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Redirection of CMV specific CTL towards CLL via CD20 targeted HLA/CMV complexes

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Abstract

Chronic lymphocytic leukemia (CLL) is a slowly progressing malignancy of CD5+ B cells, for which at present no curative treatment is available. In our current study, we apply a novel bridging reagent to redirect cytomegalovirus (CMV) specific cytotoxic T lymphocytes (CTL) to target CLL. A streptavidin fused anti-CD20 single chain variable fragment (scFv) is used in combination with biotinylated MHC class I molecules containing CMV pp65 peptide (HLA/CMV). We demonstrate that CLL cells coated with this CD20-HLA/CMV complex can be lysed by autologous CMV specific CTL with similar efficiency as CLL cells directly loaded with CMV-peptide. Killing is HLA restricted and occurs at scFv CD20 concentrations of ≥100 ng ml⁻¹ and HLA/CMV concentrations of ≥20 ng ml⁻¹. Furthermore, complex coated CLL cells induce both proliferation and cytokine production (interferon γ, tumor necrosis factor α, and macrophage inflammatory protein-1 β) in CMV specific CD8+ T cells. Hereby, a necessary step towards possible application of CD20-HLA/CMV complexes for immunotherapy of B cell malignancies is constituted.
Chapter 4

Introduction

At present there is no curative treatment for B cell chronic lymphocytic leukemia (CLL). The slow progression and rather long median survival make CLL an attractive target for immunotherapy. For adequate T cell mediated cancer immunotherapy it is necessary to induce activation and differentiation of tumor-specific T cells. However, in CLL (like in many other types of cancer) this is severely hampered by the poor antigen presenting capacity of the malignant B cells, which express low levels of co-stimulatory molecules and therefore escape immune surveillance. Furthermore, there is evidence that CLL is accompanied by T cell dysfunction(8;26). Nevertheless, CD40 stimulation of CLL cells was found to enhance costimulatory capacity of the tumor cells(31) and it has been demonstrated that not only allogenic(1;10) but also autologous(12) CLL directed T cells can be generated in vitro. Moreover, in a phase I clinical trial autologous, ex vivo CD40L transduced CLL cells induced a reduction of the tumor mass upon reinfusion(29). Although the specific antigen remains elusive, these studies show that active immunisation against CLL is possible.

A disadvantage however of the discussed strategies is the need for complex ex vivo treatment of tumor cells.

We have previously shown that in patients with CLL considerably expanded numbers of cytomegalovirus (CMV) reactive CD8+ T cells are present(15). CMV specific CD8+ T cells in a latent stage of the infection possess an effector phenotype, with high contents of perforin and granzymes(5). This contrasts with the CD8+ T cell pool against viruses which are cleared by the immune system like influenza and Respiratory Syncytial Virus (9). Recently we have demonstrated that these CMV-specific CD8+ cells are potent cytotoxic effector cells when directed against CLL cells loaded with CMV peptide(11). Importantly, these CD8+ cells do not require ex vivo (re)stimulation but display their cytotoxicity directly after isolation from peripheral blood(11). These data demonstrate the potential to redirect autologous CMV specific CTL towards CLL cells for cancer immunotherapy.

To develop this approach towards applications in vivo we investigated the possibility to deliver CMV peptides specifically to CLL cells via antibody-targeted HLA class I/peptide complexes in an in vitro study. We have used a targeted complex (TC) consisting of a streptavidin (SA) fused anti-human CD20 single chain variable fragment (B9E9 scFv) coupled to CMV-peptide loaded biotinylated HLA class I(25).
We compared CLL coated with these TC with CLL directly loaded with CMV peptide with respect to efficacy and specificity of autologous T cell mediated killing. Moreover, we analysed their capacity to induce proliferation and cytokine production by CMV-specific T cells.

**Methods**

**Patient samples**

After obtaining informed consent, 30 ml of blood was drawn from patients fulfilling the diagnostic criteria for CLL (18). All patients had Rai clinical stage 0-II and had not received prior treatment. Peripheral blood mononuclear cells (PBMC) obtained via density gradient centrifugation were directly frozen in fetal calf serum (FCS) containing 10% DMSO (Sigma Chemical Co.) and stored in liquid nitrogen before use (15). CLL PMBC fractions contained >90% of CD5+/CD19+ B cells as assessed by flowcytometry. On the day of use, cells were thawed and cultured in Iscove’s modified essential medium (IMDM) supplemented with 10% heat-inactivated FCS, 100 U ml⁻¹ penicillin and 100 mg ml⁻¹ streptomycin. B cells were obtained from a PBMC fraction of a healthy donor by depletion of T cells, monocytes and macrophages (13) using anti-CD3, anti-CD14 and anti-CD16 immunomagnetic beads and a magnetic particle concentrator (both Dynal A.S., Oslo, Norway).

**Reagents**

HLA-A2–binding NLVPMVATV CMVpp65 peptide and HLA-B7–binding TPRVTGGGAM CMVpp65 peptide (IHB-LUMC peptide synthesis library facility, Leiden, The Netherlands) were used. The B9E9 scFvSA fusion protein, consisting of the variable region of murine IgG2a anti-human-CD20 fused to streptavidin (scFv CD20) has been described before (25) and was used in combination with biotinylated human MHC class I molecules containing CMVpp65 peptide (HLA/CMV). In these experiments, CMVpp65 peptide containing MHC class I molecules HLA-A*0201/NLVPMVATV (HLA-A2) and HLA-B*0702/TPRVTGGGAM (HLA-B7), generated by ProImmune (Oxford, UK), were used. Complexes, formed by scFvSA and HLA/CMV, are hereafter referred to as targeted constructs (TC).
**Chapter 4**

**Expansion of virus-specific autologous cytotoxic T-lymphocytes (CTL)**

Thawed PBMC (>90% CLL cells) from CMV seropositive CLL patients were used for expansion of CMV specific CTL as described before (11). Briefly, PBMC (at a final concentration of $5 \times 10^6$ cells ml$^{-1}$) were stimulated with CMVpp65 peptide (1.25 μg ml$^{-1}$) loaded CLL cells and IL-2 (50 U ml$^{-1}$, Biotest Ag, Dreieich, Germany). After one week, cells were restimulated on a weekly basis with CMV peptide loaded irradiated (30 Gy) EBV transformed cell-lines expressing either HLA-A2 or HLA-B7 (5x10$^4$ cells ml$^{-1}$) in the presence of IL-2. Percentage of CMV specific CTL in culture was determined by FACS analysis staining cell culture samples with CD8-PE (Becton Dickinson, San Jose, CA) and APC-conjugated A2 or B7 CMV tetramers (Sanquin, Amsterdam, the Netherlands).

**Cytotoxicity assay**

CTL activity was measured in a standard $^{51}$Cr release assay (28). As targets, freshly thawed CLL cells and B cells were used. Target cells were labelled with 50 μl $^{51}$Cr (30 μg ml$^{-1}$, 500 μCi mg$^{-1}$, Amersham, Buckinghamshire, England). After washing, cells were incubated at 37°C either with 0.1 μg ml$^{-1}$ CMVpp65 for 1 hour or with scFv CD20 (50 μg ml$^{-1}$) for 30 min and, subsequently, with HLA/CMV (at various concentrations) for 20 min. Autologous CMV-specific effector cells (obtained as described above) were incubated with 3000 $^{51}$Cr-labeled target cells at a 4:1 effector:target ratio. After 4 hours, released radioactivity was measured and specific lysis was calculated according to the following formula: percentage of specific release = ((experimental release - spontaneous release)/(maximum release - spontaneous release)) x 100 %. Results are presented as specific lysis and represent the median of specific lysis in 6 replicate samples ± SEM.

**In vitro stability assay**

HLA class I negative Daudi cells were targeted with TC as described before (23). Briefly, Daudi cells were incubated with scFv CD20 at 50 μg ml$^{-1}$ for 30 min and, after washing, with HLA/CMV complexes at 2.5 μg ml$^{-1}$ for another 20 min. After labelling, cells were washed and resuspended in medium and grown at 37°C in a 5% CO$_2$ containing atmosphere. At various time points, samples of cells were taken, washed, stained with a FITC labelled anti-HLA class I antibody (PharMingen, San Diego, CA) and analysed by flowcytometry. Furthermore, HLA-A2 negative CLL cells were targeted with HLA-A2 TC.
CLL cells were incubated with scFv CD20 at 50 μg ml⁻¹ for 30 min and, after a washing step, with HLA/CMV complexes at 2.5 μg ml⁻¹ for another 20 min. After labelling, cells were washed and resuspended in medium and grown at 37°C in a 5% CO₂ containing atmosphere. Immediately after labelling and after 24 hours, samples were taken, washed, stained with a FITC labelled anti-HLA-A2 antibody (PharMingen, San Diego, CA) and analysed by flowcytometry. Furthermore, TC coated CLL (t=24h) and non-treated CLL cells from the same donor (t=24h) were used as targets in a standard ⁵¹Cr release assay with CMV specific HLA-A2 CTL as effector cells at a 1:1 effector:target ratio.

**TC induced proliferation of CMV specific cytotoxic T-lymphocytes**

Freshly thawed PBMCs from CMV seropositive CLL patients were labelled with CFSE (Molecular Probes, Eugene, OR). After washing, cells were incubated with scFv CD20 (50 μg ml⁻¹) and, subsequently, with HLA/CMV (500 ng ml⁻¹). The labelled cells were then cultured in medium at 37°C. On day 7, samples were taken from the cell cultures and stained with CD8 PE and APC-conjugated CMV tetramers.

**Cytokine production assays**

CMV specific effector cells were stimulated for 6 h at 37°C with TC coated CLL(3;19). All stimulations were performed in medium containing 4 μg ml⁻¹ anti-CD28 (15E8) and 2 μg ml⁻¹ anti-CD29 (TS2/16). For the last 5 h of culture, brefeldin A (Sigma-Aldrich) was added in a final concentration of 10 μg ml⁻¹. After culture, the cells were fixed in FACS lysing solution (BD Biosciences) and permeabilized (BD Biosciences). On those cells, an intercellular staining was performed using anti-IFN-γ-FITC, anti-TNF-a-FITC and anti-MIP-1β-PE (all from BD Biosciences). To discriminate CTL from CLL, anti-CD8 PerCP Cy5.5 (BD Biosciences) was used, whereas anti-C69 APC (BD Biosciences) staining was applied to visualize activated cells.

In order to analyse the cytotoxic capacity of cytokine producing CMV specific CD8 cells, CMV specific effector cells were stimulated with TC coated or CMVpp65 treated CLL cells as described above. To isolate the IFN-γ secreting cells, they were labelled using an IFN-γ capture assay as described before(2). Briefly, the cells were washed in ice cold buffer (phosphate buffered saline containing 0.5% BSA and 2 mM EDTA) and resuspended in ice cold medium. Next, IFN-γ capture reagent was added (Miltenyi Biotech, Bergisch Gladbach, Germany) and the cells were put on ice for 5 minutes. Then warm culture
medium was added and the cells were incubated at 37°C for 45 minutes under slow continuous rotation. After incubation, ice cold buffer was added and the cells were spun down. Subsequently, the pellet was resuspended in ice cold buffer. PE-conjugated IFN-γ detection antibody (Miltenyi Biotech) and FITC-conjugated antibodies against CD4, CD14, CD16 and CD19 (all BD Biosciences) were added and the cells were incubated on ice for another 10 minutes. After washing with ice cold buffer, the cells were resuspended in RPMI containing 5% FCS. CD4/14/16/19 negative IFN-γ positive cells were sorted by flowcytometry (FACS Aria, BD Biosciences) and collected. The sorted cells were cultured for 4 days (in the presence of IL-2) and subsequently used as effector cells in a standard chromium release assay at a 1:1 effector:target ratio.

Results

Lysis of HLA/CMV targeted CLL by CMV specific CTL

We tested the efficacy of in vitro expanded CMV specific CTL in a standard 4h chromium release assay against autologous CLL cells either loaded with CMVpp65 or targeted with TC formed by scFv CD20 and HLA/CMV. Using a 50 μg ml⁻¹ concentration of scFv CD20 and varying concentrations of HLA/CMV, targeted CLL cells were lysed. When diluting the HLA/CMV reagent, lysis of targeted CLL became suboptimal at concentrations ≥ 4 ng ml⁻¹ (figure 1A), whereas lysis of CMVpp65 loaded CLL was not efficient at concentrations ≥ 100 pg ml⁻¹ (data not shown). In view of the molecular weight of both constructs (HLA/CMV Mw=45kDa; CMVpp65 Mw=1kDa) at a molar base 100 pg ml⁻¹ of CMVpp65 is comparable to 4.5 ng ml⁻¹ HLA/CMV. Thus, lysis of TC targeted CLL appears as efficient as lysis of CLL presenting CMVpp65 via autologous HLA class I molecules. Pre-incubation of the effector cells with HLA/CMV molecules did not inhibit lysis of TC coated CLL (data not shown). Moreover, pre-incubation of the effector cells with complete (pre-coupled) TC induced efficient lysis of uncoated CLL (data not shown), indicating that free HLA/CMV molecules or TC do not interfere with cytotoxic T cell function. Next, scFv CD20 concentrations were varied (using a constant HLA/CMV concentration of 100 ng ml⁻¹). Lysis was limited below concentrations of 100 ng ml⁻¹ (figure 1B). Molecular weights of scFv CD20 and HLA/CMV are equal (=45kDa). scFv CD20 can bind a maximum number of 4 biotinylated HLA molecules thereby forming tetramers. Since lysis remains efficient at equal
Figure 1. CTL mediated lysis of CMV loaded CLL.

CLL cells either directly loaded with CMVpp65 or coated with CD20-HLA/CMV (TC) were used as targets for ex vivo expanded CMV specific CTL in a standard chromium release assay (E:T ratio = 4:1). In A, lysis of CMVpp65 (100 μg ml\(^{-1}\)) loaded CLL (HLA-A2) is compared to CLL coated with a fixed concentration of scFv CD20 (50 μg ml\(^{-1}\)) and varying concentrations of HLA/CMV. Results shown are the mean ± SEM of five independent experiments.

B The effect of dilution of scFv CD20 on CTL mediated lysis of TC coated CLL cells. In all conditions, the concentration of HLA/CMV was 100 ng ml\(^{-1}\).

C CTL mediated lysis of TC coated CLL cells is HLA-restricted and antigen specific. HLA-B7 CMV specific CTL were used as effector cells. Target cells were either CLL cells from a HLA-B7 donor coated with B7-TC, or CLL cells from a HLA-A2 donor coated with either A2-TC or B7-TC ([scFv CD20] = 50 μg ml\(^{-1}\); [HLA/CMV] = 500 ng ml\(^{-1}\)). The right hand bar represents activity of non-CMV specific autologous T cells against TC coated CLL.
scFv CD20 and HLA/CMV concentrations (both 100 ng ml\(^{-1}\)) this suggests that, even in a monomer form, TC facilitate killing of the targeted cells. To test whether the TC behave as genuine peptide presenting MHC molecules, CLL cells from a HLA-A2 patient were coated with either A2-TC or B7-TC. B7-TC coated HLA-A2 CLL were lysed by CMV specific HLA-B7 CTL to a comparable extent as B7-TC coated autologous (HLA-B7) CLL. In contrast, A2-TC coated HLA-A2 CLL were unaffected by CMV specific HLA-B7 CTL (Figure 1 C). Importantly, autologous, non-CMV specific CD8+ T cells (containing only 1.76% CMV specific CTL) did not induce lysis of TC coated CLL (Figure 1C). Therefore, it can be concluded that there is a restriction to lysis determined by the HLA type of the TC and the antigen specificity of the CTL, indicating MHC/peptide-TCR interactions trigger CTL activity similar to autologous MHC molecules.

The effect of CD20 surface expression on TC induced lysis

CLL cells have a low surface expression of CD20 as compared to normal B cells(20). To test the effect of CD20 surface expression on susceptibility to

![Figure 2. The effect of CD20 expression on CTL mediated lysis of CD20-HLA/CMV coated B cells.](image)

CMV specific CTL were used as effector cells in a 4 hour chromium release assay. As targets (E:T ratio = 4:1) we used either CLL cells from a donor with (unusually) high CD20 surface expression (CD20high; MFI =538), “classical” CLL cells (CD20low; MFI=88) or normal B cells (MFI = 770). A fixed concentration of ScFvCD20 was used in combination with varying concentrations of HLA/CMV.
TC mediated lysis, CLL cells (CD20 expression: mean fluorescence intensity (MFI)=88), normal B cells (MFI=770) and CLL cells with (unusually) high CD20 surface expression (CD20high; MFI=538) were coated with TC using varying HLA/CMV concentrations. Only at HLA/CMV concentrations below 4 ng ml⁻¹, the lysis of TC coated CLL by CMV specific CTL was impaired, whereas the lysis of normal B cells and CD20high CLL remained unaffected (figure 2). Therefore, the tenfold lower CD20 expression on CLL does not prevent efficient lysis by TC.

![Figure 3. In vitro stability of the CD20-HLA/CMV targeted complex (TC).](image)

**A** HLA class I negative CD20 positive Daudi cells were coated with scFv CD20 (50 μg ml⁻¹) and subsequently with HLA-A2/CMV (2.5 μg ml⁻¹). The presence of the TC was detected with a FITC labelled anti-HLA class I antibody and analysed by flowcytometry, both immediately after labelling and 24 and 48 hours after labelling (iso = isotype control). **B** HLA-A2 negative CLL were coated with scFv CD20 (50 μg ml⁻¹) and subsequently with HLA-A2/CMV (2.5 μg ml⁻¹). 24 hours after labelling, the TC coated CLL cells were used as targets in a standard 4h chromium release assay (E:T ratio = 1:1).

**In vitro stability of TC**

To evaluate the stability of the cell bound TC in time, we targeted (HLA class I negative) Daudi cells with TC. The presence of the TC on the cells was detected using a broad reacting anti-HLA class I antibody and FACS analysis (figure 3A). TC appeared to be stable for at least 24 hours when targeted to Daudi cells.

To analyse the stability of TC on CLL cells, we targeted HLA-A2 negative CLL cells with HLA-A2 TC, and used these cells after 24 hours as targets in a standard chromium release assay. The presence of TC on the cells was detected...
with an anti-HLA-A2 antibody and FACS analysis. At 24 hours after targeting, the presence of the TC on CLL as detected by FACS analysis was severely reduced (data not shown). However, these cells were still lysed effectively by CMV specific HLA-A2 CTL (figure 3B). Thus, apparently 24 hours after binding of TC there still is a sufficient amount present on CLL to induce lysis.

TC induced proliferation and cytokine production in CMV specific CTL
In order to activate the immune system in vivo, CMV specific CTL must be capable of proliferation and production of cytokines upon antigen encounter. We tested both capacities in an in vitro setting. To track antigen specific proliferation, we labelled a PBMC fraction of a CLL patient which contained CMV specific CTL (0.32% of total lymphocytes) first with CFSE and, subsequently, with a combination of scFv CD20 (50 μg ml\(^{-1}\)) and HLA/CMV (500 ng ml\(^{-1}\)).

![Graph showing proliferation of CMV specific CTL](image)

**Figure 4. Proliferation of CMV specific CTL.**
Expansion of CMV specific CD8\(^+\) T cells was assessed after stimulation of PBMC with CLL cells coated with CD20-HLA/CMV TC or directly loaded with CMVpp65, all in the presence of IL-2 (50 U ml\(^{-1}\)). At day 7 the percentage of CMV specific CTL in the total lymphocyte population was determined by CD8 and CMV tetramer double staining. Before start of the assay, PBMC were labelled with CFSE to track cell division within the CMV specific CTL population (as depicted in the lower panel of the figure).
After labelling, the cells were incubated for 7 days in the presence of IL-2 (50 U ml⁻¹). Proliferation of CMV specific CTL was measured by analysing the percentage of CD8⁺/tetramer⁺ cells in the lymphocyte population. Within the CD8⁺/tetramer⁺ fraction, we measured the intensity of the CFSE signal (figure 4). Whereas non-CMV labelled PBMC showed no increase in CMV specific CTL, an increased percentage of CMV specific CTL was observed in the HLA/CMV labelled PMBC fraction (6.0% of total lymphocytes) which was accompanied by a decrease in CFSE signal intensity in most cells (indicating proliferation has occurred). Furthermore, we measured the production of several cytokines by CMV specific CD8 cells upon 6 hours of stimulation with TC coated CLL. TC coated CLL induced activation of CMV specific CD8 cells (as indicated by CD69 up-regulation) resulting in production of IFN-γ, TNF-α and MIP-1β (figure 5A). Next, we wanted to confirm that these cytokine producing cells are indeed the same cells that can induce lysis of those TC coated cells. To this end we stimulated CMV specific CTL with either TC or CMVpp65 coated CLL. Subsequently, we labelled IFN-γ producing cells with an IFN-γ detection kit and sorted them (figure 5B). Four days later, we tested the capacity of those cells to lyse CMVpp65 loaded CLL. We noticed that both TC and CMVpp65 pre-stimulated IFN-γ secreting cells were capable to induce lysis of both CMVpp65 loaded or TC coated CLL (figure 5C). Thus, the TC are capable of inducing immune activation as assessed by proliferation and cytokine production by CMV specific CTL.

Discussion

In the present study we have shown that CLL cells, targeted with complexes consisting of a streptavidin(SA) fused anti-human CD20 single chain variable fragment coupled to CMV-peptide loaded biotinylated HLA class I molecules (= targeted complexes: TC), can be lysed by autologous CMV specific CTL with similar efficiency as CLL cells directly loaded with CMV-peptide. Furthermore, we have demonstrated that TC coated CLL induce both proliferation and cytokine production (interferon γ, tumor necrosis factor α, and macrophage inflammatory protein-1β) in CMV specific CD8⁺ T cells. Most active immunotherapies require knowledge of the eliciting antigen and ex vivo manipulation of patient cells. The first aspect can be circumvented by
Figure 5. CD20-HLA/CMV TC induced production of cytokines by CMV specific CTL. A Ex vivo expanded autologous CMV specific CTL were incubated with CLL cells that were coated with CD20-HLA/CMV TC or (as a positive control) directly loaded with CMVpp65. The negative control (-) consisted of uncoated CLL cells. After 1 hour of incubation, brefeldin A was added to prevent excretion of produced cytokines into the medium. After 6h of stimulation, the production of IFN-γ, TNF-α and MIP-1β by the CMV specific CTL (selected by CD8 staining) was determined by intracellular staining. For counterstaining, CD69 was used as a marker for cell activation. B CMV specific CTL were incubated with TC or CMVpp65 coated CLL for 4 hours. Subsequently, IFN-γ secreting cells were labelled using an IFN-γ detection kit. Double staining with a combination of FITC labelled CD4/14/16/19. C CD4/14/16/19 negative and IFN-γ positive cells were selected and incubated for four days in the presence of IL-2. Subsequently these cells were used as effectors in a standard chromium release assay. In separate experiments (indicated by the interrupted line), either CMVpp65 loaded or TC coated CLL served as targets (E:T ratio = 1:1).
applying the resident anti-CMV immunity. The second aspect was addressed in previous research by Savage et al., where the powerful effector mechanisms of virus specific cytotoxic T cells were successfully linked with the specificity of monoclonal antibodies. In vitro experiments with CD20 expressing tumor cells and influenza or EBV derived viral peptides, showed that CD20-targeted HLA class I/peptide complexes were expressed on the cultured tumor cells, and that these targeted tumor cells could be lysed very efficiently by PBMC from healthy donors, in vitro prestimulated with viral antigen(23). In vivo studies with SCID mice xenografted with the human B cell lymphoma Daudi line showed adequate protection from tumor growth when HLA-A2/influenza peptide targeted lymphoma cells were inoculated together with influenza specific CTL(22). Whether these findings can be extrapolated to a clinical situation is highly dependent on the penetration of the reagent in tissues. This aspect was addressed by Lev et al., who demonstrated the efficacy of a scFv (anti-CD25 or anti-mesothelin) / HLA-A2 chimerical fusion protein in nude mice bearing pre-established human tumor xenografts (14). It could be argued that one step targeting by such a (~65 kDa) reagent enables higher tissue penetration compared to the two step approach described here. However, in a recent phase I study in B–cell non-Hodgkin lymphoma patients, the streptavidin CD20 scFv fragment in combination with biotinylated 99Yttrium showed adequate penetration in lymph nodes, subcutaneous localisations, joints and even testes (4). Since the molecular weight of biotinylated HLA/CMV is similar (~45 kDa) it is expected that the penetration of HLA/CMV will be comparable. Although a one step approach avoids the exposure of (potentially immunogenic) streptavidin, a two-step labelling procedure enables higher flexibility for patients with different HLA types.

Our data on T cell proliferation and cytokine production induced by TC coated CLL are in line with a recent study showing that human immunodeficiency virus (HIV) and Karposi sarcoma antigen containing HLA complexes induce expansion and activation of autologous HIV and Karposi sarcoma specific CTL (27). This emphasizes the potential of antibody targeted MHC complexes as a new approach for therapeutic vaccination. In our study intracellular staining revealed antigen triggered production of IFN-γ, TNF-α and MIP-1β in CD8 positive T cells. IFN-γ is known to recruit T cells, activate macrophages and to induce upregulation of MHC class I and II(24). TNF-α also activates macrophages(21). Finally, MIP-1β is a cytokine which is thought to be involved in the activation of monocytes, macrophages and, importantly, den-
dritic cells (DC)(17). This activation of macrophages and DC may facilitate the uptake and presentation of tumor cell particles. *In vitro* it has been shown that offering apoptotic bodies of CLL cells to DC can induce autologous tumor specific CTL(12). Thus, in CLL the CD20-HLA/CMV complexes might be a double edged sword: direct induction of both T cell mediated killing and a secondary activation of CLL-specific T cell mediated immune response. CMV-specific CD8+ cells are excellent effector cells for possible use in clinical cellular immunotherapy studies. They are proven to be cytotoxic, not autoreactive and capable of homing in a broad variety of tissues. The approach is widely applicable because 70–90% of healthy adults are CMV positive. In our recent study 72% of CLL patients were found to be CMV-seropositive(15). It has to be noted that the latter figure might be a slight underestimation of the real proportion of CMV positive patients because hypogammaglobulinaemia is frequently present in (advanced) CLL.

Especially in CLL there are a number of advantages of the presented approach over the use of (radiolabeled) CD20 antibodies. Monotherapy with conventional doses of rituximab, a human/mouse chimeric CD20 monoclonal antibody, has only limited efficacy(16). This is probably due to the low expression of CD20 in CLL precluding optimal complement activation(6). Moreover, in contrast to many cell lines, freshly isolated CLL cells are resistant to CD20 mediated antibody dependent cellular cytotoxicity (ADCC) by NK cells, an important second effector mechanism *in vivo* of CD20 monoclonal antibodies(7). Radiolabeled CD20 antibodies have considerable side effects, notably myelosuppression and increased risk for secondary myelodysplasia or acute myeloid leukaemia(30).

However, unlike ADCC or complement dependent cytotoxicity, T cell activation and killing requires only a few TCR to drive the process. Notably virus specific CTL require only minute amounts of peptide presented on target cells. The potency of CMV specific CTL is emphasised by the findings that the tenfold lower CD20 surface expression on CLL compared to normal B cells hardly seems to affect the lysis of CLL (Figure 2).

The findings of our study constitute a necessary step towards possible application of CD20-HLA/CMV complexes for immunotherapy of B cell malignancies. The next step will be to test the CD20-HLA/CMV targeting complexes *in vivo* in a combined mouse B cell tumor/CMV model. It is obvious that this recently recognized capacity to redirect existing antiviral immunity towards tumor cells has a utility in cancer immunotherapy far beyond CMV and CLL.
Acknowledgments

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Reference List


Chapter 4


Novel bridging reagent for active immunotherapy in CLL


