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Expansion of natural killer (NK) and natural killer-like T (NKT)-cell populations derived from patients with B-chronic lymphocytic leukemia (B-CLL): a potential source for cellular immunotherapy

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B-cell chronic lymphocytic leukemia (B-CLL) is the most common leukemia in the Western world. It is currently an incurable disease, making new treatment options such as immunotherapy desirable. Monoclonal antibodies (Mabs) to surface antigens of the tumor cell is one option. Administration of cytotoxic cells such as natural killer (NK) and natural killer-like T (NKT) cells expanded *in vitro* might be a useful treatment modality alone or in combination with MAbs. A limiting step in the development of successful cellular immunotherapy has been the availability of appropriate cytotoxic cells. Here, we report the feasibility of expanding populations of the human killer cells, CD3−CD56+ NK and CD3+CD56+ NKT cells, from peripheral blood mononuclear cells (PBMCs) of B-CLL patients. The influence of tumor B cells on the *in vitro* expansion of killer cells was assessed by depleting B cells from PBMCs by microbead separation before culture. The 21-day cultures from both B-cell- and non-B-cell-depleted PBMC showed a marked expansion of NK cells, and also of T cells, among which almost half had the NKT phenotype. Depletion of B cells before culture did not change the expansion rates of NK and NKT cells significantly. In patients with progressive B-CLL, NK cell expansion capacity was improved after fludarabine treatment when compared to samples obtained before treatment. Repeated samples of PBMCs from individual untreated patients with both indolent and progressive disease cultured under identical conditions gave similar NK cell expansion rates. Expanded killer cell populations had cytotoxic function against the NK-sensitive target K562 cell line and expressed high levels of Granzyme B. From our studies, we conclude that NK cells as well as NKT cells from the peripheral blood of B-CLL patients can be expanded, and that these cells have cytotoxic capacity.
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Introduction

Chronic lymphocytic leukemia of the B-cell type (B-CLL) is the most common subtype of indolent lymphoma characterized by an accumulation of long-lived clonal B cells in the bone marrow, blood, lymph nodes, and spleen.[1] The clinical course of this disease is highly variable. Clinical staging systems as Rai and Binet have been used to predict the prognosis. Some patients with a low clinical stage might live for 20 years or more, while patients with an advanced stage (B or C according to the Binet classification) have considerably reduced survival time.[2,3]

Despite new treatment regimens, CLL remains incurable. Good response to fludarabine has been reported, however, without significant prolongation of survival time.[3,4] Allogeneic bone marrow transplantation may yield complete remissions,[5,6] but its applicability is limited by graft-versus-host disease (GVHD) and the low availability of donors. Autologous stem cell transplantation often leads to good responses but with a high incidence of relapse.[7] Thus, alternative methods of treatment for patients with B-CLL are needed.

Two monoclonal antibodies (Mabs), Rituximab (anti-CD20)[8] and Campath-1H (anti-CD52),[9] have recently been introduced for the treatment of CLL patients. Possible mechanisms for destruction of leukemic cells by these Mabs include antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-mediated lysis. Immunologic effector cells such as cytotoxic natural killer (NK) and T cells are important for ADCC, and cell therapy using peripheral blood-derived, IL-2-activated killer cells might enhance the efficacy of Mab therapy. However, major obstacles in developing this treatment have been the identification and expansion of such effector cells from the patients' peripheral blood in sufficient numbers.

NK cells are a subset of cytolytically active lymphocytes killing virally infected or tumor targets but to spare normal cells.[10,11] In cancer-bearing hosts, NK cells have been considered as an important component of antitumor immunity responsible for rapid elimination of blood-borne metastases.[10,11] The cytolytic activity of NK cells occurs spontaneously (ie in the absence of deliberate, prior immunization) and does not require expression of syngeneic major histocompatibility complex (MHC) antigens by target cells. NK cells do not appear to express one dominant receptor that dictates the specificity of cytolysis. Rather, triggering of NK cell cytotoxicity reflects a balance between activating and inhibitory signals mediated by cell-surface receptors that belong to several different gene families.[12–15]

In B-CLL patients, several phenotypic and functional alterations in NK cells[16,17] have been described such as defective cytotoxic activity.[16–19] The reason as to why NK activity is low in most patients with CLL is unclear. A dilutional effect of leukemic cells and intrinsic functional defects on NK cells have been implicated.[16,20,21] These abnormalities found in NK cells might allow the appearance and/or maintenance of the leukemic B-cell accumulation. However, defective NK activity has been shown to be restored by interferon (IFN)-α and IL-2.[19]

Natural killer-like T (NKT) cells were first described as a distinct subset of T cells less than a decade ago,[22,23] express surface receptors that are also found on conventional T cells, together with receptors that are characteristic of NK cells. One striking property of NKT cells is their capacity to produce a spectrum of cytokines rapidly, including interleukin (IL)-4, IL-10, IL-13, interferon (IFN)-γ, and tumor necrosis factor-α, upon engagement with T-cell receptors.[22,23] The role of NKT cells in antitumor immunity has been investigated both in models of natural immunity and in models where immunity is induced by

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the cytokine IL-12.[24] NK cells and conventional T cells contribute to the therapeutic activities of this cytokine as well.[25] Moreover, NKT cells can prevent GVHD after bone marrow transplantation[25] in an IL-4-dependent manner. Obtaining a large number of NKT cells may be instrumental in controlling certain tumors and GVHD.

Considering the potential usefulness of autologous killer cells such as CD3−CD56+ NK cells and CD3+CD56+ NKT cells in immunotherapy of B-CLL, we explored the feasibility of expanding these populations from the peripheral blood of B-CLL patients.

Materials and methods

Patients

Peripheral blood samples of six patients with phenotypically classical (CD19+CD5+CD23+) B-CLL were included in the study. All patients had a total lymphocyte count of $>5\times10^9$ Lmp/l and fulfilled all diagnostic criteria for B-CLL.[26] The patients were followed from diagnosis until January 2002 at the Clinic of Hematology, Huddinge University Hospital, Karolinska Institutet. At the time of the first blood sampling, all patients were untreated. The clinical characteristics of these patients are shown in Table 1. Four of the patients (AD, KO, LS, and YO) had indolent disease for more than 5 years requiring no treatment. Two previously untreated patients (LB and RL) showed progressive disease requiring therapy,[26] and were retested after therapy. Patient LB had a progressive disease with severe night sweats, increase in the size of the spleen, and rapid increase in the absolute number of circulating lymphocytes. The patient had blood drawn at two time points before treatment and was retested after two cycles of fludarabine/cyclophosphamide, when the disease showed a complete remission in blood. Patient RL showed a rapid progression in lymphocytosis, abdominal lymphadenopathy, and spleen size. Blood samples were drawn just before the first course of fludarabine single therapy and before the next course, when blood lymphocytes were reduced to less than half of the pretreatment value. This study was approved by the local ethics committee at Huddinge University Hospital, Stockholm, Sweden. All the patients studied were untreated unless otherwise indicated. All the samples were encoded with patients' initials. The patients having progressive disease were indicated with '#' symbol after their initials (Patients' initials#). The samples obtained from the same untreated patients were encoded with number (Patients' initials1, patients' initials2, etc); however, the samples obtained from the same patients before fludarabine treatment were encoded with 'Patients' initials and sample number^{Before}', and the samples obtained from the same patients after fludarabine treatment were encoded with 'patients' initials and sample number^{After}'.

Cell cultures and quantification

The *in vitro* culture conditions for the expansion of cytotoxic cells have previously been optimized on peripheral blood mononuclear cell (PBMC) from healthy individuals as described elsewhere.[27] In short, peripheral blood was obtained from B-CLL patients and cultures were initiated on the same day (day 0). PBMCs were isolated by gradient centrifugation, using Lymphoprep (Nyegaard, Oslo, Norway). After washing twice with phosphate-buffered saline (PBS) (Gibco, Grand Island, NY, USA), cell viability was assessed by trypan blue dye exclusion, and the cells which were plated onto six-well dishes (Falcon by Becton Dickinson, Le Pont de Claix, France) at 100×10^4 cells/ml. CellGro SCGM serum-free medium (CellGenix, Freiburg, Germany) was used in all the cultures with the addition of 5%

Table 1 Patient characteristics of the six B-CLL patients at diagnosis and the times of blood sampling

Patient name	*Samples*	*Time interval between samples (month)*	*Year of birth/ gender*	*Year of diagnosis*	*Binet stage at diagnosis*	*Binet stage at the time of the test and response to fludarabine treatment*	*Tot-lym ($\times10^9$/l)*
AD			1920/M	1988	A		
	AD1					A, nonprog.	28.5
KO			1921/F	1983	B		
	KO1					A, nonprog.	24.6
	KO2	4				A, nonprog.	21.6
LS			1932/M	1990	B		
	LS1					A, nonprog.	5.9
YO			1954/F	1996	A		
	YO1					A, nonprog.	31.0
	YO2	1				B	38.9
LB			1946/F	1993	A		
	LB1^{Before} #					B, prog.	78.5
	LB2^{Before} #	1				B, prog.	82.2
	LB1^{After} #	3				A, CR	2.4
RL			1928/M	1993	B		
	RL1^{Before} #						
	RL1^{After} #	1				B, PR	21.6

#, patients with progressive disease, samples obtained before (LB1 and RL1) and after (LB1 and RL1) fludarabine treatment, respectively.[a]Prog, progressive; nonprog, nonprogressive; CR, complete remission; PR, partial remission.

human serum (Sigma, St Louis, MO, USA) and 500 U/ml IL-2 (Peprotech, London, UK). For the first 5 days, the medium was further supplemented with anti-CD3 antibody (Orthoclone OKT-3, Ortho Biotech Inc., Raritan, NJ, USA) to a final concentration of 10 ng/ml. Total cell numbers were assessed by staining cells with trypan blue dye on days 0, 5–6, 9–10, 14–15, and 19–21. Absolute cell counts were calculated by multiplying the total number of cells in each subset with the percentage of these subsets determined by flow cytometry. On days 5–6, the OKT-3-containing medium was washed out, and fresh medium with IL-2 (500 U/ml) but without OKT-3 was added. The cultures were replenished with fresh medium every 2–3 days throughout the culture period. To prevent contact inhibition of cell growth, the cells were transferred to T25 flasks (TPP, Trasadingen, Switzerland) on days 10–11.

Flow cytometric analysis

The cell phenotype was analyzed by flow cytometry on days 0, 5–6, 9–10, 14–15, and 19–21. Three-color fluorescence was analyzed according to standard procedures. Briefly, cells were mixed with appropriate concentrations of fluorochrome-conjugated Mabs to CD3, CD4, CD5, CD8, CD16, CD19, CD25, CD45, and CD56. All antibodies were obtained from Becton Dickinson (BD, Mountain View, CA, USA). After the addition of primary antibody and incubation for 30 min at room temperature, cells were washed with PBS.

Propidium iodide (PI) staining was used for dead cell exclusion. For data acquisition and analysis, a FACSCalibur (BD) was used with Cell Quest software (BD). In each sample, a minimum of 10 000 cells was acquired in the analysis region of viable cells, using log-amplified fluorescence and linearly amplified side- and forward-scatter signals.

All samples were analyzed by setting appropriate SSC/FSC gates around the lymphocyte population, using PI-negative cells. Consistency of analysis parameters was ascertained by calibrating the flow cytometer with calibrating beads and FacsComp software, both from BD.

B-cell depletion

CD19+ B cells were depleted from PBMC using MACS colloidal super-paramagnetic microbeads conjugated to monoclonal CD19 antibody (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Briefly, PBMCs were washed with PBS and spun down at 1500 rpm for 5 min. After removal of supernatant, cells were resuspended in 80 μl of the separation buffer per 10^7 total cells. A volume of 20 μl of MACS anti-CD19 microbeads per 10^7 total cells was added, mixed, and incubated for 15 min at 4°C. After incubation, cells were washed with separation buffer in a 10–20 × labeling volume and spun down, after which supernatants were removed completely. Cell suspensions were then applied to an LS+ column, which was prewashed with the separation buffer and placed in the magnetic field of an appropriate MACS separator. The column was rinsed with 4 × 3 ml separation buffer. Negative (B-cell-depleted) and positive (B-cell-enriched) selected cells were collected. After separation, cells were washed with PBS, and viability was assessed with trypan blue dye exclusion.

Transwell cultures

B-cell- and non-B-cell-depleted fractions from one of the patients with progressive disease (RL) were cultured in a transwell (Costar, Cambridge, MA, USA; 24-mm diameter, 3.0 μm pore) with the B-cell-enriched fraction in the lower and B-cell-depleted fraction in the upper chamber. To avoid contact growth inhibition, cells from the upper and lower level fractions, respectively, were transferred on day 10 to separate flasks, and culture was continued until day 21.

Immunocytochemical analysis

On day 21, cultured cells were deposited on microscope slides by cytocentrifugation (Shandon, Cytospin II, Pittsburgh, PA, USA). Slides were frozen and stored at −20°C until staining. Before staining, slides were fixed in 4% formaldehyde in PBS, and antigen was retrieved by incubation in citric acid buffer, pH 7.2, at 60°C overnight.

A polyclonal antiserum to CD3 was obtained from Dakopatts AB (Stockholm, Sweden). The primary antibody to Granzyme B (Mab Grb-7) was obtained from Sanbio (Uden, The Netherlands). For antigen detection, a DAB-based labeled streptavin–biotin immunoperoxidase method (LSAB, Dakoplatts) was used in a Techmate Horizon staining machine (Dakoplatts) according to the manufacturer's instructions.

Cell-mediated cytotoxicity

To assess the cytotoxic capacity of cells obtained after *in vitro* expansion, cells from two patients were analyzed on day 21 of culture. The NK-sensitive K562 cell line was used as a target. The cytotoxic function of cultured cells was measured in a standard 4 h ^{51}Cr-release assay in triplicates at various effector:target ratios. Target cells were labeled with 100 μCi of ^{51}Cr for 2 h at 37°C, washed three times with PBS, and resuspended in 10 ml of RPMI medium. A total of 3×10^4 target cells in 100 μl RPMI medium were placed in triplicates into U-bottomed 96-well plates and incubated for 4 h with 100 μl of effector cells at appropriate concentrations to obtain effector:target ratios from 0.3:1 to 10:1. Cells were then collected by centrifugation, and aliquots of supernatants were counted in a counter. The percentage specific ^{51}Cr release was calculated according to the formula: percent specific release = ((experimental release − spontaneous release)/(maximum release − spontaneous release)) × 100.

Statistics

The results were compared by using nonparametric tests (Mann–Whitney *U*-test). *P*-value <0.05 was considered to be significant.

Results

In vitro *expansion of NK cells and NKT cells from patients with B-CLL*

PBMCs of all patients with B-CLL evaluated here were initially cultured in a concentration of 100×10^4 cell/ml. At the start of the culture, each patient had variable numbers of NK, T, and B cells. However, in all patients, the numbers of NK cells were

Table 2 Absolute cell counts ($\times 10^4$) of NK cells, T cells including subsets, and B cells on days 0 and 21. Cultures were initiated without prior B-cell depletion at a starting concentration of PBMC of 100×10^4 cells/ml

Patients	*AD1*		*KO2*		*LS1*		*YO2*		*LB1^{Before} #*		*LB1^{After} #*		*RL1^{Before} #*		*RL1^{After} #*	
	D0	*D21*	*D0*	*D21*	*D0*	*D21*	*D0*	*D21*	*D0*	*D21*	*D0*	*D21*	*D0*	*D21*	*D0*	*D21*
NK cells																
Total CD3−CD56+ NK cells	5	2484	3	963	11	2221	3	1704	1	43	17	1226	2	281	3	307
Subsets (of NK cells)																
CD3−CD8+	1	390	0	59	7	111	2	481	2	13	8	298	0	58	0	49
T cells																
Total CD3+ T cells	19	1531	9	1013	42	2303	5	80	3	88	49	2980	8	68	13	71
Subsets (of T cells)																
CD3+CD56−	16	545	7	713	39	1580	4	29	3	22	45	2444	8	18	12	25
CD3+CD56+	3	986	2	300	3	953	1	351	0	66	4	536	0	50	0	46
CD3+CD4+	9	401	4	399	31	1331	3	7	1	13	22	2137	5	9	9	34
CD3+CD8+	5	351	2	331	11	557	2	62	2	46	24	817	3	38	4	26
B cells																
CD19+	52	0	69	1	41	10	88	0	96	62	12	0	82	65	80	0

#, Patients with progressive disease, samples obtained before (LB1^{Before} and RL1^{Before}) and after (LB1^{After} and RL1^{After}) fludarabine treatment, respectively. For LB1, complete remission (CR), and for RL1, partial remission were obtained after fludarabine treatment.

Table 3 Absolute cell counts ($\times 10^4$) of NK cells, T cells, including subsets, and B cells on day 21.[a] Cultures were initiated after B-cell depletion at a starting concentration of PBMC of 100×10^4 cells/ml

Patients	*AD1*	*KO2*	*LS1*	*YO2*
	D21	*D21*	*D21*	*D21*
NK cells				
Total CD3−CD56+ NK cells	4008	773	1812	4333
Subsets (of NK cells)				
CD3-CD8+	629	13	84	1446
T cells				
Total CD3+ T cells	4128	1977	4000	160
Subsets (of T cells)				
CD3+CD56−	1420	1708	2456	59
CD3+CD56+	2708	269	1584	101
CD3+CD4+	533	769	3316	53
CD3+CD8+	743	868	424	88
B cells				
CD19+	0	0	4	3

[a]Owing to very low number of cells at day 0, FACS analysis was not attempted.

low (Table 2). After 21 days of culture, all PBMC samples from the patients with indolent disease showed a marked CD3−CD56+ NK cell expansion both in samples of non-B-cell- (Table 2) and B-cell-depleted PBMCs (Table 3). The expansion approached log-linearity after an initial 'resting' phase. The median NK cell number obtained after 21 days was 1963×10^4 cells/ml (range 963–2484) for non-B-cell-depleted samples and 2883×10^4 cells/ml (range 773–4333) for B-cell-depleted samples (Figure 1). The difference between NK cell expansion rates from these two samples was not significant (*P*-value >0.56).

Under the culture conditions used, not only NK cells (CD3−CD56+) but also T (CD3+CD56−) and NKT cells (CD3+CD56+) were expanded. The median NKT cell number obtained after 21 days was 553×10^4 cells/ml (range 51–986) for non-B-cell-depleted samples and 927×10^4 cells/ml (range 101–2708) for B-cell-depleted samples (Figure 2). The NKT cell expansion rate difference between these two samples was also not significant (*P*-value >0.39). Samples from some patients (AD1 and LS1) had a particularly prominent increase of NKT cells (Table 2). The absolute cell counts for NK, NKT, T, and B cells appear in Table 2 for non-B-cell-depleted cultures and in Table 3 for B-cell-depleted cultures. In cultures in which most of the expanded cells were CD3−CD56+, NK cells had NKT numbers that were relatively low (Table 2, patient YO2). In contrast, under the same conditions, cultured PBMCs from patients with progressive disease (LB1^{Before} and RL1^{Before}) had far less NK cell expansion after 21 days. Moreover, in patient LB1 the NK expansion capacity was restored after fludarabine therapy, when the patient had achieved a CR. B cells remained in the cultures, but were not expanded. B cells were more markedly reduced in cultures from patients with indolent disease (AD1, KO2, LS1, and YO2) than in samples obtained from patients with progressive untreated disease. In contrast, in samples obtained from patients after treatment, the number of B cells was markedly reduced during culture (Table 2).

To study the behavior of NK cells, we subjected PBMCs from a patient with progressive disease, RL, after receiving fludarabine treatment, to a transwell system where we cocultured B-cell-depleted PBMC with tumor B-cell-enriched cells. PBMCs cultured in a transwell system expanded to a slightly better extent than those containing non-B-cell-depleted PBMCs (data not shown).

Expansion rate variability of NK cells in samples obtained at different time points

To assess the reproducibility of our culture conditions, PBMCs obtained from the same patients at different time points were cultured under identical conditions. The time interval between the first and second samples was not longer than 1 month, except in patient KO2, for whom it was 4 months, and for LB with 3 months between the sample before therapy and the sample after therapy. As indicated in Figure 3, the absolute NK cell counts were quite similar in repeated samples, both in those

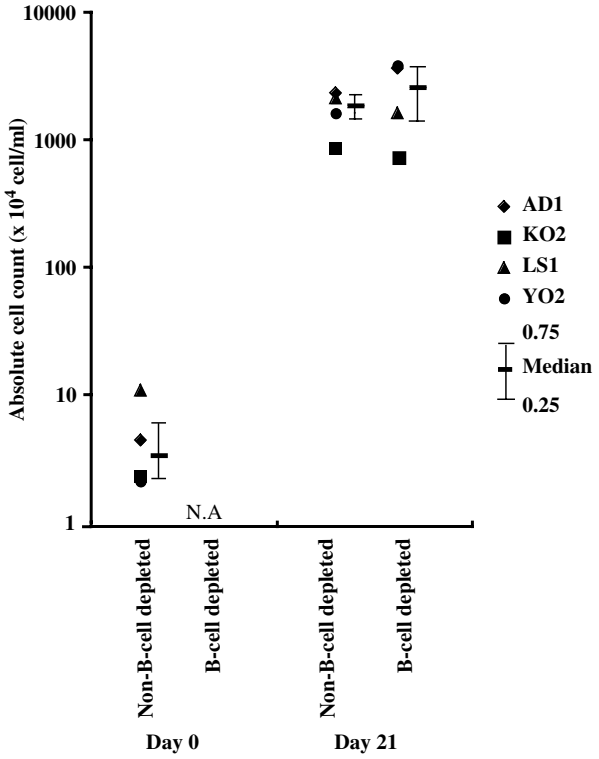

Figure 1 Absolute cell counts of CD3−CD56+ NK cells before and after *in vitro* expansion. Both non-B-cell- and B-cell-depleted PBMC from four CLL patients (AD1, KO2, LS1, and YO2) with indolent disease were cultured at 100×10^4 cells/ml in a medium supplemented with OKT-3 (first initial 5 days, 10 ng/ml) and IL-2 (500 U/ml) for 21 days. Median values and interquartile ranges are indicated. No significant difference in the NK cell count was seen when comparing cultures from non-B-cell- and B-cell-depleted samples (*P*-value >0.56). NA: not analyzed due to low number of cells.

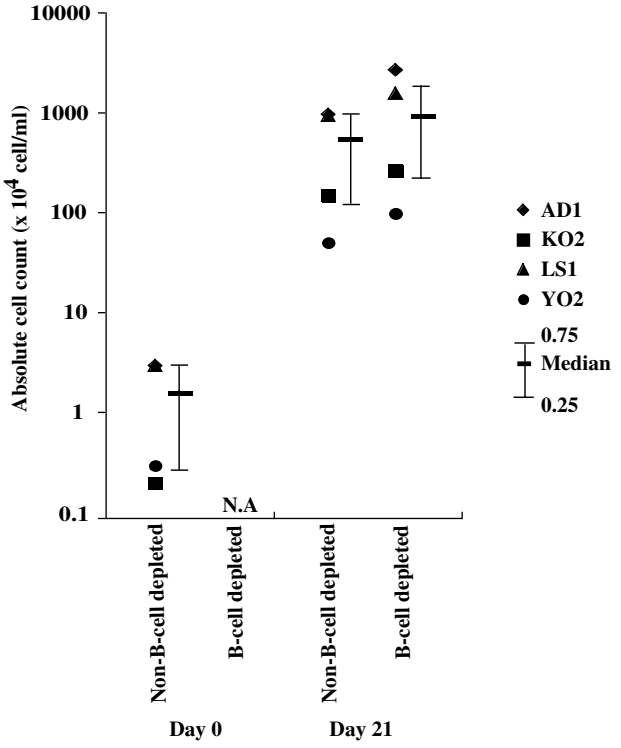

Figure 2 Absolute cell counts of CD3+CD56+ NKT cells before and after *in vitro* expansion. Both non-B-cell-and B-cell-depleted PBMC of four CLL patients (AD1, KO2, LS1, and YO2) with indolent disease were cultured at 100×10^4 cells/ml in a medium supplemented with OKT-3 (first initial 5 days, 10 ng/ml) and IL-2 (500 U/ml) for 21 days. Median values and interquartile ranges are indicated. No significant difference in the NKT cell count was seen when comparing cultures from non-B-cell- and B-cell-depleted samples (*P*-value >0.39). NA: not analyzed due to low number of cells.

obtained from patients with indolent (KO and YO) and progressive disease (LB), indicating a high degree of reproducibility in NK cell expansion capacity from the same patients at different time points.

Effect of fludarabine treatment on killer cell expansion in vitro

Samples from two patients with progressive disease (LB, RL) were obtained for culture before and after fludarabine treatment. Notably, LB received three courses of fludarabine between blood samplings, whereas RL received only one. Accordingly, the response status (response to fludarabine) in LB was better (Table 1). The numbers of NK and T cells obtained after treatment increased markedly more than those cells from samples obtained before treatment, especially for patient LB ($LB1^{After}$, Table 2). Similarly, LB had a lower post-treatment level of B cells compared to RL (Table 2).

NK and T-cell proportions and subset distribution

The percentages of NK cells were rather low on day 0 for all patients (except patient LS1) and even lower for both patients with progressive disease (LB^{Before}, RL^{Before}). However, after 21 days of incubation, samples from patients with indolent disease contained median 74% of NK cells (range 49–94). On day 21, patients who had relatively low proportions of NK cells manifested a high percentage of NKT cells. In general, the percentages of CD3+ T cells expressing the NK marker CD56, in patients with indolent disease (median 53% (range 17–75)), markedly increased by day 21 (data not shown).

Cytotoxicity

Granzyme B staining: In cytospin preparations, both from CD19- and nondepleted cultures obtained at the test period's end (day 21), a high level of Granzyme B was seen in the majority of the cells (data not shown).

Cell-mediated cytotoxicity: Cytotoxicity of the expanded cell populations from the cultured peripheral blood of two patients with indolent disease (AD1 and YO2) was evaluated by Cr-release assay. The NK-sensitive tumor cell line K562 served as a target. On day 21 of expansion, specific lysis of the target cell line could be shown in samples from both patients (Figure 4). At a 1:1 effector:target cell ratio, the median cytotoxic activity was 26 and 44% (percent specific release) in cultures of non-B-cell-depleted cells (Figure 4a) and 17 and 47% in B-cell-depleted cultures (Figure 4b) for patients AD1 and YO2,

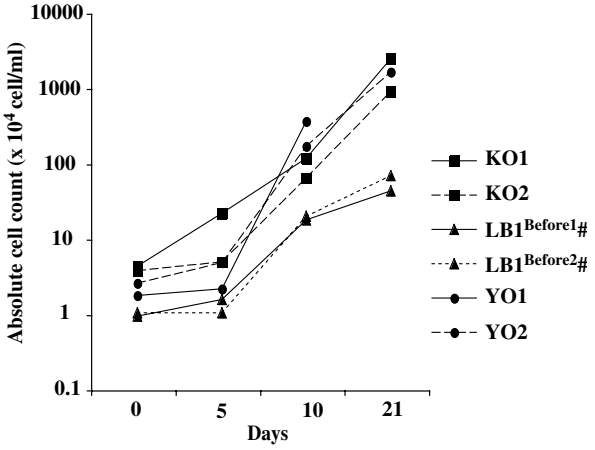

Figure 3 CD3−CD56+ NK cell expansion rates in repeated samples obtained from three individuals and cultured under identical conditions. Two patients had indolent (KO and YO) and one patient had progressive (LB) disease. Samples from the patient with progressive disease were labeled with the symbol '#'. 'Before' after patients' initials refers to samples obtained before fludarabine treatment. Numbers after patients' initials refer to sample numbers. Time interval between first and second samples of the same patients was 1 (LB, YO) to 4 (KO) months.

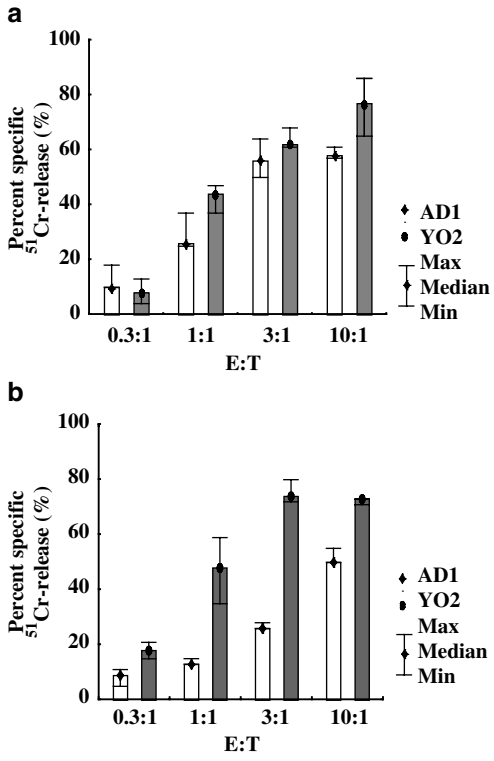

Figure 4 ^{51}Cr-release assay for cytotoxicity of day 21 cultures from (a) non-B-cell- and (b) B-cell-depleted PBMC samples. K562 served as target cells at the effector:target ratios indicated. Values are presented as median with ±min/max.

respectively. The proportion of NK cells in samples AD1 and YO2 differed in proportion (61% for AD1 and 94% for YO2) in unseparated PBMC and 47 and 93%, respectively, in samples from B-cell-depleted PBMC, that is, reflecting slightly higher cytotoxicity for the YO2 sample.

Discussion

We report here the feasibility of expanding killer cell populations of the CD3−CD56+ NK and CD3+CD56+ NKT phenotypes by using anti-CD3 antibody and cytokine (IL-2) induction of PBMC of patients with indolent or progressive B-CLL. To our knowledge, this is the first such study of NK cell as well as NKT cell expansion from peripheral blood of B-CLL patients.

NK cells are known to play a role in the control of solid tumor metastases[28] and also of leukemia.[29] The observation that NK activity is significantly reduced in peripheral blood of CLL patients as compared to healthy donors could argue for an NK cell dysfunction contributing to tumor cell accumulation *in vivo*.[16–19] Reasonably, then, increased numbers and/or activity of killer cells might be therapeutically beneficial for CLL, as it has been shown that NK cells have low cytotoxic capacity in B-CLL patients.[16–19] However, with all leukemic patients, autologous antileukemic effector cells are difficult to collect because of their limited number in the peripheral blood. Previously, others studied expansion of NKT but not of NK cells from the peripheral blood of B-CLL patients.[30] In that report, the T cells that expanded *in vitro* partly coexpressed the NK marker CD56. Moreover, the expansion of CD3+CD56+ T cells from normal donors,[31] patients with CML,[32] and patients undergoing autologous hematopoietic cell transplantation[33] has also been reported.

Recently, we successfully generated cultures of NK cells isolated from the peripheral blood of healthy donors.[27] Therefore, investigating whether PBMC from B-CLL patients could similarly serve as a source of killer cells was of significant interest. In the present study, consistent with those previous results, we successfully expanded NK and NKT cells from patients with indolent phase B-CLL at levels similar to those from healthy donors despite extremely low starting numbers of patients' NK and NKT cells. Our findings that depletion of B cells prior to culture did not significantly influence NK and NKT cell expansion rates indicates that in indolent phase, malignant B cells have no negative effect on the expansion of both NK and NKT cells. Additionally, the average proportion of NK cells achieved after culture was actually higher (74%) in CLL patients than what we have previously seen in healthy donors (55%).[27] In accordance with our previous results, we found a high percentage of T cells coexpressing the NK marker CD56. Our finding that expansion rates were lower for the patients with progressive disease, especially before fludarabine treatment, may indicate that the very low number of NK cells at the start of culture may have a negative effect on the expansion rate. However, since lowering the B-cell count by fludarabine treatment increased the expansion yield, this cytostatic regimen apparently does not negatively influence the post-therapeutic, *in vitro* proliferative rate of NK and T cells.

The analysis of samples obtained from the same patients at different time points revealed that the expansion rates were stable over time and were related to patients' characteristics and tumor burden/therapy. Furthermore, our tumor B-cell depletion and coculture experiments in a transwell system indicated that initiating cultures with large amounts of tumor cells had only a modest influence on the killer cell expansion capacity. Of interest, a small proportion of the cells present when the culture period ended were still B cells. In samples obtained from patients with progressive disease, before therapy, only moderate

reductions in absolute B-cell counts were seen upon culture. Since previous studies have shown that non-neoplastic lymphocytes delay tumor B-cell apoptosis by secreting cytokines and growth factors,[34] it remains to be determined whether the B cells remaining at the end of the culture were neoplastic or active and if they were dependent on NK and T cells for their survival.

Since preceding cytostatic therapy might negatively influence the yield when collecting peripheral blood stem cells for transplantation, a negative effect of fludarabine on the subsequent growth of killer cells *in vitro* might be anticipated. Contrary to that possibility, we found better NK cell expansion in post-therapy in cultures from the patient LB who achieved CR after three courses of fludarabine. Moreover, patient LB had a better killer cell expansion capacity post-therapy than the patient RL, who received only one course of fludarabine, and only achieved PR. It remains to be determined if successful fludarabine therapy consistently enhances the *in vitro* expansion capacity of cytotoxic cells.

Our finding that cells with the NK as well as the NKT phenotype can be expanded in culture indicates that multiple subsets of killer lymphocytes, utilizing partly different killer mechanisms, are generated simultaneously, which may be therapeutically beneficial.

In our cultures, the expression of CD16 was high on NK cells indicating their activated status (data not shown). In as much as CD16 is an Fc receptor involved in ADCC,[16–18] the expanded cell populations generated here could be instrumental as effectors following Mab therapy. The Mab, Campath-1H, had only a marginal effect on CD16 + NK cells compared to that on T and B cells,[35] but deficiency in the number of NK and NKT cells remains several months after Campath-1H therapy (personal communication). Thus, following Mab therapy, the infusion of IL-2-activated killer cells may be of clinical value. Still another phenotypic indicator of the cytotoxicity of the expanded killer cells was the presence of high levels of Granzyme B within cytoplasmic granules. Additonally, the NK-sensitive CML tumor cell line K562 was lysed at a 1:1 effector:target ratio resulting from a non-B-cell-depleted population of killer cells. Compared to NK cells taken from healthy donors and expanded, killer cells derived from CLL patients appeared to be equally cytotoxic after expansion *ex vivo*.[33]

In conclusion, our results clearly demonstrate that several potentially cytolytic subtypes of lymphocytes from B-CLL patients can be expanded *in vitro*. These cells express CD16 and Granzyme B and have cytolytic capacity, indicating that their use alone or in combination with Mab may be a feasible clinical strategy for controlling B-CLL.

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