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ARTICLE

Antibodies conjugated with viral antigens elicit a cytotoxic T cell response against primary CLL ex vivo

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Abstract

Chronic lymphocytic leukemia (CLL) is the most frequent B cell malignancy in Caucasian adults. The therapeutic armamentarium against this incurable disease has recently seen a tremendous expansion with the introduction of specific pathway inhibitors and innovative immunotherapy. However, none of these approaches is curative and devoid of side effects. We have used B-cell-specific antibodies conjugated with antigens (AgAbs) of the Epstein–Barr virus (EBV) to efficiently expand memory $CD4^+$ cytotoxic T lymphocytes (CTLs) that recognized viral epitopes in 12 treatment-naive patients with CLL. The AgAbs carried fragments from the EBNA3C EBV protein that is recognized by the large majority of the population. All CLL cells pulsed with EBNA3C–AgAbs elicited EBV-specific T cell responses, although the intensity varied across the patient collective. Interestingly, a large proportion of the EBV-specific $CD4^+$ T cells expressed granzyme B (GrB), perforin, and CD107a, and killed CLL cells loaded with EBV antigens with high efficiency in the large majority of patients. The encouraging results from this preclinical ex vivo study suggest that AgAbs have the potential to redirect immune responses toward CLL cells in a high percentage of patients in vivo and warrant the inception of clinical trials.

Introduction

Chronic lymphocytic leukemia (CLL) is the most common type of leukemia in the elderly. The disease is generally indolent and usually requires only delayed therapeutic intervention. However, a minority of patients experience a more aggressive course characterized by resistance against standard chemoimmunotherapy and limited life expectancy. Although the recent advent of targeted drugs, such as inhibitors of Bruton's tyrosine kinase and antagonists of Bcl-2, has largely improved the outcome of poor-risk CLL, the disease remains incurable by pharmacological treatment with a particularly poor outlook for those patients who become refractory against pathway inhibitors. In contrast, cellular immunotherapy strategies, such as allogeneic hematopoietic cell transplantation (HCT) or chimeric antigen receptor-modified T cells (CAR T), may result in sustainable disease eradication, but confer significant risks of morbidity and mortality [1, 2]. Antigen-armed antibodies (AgAbs) are chimeric fusion proteins that combine monoclonal antibodies (mAbs) with antigens from infectious agents [3, 4]. The antibody serves as a vehicle that delivers the attached antigen moiety to target cells, usually antigen-presenting cells (APCs). When the bound antibody is

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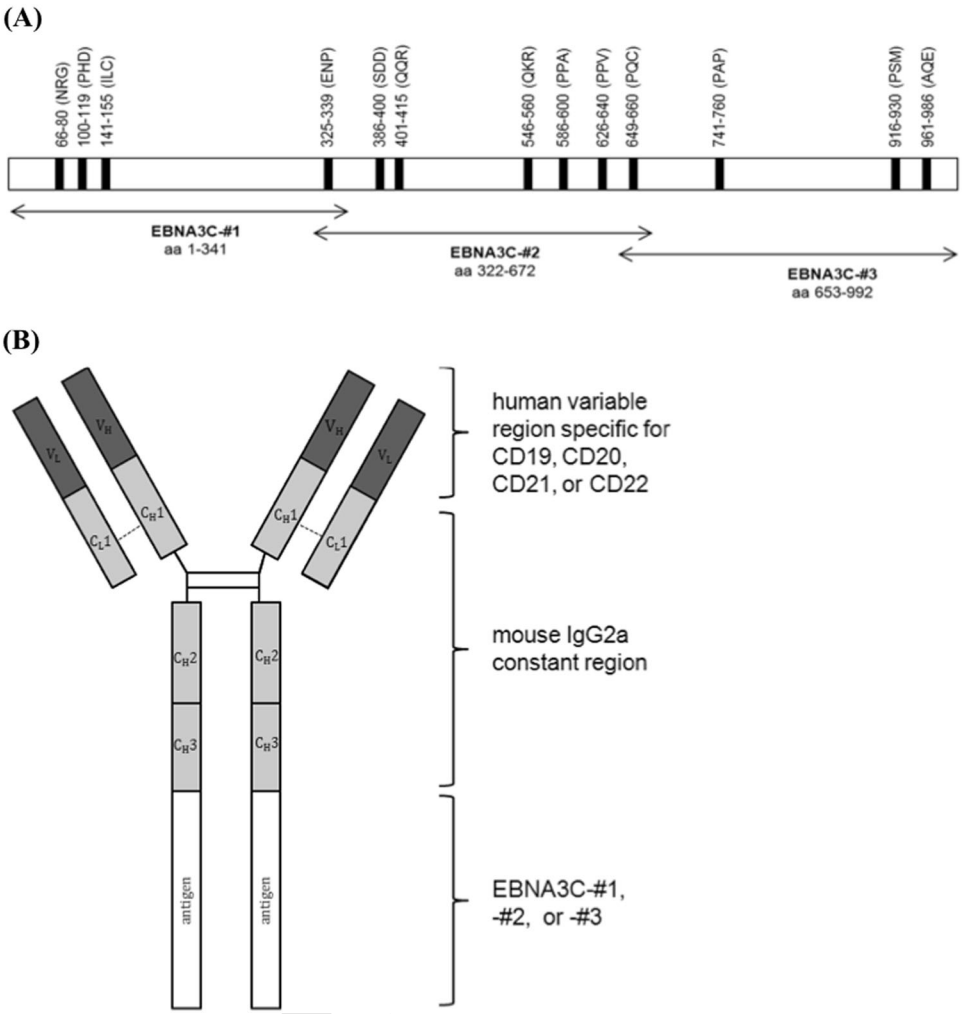

Fig. 1 Structure of EBNA3C–AgAbs. **a** Schematic representation of the EBNA3C protein showing previously described CD4$^+$ T cell epitopes with acronyms. The antigenic subdomains are referred to as EBNA3C-#1, -#2, and -#3. **b** Assembled EBNA3C–AgAbs consist of two light and two heavy chains with their conjugated antigen moiety at the C termini. C: constant; V: variable; H: heavy; L: light

internalized into its target cell by receptor-mediated endocytosis, the coupled antigens are processed and presented through the HLA class-II pathway. This results in the recognition and killing of the APC by activated memory CD4$^+$ cytotoxic T lymphocytes (CTLs) [4, 5]. Diverse bacterial and viral antigens or mutated oncogenes can be incorporated into AgAbs [3, 6–10]. Based on the high EBV seroprevalence in the adult human population worldwide (>90%) [11], we have generated AgAbs that carry antigens of the Epstein–Barr virus (EBV). The lifelong persistence of the virus in infected individuals guarantees that EBV-specific T cell immunity is constantly activated to prevent the development of EBV-induced lymphoproliferative disorders (LPD) [12, 13]. We previously explored the immunotherapeutic potential of EBV–AgAbs by demonstrating their ability to recognize and kill Burkitt's lymphoma cells in vitro [3]. Immunotherapy approaches in CLL have been hampered by a deficient T cell response in most patients [14–17]. However, EBV-specific CD8$^+$ T cells from CLL patients have recently been found to be functional in terms of cytokine production and cytotoxicity, suggesting that T cell exhaustion in CLL is heterogeneous [18]. Moreover, effective CD4$^+$ CTLs have been described in CLL [19, 20]. In principle, several transgenic mouse models of CLL could be used to test the therapeutic potential of AgAbs in vivo, however, they have a long latency phase and these mice are not susceptible to infection with EBV [21]. Therefore, we have evaluated the efficacy of AgAbs ex vivo by inducing multiplication of EBV-specific CD4$^+$ CTLs from patients with CLL and found that they are able to kill autologous primary CLL cells.

Materials and methods

Blood samples

Heparinized peripheral blood samples (50 mL) were obtained from therapy-naive patients with CLL ($n = 12$). All patients provided informed written consent in

Table 1 Clinical characteristics of patients with CLL

CLL patient	Sex	Age	Diagnosis to study inclusion, months	WBC count, 10^9/L	FISH	CLL cells		CD19+ cells			T cells	
						$CD19^+CD5^+$, % of PBMC	HLA class II+, % of $CD19^+CD5^+$	CD20, %	CD21, %	CD22, %	$CD3^+$, % of PBMC	Ratio of CD4/CD8
P1	F	68	63	22	Normal	80.7	99.9	95.5	99.0	54.8	9.6	2.3
P2	F	73	55	49	del 13q14, trisomy 12q13	73.5	99.9	99.7	99.7	86.5	20.8	2.4
P3	F	76	73	227	del 14q32	92.3	100.0	98.4	93.5	96.9	3.3	0.8
P4	F	58	96	61	del 13q14	91.2	99.9	99.4	74.0	93.4	3.9	0.9
P5	F	75	323	45	ND	73.3	99.8	98.5	91.4	96.8	20.8	0.8
P6	M	77	75	38	ND	81.7	99.8	98.4	94.4	97.6	16.6	0.1
P7	M	74	82	38	del 14q32	86.8	100.0	99.8	98.1	98.6	11.5	5.0
P8	M	68	123	66	Normal	73.7	99.9	97.1	93.7	99.7	2.4	2.3
P9	M	68	165	35	del 13q14, nullisomy 13q14, and del of distal IgH gene locus	94.2	100.0	99.0	98.3	99.2	3.5	4.3
P10	M	65	85	107	ND	91.6	99.8	98.2	92.2	97.4	3.8	1.4
P11	M	63	51	25	del 13q14	88.1	99.8	89.8	95.1	87.7	10.3	2.6
P12	M	70	54	88	del 13q14, trisomy 14q32	92.5	99.8	97.9	97.5	57.4	2.5	1.1

M male, *F* female, *ND* not determined, *del* deletion

compliance with the Declaration of Helsinki [22]. As controls, we collected mononuclear cell fractions from blood of healthy donors ($n = 5$) without evidence of lymphoproliferation, some of whom were older than 50. The study was approved by the institutional review board (Ethikkommission der Medizinischen Fakultät Heidelberg, S603/2015).

Ex vivo expansion of EBNA3C-specific $CD4^+$ T cells

EBNA3C-specific $CD4^+$ T cells were ex vivo expanded from PBMCs through the repetitive challenge with all 12 EBNA3C–AgAbs (anti-CD19, anti-CD21, anti-CD20, and anti-CD22 antibodies conjugated with EBNA3C-#1 (aa 1–341), EBNA3C-#2 (aa 322–672), and EBNA3C-#3 (aa 653–992), respectively) every 2 weeks. A total of 5×10^6 PBMCs in 2 mL of AIM V® Medium (Gibco, Grand Island, NY, USA) supplemented with 10% human serum (HS), 2 mM L-glutamine (Gibco), 10 mM HEPES (Gibco), 50 µg/mL gentamicin (Gibco), and 10 µg/mL ciprofloxacin (Sigma-Aldrich, St. Louis, MO, USA) without interleukin-2 (IL-2) (Novartis Pharma, Basel, Switzerland) were pulsed with 1–5 ng/mL per EBNA3C–AgAb in a 24-well plate (TPP, Trasadingen, Switzerland). After 2 weeks, 5×10^6 autologous PBMCs were loaded with the same AgAb concentrations, irradiated (40 Gy), and mixed with the previously treated cells in a medium with 10 U/mL IL-2. The procedure was repeated biweekly using 2×10^6 autologous PBMCs from week 4 to 10. From week 12, autologous LCLs were pulsed with 10 ng/mL per EBNA3C–AgAb, irradiated (80 Gy), and used as APCs, together with irradiated PBMCs (1×10^6) from unrelated buffy coats as feeder cells. In some cases, $CD8^+$ T cells were depleted using CD8a PE antibodies and PE MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Ethical approval to use HS from voluntary donors was obtained from the Ethikkommission of the Medizinische Fakultät Heidelberg (S-36/2011).

T cell activation assays

A total of 5×10^4 target cells were incubated with RPMI medium 1640 (Gibco) only, unmodified antibodies, EBNA3C–AgAbs, or peptides in a U-bottom well plate for 8 h at 37 °C. Effector T cells were added at various effectors to target cell (E:T) ratios and incubated for 16 h. Interferon-γ (IFN-γ) and granzyme B (GrB) released into the supernatants was quantified using ELISA (Mabtech, Nacka Strand, Sweden). One representative result out of at least two independent experiments performed on each patient's sample in triplicates is shown. Means and standard deviations are displayed in bar charts.

Results

EBV–AgAbs can induce the proliferation of specific T cells in patients with CLL

We generated 12 AgAbs that consist of three overlapping segments of the EBNA3C protein combined with CD19, CD20, CD21, or CD22-specific antibodies, here referred to as EBNA3C-#1, -#2, and -#3 (Fig. 1). EBNA3C is a strongly immunogenic protein that carries multiple epitopes and is recognized by the majority of the infected population (Table S1) [23]. Thus, these AgAbs should in principle be able to stimulate EBV-specific T cells in any EBV-positive individual [13]. The AgAbs efficiently bound to B cells and induced a potent cytotoxic T cell response in healthy individuals ($n = 5$) (examples are shown in Supplemental Results, Figure S1, Figure S2). Peripheral blood samples were collected from treatment-naive CLL patients ($n = 12$), whose clinical data are given in Table 1. EBNA3C-specific $CD4^+$ T cells were expanded ex vivo by repetitive T cell stimulation with autologous PBMCs pulsed with EBNA3C–AgAbs (Fig. 2a, b). However, in nine subjects, this expansion was accompanied by the outgrowth of $CD8^+$ T cells, which usually waned with time, but in two cases were negatively selected to establish a pure $CD4^+$ T cell culture (P1 and P6 shown in Fig. 2b). In parallel, we transformed normal B cells from each patient with EBV to obtain lymphoblastoid cell lines (LCL) that can be used as APCs.

$CD4^+$ T cells from patients with CLL expanded ex vivo with AgAbs are specific for EBNA3C

We then assessed the specificity of the $CD4^+$ T cell lines established from the blood of CLL patients. Pulsing autologous LCLs with CD20-specific AgAbs led to a robust response in all samples (examples are shown in Figure S3). Three of those recognized EBNA3C-#1, eight EBNA3C-#2, and 10 EBNA3C-#3. More than one T cell line could be expanded from most patient blood samples, and specificity for two different EBNA3C segments was detected in most patients (Table 2). We confirmed the specificity of these T cells after stimulation with a set of four HLA class II-restricted EBNA3C peptides (Table S1). A specific response was found for three of these peptides in six out of twelve patients (Table 2 and Figure S3).

EBNA3C-specific $CD4^+$ T cells recognize primary CLL cells

We tested the ability of the EBV-specific T cells to recognize AgAb-treated autologous primary CLL cells by measuring IFN-γ responses. We cocultured the T cells with CD5-purified CLL cells from P11 that were treated with AgAbs. In parallel, we tested the ability of total PBMCs to serve as APCs (Fig. 3a). Both target cell samples elicited a T cell response, and there was no substantial difference in the intensity of recognition between purified and non-purified CLL cells. Therefore, we limited our further analyses to patients' PBMCs. We could detect T cell responses against all 12 CLL samples using CD20-specific AgAbs, however, with highly variable intensities (Fig. 3a–d and Table 2). CLL cells not loaded with EBV antigens or CLL cell lysates did not induce IFN-γ responses in these $CD4^+$ T cells (Figure S4). Thus, there was no evidence of antigen spreading. We assessed CD80 and CD86 expression on ten LCL and ten CLL cell samples. CLL cells expressed much lower levels of these costimulatory molecules (14.4% and 26.2%, respectively) than LCLs (99.2% and 94.9%, respectively) (Table S2). This could contribute to the generally weaker T cell responses elicited by CLL cells as compared to autologous LCLs (Fig. 3 and Figure S3).

Patient-derived EBNA3C-specific $CD4^+$ T cells are capable of killing primary CLL cells

We evaluated the cytotoxic potential of the T cell lines against primary CLL samples and observed highly efficient levels of killing that in some cases reached a plateau of 80% at E:T = 10:1 (Fig. 4a–d). This is an intriguing result as CLL cells are known to be frequently resistant to apoptosis through Bcl-2 overexpression and indeed, treatment of CLL cells with venetoclax led to dose-dependent cell death that was amplified by simultaneous exposure to EBNA3C-specific $CD4^+$ T cells (Figure S5). However, T cell killing itself was not improved by the Bcl-2 block, possibly because of T cell inhibition by the drug. We could also observe potent killing of autologous LCLs after treatment with EBNA3C–AgAbs (Figure S6). EBNA3C-specific $CD4^+$ T cells of some samples (e.g., P6) showed weaker IFN-γ responses against CLL cells than against LCLs after AgAb stimulation (Fig. 4e). However, the same T cells efficiently killed both CLL cells and LCLs (Fig. 4f). All T cell lines displayed a potent cytotoxic response after AgAb stimulation, irrespectively of the level of IFN-γ secretion (Table 2). Addition of concanamycin A (CMA), a vacuolar type H^+-ATPase (V-ATPase) inhibitor, prevented killing of all target cells treated with EBNA3C–AgAbs (Fig. 4a–d). Thus, AgAbs elicited a perforin-based cytotoxic response triggered by EBNA3C-specific $CD4^+$ T cells. Similar results were obtained with EBNA3C–AgAbs against CD19, CD21, or CD22 (Supplemental Results, Figure S7). We also performed IFN-γ and calcein-AM assays with $CD8^+$ cells that were amplified from some samples, using PBMCs as APCs pulsed with EBNA3C–AgAbs or two HLA class I-restricted EBNA3C epitopes as a source of antigen, but

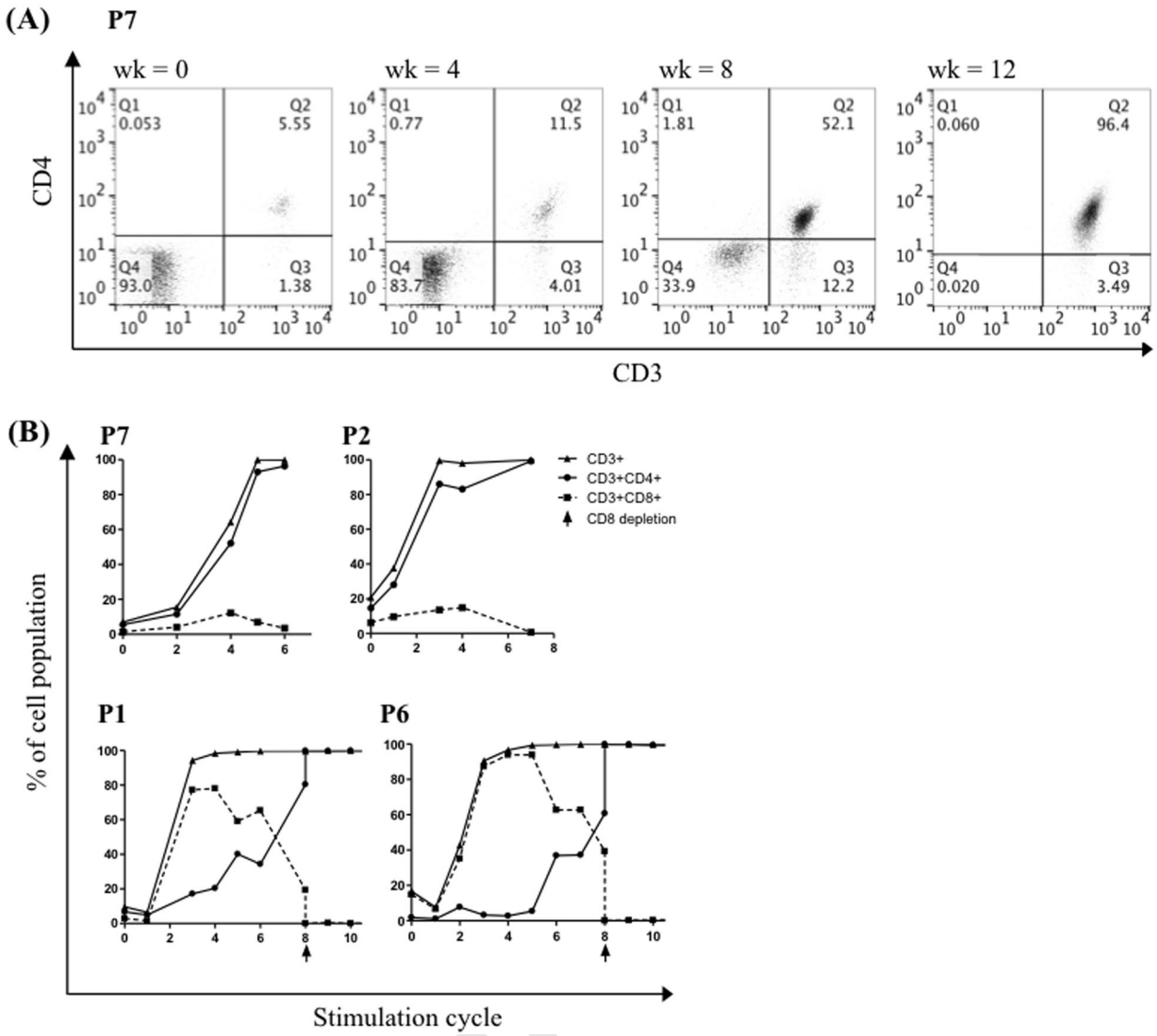

Fig. 2 Treatment with EBNA3C–AgAbs leads to ex vivo expansion of $CD4^+$ T cells from patients with CLL. **a** Outgrowth of $CD3^+CD4^+$ T cells from PBMCs of P7 through stimulation with EBNA3C–AgAbs was monitored by FACS. **b** Enrichment of T cells from four patients is shown, as defined by FACS. In P1 and P6, $CD8^+$ T cells were depleted at week 16 using MACS beads. wk: week

could not identify any specific response (Figure S8, data not shown). Thus, the response against EBNA3C is restricted to the $CD4^+$ T cell subset. We then assessed the expression of CD107a, a surrogate marker of cytotoxic granule secretion, on T cells upon stimulation with AgAbs. EBNA3C-specific $CD4^+$ T cells from P11, which are specific to EBNA3C-#2 and -#3, showed time-dependent increase of CD107a after co-culture with autologous PBMCs loaded with EBNA3C-#2 or EBNA3C-#3 AgAbs. Thus, up to 50% of these T cells are endowed with potential cytotoxic functions (Fig. 5a, b). The percentage of $CD4^+$ CTLs among total T cells varied between 14 and 59% with an average of 35% after a co-culture period of 8 h (Table 2). Importantly, $CD107a^+$ cytotoxic EBNA3C-specific $CD4^+$ T cells can be detected in vivo at low frequency in the peripheral blood of CLL patients after AgAb stimulation (Figure S9). CD107a-negative $CD4^+$ T cell lines displayed high expression of CD28 (92.3%, average of seven cases) (Figure S10) but not of Foxp3 (data not shown). These cells expressed CD69 (90.6%, average of seven cases) (Figure S10), a typical T cell activation marker. Thus, they are neither anergic nor T regulatory (Treg) cells. A subset of T cell lines (five out of nine tested) secreted IL-4 after AgAb stimulation, indicating that they contain Th2 cells (Figure S11), that can act as helper cells but also have the potential to become cytotoxic [24]. We also quantified direct secretion of GrB from expanded $CD4^+$ T cells (Fig. 5c and Figure S12). T cells strongly expressed GrB upon stimulation, and at much higher levels than IFN-γ. Nevertheless, the intensity of the response varied across the CLL samples. We conclude that

Table 2 Characterization of ex vivo expanded $CD4^+$ T cells with EBNA3C specificity

CLL patient	$CD4^+$ T cell specificity (EBNA3C)	IFN-γ release	GrB release	Calcein-AM release	CD107a surface expression, %	EBNA3C $CD4^+$ T cell epitope specificity
P1	-#2, -#3	Medium	Medium	High	49	PPV
P2	-#2, -#3	Low	Medium	High	22	ND
P3	-#2, -#3	High	Medium	High	20	AQE
P4	-#2, -#3	Medium	High	High	14	AQE
P5	-#1, -#2	High	High	High	28	PPV, SDD
P6	-#3	High	Medium	Medium	45	ND
P7	-#3	Low	Low	High	30	ND
P8	-#2, -#3	High	Medium	Medium	59	AQE
P9	-#1, -#3	Medium	Medium	High	58	ND
P10	-#3	Low	Medium	Low	15	ND
P11	-#1, -#2, and -#3	High	High	Medium	41	ND
P12	-#2	High	Medium	Medium	41	SDD

The table indicates the specificity of the $CD4^+$ T cells from each patient, the level of IFN-γ or GrB secretion, the magnitude of the cytotoxic response, and the response to EBNA3C peptides. IFN-γ levels were divided into high (>2000 pg/mL), medium (500–2000 pg/mL), and low (0–499 pg/mL). GrB levels were divided into high (>20,000 pg/mL), medium (5,000–20,000 pg/mL), and low (0–4999 pg/mL). Levels of CTL responses were divided into high (>50%), medium (16–50%), and low (5–15%). The proportion of $CD4^+$ T cells expressing CD107a on their cell surface upon AgAb stimulation is given in %. $CD4^+$ T cell specificities against HLA class II-restricted EBNA3C epitopes were tested with the four synthesized EBNA3C peptides ENP, SDD, PPV, and AQE

ND not determined

all CLL cell samples exposed to AgAbs elicit a potent cytotoxic $CD4^+$ T cell response with activation of the perforin/GrB pathway.

Discussion

The present ex vivo study reports the results of a novel immunotherapy approach aimed at redirecting EBV-specific endogenous $CD4^+$ T cells devoid of genetic modification to leukemic cells labeled with EBV antigens. This approach was applied to a group of therapy-naive unselected CLL subjects and could in principle be extended to antigens from other ubiquitous viruses such as herpes simples virus (HSV), cytomegalovirus (CMV), or even bacterial antigens, e.g., from *H. pylori*. In all patients, the EBNA3C–AgAb triplet induced growth of EBNA3C-specific T cells, which indicates that it probably covers a large proportion of the population, possibly its entirety. This eliminates the need to identify haplotype-matching epitope restrictions in the individual CLL patient, while decreasing the risk of tumor escape through exhaustion of single EBNA3C peptide-specific T cell clones. We noted that the amounts of IFN-γ released by endogenous T cells were generally lower, and sometimes much lower after stimulation with CLL cells relative to LCLs. This fits with lower expression of the costimulatory molecules CD80 and CD86 on CLL cells, relative to LCLs, and confirms reports that LCLs efficiently present antigens and that CLL cells do not, despite high-expression levels of HLA class-II molecules [25–28]. The T cell response was exclusively directed against viral antigens as CLL cells or CLL lysates did not elicit any T cell response. This suggests that our strategy does not lead to antigen spreading, although the present study cannot decide whether this could happen in vivo [29]. AgAbs directed against CD19, CD20, CD21, or CD22 showed a very similar efficacy in inducing recognition and destruction of CLL cells. This leaves the opportunity of tailoring the treatment according to the expression level of these molecules in different CLL cases. It also offers alternatives if a clone of tumor cells lacking one of these molecules emerges. In nine out of twelve cases, we observed co-expansion of $CD3^+CD8^+$ and $CD3^+CD4^+$ cells. However, EBNA3C-specific T cell responses were confined to the $CD4^+$ subsets. The specificity of the $CD8^+$ cells remains unclear, although they could be autoimmune in nature, as has been previously described in patients with CLL [30, 31].

The vast majority of primary CLL cell samples treated with EBNA3C–AgAbs were sensitive to cytolysis by EBNA3C-specific $CD4^+$ T cells. We could ascribe the T cell cytotoxicity to the perforin/GrB pathway as CTL activity was blocked by inhibition of granule exocytosis with CMA. Moreover, CD107a staining showed that, on average, approximately one-third of the $CD4^+$ T cells had cytotoxic functions. We could also detect peripheral memory EBNA3C-specific CD107a-positive $CD4^+$ T cells in the

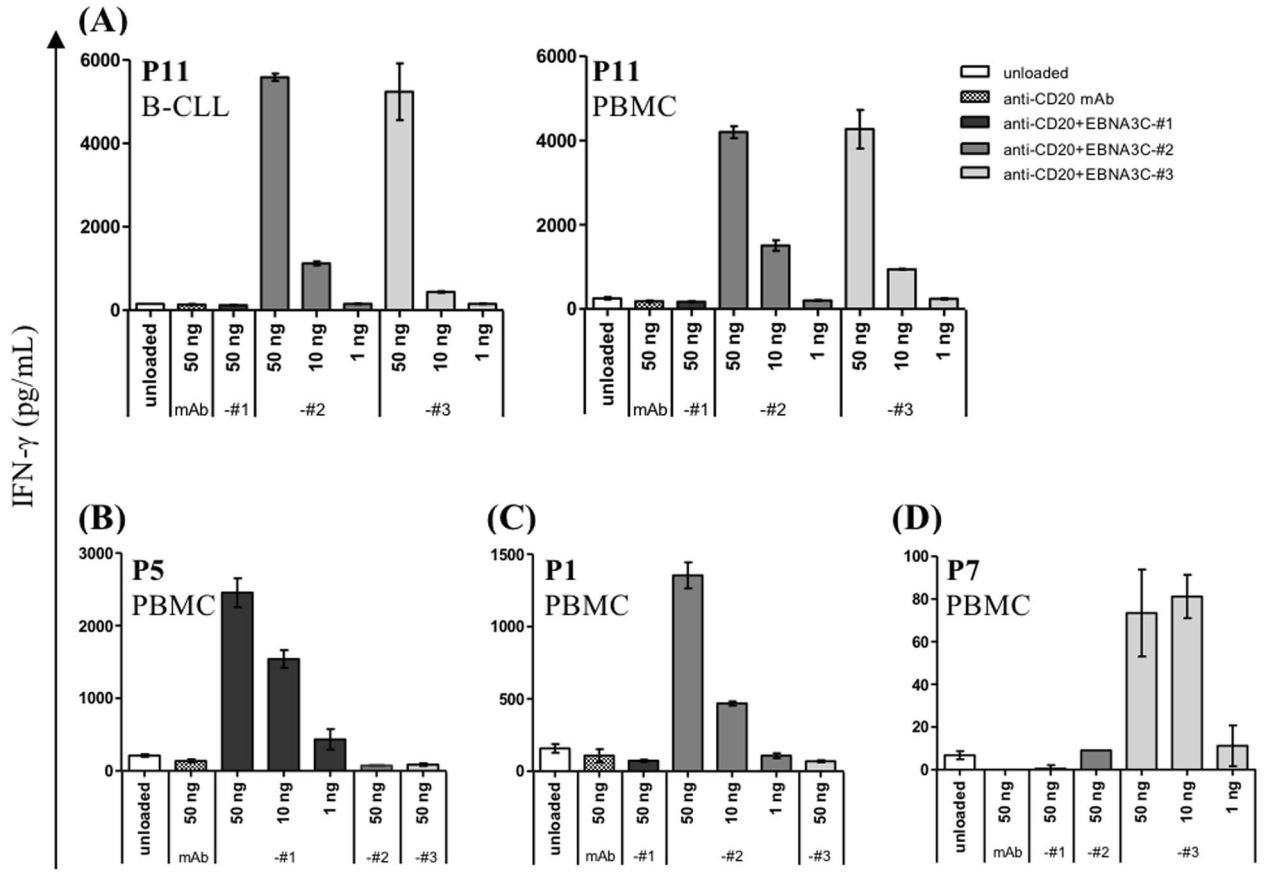

Fig. 3 Primary CLL cells treated with AgAbs are recognized by EBNA3C-specific CD4$^+$ cells. IFN-γ release assays were performed at E:T = 5:1 with EBNA3C-specific CD4$^+$ T cells and **a** CD5-purified CLL cells or PBMCs from P11 as APCs; **b–d** PBMCs from three other patients as APCs

blood of several CLL patients prior to expansion. Thus, the remarkable ability of EBV antigens to elicit CD4$^+$ cytotoxic responses make them very attractive candidates for anti-cancer T cell therapy [32, 33]. The nature of the CD107a-negative population and whether it contributes to tumor cell elimination through helper function remains to be determined. The intensity of the cytotoxic response against CLL cells and the strength of the GrB signals both appeared to be much stronger than the IFN-γ response. It has been previously reported that T cells with cytotoxic activity do not always secrete IFN-γ, whereas non-cytotoxic T cells may release IFN-γ [34, 35]. LCLs express viral and cellular anti-apoptotic proteins that limit the intensity of the cytotoxic response [36]. However, our direct target cell lysis assays showed that the EBV-specific T cells killed AgAb-treated LCLs and CLL cells with similar efficiency. This is interesting as CLL cells frequently overexpress Bcl-2, an anti-apoptotic protein that may inhibit perforin-dependent pathways, and we could confirm in the present study that these tumor cells can be killed by potent apoptosis inducers [37]. EBV-specific T cells have previously demonstrated their ability to eradicate post-transplant lymphoproliferative disorders (PTLDs) in vivo [28]. This suggests that EBV-specific T cells could also efficiently kill CLL cells in vivo, providing a rational basis for future clinical trials with AgAbs. We found no correlation between the levels of T cell activation or CTL function, and the investigated clinical parameters including leukocyte number, CD4/CD8 ratio, time from diagnosis, and the CLL burden in the peripheral blood. A potential limitation of the study is its restriction to therapy-naive patients. The efficacy of the approach critically depends on the presence of EBV-specific CTLs and is likely to show better results in patients that have not yet been subjected to intensive cytotoxic treatment. The potential presence of pre-existing antibodies against EBNA3C peptides in the serum of patients that could build a complex with the AgAbs may cause concern. These conjugates might prevent efficient targeting of CLL cells. However, human serum was constantly used for T cell maintenance and stimulation in the present study and we found no evidence of adverse effects on AgAbs. Moreover, CLL patients frequently have low or even null IgG titers against EBV. This is a potential advantage of choosing EBV antigens versus, e.g., CMV antigens, as CMV-specific

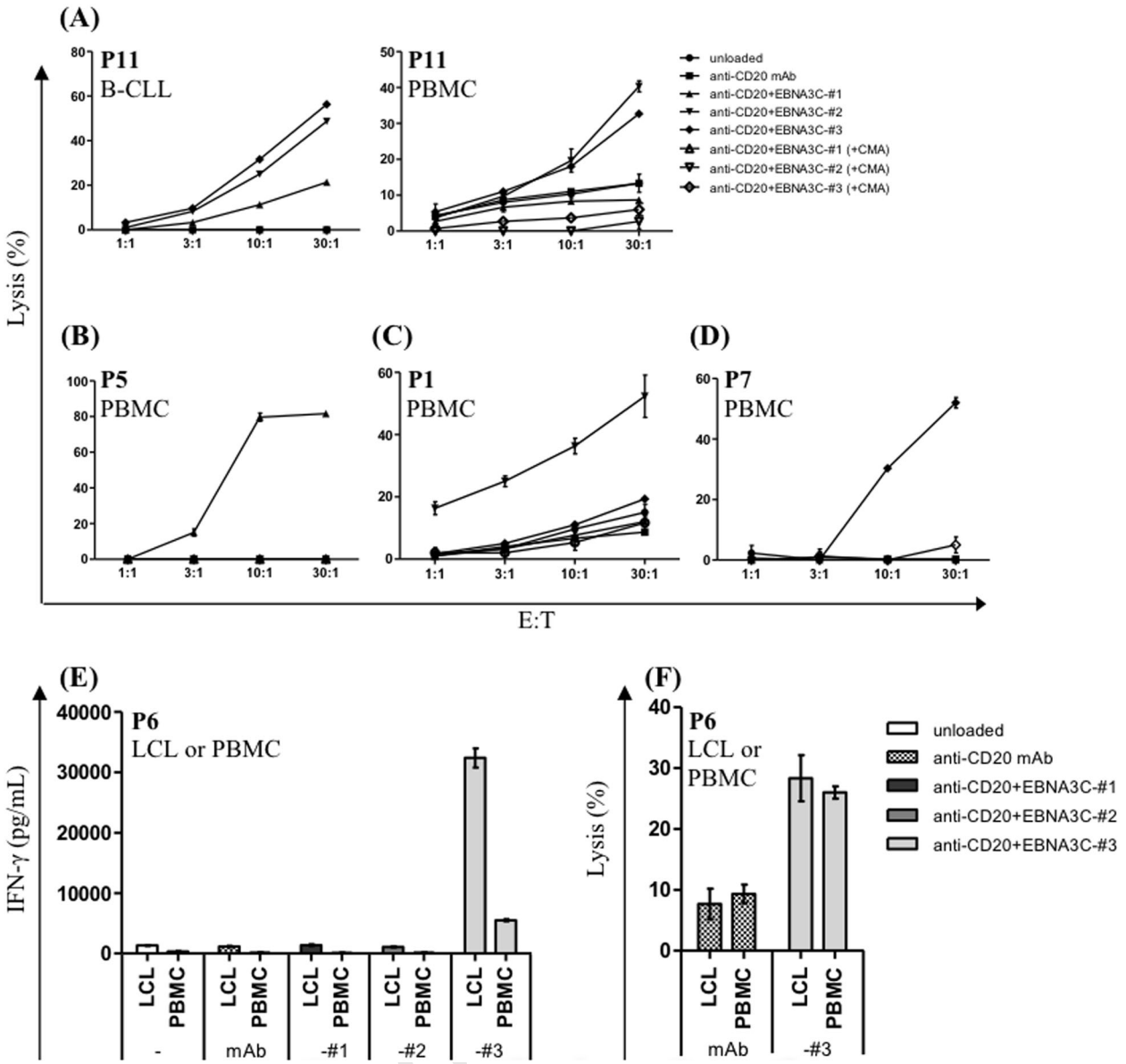

Fig. 4 EBNA3C-specific CD4$^+$ T cells kill primary CLL cells exposed to EBNA3C–AgAbs via the perforin/GrB pathway. **a**, **b** Calcein-AM assays were performed with EBNA3C-specific CD4$^+$ T cells and **a** CD5-purified CLL cells or PBMCs from P11 as APCs; **b**–**d** PBMCs from three other patients as APCs. **e**, **f** EBV-specific CD4$^+$ cells efficiently kill CLL cells despite a weaker cytokine release. **e** IFN-γ release assay was performed at E:T = 5:1 with autologous PBMCs or LCLs using 50 ng AgAbs. **f** Same as **e** for a 3 h calcein-AM assay performed at E:T = 30:1

antibody responses are better preserved [38]. Thus, it is unlikely that CLL patients will be able to mount an efficient response against the antigen moiety in the AgAbs used in this study, even if the EBV antigens could potentially stimulate it through a hapten-carrier effect. This potential drawback could be reduced by limiting the ability of AgAbs to bind to Fc receptors through mutagenesis [39]. Thus, it is likely that repetitive injections of the antibodies to progressively reduce the tumor cell burden will remain effective over time. Some CLL patients have difficulties in controlling EBV-infected cells and fludarabine- and alemtuzumab-based treatments may predispose patients to EBV-driven lymphoproliferations [40–42]. Regular monitoring of EBV loads in the peripheral blood should enable identification of these patients who are less likely to respond to AgAb treatment, although this therapy should not have deleterious consequences for the control of EBV-infected cells, but instead possibly stimulate EBV-specific T cell responses.

Our results might appear at first sight to contradict the current view that CLL is accompanied by T cell exhaustion and dysfunction with impaired immunological synapse

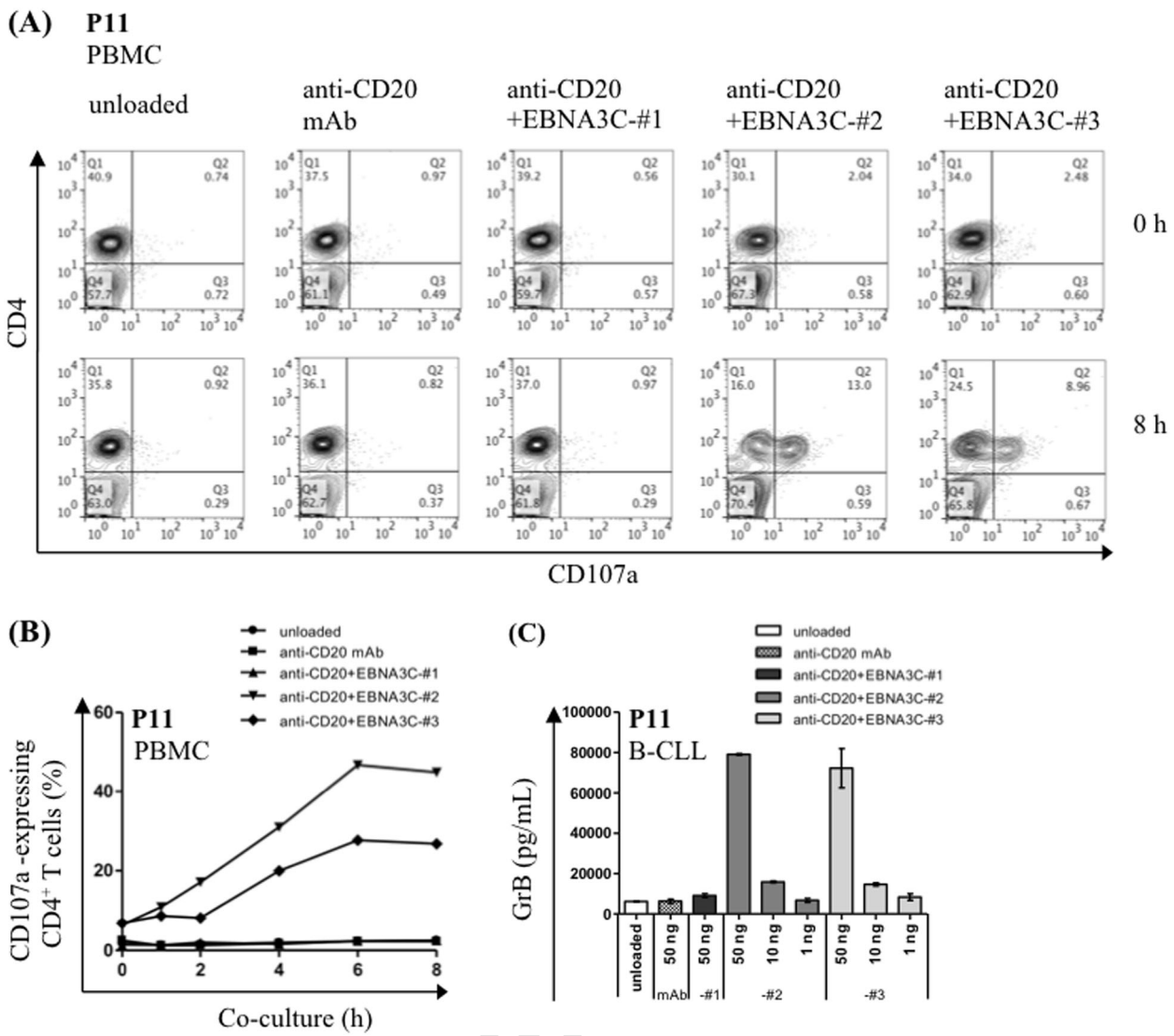

Fig. 5 Treatment with AgAbs activates granule exocytosis with GrB release from EBV− EBNA3C-specific $CD4^+$ T cells. **a**, **b** Evolution of CD107a expression on activated $CD4^+$ T cells specific to EBNA3C-#2, and -#3. Effector T cells, cocultured with PBMCs as APCs, were analyzed using FACS. **a** Expression of CD107a on $CD4^+$ T cells before and after 8 h co-culture. **b** Kinetics of CD107a expression on $CD4^+$ T cells after stimulation (0–8 h). **c** GrB release assay was performed at E:T = 5:1 with autologous CD5-purified CLL cells

formation [14–17]. However, these studies were restricted to global T cell populations. EBV-specific $CD8^+$ T cells from CLL patients have recently been found to retain intact functionality [18]. Similar observations were made on CMV-specific $CD8^+$ T cells from CLL patients [43]. It remains unclear whether T cells directed against viral antigens are an exception or whether T cell exhaustion is more heterogeneous in CLL patients than previously assumed [18]. In any case, $CD4^+$ and $CD8^+$ virus-specific T cells appear to represent ideal candidates for immunotherapy against CLL, provided that they can attack tumor cells, for example after AgAb treatment.

CLL has a limited sensitivity to rituximab monotherapy [44, 45]. Typically, approximately half of patients treated with rituximab only do not respond, or suffer early relapses [45]. The resistance to rituximab in CLL may be explained by a relatively weak CD20 surface expression coupled to relatively high CD20 internalization rates [46, 47]. However, AgAbs will benefit from efficient internalization as it will increase EBV antigen presentation on CLL cells.

In conclusion, the AgAbs triplet can reliably induce a powerful CTL response against autologous CLL cells ex vivo, thereby combining the benefits of antibody treatment with the high therapeutic potential of cellular immunotherapy. These results warrant the inception of clinical trials that aim at evaluating their clinical benefit in patients with CLL.

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Author contributions VS performed the experiments, analyzed the data, and wrote the manuscript. MI and DGvZ undertook research and provided technical support. PD designed the clinical protocol and provided blood samples. SF designed the clinical investigation protocol and undertook research. JM provided T cell clones and oversaw research. HJD planned the study, oversaw the research, and wrote the manuscript. All authors approved the article.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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