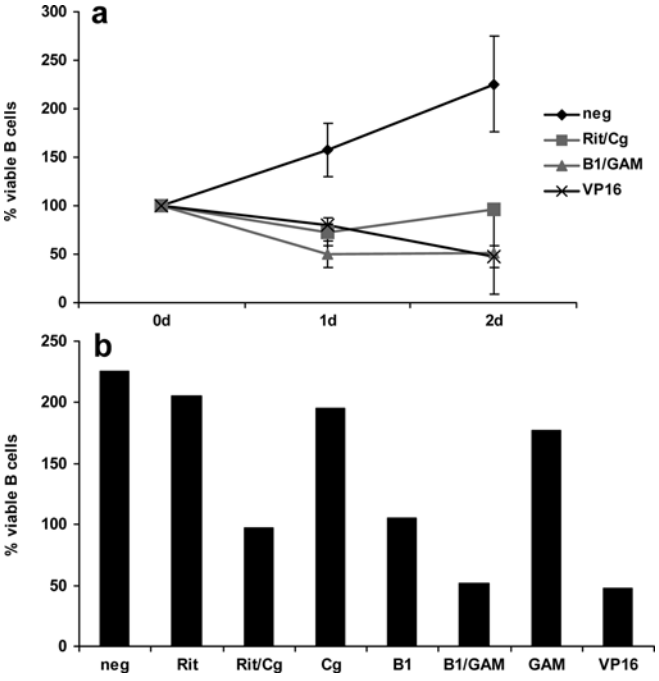

Fig. 2 Time-dependent reduction of cell viability by rituximab and B1. **a** 6×10^5 Raji (100%) cells were incubated with 10 μg/ml rituximab or B1 and 50 μg/ml crosslinking MoAbs for 1 and 2 days. Cell viability was determined by trypan blue exclusion. **b** Cell viability with or without crosslinking antibodies

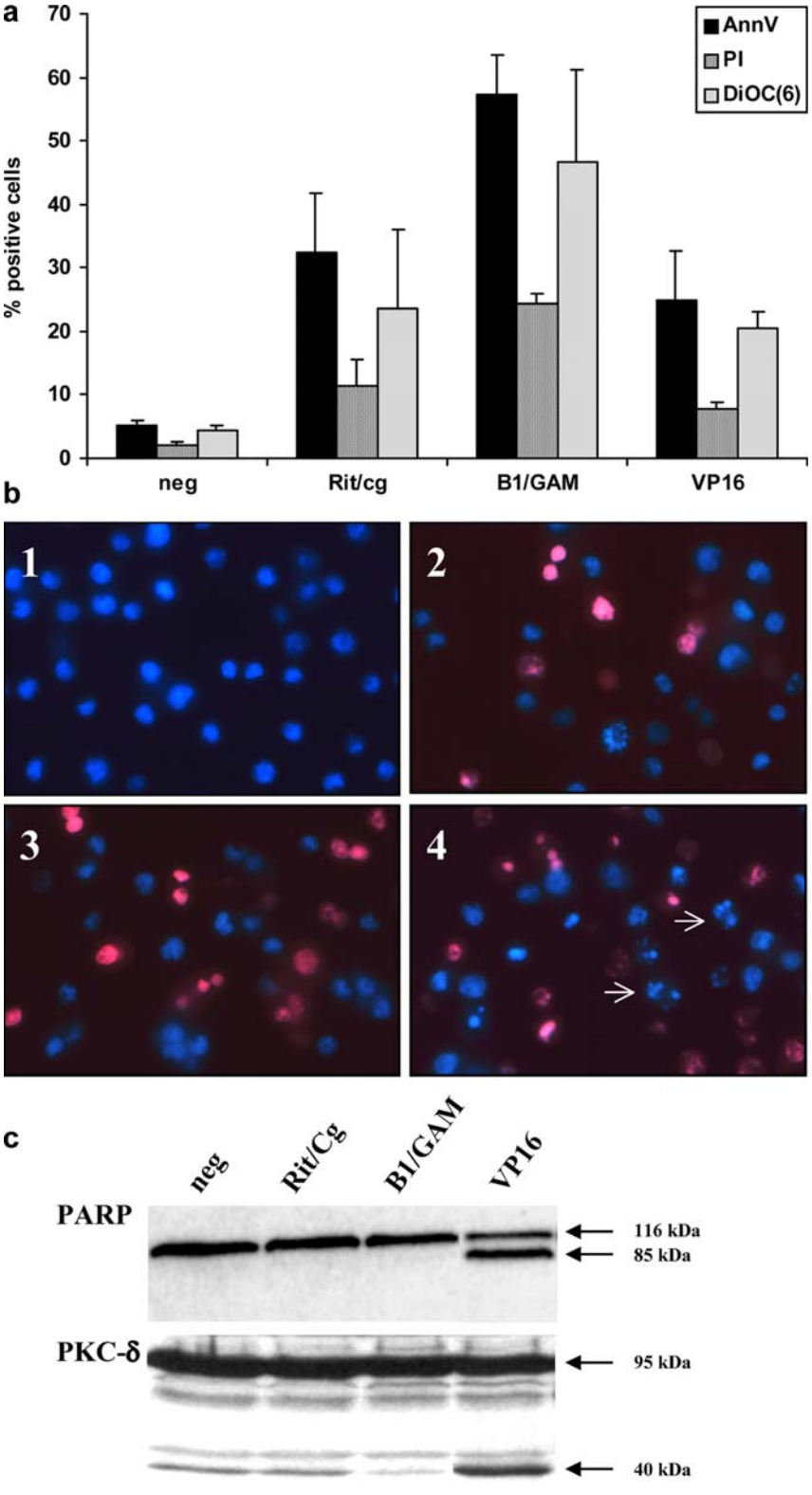

Fig. 3 Induction of cell death in Raji cells by rituximab and B1. **a** Raji cells were incubated with 10 μg/ml rituximab or B1 and 50 μg/ml crosslinking MoAbs or VP-16. After 24 h dead cells were determined by annexin V (*AnnV*), propidium iodide (*PI*), and DiOC(6) staining and analyzed by FACS measurement. **b** Determination of cell morphology by Hoechst 33342 (*red cells*) and propidium iodide (*blue cells*) of (1) untreated cells, (2) rituximab-, (3) B1-, and (4) VP-16-treated cells. Apoptotic cells after VP-16 incubation (4) are marked by *arrows*. Their nucleus is condensed and fragmented. **c** Western blot analysis of PARP and PKC-δ cleavage. PARP is degraded to a 85-kDa fragment in apoptotic cells and PKC-δ is cleaved to an active 40-kDa fragment

(MFI 404) (data not shown). These results suggested that the higher expression of the complement inhibitor CD59 in Ramos cells might cause the resistance of this cell line to CDC caused by rituximab.

Cell death induced by anti-CD20 antibodies occurs in a time-dependent manner

To study the effect of anti-CD20 MoAbs on cell proliferation and induction of cell death in more detail, we performed time kinetics with crosslinked rituximab and

B1. Again VP-16 was used as a positive control (Fig. 2). Rituximab induced rapid cell lysis within 4 h (data not shown) reaching its maximum after 1 day. Thereafter the number of viable cells increased from 73% compared to the starting value to 97% (Fig. 2a) after 48 h and stayed at a constant level until 72 h (data not shown). These results suggest that some cells are resistant to rituximab and retain the ability to proliferate after contact with the antibody. Crosslinked B1 antibody showed a more pronounced effect compared to rituximab with viable cells decreasing to 50% at day 1 and 52% at day 2. In contrast, the number of viable cells after incubation with VP-16 decreased to 79% at day 1 and to 48% at day 2 and further decreased to 18% at day 3 (data not shown). A more detailed analysis of day 2 (Fig. 2b) showed that B1 as single agent induced cell death comparable to rituximab with crosslinking.

Both anti-CD20 antibodies, rituximab and B1, induce cell death by a caspase-independent apoptotic mechanism

Next we analyzed the mode of cell death induced by rituximab and B1. It was shown that B1 might induce apoptosis in Raji and Ramos cells [18, 19]. Therefore, the induction of apoptotic cell death was measured by annexin V-FITC/propidium iodide and DiOC(6) staining and FACS analysis in both cell lines. As shown in Fig. 3a, crosslinked rituximab and B1 clearly induced apoptotic cell death after 1 day of incubation. As in the experiments described above, B1 exhibited stronger effects than rituximab (57% annexin V^+ cells vs 32%). The DiOC(6) staining (47% for B1 and 24% for rituximab), which measures loss of mitochondrial membrane potential in the early phase of apoptosis, showed a good correlation with these results. As expected, VP-16 also induced apoptotic cell death, as detected both by DiOC(6) and annexin V/propidium iodide staining. Cell death was detected as early as 4 h of antibody and VP-16 incubation, respectively (data not shown). Moreover, these analyses showed a good correlation to the determination of viable cells by trypan blue exclusion (Fig. 1). Also flow cytometric changes in FSH (forward scatter) and SSH (sideward scatter), characteristic for apoptosis, could be detected (data not shown).

To analyze the induction of cell death in further detail, the morphological changes associated with cell death were investigated. Cells were stained with Hoechst 33342 and propidium iodide (Fig. 3b). While Hoechst 33342 penetrates intact cell membranes, propidium iodide only crosses damaged membranes in late apoptotic or necrotic cells. This allows the discrimination of healthy cells (faintly blue, Fig. 3b-1) from early apoptotic cells (discrete blue apoptotic bodies, Fig. 3b-4, arrows), late apoptotic cells, and necrotic cells (red).

Surprisingly, we could not detect the existence of early apoptotic cells, characterized by the formation of discrete $Hoechst^+$ apoptotic bodies in rituximab- and B1-treated cells [Fig. 3b-2 (rituximab), b-3 (b1)]. Only propidium iodide-stained cells were seen as an indicator for late apoptosis. The number of these cells correlated well with the number of annexin V^+ cells (data not shown). These observations were not due to experimental procedures, because VP-16-treated cells showed the typical picture of early apoptosis as described by the formation of apoptotic bodies as visualized by the Hoechst dye (Fig. 3b-4, arrows).

We then performed Western blot analyses to define the degradation of further downstream proteins such as PARP or PKC-δ. Apoptosis leads to cleavage of the 116-kDa native PARP to a 85-kDa fragment. Cell lysates of control cells, rituximab-, B1-, and VP-16-treated Raji cells were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and PARP was visualized by anti-PARP immunoblotting. As shown in Fig. 3c, treatment with VP-16 led to a markedly enhanced PARP degradation, as seen by the 85-kDa PARP fragment. In contrast to a recent publication [19, 28], we did not detect any PARP degradation in rituximab- and B1-treated cells. To further confirm this result, we also analyzed the proteolytic activation of another protein, PKC-δ that is involved in apoptosis [29]. PKC-δ is cleaved by caspase 3 to generate a 40-kDa kinase active fragment. As with PARP we could detect a cleavage of PKC-δ in the VP-16-treated cells, but not in the anti-CD20 antibody-treated cells. Taken together, these results indicate that anti-CD20 MoAbs seem to induce a nonclassic pathway of programmed cell death that is independent of the activation of caspases. This pattern is known for other antibodies such as anti-CD47 [30] and anti-HLA-DR antibodies [31].

Induction of cell death by alemtuzumab

Alemtuzumab was shown to deplete B cells in vivo. Similar to rituximab, potential mechanisms are antibody-dependent cellular cytotoxicity (ADCC) and CDC [15]. However, the mechanisms of cell destruction are even less understood than for anti-CD20 MoAbs. Because alemtuzumab is a promising agent for the treatment of B-cell neoplasias, we were interested to learn more about its mechanism of action. As the expression of CD52 on Raji cells was very low, we had to use Ramos cells. As shown in Fig. 4a, alemtuzumab alone (concentration 10 μg/ml) had no effect on the viability of Ramos cells. Crosslinking alemtuzumab with a goat antihuman antibody (Cg) decreased cell viability by about 35% as determined by trypan blue exclusion. Again, the crosslinking antibody alone had no effect on cell viability.

To examine whether these results were due to a stop in proliferation or to an induction of cell death, FACS analyses were performed (Fig. 4b). Again we could detect an increase of annexin V^+ cells from 13% (untreated cells) to 34% after treatment with alemtuzumab and crosslinking antibody, indicating that alemtuzumab-treated cells were killed by a mechanism involving the presentation of annexin V at the surface. Similarly, increased numbers of dead cells were also detected in the forward/sideward

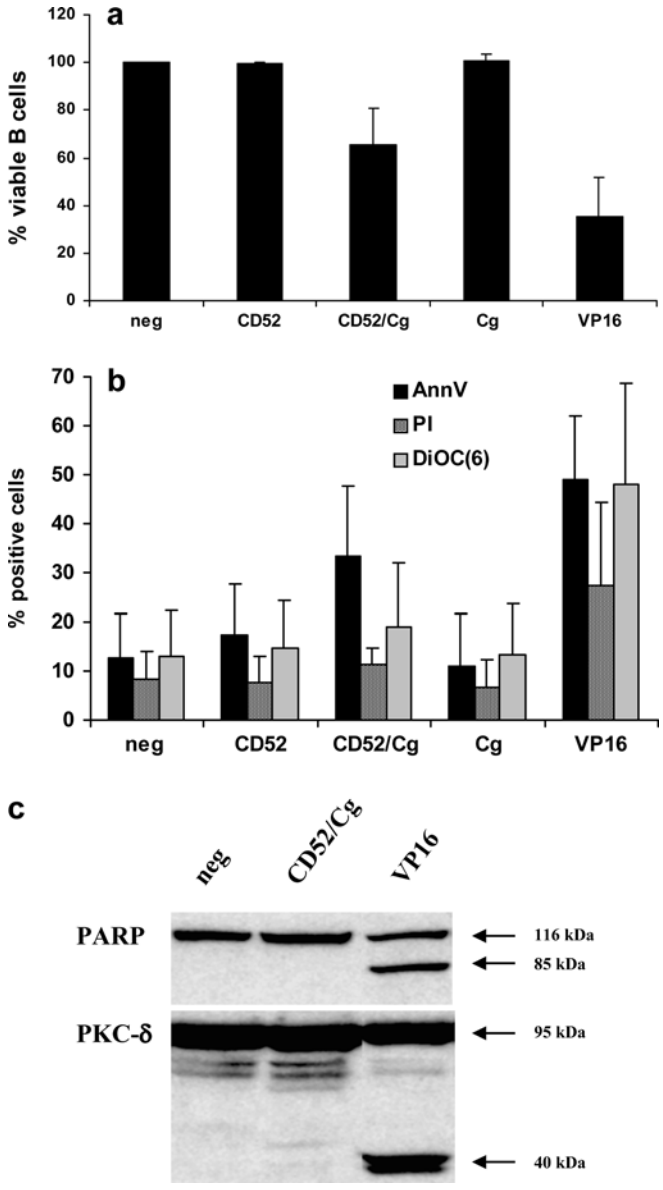

Fig. 4 Induction of cell death in Ramos cells by alemtuzumab. **a** 6×10^5 Ramos cells well incubated with 10 μg/ml alemtuzumab, with or without 50 μg/ml crosslinking antibody goat antihuman (*Cg*) or goat or crosslinking antibody alone. As positive control 68 μM VP-16 was used. Cell viability was determined after 24 h of incubation at 37°C by trypan blue exclusion. **b** Comparison of annexin V/propidium iodide and DiOC(6) staining. **c** Western blot analysis of PARP and PKC-δ cleavage. PARP is degraded to a 85-kDa fragment in apoptotic cells and PKC-δ is cleaved to an active 40-kDa fragment

scatter (data not shown). Again incubation with alemtuzumab alone or crosslinking antibody alone showed no effect (17% and 11% annexin V^+ cells, respectively). When using DiOC(6) staining, an increase of cells with loss of mitochondrial membrane potential was also detected, although the number of cells undergoing apoptosis recognized by this method was significantly lower (19%) than with annexin V staining (34%). Western blot analyses with anti-PARP and anti-PKC-δ MoAbs (Fig. 4c) were performed. As with anti-CD20 MoAbs, no PARP cleavage and no PKC-δ activation were detected in alemtuzumab-treated cells. These results indicated that comparable to rituximab alemtuzumab used a caspase-independent mode to induce cell death.

Cell death induction by anti-CD20 and anti-CD52 antibodies in freshly isolated CLL cells

Next, we determined whether the same mechanisms played a role in the killing of primary CLL cells. CLL cells were purified from peripheral blood of eight CLL patients as described under "Methods." Expression of CD20 and CD52 was analyzed by FACS measurement (Fig. 5a). It is known that CD20 expression in CLL patients is relatively low when compared with CD52 [32]. In our samples, the MFI varied between 14 and 280 for CD20, and between 43 and 392 for CD52.

Because CLL cells proliferate slowly in vitro, a reduction of viable cells could only be caused by cell lysis. We first compared trypan blue staining and annexin V/propidium iodide staining. Figure 5b demonstrates that both methods yielded similar results. For further analysis we performed annexin V/propidium iodide measurements. First, we analyzed the influence of anti-CDC20 and anti-CD52 MoAbs on the cell viability after 24 h of cell incubation. Again we compared MoAbs alone, MoAbs with a secondary crosslinking antibody, and the crosslinking MoAbs alone. As a positive control for cell death induction we used dexamethasone. As seen in Fig. 5c, incubation of CLL cells with rituximab alone or together with a crosslinking antibody had only minor effects on cell viability of CLL patients 1, 2, 5, and 6. The same results were obtained when B1 and a goat antimouse antibody were used. This failure of inducing cell death might be due to the low expression of CD20 on the malignant B cells, especially for patients 2 and 5. Treatment of CLL cells isolated from patient 8 clearly induced cell death depending on the crosslinking of rituximab (56% annexin V-positive cells) and B1 (60%), respectively, compared to 30% annexin V-positive cells for untreated cells. Although CLL cells of this patient had the highest CD20 expression (MFI 294) of the samples analyzed, the additional cell death induction was much lower (twofold) than with patient 4 (threefold), for whom the number of annexin V-positive cells increased from 12 to 31% (Rit/Cg) and 34% (B1/GAM). This might be due to the high basic level of apoptotic cells in the B-cell preparation of patient 3 (30%).

Next, we treated CLL cells with alemtuzumab. This antibody alone also showed no effect, except for patient 4, in whom all three MoAbs analyzed induced cell death without crosslinking (Fig. 5c). Crosslinking the alemtuzumab antibody enhanced the number of annexin V-positive cells from 32 to 42% compared to 12% for untreated cells. In all patient samples except one, cell death induction by alemtuzumab was strictly dependent on crosslinking of the antibody. We then asked whether the loss of mitochondrial membrane potential observed in antibody-treated cell lines also occurred in the CLL cells.

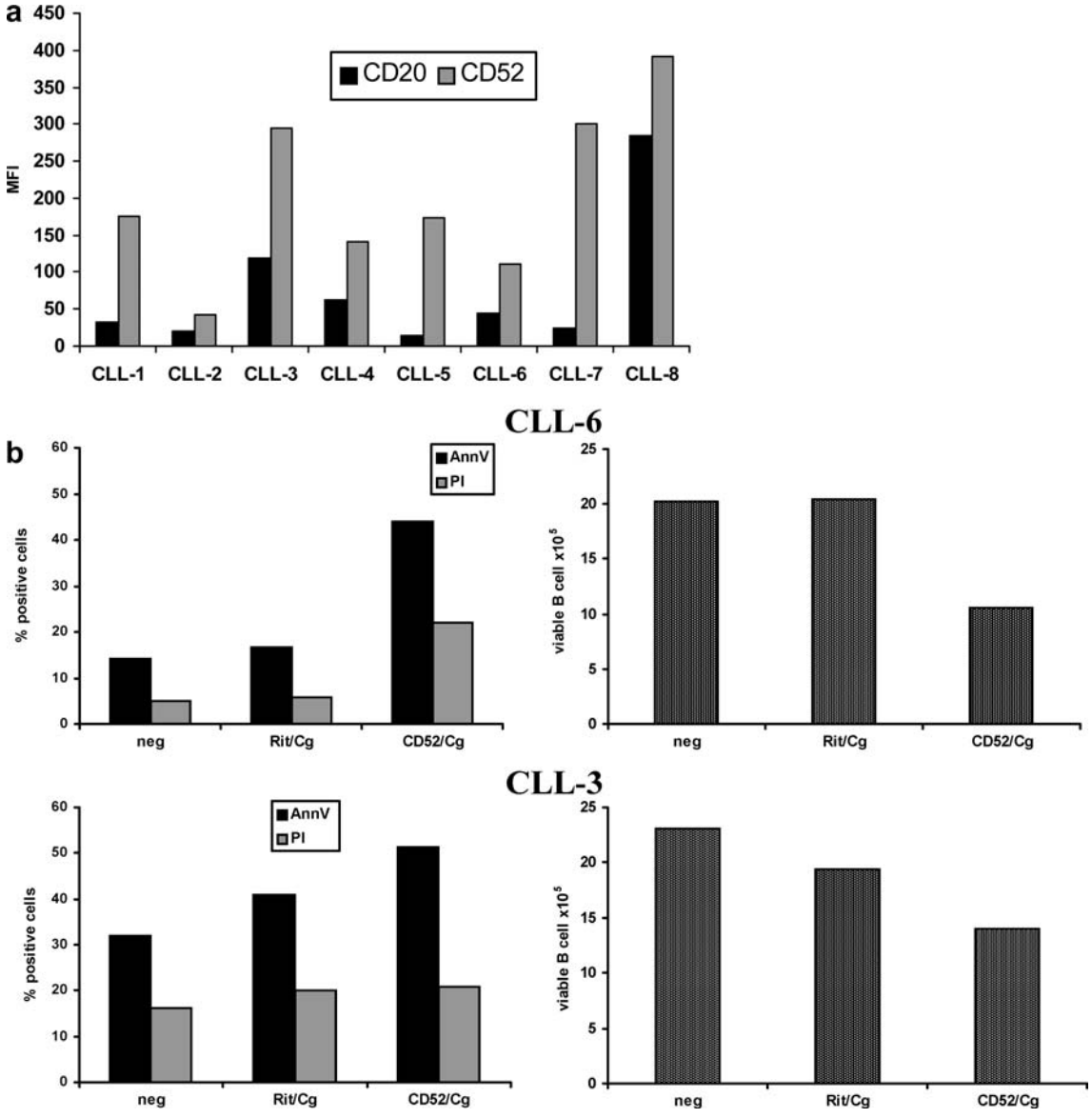

Fig. 5 Cell death induction in CLL cells in vitro. **a** Expression of CD20 and CD52 on CLL cells was measured by FACS analysis (*MFI* mean fluorescence intensity). **b** Purified CLL cells were incubated with 10 μg/ml rituximab and 10 μg/ml alemtuzumab, respectively, together with 50 μg/ml of the corresponding cross-linking antibody. Cell viability was determined after 24 h of incubation at 37°C by trypan blue exclusion (viable cell $\times 10^5$). Cell death was analyzed by annexin V (*AnnV*) and propidium iodide (*PI*) measurement (% positive cells). Examples of CLL patients 3 and 6 are shown

Figure 5d shows that this observation could be confirmed in freshly isolated CLL cells, although the number of DiOC(6)-negative cells characteristic for cells undergoing programmed cell death was higher than the number of annexin V-positive cells. The percentage of apoptotic cells without any treatment was 5.6 vs 15.6% as determined by annexin V measurement vs DiOC(6) staining, respectively. After alemtuzumab treatment, 33.6 or 44.2% of the cells were apoptotic as determined by annexin V or DiOC(6) staining, respectively.

Finally, cell lysates of freshly isolated CLL cells were analyzed by Western blotting and probed with an anti-PARP antibody. As in cell lines, no additional PARP cleavage could be detected in the antibody-treated cells (Fig. 5e). Using dexamethasone as positive control, a significant PARP cleavage was seen following dexamethasone-induced apoptosis. Taken together, the results indicate that both in neoplastic B-cell lines and in primary CLL cells, cell death induced by the MoAbs rituximab, B1, and alemtuzumab is induced by a nonclassic pathway of cell death, which does not use caspases and cannot be described by the morphological pattern of early apoptosis.

Discussion

Rituximab and alemtuzumab show promising results in the treatment of non-Hodgkin's lymphomas (NHL) including

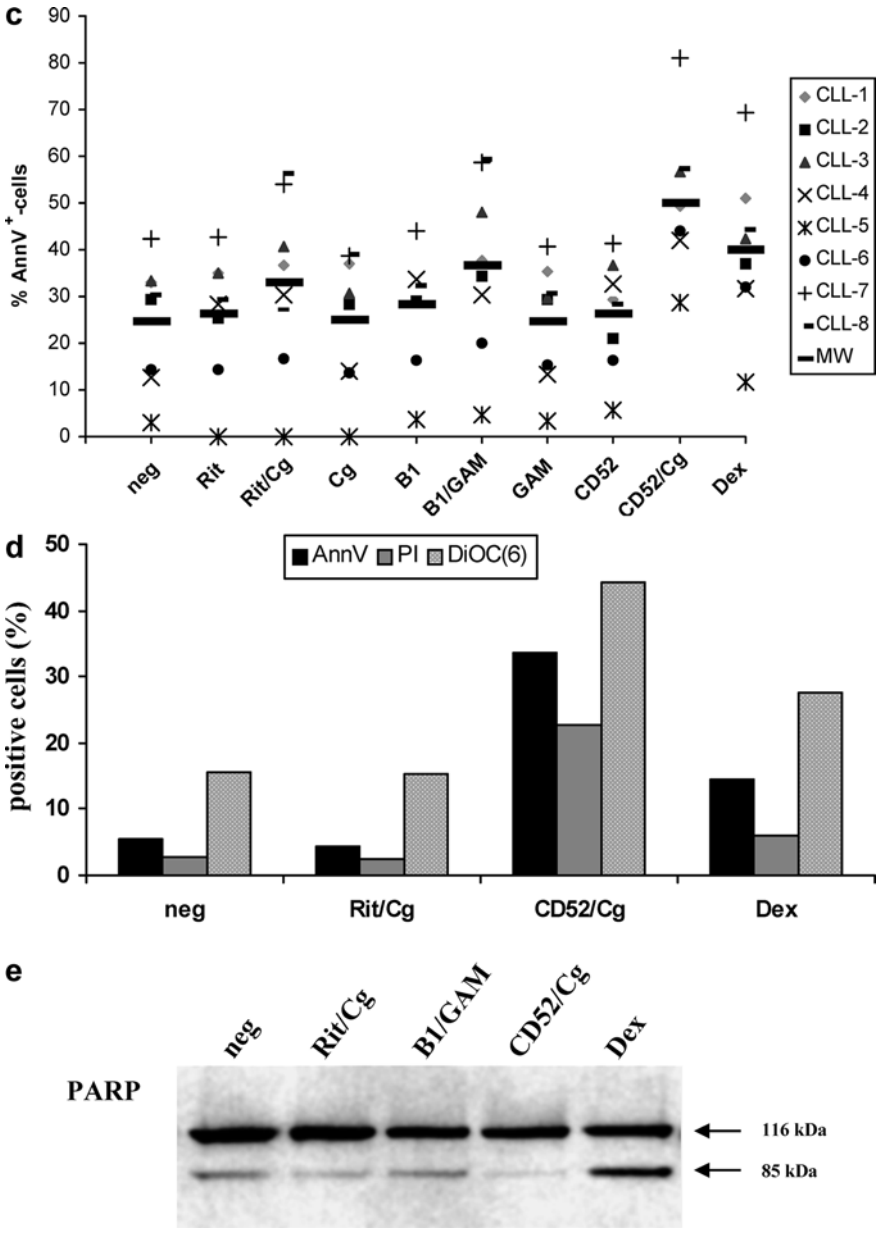

Fig. 5 **c** Purified CLL cells were incubated with the indicated MoAbs (10 μg/ml) and crosslinking MoAbs (50 μg/ml) and annexin V/propidium iodide staining was measured after 24 h. **d** Comparison of annexin V/propidium iodide and DiOC (6) staining, as demonstrated for CLL patient 6. **e** Western blot analysis of PARP cleavage of cell lysates of CLL patient 3. PARP is degraded to a 85-kDa fragment in apoptotic cells

CLL. Involvement of apoptosis, complement-dependent cytotoxicity (CDC), and antibody-dependent cytotoxicity (ADCC) are discussed as potential mechanisms of action [17]. However, the precise mechanism of action of both MoAbs remains to be elucidated. In our study we analyzed the direct, effector cell-independent cell killing activity of two anti-CD20 MoAbs rituximab and B1 and of the anti-CD52 alemtuzumab, in vitro. Rituximab alone had no effect on cell viability of the B-cell lines tested. Crosslinking with a secondary antibody was a mandatory prerequisite to achieve a reduction of viable cells, while the crosslinking antibody alone had no effect. In marked contrast, B1 alone induced a marked reduction of cell viability that could be further enhanced by using a secondary antibody and was stronger than with crosslinked rituximab, even if analyzed for a longer period of time. Similar results were recently described by Cardarelli et al. [33] with a different B-cell line (BALL-1) and Chan et al. [34]. In contradiction to the results obtained by Cardarelli et al. and by our group, the induction of apoptosis in Ramos cells by rituximab without crosslinking was observed by Hofmeister et al. [18] and Shan et al. [19].

The same response was obtained in the presence of an artificial or heat-inactivated serum to exclude effects due to complement. Concomitant incubation of rituximab and human serum led to an increased reduction of cell viability of Raji but not of Ramos cells. Analysis of the expression of complement inhibitors CD55 and CD59 showed that the expression of CD55 was comparable in Ramos and Raji cells, while expression of CD59 was significantly lower on the latter cell line. This indicates that the reduced CD59 expression is responsible for the reduced viability of Raji cells. These results are in accordance with observa-

tions by Treon et al. [35] who showed that multiple myeloma and NHL cell lines with low CD59 expression were sensitive to CDC induced by rituximab, while $CD59^+$ cell lines were not. The importance of complement inhibitors was further demonstrated by use of anti-CD55 and anti-CD59 MoAbs, which increased complement-mediated cell lysis [20, 36]. However, expression of these complement inhibitors had no impact on the clinical outcome of rituximab treatment of follicular lymphoma [37] or CLL patients [38], nor did it allow the prediction of complement susceptibility of CLL, PLL, or MCL patient samples in vitro. Therefore, the importance of CDC in vivo is still not entirely clear. CDC might contribute to tumor cell killing in vivo, as an IgG4 version of the antibody is unable to deplete normal B cells in primates [39]. Complement activation also seems to play a key role with regard to the clinical side effects of rituximab [40]. Using the mouse anti-CD20 antibody B1 alone or in combination with a crosslinking antibody, no complement-mediated cell killing could be observed. A recent publication by Cragg and Glennie [41] indicates that this difference between rituximab and B1 might be due to a different binding of C1q, the first component of the complement cascade. The redistribution of CD20 by rituximab to membrane rafts seems to be a prerequisite for C1q binding. Antibodies unable to be redistributed such as B1 cannot activate the complement cascade.

Further analysis showed good agreement between the reduction of cell viability and the induction of cell death as reflected by assessment of the hallmarks of early apoptosis (phosphatidyl exposure on the outer cell membrane or reduction of mitochondria membrane potential $\Delta\psi_m$ [18, 26]). In contrast to the observations described in these publications, a more detailed analysis by Western blotting in our investigations showed no features of a classic, caspase-dependent apoptosis such as PARP or PKC-δ degradation. In addition, morphological studies also did not show typical apoptotic features such as the formation of apoptotic bodies characterized by Hoechst 33342 staining, but exclusion of propidium iodide. Cell death induced by alemtuzumab showed similar characteristics. This was not only seen in B-cell lines, but also with freshly isolated CLL cells in vitro (Fig. 5b, c, d, e).

There is accumulating evidence that antibody-induced cell death may not follow the route of classic apoptosis. Van der Kolk et al. recently described that rituximab induced a caspase-independent cell death in Ramos cells [42]. These authors showed that the use of the broad-spectrum caspase inhibitor zVAD-fmk inhibited caspase activation and PARP degradation but not induction of cell death. Similar apoptotic mechanisms were also observed for the cell death induction in T cells by MoAbs against CD45 [43]. Again, early apoptotic events could be observed, but no nuclear events such as chromatin condensation or DNA fragmentation. In addition, the production of reactive oxygen species (ROS) was shown. Bellosilo et al. showed that the incubation of lymphoma cells in vitro with rituximab and human complement led to caspase-independent cell death induction and involvement of ROS [44]. Finally, induction of apoptosis in CLL cells in vitro without nuclear features was also described for a MoAb against CD47 [30]. So far, the exact mechanisms of these apoptotic pathways induced by the different antibodies are not understood, but their biochemical and morphological characteristics clearly differ from the classic pattern of apoptosis [30, 43]. Also in vivo the contribution of apoptosis and caspase activation for the effects of rituximab is not clear. In a recent publication, Byrd et al. observed caspase-3 activation and PARP degradation in vivo in three CLL patients with partial response, but not in seven other patients with stable disease after rituximab treatment [4].

Activation of MAP kinases might be involved in cell death induction, as both crosslinked rituximab and B1 induce tyrosine phosphorylation and activation of MAP-kinase ERK-2 in Raji cells [45]. Activation of ERK-2 with immobilized rituximab was recently observed in the B-cell line BL60-2 by Mathas et al. [28]. Soluble rituximab induced a much weaker phosphorylation, but this might be due to the fact that the antibody was not crosslinked in these experiments. Phosphorylation of the MAP-kinases JNK, ERK, and p38 could also be shown in CLL cells [46]. Again phosphorylation was dependent on rituximab crosslinking.

Much less is known about the effects of alemtuzumab so far. Our results obtained in Ramos cells showed that alemtuzumab led to reduction of viable cells, but only after crosslinking of the antibody. Alemtuzumab was described to induce growth arrest in the B-cell line Wien 133 [23]. FACS analysis of phosphatidyl serine exposure and reduction of mitochondria membrane potential clearly showed that alemtuzumab can also induce cell death (Fig. 4b). Alemtuzumab alone showed no effect. Analysis of PARP and PKC-δ degradation demonstrated that alemtuzumab induced nonclassic apoptosis in Ramos cells in vitro, similar to rituximab. Contrary to rituximab-induced apoptosis, the induction of cell death as measured by DiOC(6) staining after crosslinking of alemtuzumab was significantly lower compared to annexin V staining. This indicates that the mitochondrial pathways might not play a major role in alemtuzumab-induced apoptosis. Interestingly, Chan et al. [34] could recently demonstrate that Bcl-2 overexpression strongly reduced the CD20 antibody-induced mitochondria membrane depolarization, but not the amount of annexin V-positive cells. This indicates that also CD20-induced apoptosis can also bypass the mitochondrial pathway. Alemtuzumab also induced cell death in freshly isolated, primary CLL cells. In all CLL samples analyzed, alemtuzumab induced higher apoptosis compared to rituximab or B1. This at least in part might be due to the higher expression of CD52 compared to CD20 in all patient samples analyzed (Fig. 5a). Due to the small number of cases analyzed, the relationship to pretreatment of CLL patients, cytogenetic abnormalities, or to the expression of CD20 and CD52 could not be analyzed. Taken together, our results indicate that the induction of atypical apoptosis might also

play a role in reduction of tumor cells in vivo by alemtuzumab treatment.

In conclusion, the observations presented in this manuscript suggest that the activation of a caspase-independent apoptotic pathway is a potential mechanism of cell killing by both MoAbs and explains why both rituximab and alemtuzumab can lyse tumor cells in patients who have become resistant to chemotherapeutic agents, which depend on activation of classic caspase-dependent apoptotic pathways to exert their function.

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