

## Brief report

## Transduction of CLL cells by CD40 ligand enhances an antigen-specific immune recognition by autologous T cells

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Several features of chronic lymphocytic leukemia (CLL) suggest that immune-based strategies may have therapeutic potential. A promising approach is provided by the transduction of CLL cells with CD40 ligand (CD40L) by viral vectors to enhance their immunogenicity. We compared the antigen-presenting capacity of CD40L-transduced CLL cells with mock-transduced or CD40L-stimulated CLL cells (CD40-CLL). A signifi-

cantly higher number of T cells could be expanded using CD40L-transduced CLL cells as antigen-presenting cells (APCs) compared with the control group ( $P = .008$ ). Using 5 different CLL-associated tumor antigens, including fibromodulin, MDM2 (murine double minute 2), survivin, p53, and KW-13, we show in interferon- $\gamma$  (IFN- $\gamma$ ) enzyme-linked immunospot (ELISPOT) assays after 35 days of in vitro culture that the number

of antigen-specific autologous T cells was also significantly higher when CD40L-transduced CLL cells were used as APCs ( $P < .001$ ). Thus, CD40L-transduced CLL cells are able to induce an antigen-specific T-cell response and might be superior to CD40-CLL cells for immune-based therapeutic strategies in CLL. (Blood. 2005;106:3223-3226)

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## Introduction

Despite recent advances, chronic lymphocytic leukemia (CLL) as the most common leukemia remains a largely incurable disease. Several features of this disease suggest that immune-based strategies may have therapeutic potential.<sup>1</sup> CLL cells express major histocompatibility complex (MHC) class 1 and 2 molecules and several tumor-associated antigens (TAAs).<sup>2-5</sup> Although most CLL cells are ineffective antigen-presenting cells (APCs) with a low expression of costimulatory molecules, stimulation with CD40 ligand (CD40L) restores their poor antigen-presenting capacity.<sup>6,7</sup> Transduction of CLL cells with CD40L by viral vectors presents an alternative strategy to enhance their immunogenicity with promising results in a first phase 1 clinical trial.<sup>1,8,9</sup>

Adeno-associated virus (AAV), a nonenveloped human parvovirus, has gained attention for human gene therapy because of its favorable safety features. High transfection rates of CLL cells are achieved using an adenovirus-free packaging system with recombinant AAV.<sup>10</sup> Thus far, enhanced proliferation of T cells and interferon  $\gamma$  (IFN- $\gamma$ ) production was demonstrated using virally transduced CLL cells, but a CLL-specific (ie, TAA-specific) immune assessment has been lacking. In the present study we show that, based on several CLL-associated tumor antigens, CD40L-transduced CLL cells are indeed able to evoke an antigen-specific T-cell response in an autologous setting, and they seem to be more potent APCs than CD40-stimulated CLL cells. Therefore, we

provide an in vitro model for the principle of action of a CD40L-based vaccine in CLL.

## Study design

## Patients and peptides

After informed consent was provided, peripheral blood was obtained from 5 HLA-A0201-positive patients who satisfied diagnostic criteria for B-cell CLL (B-CLL).<sup>11</sup> Patients' characteristics were as follows: median age, 64 years (range, 48-84 years); Binet A, one patient; Binet B, one patient; Binet C, 3 patients; median leukocyte counts,  $103 \times 10^9/L$  ( $29.2-126 \times 10^9/L$ ); median hemoglobin level, 129 g/L (118-158 g/L); median platelet count,  $99 \times 10^9/L$  ( $57-235 \times 10^9/L$ ); median  $\beta_2$ -microglobulin concentration, 312 nM (155-396 nM). Three of 5 patients were tested for CD38 expression, and all findings were negative. Cytogenetic analyses by interphase fluorescence in situ hybridization (FISH) revealed one patient with a normal karyotype and 3 patients with a deletion of 13q14. Two patients had received previous treatment with chlorambucil. All peptides used were HLA-A0201 binding, synthesized, purified by high-performance liquid chromatography (HPLC) by G.J. Arnold (Ludwig Maximilians University, Munich, Germany), and dissolved in dimethyl sulfoxide (DMSO). Four fibromodulin-derived peptides, one MDM2 (murine double minute 2)-derived peptide (81-88), 4 p53-derived peptides (p1(65-73), p2(264-272), p3(139-148), p4(149-157)), 2 survivin-derived peptides (S8, S9), and the SEREX (serologic identification by recombinant expression cloning)-derived peptide KW-13 were

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Submitted April 28, 2005; accepted June 29, 2005. Prepublished online as Blood First Edition Paper, July 12, 2005; DOI 10.1182/blood-2005-04-1742.

Supported by grants from the Deutsche Forschungsgemeinschaft (SFB 455) (C.M., M.H., C.-M.W.), Wilhelm-Sander-Stiftung (95-056-2) (C.M., M.H., C.-M.W.), and Else-Kröner-Fresenius-Stiftung and Friedrich Baur-Stiftung, Germany (C.M., M.H., C.-M.W.).

C.M., M.H., and C.-M.W. were responsible for the conception and design of

the presented work. C.M. performed the research. H.B., D.B., and D.M.K. contributed analytical tools. C.M. and C.-M.W. analyzed the data and wrote the paper.

D.M.K. and H.B. contributed equally to this study.

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used.<sup>2,12-18</sup> Approval for this study was obtained from the institutional review board of the Ludwig Maximilians University, Munich and the University of Cologne, Germany.

### AAV transduction

Recombinant AAV (rAAV) vectors (AAV/mCD40L and AAV/EGFP) were produced as previously described.<sup>10</sup> CLL cells ( $1.2 \times 10^6$  cells/well in 24-well plates) were cocultured with  $\gamma$ -irradiated CD40L-expressing mouse fibroblast L cells and were incubated in 500  $\mu$ L basal Iscove medium (Biochrome, Berlin, Germany) supplemented with 10% fetal calf serum (FCS), and infectious AAV was added for 72 hours, resulting in a multiplicity of infection (MOI) between 20 and 50.<sup>19</sup>

### Generation of effector T cells

CD40L-transduced, green fluorescence protein (GFP)-transduced, CD40L-stimulated or native CLL cells, which were all cultured in basal Iscove medium supplemented with 10% FCS, were used as APCs for T-cell stimulation. Autologous T cells were cultured as previously described.<sup>2</sup> They were incubated in the presence of interleukin-2 (IL-2; 40 U/mL; Cellconcepts, Umkirch, Germany) and IL-7 (10 ng/mL; Cellconcepts). Stimulation with autologous APCs was performed on days 0, 7, 14, 21, and 28 without further adding of antigenic peptides.

### ELISPOT assay

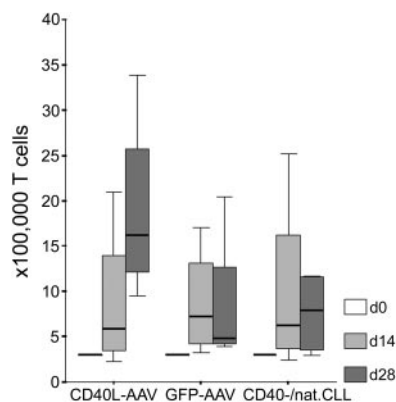
IFN- $\gamma$  enzyme-linked immunospot (ELISPOT) assays were performed as described.<sup>20</sup> On days 14 and 35, stimulator and effector cells were added in duplicate, together with the peptides (10  $\mu$ g/mL) and, as indicated, a major histocompatibility complex (MHC) class I-specific antibody (W6/32; Acris, Hiddenhausen, Germany), and incubated overnight. As stimulator cells, APCs corresponding to the APC used for T-cell culture were used. Spots were counted in a blinded manner. As a negative control, cells were incubated in the presence of DMSO, and the spots were subtracted from the experimental values to obtain the number of TAA-specific spots.

### Statistical analysis

Because of nonnormality, we used the Kruskal-Wallis (more than 2 subgroups) and the Mann-Whitney *U* test (2 subgroups) for independent subgroups and the Wilcoxon test for dependent subgroups (version 11.5; SPSS, Chicago, IL). Statistical significance was accepted for *P* values below .05.

## Results and discussion

Fibromodulin, survivin, and KW-13 are known TAAs in CLL.<sup>2,3,5</sup> MDM2 and p53 have recently been shown to serve as general TAAs in



**Figure 1. Proliferation of autologous T cells on stimulation with CLL cells in the presence of IL-2 and IL-7.** Shown are T-cell numbers ( $\times 100\,000$ ) after 0, 14, and 28 days of in vitro culture using CD40L-transduced (CD40L-AAV), GFP-transduced (GFP-AAV), or CD40-stimulated native CLL cells (CD40/nat.CLL) as APCs without antigenic peptide pulsing.

**Table 1. Higher immunogenicity of CD40L-transduced CLL cells compared with CD40-stimulated CLL cells (CD40-CLL) shown by IFN- $\gamma$  ELISPOT assays**

Sample, antigen specificity as tested in ELISPOT assay, and type of stimulation for T-cell expansion	ELISPOT, d35
<b>CLL-1</b>	
MDM2	
CD40L-AAV	8065
GFP-AAV	0
CD40-CLL	0
p53	
CD40L-AAV	534
GFP-AAV	0
CD40-CLL	0
<b>CLL-2</b>	
Survivin	
CD40L-AAV	3137
GFP-AAV	1646
CD40-CLL	1455
KW-13	
CD40L-AAV	4750
GFP-AAV	984
CD40-CLL	616
Fibromodulin	
CD40L-AAV	4287
GFP-AAV	859
CD40-CLL	0
<b>CLL-3</b>	
MDM2	
CD40L-AAV	2280
GFP-AAV	680
Native CLL	840
Fibromodulin	
CD40L-AAV	1080
GFP-AAV	220
Native CLL	260
p53	
CD40L-AAV	479
GFP-AAV	320
Native CLL	120
<b>CLL-4</b>	
MDM2	
CD40L-AAV	1800
GFP-AAV	485
Native CLL	0
Fibromodulin	
CD40L-AAV	2920
GFP-AAV	730
Native CLL	210
<b>CLL-5</b>	
MDM2	
CD40L-AAV	198
GFP-AAV	110
Native CLL	0

T cells from patients with CLL were expanded using autologous CD40L-transduced (CD40L-AAV), GFP-transduced (GFP-AAV), CD40-stimulated (CD40-CLL), and native CLL cells as APCs. IFN- $\gamma$  ELISPOT assays were performed on day 35 (d35). Given are the numbers of TAA-specific spots per 100 000 T cells. APCs in the IFN- $\gamma$  ELISPOT assay corresponded to the APCs used for the stimulation of T cells.

several types of malignancies, including leukemias.<sup>13-15,21-23</sup> We wanted to test whether CD40L-transduced CLL cells are, in fact, better APCs than CD40-stimulated (CD40-CLL) or native CLL cells in terms of a higher specific recognition by autologous T cells. We generated antigen-specific T lymphocytes from 5 patients with CLL using different kinds of autologous APCs. A significantly

higher number of T cells could be expanded using CD40L-transduced CLL cells as APCs compared with the control group consisting of GFP-transduced, CD40-CLL, or native CLL cells (Mann Whitney *U* test: *z* score,  $-2.593$ ;  $P = .008$ ). Starting with  $3 \times 10^5$  T cells, we obtained a median number of  $16.2 \times 10^5$  (range,  $9.5\text{--}33.9 \times 10^5$ ) T cells with CD40L-transduced CLL cells as APCs after 28 days of *in vitro* culture. Using GFP-transduced CLL cells as APCs, we obtained between  $3.9$  and  $20.5 \times 10^5$  T cells (median,  $4.8 \times 10^5$  T cells), and with CD40-CLL or native CLL cells as APCs, we generated between  $3.0$  and  $11.7 \times 10^5$  T cells (median,  $7.9 \times 10^5$  T cells) after 4 weeks of *in vitro* culture (Figure 1). Notably, the rate of apoptotic CLL cells within these weekly restimulation assays was not different using different APCs (CD40L or GFP-transduced or CD40-CLL/native CLL cells; Kruskal-Wallis test:  $\chi^2$ ,  $0.642$ ;  $P = .725$ ) arguing that differences in T-cell proliferation are induced by different stimulatory capacities of APCs.

After 14 and 35 days of *in vitro* culture, IFN- $\gamma$  ELISPOT assays were performed. We used the same kind of APCs as stimulator cells that we used for T-cell cultivation. In the assay, CLL cells were pulsed with different antigenic peptides derived from fibromodulin, survivin, KW-13, MDM2, and p53, respectively, to define the number of T cells that specifically recognize autologous CLL cells. After 14 days of *in vitro* culture with CD40L-transduced CLL cells, we detected 90 to 916 spots (median, 456 spots) of 100 000 T cells that were antigen specific. With APCs of the control group, we counted 0 to 584 spots (median, 258 spots) of 100 000 T cells, which was not different from results obtained with CD40L-transduced CLL cells (Mann-Whitney *U* test: *z* score,  $-1.339$ ;  $P = .194$ ).

After 35 days of *in vitro* culture with CD40L-AAV transduced CLL cells, we detected 198 to 8065 spots (median, 2280 spots) of 100 000 T cells that were antigen specific. With APCs of the control group, we determined only 0 to 1646 spots (median, 240 spots) of 100 000 T cells (GFP-AAV-transduced CLL cells: median, 485 spots [range, 0-1646 spots] of 100 000 T cells) (CD40-stimulated/native CLL cells: median, 120 spots [0-1455 spots] of 100 000 T cells). Taken together, after 35 days of *in vitro* culture, a significantly higher number of antigen-specific T cells was obtained using CD40L-transduced CLL cells as APCs compared with APCs from the control group, which is shown for different TAAs in CLL (Mann-Whitney *U* test: *z* score,  $-3.415$ ;  $P < .001$ ) (Table 1).

To test the antigen specificity, blocking experiments were performed with an MHC class I-specific monoclonal antibody (W6/32) in 4 of 5 patients in both ELISPOT assays at day 14 and day 35. Adding the antibody resulted in a significant decrease in the number of spots detected (Wilcoxon test: day 14, *z* score,  $-2.666$ ;  $P = .008$ ; day 35, *z* score,  $-2.934$ ;  $P = .003$ ) (data not shown).

By using CD40L-transduced CLL cells as APCs, we observed a significant increase in the number of specific T cells detected in the ELISPOT assay at day 35 compared with day 14 (Wilcoxon test: *z* score,  $-2.666$ ;  $P = .008$ ), whereas APCs of the control group showed no significant increase (Wilcoxon test: *z* score,  $-1.799$ ;  $P = .072$ ). From previous studies we know that a certain threshold number of APCs must be reached to successfully expand autologous T cells in this experimental setting.<sup>2</sup> For T-cell priming, an APC/T cell ratio of 7:1 was sufficient, which could then be decreased to a ratio of 1:1 in further T-cell stimulations. Here, in the described experiments, we used approximately half the number of APCs needed for adequate T-cell stimulation. Therefore, in most expansions using APCs of the control group, the critical threshold of activation was not reached and autologous T cells could not be further expanded later than day 21. In contrast, probably because of the activation of bystander CLL cells, the smaller number of APCs at the time of restimulation in the group with CD40L-transduced CLL cells was sufficient for T-cell expansion. We and other investigators could show that CD40L transduction of CLL cells has the unique potential to activate native bystander CLL cells regarding the expression of adhesion and costimulatory molecules, thus enhancing immunostimulatory effects, compared with CD40-activated CLL cells.<sup>8,10</sup> In our view, this is at least one of the crucial processes that also results in a more potent antigen-specific T-cell response using CD40L-transduced CLL cells compared with CD40-CLL cells. In summary, we show that CD40L-transduced CLL cells are highly immunogenic and are able to induce an antigen-specific immune response superior to that of CD40-CLL cells and unstimulated native CLL cells. Besides a model system based on CD95-mediated apoptosis, we provide an antigen-specific, T cell-based explanation for the possible mode of action of a CD40L-based vaccine *in vivo*.<sup>24</sup>

## References

- Wierda G, Cantwell MJ, Woods SJ, Rassenti LZ, Prussak CE, Kipps TJ. CD40-ligand (CD154) gene therapy for chronic lymphocytic leukemia. *Blood*. 2000;96:2917-2924.
- Mayr C, Bund D, Schlee M, et al. Fibromodulin as a novel tumor-associated antigen (TAA) in chronic lymphocytic leukemia (CLL), which allows expansion of specific CD8+ autologous T lymphocytes. *Blood*. 2005;105:1566-1573.
- Schmidt SM, Schag K, Muller MR, et al. Survivin is a shared tumor-associated antigen expressed in a broad variety of malignancies and recognized by specific cytotoxic T cells. *Blood*. 2003;102:571-576.
- Trojan A, Schultze JL, Witzens M, et al. Immunoglobulin framework-derived peptides function as cytotoxic T-cell epitopes commonly expressed in B-cell malignancies. *Nat Med*. 2000;6:667-672.
- Krackhardt AM, Witzens M, Harig S, et al. Identification of tumor associated antigens in chronic lymphocytic leukemia by SEREX. *Blood*. 2002;100:2123-2131.
- Van den Hove LE, Van Gool SW, Vandenberghe P, et al. CD40 triggering of chronic lymphocytic leukemia B cells results in efficient alloantigen presentation and cytotoxic T lymphocyte induction by up-regulation of CD80 and CD86 costimulatory molecules. *Leukemia*. 1997;11:572-580.
- Schultze JL, Michalak S, Seamon MJ, et al. CD40-activated human B cells: an alternative source of highly efficient antigen presenting cells to generate autologous antigen-specific T cells for adoptive immunotherapy. *J Clin Invest*. 1997;100:2757-2765.
- Kato K, Cantwell MJ, Sharma S, Kipps TJ. Gene transfer of CD40-ligand induces autologous immune recognition of chronic lymphocytic leukemia B cells. *J Clin Invest*. 1998;101:1133-1141.
- Cantwell M, Hua T, Pappas J, Kipps TJ. Acquired CD40-ligand deficiency in chronic lymphocytic leukemia. *Nat Med*. 1997;3:984-989.
- Wendtner CM, Kofler DM, Theiss HD, et al. Efficient gene transfer of CD40 ligand into primary B-CLL cells using recombinant adeno-associated virus (rAAV) vectors. *Blood*. 2002;100:1655-1661.
- Cheson B, Bennett JM, Grever M, et al. National Cancer Institute-sponsored Working Group guidelines for chronic lymphocytic leukemia: revised guidelines for diagnosis and treatment. *Blood*. 1996;87:4990-4997.
- Stanislowski T, Voss RH, Lotz C, et al. Circumventing tolerance to a human MDM2-derived tumor antigen by TCR gene transfer. *Nat Immunol*. 2001;2:962-970.
- Barfoed AM, Petersen TR, Kirkin AF, Thor Straten P, Claesson MH, Zeuthen J. Cytotoxic T-lymphocytes clones, established by stimulation with the HLA-A2 binding p53<sub>65-73</sub> wild type peptide loaded on dendritic cells *in vitro*, specifically recognize and lyse HLA-A2 tumour cells overexpressing the p53 protein. *Scand J Immunol*. 2000;51:128-133.
- Houbiers JGA, Nijman HW, von der Burg SH, et al. *In vitro* induction of human cytotoxic T lymphocyte responses against peptides of mutant and wild-type p53. *Eur J Immunol*. 1993;23:2072-2077.
- Wurtzen PA, Claesson MH. A HLA-A2 restricted human CTL line recognizes a novel tumor cell

- expressed p53 epitope. *Int J Cancer*. 2002;99:568-572.
16. Theobald M, Biggs J, Dittmer D, Levine AJ, Sherman LA. Targeting p53 as a general tumor antigen. *Proc Natl Acad Sci U S A*. 1995;92:11993-11997.
  17. Andersen MH, Pedersen LO, Becker JC, Straten PT. Identification of a cytotoxic T lymphocyte response to the apoptosis inhibitor protein survivin in cancer patients. *Cancer Res*. 2001;61:869-872.
  18. Andersen MH, Pedersen LO, Capeller B, Brocker EB, Becker JC, Straten P. Spontaneous cytotoxic T-cell responses against survivin-derived MHC class I-restricted T-cell epitopes *in situ* as well as *ex vivo* in cancer patients. *Cancer Res*. 2001;61:5964-5968.
  19. Garrone P, Neidhardt EM, Garcia E, Galibert L, von Kooten C, Banchereau J. Fas ligation induces apoptosis of CD40-activated human B lymphocytes. *J Exp Med*. 1995;182:1265-1273.
  20. Tan LC, Gudgeon N, Annels NE, et al. A re-evaluation of the frequency of CD8+ T cells specific for EBV in healthy virus carriers. *J Immunol*. 1999;162:1827-1835.
  21. Bendle GM, Holler A, Pang LK, et al. Induction of unresponsiveness limits tumor protection by adoptively transferred MDM2-specific cytotoxic T lymphocytes. *Cancer Res*. 2004;64:8052-8056.
  22. Soruri A, Fayyazi A, Gangl S, Griesinger C, Albrecht CA, Schlott T. In vitro generation of cytolytic T cells against human melanoma cells overexpressing HDM2. *Anticancer Res*. 2004;24:2141-2151.
  23. Chikamatsu K, Albers A, Stanson J, et al. P53(110-124)-specific human CD4+ T-helper cells enhance in vitro generation and antitumor function of tumor-reactive CD8+ T cells. *Cancer Res*. 2003;63:3675-3681.
  24. Chu P, Deforce D, Pedersen IM, et al. Latent sensitivity to Fas-mediated apoptosis after CD40 ligation may explain activity of CD154 gene therapy in chronic lymphocytic leukemia. *Proc Natl Acad Sci U S A*. 2002;99:3854-3859.