Cytotoxic effects of the trifunctional bispecific antibody FBTA05 in ex-vivo cells of chronic lymphocytic leukaemia depend on immune-mediated mechanisms

Simone Boehrer[a], Petra Schroeder[b], Tina Mueller[c], Judith Atz[c] and Kai Uwe Chow[d]

Monoclonal antibodies such as rituximab and alemtuzumab show considerable therapeutic efficacy in chronic lymphocytic leukaemia (CLL). Aiming to further improve antineoplastic efficacy, the trifunctional bispecific antibody FBTA05 was developed. FBTA05 is thought to function by simultaneously binding B cells and T cells by its variable regions and by recruiting FcγR-positive accessory immune cells by its intact Fc region. As it was previously shown that this antibody shows considerable cytotoxicity towards a spectrum of B-cell lymphoma cell lines, we here tested its potential efficacy *ex vivo* against malignant B-CLL cells. Therefore, we assessed the capacity of increasing concentrations of FBTA05 to bind to neoplastic cells, to induce cytotoxicity (comparing it with rituximab and alemtuzumab) and cytokine release. We evaluated the results with respect to the extent of CD20 expression, the effector:target cell ratio as well as with the patients' overall effector cell status. Thus, we show that, although FBTA05-elicited cytotoxicity was comparable with that induced by alemtuzumab, it considerably exceeded the antineoplastic effects of rituximab. Noteworthy, FBTA05 shows effective elimination of malignant B cells even if CD20 surface expression is low. Importantly, a high grade of cytotoxicity was associated with the induction of T-cell proliferation and the concomittant release of interferon-γ and interleukin-6, thus overcoming the detrimental effects of an unfavourable effector:target cell ratio. In conclusion, we here present novel evidence for the therapeutic efficacy of the trifunctional, bispecific antibody FBTA05 in CLL and provide evidence for the importance of immune-mediated mechanisms conveying the cytotoxic effects against malignant B lymphocytes. *Anti-Cancer Drugs* 22:519–530 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2011, **22**:519–530

Keywords: chronic lymphocytic leukaemia, cytotoxicity, FBTA05, trifunctional bispecific antibody

[a]Service d'Hématologie Clinique, Hôpital Avicenne, AP-HP/Université Paris XIII, Bobigny, France, [b]EUFETS GmbH, Idar-Oberstein, [c]Fresenius Biotech GmbH, Preclinical Research and Development, Munich and [d]Department of Hematology and Oncology, Private Practice Center, Frankfurt, Germany

Correspondence to Professor Dr Kai Uwe Chow, Department of Hematology and Oncology, Private Practice Center, Schaubstrasse 16, 60596 Frankfurt, Germany
Tel: +49 69 6500 73 134; fax: +49 69 6500 73 150;
e-mail: chow@ifs-frankfurt.de

Received 29 November 2010 **Revised form accepted** 11 January 2011

Introduction

Non-Hodgkin's lymphoma (NHL) and chronic lymphocytic leukaemia (CLL) are among the most prevalent malignancies. NHL is the fifth most common malignant disease in the USA with an annual incidence rate of 18–20 per 100 000 individuals. The incidence of NHL increased annually by 5–10% until the mid-1990s, but has remained constant since then [1]. CLL belongs to the frequent NHLs and furthermore is the most common leukaemia in the Western world with an annual incidence of three to four per 100 000 individuals.

The monoclonal antibodies rituximab and alemtuzumab considerably enlarged the therapeutic armentarium for these diseases and provided proof of principle for the therapeutic efficacy of antigen-directed treatment with antibodies. Nevertheless, a considerable percentage of patients show primary or secondary resistance towards these agents [2,3]. It thus seems feasible to exploit this treatment principle while increasing therapeutic efficacy. To this aim, the trifunctional bispecific antibody FBTA05 was designed [4]. The antibody is directed against human CD20 and human CD3 and exerts its activity by simultaneous binding of B cells and T cells by its variable regions and by recruiting FcγR-positive accessory immune cells by its intact Fc region. As previous studies demonstrated the in-vitro efficacy of this antibody against lymphoma-derived tumour cell lines and primary cells of patients with CLL as well as its favourable toxicity profile and antitumour activity *in vivo* [4,5], we here aimed to further define the antibody's in-vitro effects on primary cells of patients with CLL in more detail, including the assessable immunological determinants.

Materials and methods

Antibodies

FBTA05 was supplied by Fresenius Biotech GmbH (Munich, Germany). The monoclonal antibody rituximab was obtained from Roche (Penzberg, Germany) and

0959-4973 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/CAD.0b013e328344887f

alemtuzumab was purchased from Genzyme Europe (Massachusetts, USA). Antibodies were used at the dosages depicted in the respective experiment.

Collection of peripheral blood from patients with CLL and isolation of mononuclear cells

After patients had given informed consent, blood samples were collected as heparinized samples by the Private Practice Center, Frankfurt, Germany. Subsequently, peripheral blood mononuclear cells (PBMCs) were purified by density gradient centrifugation using lymphocyte separation medium (PAA, Coelbe, Germany). The diagnosis of CLL was based on standard clinical and laboratory criteria. After purification, CLL cells were immediately used for the respective experiments. The experimental part was conducted at EUFETS GmbH on behalf of Fresenius Biotech GmbH.

Immunological phenotyping of the lymphatic subpopulations

Phenotyping of PBMC subpopulations was performed by flow cytometry. Therefore, samples were stained with fluorescein isothiocyanate (FITC)-conjugated, phycoerythrin (PE)-conjugated, PerCP-conjugated or allophycocyanin-conjugated antibodies (Becton Dickinson, Heidelberg, Germany; Beckmann Coulter, Krefeld, Germany; Immunostep, Salamanca, Spain) directed against CD45 (leucocytes), CD3 (T lymphocytes), CD4 (helper T lymphocytes), CD8 (cytotoxic T lymphocytes), CD19 (B lymphocytes), CD16 (natural killer cells) and CD14 (monocytes) after lysis of erythrocytes using TQ-Prep (Beckmann Coulter). CLL diagnosis was confirmed by analysing the pathologic coexpression of CD20/CD5, CD19/CD5 and CD19/CD23, respectively. Differences in cell surface expression of individual antigens depicted in Tables 1 and 2 are due to the fact that measurements were carried out on different samples and gating, on the basis of live cells by forward and side scatter, slightly differed. The absence or low expression of the adhesion molecules CD11c and CD103, of the progenitor marker CD38 and the B lymphocytic antigens CD138 and FMC7 was determined by fluorochrome-conjugated antibodies. The antibody directed against CD23 was purchased from Immunostep, and all other antibodies from Beckman Coulter. Lymphocytes were gated to exclude dead cells and debris. Data acquisition and analysis of the immune status and CLL diagnosis were performed using the CellQuest Pro Software (Becton Dickinson). The percentage of positive cells of the respective subpopulation was correlated to the total counts of nucleated cells.

The specific CD20-expression of B lymphocytes was calculated as follows:

$$\text{specific CD20 expression} = \text{MFI of CD20} - \text{MFI of IgG},$$

where MFI is the median fluorescence intensity. The effector:target ratio (E:T ratio) was defined as the ratio of CD3-positive T lymphocytes (effector cells) to CD20-positive B lymphocytes (target cells). Consequently, the effector:target ratio was calculated as follows:

$$\text{E:T ratio} = \frac{\%\ \text{CD3 positive cells}}{\%\ \text{CD20 positive cells}}$$

Assessment of FBTA05-induced activation and proliferation of T lymphocytes

PBMC were stained after one washing step using fluorescence-activated cell sorting (FACS) buffer with fluorochrome-conjugated antibodies binding to CD3, CD4 and CD8. Activation status of T lymphocytes was evaluated by co-staining of CD25 on CD4-positive and CD8-positive cells, respectively. Assessment of changes in the proliferation and activation pattern was carried out only on viable cells as determined by FACS analysis (see above) under the indicated concentrations of the FBTA05-antibody. Cell numbers were evaluated using an automated lymphocyte counting system and

Table 1 Immunophenotypic analysis of patient samples

CLL patient number	CD5+/CD19+	CD5+/CD20+	CD23+/CD19+	CD103+/CD19+	CD11c+/CD19+	FMC7+/CD19+	CD38+/CD19+	CD138+/CD19+
01-001	95	57	90	1	58	1	1	3
01-002	52	4	75	2	2	8	20	73
02-001	95	76	91	0	6	24	0	1
03-001	93	90	80	0	10	13	0	1
04-001	81	55	69	1	60	3	73	2
05-001	90	90	89	1	24	32	0	0
06-001	96	78	92	0	7	2	31	0
07-001	90	84	27	1	77	42	0	0
08-001	27	28	29	0	15	29	7	0
09-001	70	70	60	1	13	10	47	0
10-001	90	90	89	0	50	12	1	0
11-001	87	87	85	0	53	56	0	0
12-001	81	79	85	1	10	58	0	0
13-001	79	78	ND	ND	ND	ND	0	ND

Shown are the percentages of positive cells (+) with coexpression of the indicated antigens.
CLL, chronic lymphocytic leukaemia; ND, not determined.

Table 2 Immune status of chronic lymphocytic leukaemia patients as determined by immunophenotypic analysis

CLL patient number	CD45+	CD3+	CD4+/ CD3+	CD8+/ CD3+	CD16+/ CD3−	CD19+	CD20+	CD14+	CD3+ : CD20+
01-001	100.00	3.72	0.54	2.66	1.50	96.70	68.88	0.41	1 : 18.5
01-002	97.53	2.00	0.86	0.52	0.37	94.87	3.87	7.16	1 : 1.9
02-001	100.00	1.31	1.00	0.57	0.31	97.62	97.38	0.17	1 : 74.3
03-001	99.96	8.89	3.65	4.61	3.05	89.66	90.53	0.55	1 : 10.2
04-001	99.64	22.86	11.02	11.32	ND	72.67	57.15	4.80	1 : 2.5
05-001	99.83	3.44	1.57	1.20	4.19	89.65	88.90	0.24	1 : 25.8
06-001	99.30	3.45	1.73	1.56	0.49	95.68	75.68	0.10	1 : 21.9
07-001	99.70	10.22	4.17	6.35	1.50	88.09	82.31	1.68	1 : 8.1
08-001	98.43	65.74	45.34	21.40	2.44	27.48	26.01	3.93	1 : 0.4
09-001	99.71	19.47	13.22	5.88	6.50	72.15	69.39	3.49	1 : 3.6
10-001	99.67	6.96	4.66	1.50	2.13	90.80	88.60	0.51	1 : 12.7
11-001	97.53	5.52	3.26	2.34	1.94	88.94	88.14	1.76	1 : 16.0
12-001	97.72	8.35	4.93	3.56	1.61	88.65	81.11	1.28	1 : 9.7
13-001	97.96	8.19	4.97	3.38	2.00	88.37	80.64	2.75	1 : 9.8

Depicted are the percentages of positive cells (+) with coexpression of the indicated antigens as well as the effector to target cell ratio (CD3+ : CD20+).
CLL, chronic lymphocytic leukaemia; ND, not determined.

proliferation or activation of T lymphocytes was calculated as follows:

$$\text{T lymphocytes}\,(\%) = \left(\frac{\text{T lymphocyte counts of sample}}{\text{T lymphocyte counts of control}}\right) \times 100$$

Assessment of the binding capacity of FBTA05 and of antibody-mediated cytotoxicity

FBTA05 binding was evaluated by flow cytometry. To this end, 1×10^6 PBMCs were incubated for 30 min at 2–8°C with increasing concentrations of FBTA05 (0.1, 1, 10 μg/ml) as well as with a PE-conjugated anti-CD19 antibody. Control samples without FBTA05 were analysed in parallel. After one washing step, cells were incubated for 30 min at 2–8°C with a 1 : 50 prediluted FITC-conjugated anti-rat immunoglobulin G antibody (Dianova, Hamburg, Germany). After the incubation, cells were washed with PBS and FBTA05 binding to B lymphocytes was analysed by flow cytometry. FBTA05 binding was calculated as follows:

% positive cells for FBTA05 binding =
% positive cells at indicated FBTA05 concentration −
% positive cells of control sample

FBTA05-mediated cytotoxicity of patient immune effector cells against autologous B lymphocytes was analysed after two incubation periods. Depending on the time of arrival of the patients' blood samples to the laboratory, the first analysis of cytotoxicity was carried out on day 3 or 4 and the second analysis 3 days after the first assessment, that is, on day 6 or 7 of the incubation period. At the beginning of the incubation period, 2×10^6 PBMCs were cultured in 48-well culture plates in media supplemented with 20% native human serum [in order to enable complement-dependent cytotoxicity (CDC)] and the indicated concentrations of FBTA05 (0.1, 1, 2, 5, 10, 20, 50, 100, 200 ng/ml), rituximab and alemtuzumab (0.1, 0.2, 1, 10, 100 μg/ml), respectively. Provided that patient material was sufficient, each antibody concentration was evaluated two-fold. Cells were incubated at 37°C and at 5% CO_2. For the second assessment of cytotoxicity, the respective antibody was added again (concentrations as indicated above) including an exchange of 50% of the medium on the day of the first analysis. The number of B lymphocytes was evaluated after the first as well as after the second incubation period as follows: the absolute number of cells was determined using an automated lymphocyte counting system (Ac-T diff, Beckman Coulter). Dead cells were discerned by 7-aminoactinomycin D (7-AAD, Beckmann Coulter) staining and by subsequent flow-cytometric analysis using the CellQuest Pro Software. The residual numbers of B lymphocytes, T lymphocytes as well as T helper and cytotoxic T lymphocytes were assessed after staining with the respective antibodies within the 7-aminoactinomycin D-negative cell population and was calculated as follows:

$$\text{Residual B lymphocytes}\,(\%) = \left(\frac{\text{B lymphocyte counts of sample}}{\text{B lymphocyte counts of control}}\right) \times 100$$

Release of the cytotoxic mediators perforin and granzyme B

Patient cells were preincubated at 4×10^5 cells per well in microtitre plates for 24 h with the indicated FBTA05 concentrations at 37°C and at 5% CO_2 in a humidified atmosphere. To determine nonspecific activation of lymphocytes, samples without FBTA05 were incubated with phytohaemagglutinin (Sigma-Aldrich, Taufkirchen, Germany) at a concentration of 5 μg/ml. Subsequently, cells were transferred to the respective ELISPOT microtitre plates, which had been precoated overnight with antigranzyme B or antiperforin antibodies following the manufacturer's instructions (Diaclone, Hoelzel Diagnostics, Cologne, Germany). Microtitre plates were incubated overnight at

37°C and at 5% CO_2 in a humidified atmosphere. Granzyme B-specific and perforin-specific spots in the plates were evaluated after removal of the cells by several washing steps and incubation with the respective biotinylated detection antibodies and visualized by a streptavidin–alkaline phosphatase reaction in the presence of the substrate 5-bromo-4-chloro-3-indolyl-phosphate/nitro tetrazolium blue chloride using an ELISPOT reader. In parallel, the same analysis was carried out on cell-free wells (containing only medium) serving as controls. Thus, specific FBTA05-mediated release of granzyme B and perforin was calculated as follows:

$$\text{Specific spots}\,(\%) = \left(\frac{\text{Spot counts of control}\,(0\,\text{ng/ml})}{\text{Spot counts of sample}}\right) \times 100$$

Quantification of cytokine release

The release of the cytokines tumour necrosis factor α (TNF-α), interferon-γ, interleukin (IL)-10, IL-6, IL-4 and IL-2 was quantified by flow cytometry on day 3 or 4 as well as on day 6 or 7, after the initial incubation period. Therefore, cell-free supernatants were collected after centrifugation of samples for 5 min at 350 g and stored at −80°C until further analysis. Cytokines were quantified using the human T_H1/T_H2 cytometric bead array kit II (Becton Dickinson) according to the manufacturer's instructions. Briefly, six bead populations with distinct fluorescence intensities were coated with murine capture antibodies specific for the above-mentioned cytokines. The bead populations were mixed and incubated with recombinant standards or test samples and PE-conjugated anti-mouse antibodies to form sandwich complexes. Acquisition and analysis of sample data were performed by using flow cytometry and the appending cytometric bead array analysis software (Becton Dickinson).

Results

Characterization of the patient population

The diagnosis of CLL is based on morphology of the blood smear as well as on immunophenotyping most often assessed as a consequence of an elevated leucocyte count. The leucocyte counts of the patients assessed in this study are shown in Fig. 1a, and the results of immunophenotyping are summarized in Table 1.

Upon assessment, all but two patients (CLL-01-001 and CLL-08-001) were treatment naive. One patient was assessed at two different time points: under treatment with fludarabine, epirubicine and rituximab (CLL-01-001, the patient had at this time obtained a partial remission), and during maintenance treatment with rituximab (CLL-01-002). Sample CLL-08-001 was evaluated after treatment with bendamustine. At the time of assessment, nearly all patients showed abnormally increased leucocyte counts. Only patient samples CLL-01-002 and CLL-08-001 showed leucocyte counts below 4500 per microlitre owing to the previously administered treatment. Nevertheless, signs of clinical progression as well as immunophenotypic characterization were in favour of a (persisting/increasing) malignant lymphatic population.

As depicted, all patient' samples show the characteristic pathological coexpression pattern of CD5/CD19, CD5/CD20 and CD23/CD19 on the majority of lymphocytes (Table 1). Consistent with the diagnosis of CLL, the expression of CD103 was absent in all samples and the expression of CD138 was below 10% in all but one sample (CLL-01-002). In some patient samples, the expression of CD11c, FMC7 and CD38 on CD19-positive B lymphocytes was found to exceed 10%.

Characterization of the immune status of the patients with CLL

To assess the immune status in 13 different patients with CLL, their lymphatic and monocytic subpopulations were quantified by FACS analysis using the marker panel described in the Materials and methods section. Table 2 summarizes the percentages of subpopulations within the PBMC compartment.

In all but one patient sample (CLL-08-001), the majority of lymphocytes were B lymphocytes (> 70%) and the counts of T lymphocytes were pathologically reduced (1–23%). In the sample CLL-08-001, the amount of B lymphocytes was only slightly increased (27%) and T lymphocyte counts were with 66% within the physiological range. Nevertheless, as mentioned above, this sample was assessed after previous chemotherapeutic treatment and does thus not depict the original cell populations in the treatment-naive patient. Natural killer cell counts as well as the percentage of monocytes were reduced in all patient samples (Table 2).

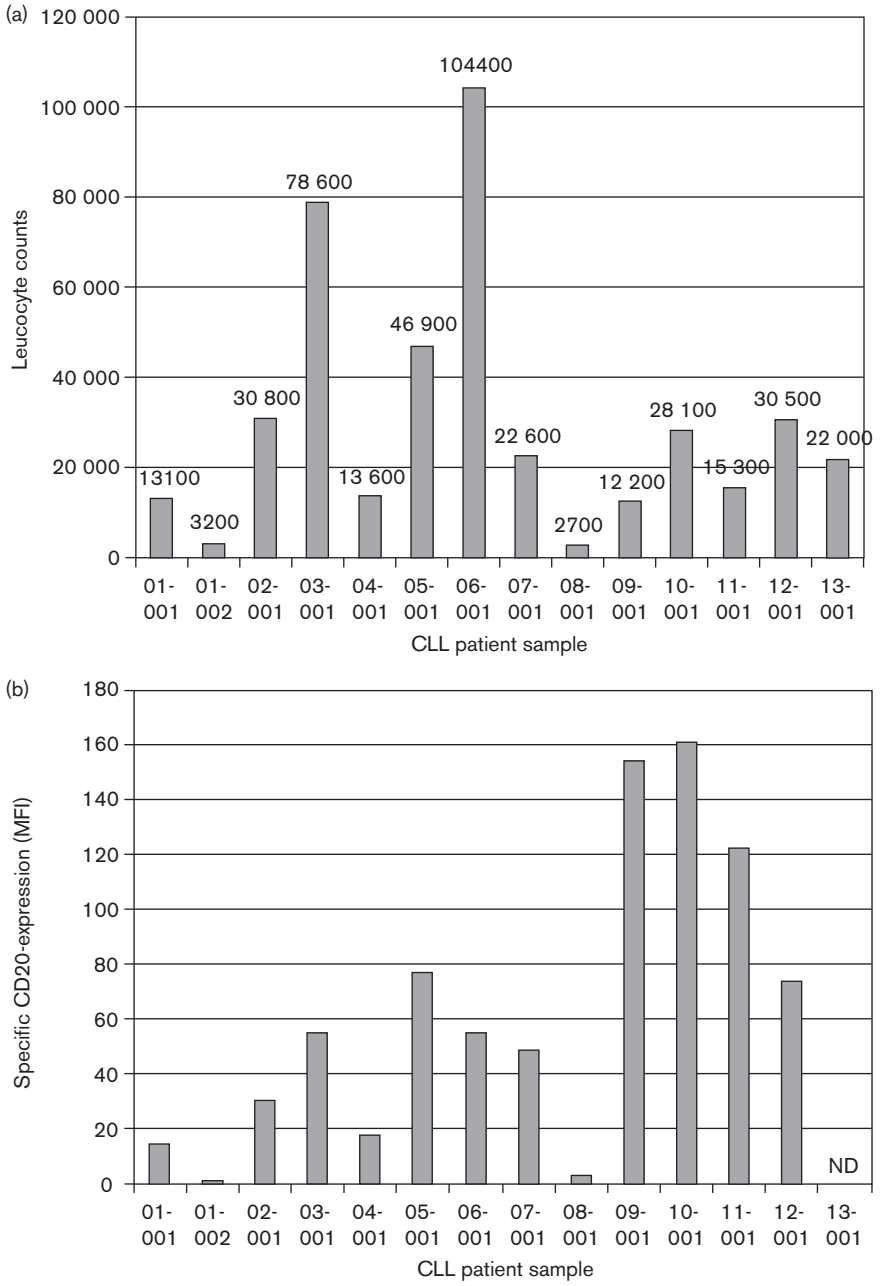
The ratio of helper and cytotoxic T lymphocytes was inversed in patient samples CLL-01-001, CLL-03-001 and CLL-07-001 as indicated by CD8-positive T lymphocyte counts exceeding the CD4-positive T lymphocyte counts. Comparable amounts of CD4-positive and CD8-positive T lymphocytes were found in samples CLL-04-001, CLL-05-001 and in CLL-06-001 (Table 2).

Noteworthy, the percentage of cells expressing CD19 was not always corresponding to the percentage of cells expressing CD20. Thus, CD20 expression was considerably lower than CD19 expression in the patient samples CLL-01-001, CLL-04-001 and CLL-06-001, and the CD20-expressing population was very small in the sample CLL-01-002 (derived at a later time point from the same patient providing sample CLL-01-001) despite a population of more than 90% of CD19-positive cells (Table 2). At least for the patient CLL-01 (that is samples CLL-01-001 and CLL-01-002), these differences in CD19 and CD20 might be explained by the previous treatment with the CD20-antibody rituximab, which may have masked CD20 molecules on the cell surface.

Effector:target cell ratio and mean fluorescence intensity of CD20 in samples of patients with CLL

As it is postulated that FBTA05 works by inducing T cell-mediated immune effector functions [4], we assessed the pretreatment effector:target cell ratio in our patient samples. Table 2 thus depicts the ratio of CD3-positive versus CD20-positive cells within the PBMC population. It thus becomes evident that in CLL the ratio of effector:target cells shows a high interindividual variability. Noteworthy, in all samples the number of CD20-positive cells exceeds the number of CD3-positive cells, showing an unfavourable effector:target ratio (see Table 2). To more clearly define the importance of CD20 expression and the in-vitro response to the trifunctional antibody, we also determined the MFI of specific CD20 expression (as described in the Materials and methods section). As depicted in Fig. 1b, the samples derived from treatment-naive patients showed a considerable

Fig. 1



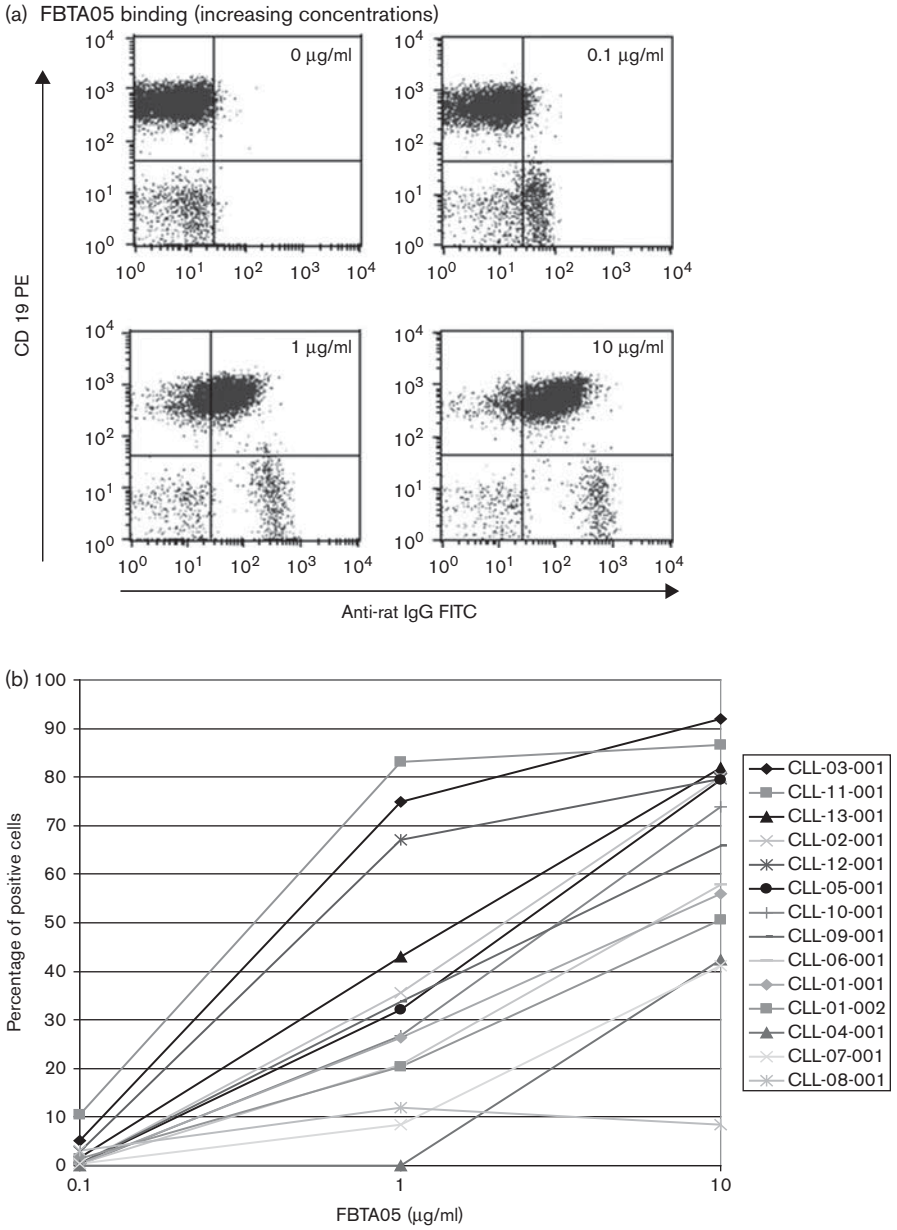
Absolute leucocyte counts per microlitre of the assessed patient samples (a) and mean fluorescence intensities (MFIs) of CD20 expression (b). Numbers above the bars within graph (a) depict the absolute number of leucocytes per microlitre (ND, not determined). CLL, chronic lymphocytic leukaemia.

interindividual variability of fluorescence intensity of CD20. The low intensity of patient samples CLL-01-001/02 and CLL-08-001 is most likely attributable to the previous chemoimmunotherapeutic treatment.

Binding capacity of FBTA05 to malignant B lymphocytes

To assess the binding capacity of the bispecific antibody FBTA05 to malignant B lymphocytes, we incubated patient cells with increasing concentrations of 0.1, 1 and 10 µg/ml of FBTA05. Samples incubated in the absence of FBTA05 were used as control. For flow-cytometric analysis, cells were stained with CD19-PE (evaluation of B cells) in parallel to anti-rat-immunoglobulin G FITC (staining of bound FBTA05). Figure 2 depicts the original FACS plots of the patient sample CLL-12-001 (Fig. 2a) showing a dose-dependent binding of FBTA05 to the B lymphocytes, as well as the quantitative analysis for all patient samples (Fig. 2b).

Fig. 2



Flow-cytometric analysis of FBTA05 binding to malignant B lymphocytes. Original fluorescence-activated cell sorting plots from the patient sample chronic lymphocytic leukaemia (CLL)-12-001 showing fluorescent staining at different concentrations of FBTA05 (a) and depiction of FBTA05 binding for all patient samples (b). Percentages of FBTA05-bound B lymphocytes were determined by binding of anti-rat immunoglobulin G (IgG) fluorescein isothiocyanate (FITC) to FBTA05 and concomitant counterstaining with anti-CD19-PE.

These data show that, except for one patient sample (CLL-08-001, assessed after previous treatment), the FBTA05 antibody binds in a strictly dose-dependent manner (Fig. 2).

In addition, these data once more demonstrate a considerable interindividual variability of the malignant cells in the capacity to bind FBTA05. Thus, the percentage of antibody-bound CD19-positive cells (at the concentration of 10 μg/ml FBTA05) ranges from approximately 90% (CLL-03-001, CLL-11-001) to only approximately 40% (CLL-04-001, CLL-07-001).

In conclusion, these data demonstrate that the capacity of FBTA05 to bind to malignant lymphocytes increases with the extent of CD20 expression in the individual patient samples. Nevertheless, even upon low CD20 surface expression on CLL cells, the FBTA05 antibody effectively binds to its antigen. Both observations foster the notion that a potential beneficial effect of the bispecific antibody is not (solely) dependent on the intensity of CD20 expression of the malignant cells.

Cytotoxicity of FBTA05 against malignant B lymphocytes

In order to assess the antineoplastic activity of FBTA05 *in vitro*, we evaluated the FBTA05-induced tumour cell killing by flow-cytometric quantification at two time points. As described in the Materials and methods section, patient samples were incubated with increasing concentrations of the trifunctional antibody. In a first analysis, cytotoxicity was assessed after 3/4 days of the incubation with the antibody. Aiming to recapitulate the clinical setting (with several infusions of the antibody) as close as possible, we carried out a second analysis of cytotoxicity on day 6/7. To this aim, additional antibody was added again at the time of first analysis, including a change of (50%) the medium.

To more precisely describe the cytotoxic capacity of the antibody FBTA05, CLL samples were incubated with a broad concentration range. In order to more clearly depict results, we defined a 'responder' and a 'nonresponder' group. Responders were defined as showing a reduction of malignant B lymphocytes by at least 80% (as compared with the untreated control sample) at any time point of the incubation period. Accordingly, nonresponders did not reduce malignant cells below the threshold of 80% at any given concentration and at any given time point (that is, after the first or second incubation period).

As depicted in Fig. 3, the antibody FBTA05 induces a concentration-dependent and time-dependent depletion of malignant B cells in the majority of patient samples. It thus becomes evident that, at least for in-vitro responses in this autologous CLL setting, concentrations exceeding 10 ng/ml need to be applied. In addition, only three patient samples showed a considerable decrease of cell viability after 3/4 days (Fig. 3a, left graph), whereas the majority of samples showed a response upon the second incubation with the antibody (Fig. 3b, left graph). Nevertheless, two patient samples (CLL-01-002 and CLL-11-001) did not show a response towards the antibody (Fig. 3b, right graph). In the case of sample CLL-01-002, the binding of FBTA05 might have been hindered by the previous treatment with rituximab.

Comparing FBTA05's efficacy to eliminate malignant B lymphocytes with the expression of CD20 on the cell surface (MFI), it thus becomes evident that the antibody's effectiveness does not depend on high CD20 levels. Thus, a wide range of MFI for CD20 was found in the responder group (ranging from an MFI of 14 in sample CLL-01-001 to an MFI of 161 in sample CLL-10-001). Underscoring the notion that the extent of CD20 expression is not of primary importance for FBTA05's cytotoxic effects is the observation that neither responders nor nonresponders could be discerned solely by the extent of antibody binding to the malignant cells.

In order to put the observed antitumour effect into perspective, samples were concomitantly assessed for their response to rituximab and alemtuzumab. Consequently, if cell numbers were sufficient we compared the FBTA05-elicited effects with those observed under a concentration range of rituximab and alemtuzumab. Noteworthy, the presence of native human serum allowed CDC, as CDC is described as a mode of action of rituximab as well as of alemtuzumab [6–8]. Accordingly, the patient samples CLL-01-001 to CLL-05-001 could be tested for the effects induced by rituximab, and patient samples CLL-06-001, CLL-07-001 and CLL-09-001 to CLL-13-001 could be tested under incubation with alemtuzumab.

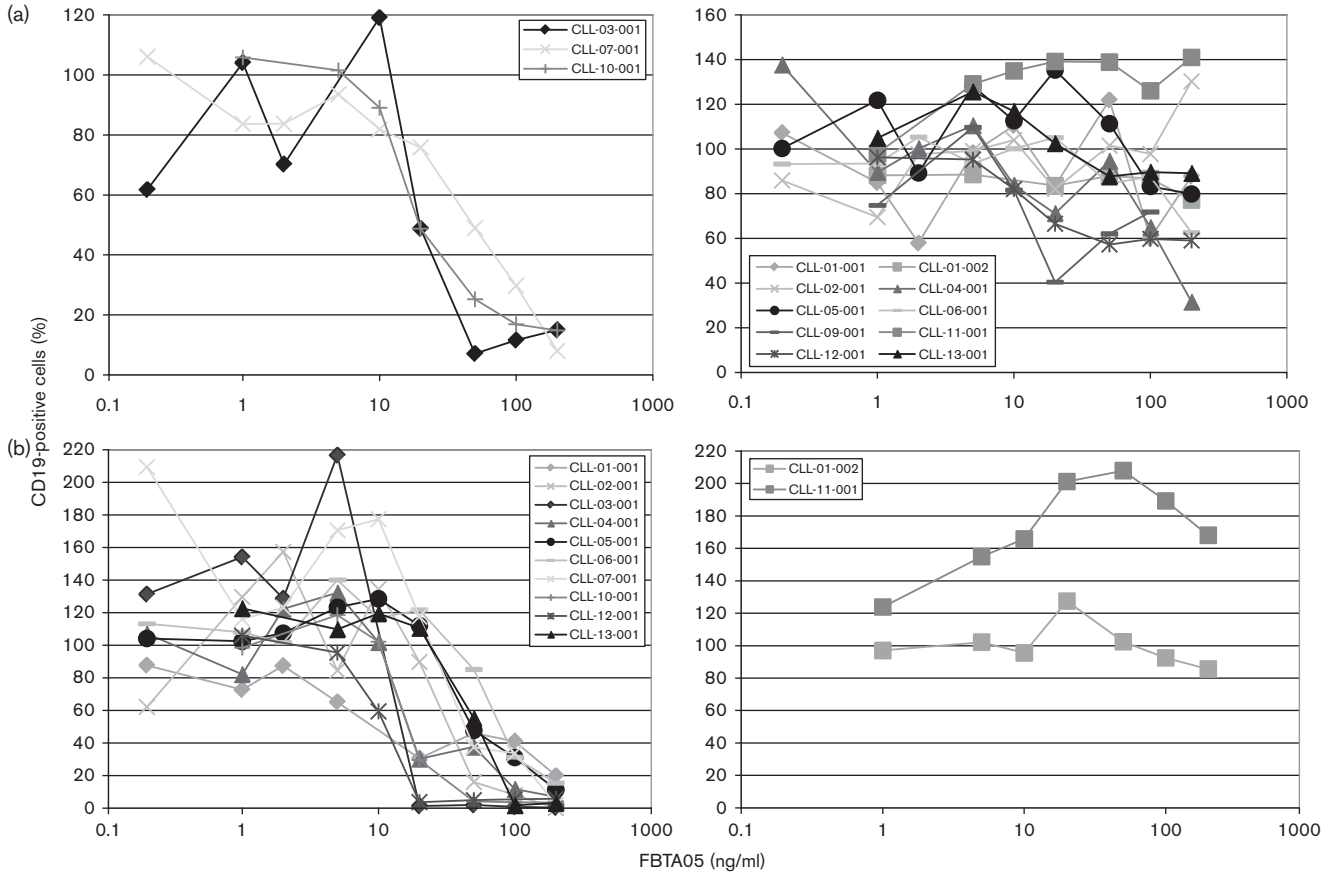
Recapitulating thus the experiments carried out with the antibody FBTA05, we assessed the drugs' antitumour effects after a first (3/4 days) and a second incubation period (6/7 days) with the respective antibody. Thus, we show that rituximab was not able to deplete malignant B cells beyond 50%, not even after a second incubation period (Table 3). Notably, a considerable number of patient samples were completely resistant towards rituximab, and even increased the number of B lymphocytes despite the presence of rituximab (such as CLL-03-001).

In contrast, alemtuzumab efficiently killed B lymphocytes in a dose-dependent and time-dependent manner (Table 4). As depicted, alemtuzumab showed in most cases (except for CLL-11-001) a considerable cytotoxic effect already after the first incubation and increased its efficacy in all patient samples after the second incubation period (Table 4).

FBTA05-induced proliferation and activation of T lymphocytes

As FBTA05 is thought to function by modulating the immune response by using T cells, we next assessed its

Fig. 3



Cytotoxic effects of increasing concentrations of the FBTA05-antibody on malignant B lymphocytes. (a) Assessment of cytotoxicity after 3/4 days in the presence of the indicated concentrations in responders (defined as showing a tumour cell kill exceeding 80%, left graph) and nonresponders (right graph) and (b) after incubation for 6/7 days (left graph: responders, right graph: nonresponders). CLL, chronic lymphocytic leukaemia.

Table 3 Rituximab-mediated depletion of malignant B-cells in chronic lymphocytic leukaemia patient samples after the first (upper table) and second (lower table) incubation period with the indicated concentrations

Rituximab (μg/ml)	CLL-01-001 (%)	CLL-02-001 (%)	CLL-03-001 (%)	CLL-04-001 (%)	CLL-05-001 (%)
0.1	100	119	73	120	92
0.2	74	140	98	115	115
1.0	71	151	78	94	109
10	57	146	94	123	138
100.0	57	139	67	117	121
0.1	105	91	114	95	99
0.2	50	81	79	95	121
1.0	73	91	141	85	107
10.0	74	86	140	95	105
100.0	51	81	174	104	108

Depicted are the percentages of residual B lymphocytes for each antibody concentration relative to nontreated control.
CLL, chronic lymphocytic leukaemia.

impact on the rate of proliferation of CD3-positive cells. To facilitate interpretation of results, we once more depict the effect of FBTA05 on T-cell proliferation separately for the responders and nonresponders (as defined above). Once more, assays were carried out with the indicated concentrations of the antibody (assessment of T-cell proliferation on day 3/4, after the incubation) as well as after a second incubation period (assessment on day 6/7).

It thus becomes evident that both nonresponders (in the cytotoxicity assay, CLL-01-002 and CLL-11-001) are unable to induce proliferation of T cells at any time point of assessment (Fig. 4a and b, right panel), and that two of

Table 4 Alemtuzumab-mediated B-cell depletion in chronic lymphocytic leukaemia patient samples after the first (upper panel) and second incubation period (lower panel) with the indicated concentrations

Alemtuzumab (μg/ml)	CLL-06-001 (%)	CLL-07-001 (%)	CLL-09-001 (%)	CLL-10-001 (%)	CLL-11-001 (%)	CLL-12-001 (%)	CLL-13-001 (%)
0.1	100	102	99	82	123	90	116
0.2	103	92	93	69	125	71	113
1.0	89	68	109	31	96	55	87
10.0	36	8	72	14	74	8	51
100.0	16	6	29	9	69	1	29
0.1	114	74	ND	100	100	100	100
0.2	105	76	ND	59	106	89	96
1.0	94	22	ND	51	87	104	89
10.0	51	3	ND	3	68	37	33
100.0	21	2	ND	5	49	8	7

Depicted are the percentages of residual B lymphocytes for each antibody concentration.
CLL, chronic lymphocytic leukaemia; ND, not determined.

the three 'early responders' (i.e. tumour cell killing exceeding 80% after a single incubation with the antibody) show concomitantly an early and considerable proliferation of T lymphocytes (Fig. 4a, left graph), whereas the 'third responder' does induce a considerable T-cell proliferation detectable at the later time point (Fig. 4b, left graph). These results thus link the ability of the FBTA05 antibody to effectively eliminate malignant lymphocytes to its capacity to induce a proliferation of T cells and potentially T cell-mediated immunemechanisms.

Previously described bispecific antibodies show cytotoxicity against B cells only after prestimulation (for example with anti-CD28 antibodies, [9,10]). As the above-described data provide evidence that FBTA05 might be able to use T cells without prestimulation, we thought to corroborate this conclusion by assessing changes in the activation status of CD3-positive cells incubated with the antibody FBTA05. To this aim, we measured the upregulation of the activation marker CD25 in the CD4-positive as well as in the CD8-positive T cell population. As depicted in Fig. 4c and d, FBTA05 induced a considerable upregulation of the activation marker CD25 in both T cell subpopulations (left panel: analysis on day 3/4, right panel: analysis on day 6/7).

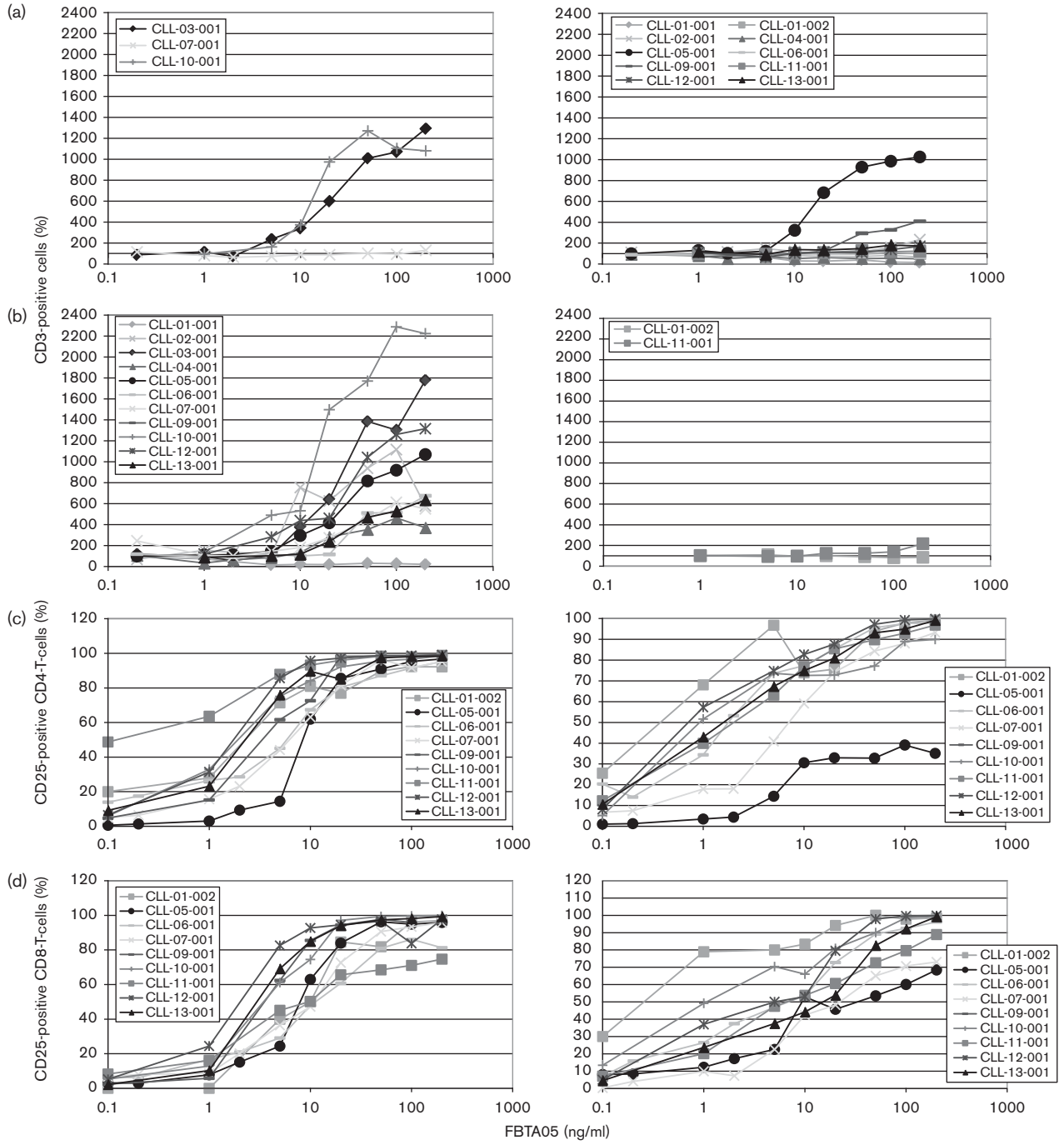
FBTA05-mediated release of the effector molecules granzyme B and perforin

As the FBTA05 antibody is thought to act by using effector molecules, we also assessed its capacity to induce a release of granzyme B and perforin by immune effector cells. To this end, patient samples were incubated with a broad dose range of the antibody, and the release of effector molecules was assessed 24 h after the incubation. As depicted in Fig. 5a in an exemplary manner for the patient sample CLL-02-001, the antibody elicited a release of both molecules, albeit to a different degree, in all patient samples. It thus became evident that the antibody induced almost unanimously a higher degree of granzyme B than perforin release (exception: patient sample CLL-06-001 in which perforin release exceeds the release of granzyme B, and patient sample CLL-13-001 where the extent of the release of both molecules is comparable, data not shown). In addition, the majority of samples show a parallel release of both molecules, supporting the notion that the capacity to induce an effector molecule release is not dependent on the individual molecules, but rather a characteristic of the targeted and thus activated cell. The evaluation of a broad concentration range of the antibody enabled us moreover to demonstrate that the new therapeutic agent induces a steep dose–response curve already observed at low concentration levels before the release of effector molecules reaches a plateau where it cannot be augmented any further (exception: patient samples CLL-06-001 and CLL-07-001, data not shown). Noteworthy, the majority of patient samples showed the peak of effector molecule release already in dose levels below 50 ng/ml (data not shown).

FBTA05-mediated release of inflammatory cytokines

In order to further describe the antibody's capacity to elicit an immune-mediated response, we moreover assessed the pattern of cytokine release. Thus, the amounts of cytokines released by the individual patient's PBMC were quantified by flow cytometry on day 3/4 and day 6/7 of the initial incubation with the FBTA05 antibody. Measurement of the cytokines interferon-γ, TNF-α, IL-2, IL-4, IL-6, and IL-10 was thus carried out on cell-free culture supernatants. In addition, we detected the same cytokines in six patient samples treated with increasing concentrations of rituximab, and the IL-6 release induced by increasing concentrations of alemtuzumab in seven patient samples. As depicted in an exemplary way in Fig. 5b, FBTA05 induced a dose-dependent increase in inflammatory cytokines, notably augmenting the level of interferon-γ, TNF-α, and to a lesser extent the level of IL-10 and IL-6, whereas the majority of patients' samples did neither increase IL-2 nor IL-4 (data not shown). Unanimously, cytokine release was more pronounced on day 3/4 of the incubation period, whereas cytokine levels decreased (or remained on baseline levels) on day 6/7 of the incubation period (an effect that might also be due to the short half-life of these cytokines in culture). As already demonstrated for the release of effector molecules, the antibody FBTA05 showed a steep dose–response curve already at low concentration levels. In addition, our data demonstrate

Fig. 4



FBTA05-elicited proliferation and activation of T cells in samples of patients with CLL. Degree of T-cell proliferation after 3/4 days (a) and 6/7 days (b) of incubation with increasing dosages of FBTA05 in responders (left panel) and nonresponders (right panel). FBTA05-induced activation of CD4-positive T cells (c) and CD8-positive T cells (d) in all assessable samples after 3/4 days (left panels) and 6/7 days (right panels). Activation was assessed by flow-cytometric quantification of the CD25-positive cells within the CD4-positive and CD8-positive compartments, respectively. CLL, chronic lymphocytic leukaemia.

that the antibody FBTA05 is able to induce the release of inflammatory cytokines most likely contributing to its mode of action.

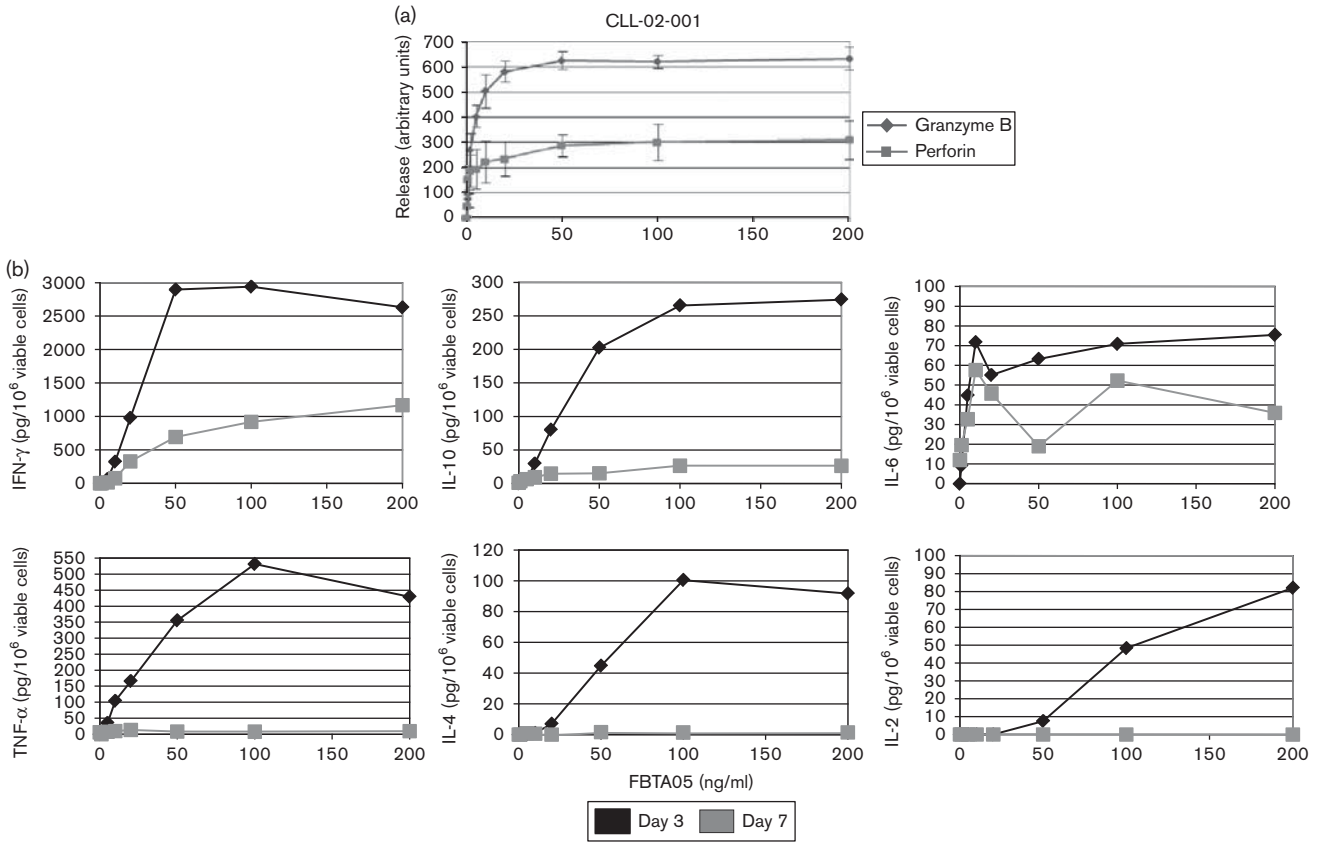
Correlating once more the pattern of cytokine release with the concomitantly assessed cytotoxic efficacy of the trifunctional antibody, it becomes evident that the early

'responder' group (those showing a tumour cell killing of at least 80% on day 3/4 of the incubation period) shows a steep increase in interferon-γ and IL-6 (and to a lesser extent in IL-10) levels, whereas 'nonresponders' (those failing to achieve an 80% reduction in malignant cells at any time point) are unable to release these cytokines.

Discussion

Treatment of lymphoproliferative malignancies with anti-CD20-directed antibodies proved to be highly efficient [7]. Nevertheless, in the majority of patients the disease relapses and becomes eventually resistant to this treatment [8]. The mechanisms of resistance are still incompletely elucidated, but are most likely due to tumour cell-related as well as host-factor-related changes. Thus, lymphoma cells may become 'intrinsically' resistant by decreasing the CD20 expression on the cell surface and/or by enforcing signalling of antiapoptotic pathways [11,12]. 'Extrinsic', that is host-related mechanisms of resistance, consist of impaired functioning of effector cells, of antibody-dependent cellular cytotoxicity, and of the reticulo-endothelial system failing to remove antibody-loaded tumour cells [11,12]. Aiming to exploit the successful treatment principle of antibody-directed therapy, bispecific antibodies hold the promise to overcome these above-described mechanisms of resistance. The trifunctional bispecific antibody FBTA05 connects CD20-positive B lymphocytes with CD3-positive T lymphocytes through its variable regions and moreover recruits Fc-γ receptor-positive accessory immune cells by its Fc region. So far, there are two reports assessing the in-vitro and in-vivo properties of FBTA05 [4,5]. Buhmann *et al.* [5] assessed the efficacy and toxicity of the trifunctional antibody in a pilot trial of six patients refractory to standard therapy (including rituximab in four patients and alemtuzumab in two patients) and with relapsing disease after allogenic stem cell transplantation. Three patients with p53-mutated CLL and three patients with high-grade NHL were thus treated with escalating doses of FBTA05, followed by donor-lymphocyte infusions and secondary stem cell transplantation. This group thus showed that, with manageable side effects, three patients with CLL achieved a prompt but transient clinical response, and that one high-grade

Fig. 5



FBTA05-mediated release of cytotoxic molecules and of inflammatory cytokines. (a) FBTA05-mediated release of the cytotoxic molecules granzyme B and perforin after 24 h of incubation depicted for the patient sample chronic lymphocytic leukaemia (CLL)-02-001. (b) FBTA05-mediated release of the inflammatory cytokines interferon γ (IFN-γ), tumour necrosis factor α (TNF-α), interleukin (IL)-2, IL-4, IL-6 and IL-10 after 3 and 7 days of incubation. The figure shows representative results of one patient sample (CLL-12-001).

NHL patient remained without disease progression for 4 months. Previously, Stanglmaier *et al.* [4] tested the in-vitro properties of the FBTA05 antibody on lymphoma cell lines as well as on patient-derived cells providing first in-vitro evidence for a potential therapeutic benefit. We extended these studies and increased the sample size as well as the experimental setting, but similarly used ex-vivo patient cells in order to most closely mimic the clinical setting.

We thus aimed in this study to more clearly define the in-vitro characteristics of FBTA05 on ex-vivo cells from patients with CLL. As the antibody tries to overcome tumour cell-related as well as host-related mechanisms of resistance, we evaluated crucial components of both aspects. Although the validity of our conclusions is limited by the small sample number, several preliminary conclusions can be drawn: a side-by-side comparison demonstrated that at least *in vitro* the cytotoxic activity of the antibody FBTA05 exceeds the antitumour effects of rituximab, which functions by aiming at the same target. Determining the underlying mechanisms, we provide evidence that the cytotoxic efficacy of FBTA05 does neither depend on the density of CD20-expression on the malignant lymphocyte, nor on the extent of FBTA05-antibody binding to the cell. In contrast, immune-mediated mechanisms seem to be crucial for the antibody-mediated elimination of B lymphocytes. Thus, our data demonstrate that a high grade of cytotoxicity is associated with the capacity to induce the proliferation of T lymphocytes (which is absent in the 'nonresponders'). Furthermore, our data favour the notion that the FBTA05-mediated ability to increase the number of T lymphocytes is of paramount importance, as it might overcome the detrimental effects of an unfavourable effector:target ratio. This is shown by the fact that samples with a very advantageous effector:target ratio, but unable to increase proliferation of T cells did show a low degree of cytotoxicity, whereas the antibody-mediated T-cell proliferation might be responsible for the fact that the rather unfavourable effector:target ratio in the 'responder' group did not negatively impact on the cytotoxic efficacy. In addition, the stimulation of T cells was associated with the release of interferon-γ and IL-6 (and to a lesser extent IL-10), whereas nonresponders failed to do so. Thus, cytotoxicity of FBTA05 seems to rely on activation of T lymphocytes and the concomitant release of interferon-γ and IL-6. Noteworthy, FBTA05 induced a release of granzyme B and perforine from effector cells in all tested patient samples. Moreover, the predominant pattern of release was a parallel liberation of both molecules, fostering the observation that FBTA05 might thus overcome an unfavourable effector:target cell ratio.

In conclusion, we define here the in-vitro characteristics of the new trifunctional, bispecific antibody FBTA05 and provide first evidence for crucial mechanistic components underlying its beneficial therapeutic effects.

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