Adequate synapse formation between leukemic B cells and effector T cells following stimulation with artificial TCR ligands

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Artificial T cell receptor (TCR) ligands can be used to direct virus specific cytotoxic T lymphocytes (CTL) towards tumor cells. Because of their size, these constructs may differ from cognate peptides in their ability to induce T cell activation. We here analyzed signaling outcomes upon synapse formation between human cytomegalovirus (CMV)-specific CTL and chronic lymphocytic leukemia (CLL) cells through targeted complexes (TC) containing anti-CD20 single-chain variable fragment and biotinylated major histocompatibility complex (MHC) class I molecules presenting peptides from CMVpp65. TC coated CLL cells were effectively lysed by CMVpp65-specific CTL but induced less interferon gamma (IFN-γ) production than peptide loaded targets. Confocal microscopy revealed that TC induced a redistribution of TCR/CD3 but not CD2 towards the immunological synapse. Furthermore, morphological examination of immunological synapses showed smaller and “patchy” interactions between TC coated B cells and CTL as compared to peptide coated targets. Finally, pre-incubation of CTL with CD2 antibodies led to a Fc-dependent redistribution of CD2 into TC-induced synapses and restored IFN-γ production by CMV-specific CTL.

Thus, redistribution of CD2 towards the immunological synapse appears to be essential for full T cell activation. However, CD2 triggering is not required for efficient lysis of tumor cells, demonstrating that CTL require only minimal stimulation to release their cytotoxic content.


Chapter 5

Introduction

Like in other types of cancer, non-responsiveness to chemotherapy due to dysfunction of p53 is a serious problem in the treatment of CLL (1;4;10). Therefore, an urgent need exists for therapies that do not rely on functional p53. One possible way to circumvent p53 dysfunction may be passive immunotherapy. In this respect, treatment of CLL with the CD52 monoclonal antibody alemtuzumab seems promising, since it showed to be effective against CLL with mutated p53 (32).

Besides the use of monoclonal antibodies, active immunotherapy for CLL is also the topic of investigation in the search for p53 independent therapeutic strategies. In this perspective, several groups have described techniques to elicit CLL specific T cell responses in vitro, either via dendritic cells transfected with CLL mRNA (24) or by enhancing immunogenicity of CLL cells via CD40 ligation (16;34;37). However, the induction of T cell responses against CLL cells in vivo may be hampered by immuno-tolerance towards the tumor. To solve this problem, we and others have attempted to exploit the powerful cytotoxic capacities of antiviral CTL by redirecting them to tumor cells. Previously, we have observed that CLL patients have an increased number of CTL directed against CMV without any signs of viral reactivation (22). Furthermore, we have demonstrated that the cytolytic capacity of these CTL can be used to lyse autologous CMV peptide-loaded CLL cells (18). Recent studies indicate that this strategy may also be applicable in vivo, since we could show that CLL cells were effectively lysed by CMV-specific CTL when targeted with targeted complexes (TC) consisting of MHC class I molecules containing CMVpp65 peptide and a CD20 single chain variable antibody fragment (23).

A potential danger of using a strong TCR agonist specific for expanded populations of effector T cells is the massive release of cytokines that can lead to the cytokine release syndrome (2;7;33). To evaluate the efficacy and potential risk of CTL activation induced by TC we compared T cell activation parameters induced by targets loaded with either CMVpp65 peptide or TC. Furthermore, the immunological synapse formation between CMV-specific CTL and either TC–coated or CMV-peptide-loaded CLL cells were compared as to the role of accessory molecules as well as to the dimensions of the synapse.
Methods

Patient samples
After obtaining informed consent, 30 ml of blood was drawn from patients fulfilling the diagnostic criteria for CLL (26). Freshly isolated PBMC were frozen in IMDM supplemented with 20% fetal calf serum (FCS) and 10% DMSO (Sigma Chemical Co.) and stored in liquid nitrogen before use. On the day of use, cells were thawed (viability after thawing > 90%) and cultured in RPMI 1640 containing 10% v/v heat-inactivated FCS, 100 U ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin. All procedures concerning recruitment of patients and collection of patient materials were approved by the Medical Ethical Committee of the Academic Medical Center.

Reagents
HLA-A2-binding NLVPMVATV CMVpp65 peptide and HLA-B7-binding TPRVTGGEAM 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) (29) was used to target CD20 positive cells with biotinylated human MHC class I molecules containing CMVpp65 peptide (HLA/CMV). In our current studies, CMVpp65 peptide containing MHC class I molecules HLA-A*0201/NLVPMVATV (HLA-A2) and HLA-B*0702/TPRVTGGEAM (HLA-B7), generated by ProImmune (Oxford, UK), were used. Complexes, formed by scFv CD20 and HLA/CMV are hereafter referred to as targeted constructs (TC).
Antibodies against CD2 (CLB-CD2.1, CLB-T11/1), LFA1 (CLB-54) and CD80 (DAL 1) were purified from hybridoma culture supernatants. F(ab’)₂ fragments from CLB-CD2.1 and CLB-T11/1 were produced by pepsin digestion. All antibodies were used at a final concentration of 10 μg ml⁻¹.

In vitro expansion of CMV specific cytotoxic T lymphocytes (CTL)
Thawed PBMC from three HLA-A2 or HLA-B7 CMV seropositive CLL patients (all Rai stage II and untreated) were used for expansion of CMV-specific CTL as described before (18). Briefly, PBMC (at a final concentration of 5-10x10⁶ cells ml⁻¹) were cultured in IMDM containing 10% v/v Human Pooled Serum (HPS; Cambrex, East Rutherford, NJ), CMVpp65 peptide (1.1 μM) and IL-2 (50 U ml⁻¹ Biotest Ag, Dreieich, Germany). After one week of culture, cells
were restimulated every 7 days with CMV peptide loaded irradiated (30 Gy) Epstein Barr virus (EBV) transformed B cell-lines expressing either HLA-A2 or HLA-B7 (5x10⁴ cells ml⁻¹) in the presence of IL-2. The 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 flow cytometry-based assay. As targets, freshly thawed CLL cells were used. Target cells were labeled with CellTrace Far Red DDAO-SE (Molecular Probes, Eugene, OR) to distinguish them from effector cells. After washing, the labeled CLL cells were incubated at 37°C either with CMVpp65 peptide for 1 hour or with scFv CD20 (1.1 μM) for 30 min and subsequently (after one washing step) with HLA/CMV for 20 min. Autologous CMV-specific effector cells (obtained as described above) were incubated with the labeled target cells at a 1:1 effector:target ratio. After 4 hours, target cell death was determined by 3,3′-dihexyloxacarbocyanine iodide (DiOC₆(3)) staining. Gating on DDAO-SE positive cells, cell death was measured by determining the percentage of DiOC₆(3)low cells within the target population. In some conditions, effector cells were pre-incubated for 2 hours with concanamycin A (100nM; Sigma-Aldrich) or target cells were pre-treated with CD95 blocking antibody (Fas2; 10 μg ml⁻¹) to determine the role of granule excretion and Fas ligation in target cell killing, respectively.

Calcium release assay
EBV transformed B cells were either loaded with CMVpp65 peptide (250 nM) or coated with TC (HLA/CMV concentration 250 nM). Next, these cells were allowed to adhere to the bottom of poly-L-lysine coated 96 wells flat bottom culture plates for 10 minutes at 37°C. Subsequently, CMV-specific CTL loaded with fluo-4 AM (Molecular Probes, Eugene, OR; final concentration 2μM) were added to the wells and the plates were inserted into a Wallac 1420 Victor₃ plate reader (PerkinElmer, Waltham, MA). Over time, calcium release was determined by measuring Fluo-4 AM fluorescence (Eₑₚ) with intervals of 10 seconds (excitation filter 488nm, emission filter 535nm). At the end of each assay, the maximal calcium signal (Eₘₚₓ) was determined by adding 0.1% Triton-X to each well and subsequently the zero signal (E₀) was determined by adding 4M EDTA to each well. The emission values were recalculated to per-
Cytokine production assays
CMV-specific effector cells were stimulated for 6 h at 37°C with CMVpp65 peptide loaded or TC coated CLL cells. All stimulations were performed in medium containing 4 μg ml⁻¹ anti-CD28 (CLB-CD28/1) and 2 μg ml⁻¹ anti-CD29 (TS2/16)(9;27). For the last 5 h of culture, brefeldin A (Sigma-Aldrich) was added in a final concentration of 10 μg ml⁻¹. After stimulation, the cells were fixed in FIX&PERM Solution A (An der Grub, Kaumberg, Austria) and permeabilised in Solution B (An der Grub) in the presence of anti-IFN-γ FITC, anti-CD69 PE, anti-CD3 PerCP Cy5.5 and anti-CD8 APC (all BD Biosciences). IFN-γ production and CD69 expression in CMV-specific CTL (gate on CD3⁺/CD8⁺ cells) were evaluated via flow cytometry.

Transmission electron microscopy
EBV transformed B cell lines were either loaded with CMVpp65 peptide (250 μM) or coated with TC (HLA/CMV concentration 250 μM). Next, the labeled EBV transformed B cells were added to CMV-specific CTL in a 96 wells round bottom plate. The plates were then centrifuged and incubated at 37°C/ 5% CO₂ for 10 minutes to enable conjugate formation. Subsequently, preparation for electron microscopy was performed as previously described(9). Briefly, upon fixation in Karnovsky’s fixative (0.1 M cacodylate buffer pH 7.4 containing 4% w/v formaldehyde and 2.5% w/v glutaraldehyde) samples were treated with 1% osmium tetroxide plus 0.5% uranyl acetate and embedded in Epon Resin (Hexion Specialty Chemicals, Hoogland, The Netherlands). Ultra-thin sections were stained with uranyl acetate and lead citrate, and subsequently examined under a Philips CM10 electron microscope; images were obtained and measured with iTEM software (Soft Imaging System GmbH). For determination of intermembrane separation distance, several conjugates were analyzed and > 100 measurements were performed (CMVpp65 peptide as well as TC) on tight junctions where both membranes had a trilaminar appearance.

Confocal fluorescence microscopy
Imaging of conjugates between target and effector cells was performed as previously described (20). Peptide and TC loading of EBV transformed B cells
was performed as for electron microscopy. During the last 15 minutes of loading, CMTMR Orange (0.5 μM; Molecular Probes, Eugene, OR) was added to discriminate target cells from effector cells. Next, loaded targets were incubated with CMV-specific CTL to induce conjugate formation. In some conditions, antibodies against CD2 (CLB-CD2.1) or CLB-CD2.1-derived F(ab’)2 fragments were added to the CTL prior to conjugate formation. Subsequently, the cells were gently resuspended and dropped onto poly-L-lysine coated glass coverslips. After 5 minutes, the coverslips were washed two times with phosphate-buffered saline (PBS) and subsequently the attached cells were fixed with 3% v/v paraformaldehyde. Cells were stained with CD3 FITC (Pharmingen, San Diego, CA) or FITC labeled CLB-CD2.1 antibody (CD2). Blocking antibodies and F(ab’)2 fragments against CD2 (CLB-CD2.1) were visualized using FITC labeled goat-anti-mouse F(ab’)2 fragments (Jackson ImmunoResearch Laboratories, West Grove, PA). Next, the coverslips were washed with PBS and briefly incubated in PBS with 0.1% w/v saponin (Calbiochem, San Diego, CA) and 3% v/v FCS. Finally the coverslips were embedded in Vectashield with DAPI (Vector Laboratories, Burlingame, CA). Imaging was performed with a Leica TCS-SP2 confocal microscope and Leica LCS software. Excitation/detection of the 3 fluorophores was performed as follows: FITC: 488nm/500-520nm, CMTMR orange: 594nm/600-630nm and DAPI 405nm/420-475nm. To avoid crosstalk between the fluorophores sequential line scan was used. In each microscopy slide, at least 30 conjugates between T cells and target cells were analyzed and the localization of CD3 and CD2 (randomly distributed or focused to the point of interaction between two cells) was assessed by eye in single plane scans. Additional Z-stack imaging was performed on most slides to confirm the position of CD3 and CD2 (focused or randomly distributed) observed in single plane scans.

Results

**TC induce submaximal cytokine production by CMV-specific CTL**

In previous studies we established that CD20-targeted HLA/CMVpp65 complexes (TC) were able to induce lysis of targeted CLL cells and to trigger cytokine production by autologous CMV-specific CTL (23). Here, we compared the limiting concentrations of CMV pp65 peptide and TC for lysis of CLL cells as well as target cell-induced cytokine production by CTL. In line with our
Figure 1. Both CMVpp65 peptide and TC coated CLL cells induce granule-mediated lysis and IFN-γ production by CMV specific CTL. CLL cells were coated with either CMVpp65 peptide or CD20 targeted HLA/CMVpp65 complexes (TC). Next, the limiting peptide concentration for lysis of coated CLL cells and IFN-γ production by CMV-specific CTL were determined (E:T ratio = 1:1). A The effect of peptide concentration on lysis of coated CLL cells. The efficacy of lysis is reflected as % of the maximal effect (maximal specific lysis for pp65: 45% and TC: 40%). The average of three independent experiments is shown. Error bars indicate SEM. B CLL cells were pre-incubated with a blocking antibody against CD95 (Fas2; 10 μg ml⁻¹) or CMV-specific CTL were pre-treated with concanamycin A (CMA; 100 ng ml⁻¹). Subsequently, lysis of either CMVpp65 peptide- or TC-coated CLL was determined (peptide concentration 250 nM). Results are from a single experiment; maximum specific lysis induced in this experiment was 47% for CMVpp65 peptide- and 39% for TC-coated CLL. Lysis of non-coated CLL cells by CMV specific CTL was <5% compared to control C The effect of peptide concentration on IFN-γ production by CMV-specific CTL triggered by CLL cells either coated with CMVpp65 peptide or TC. Mean of three independent experiments, error bars indicate SEM. D Activation (indicated by upregulation of CD69) of CMV-specific CTL by either CMVpp65 peptide or TC coated CLL. Mean of three independent experiments, error bars indicate SEM.
previous observations, CMVpp65 peptide and TC induced equally efficient lysis of targeted CLL cells. At concentrations above 1nM both CMVpp65 peptide and TC gave maximal lysis of coated CLL (45% vs 40%, respectively; figure 1A). To explore the mechanism(s) of cytotoxicity by both agonists, either CMV-specific CTL were pre-treated with concanamycin A or the target cells were pre-incubated with the CD95 blocking antibody Fas2. Subsequently, the effect on lysis of CMVpp65 or TC coated CLL cells was tested. As depicted in figure 1B, lysis of both CMVpp65 peptide and TC coated CLL could only be blocked by pre-treating CMV-specific CTL with concanamycin A, demonstrating that lysis of both CMVpp65- and TC-coated target cells is mediated by release of cytolytic granules by CMV-specific CTL.

A quantitative difference between peptide and TC became apparent when cytokine production capacity was tested. CMV-specific CTL were triggered to produce IFN-γ when stimulated by CLL cells loaded with CMVpp65 peptide and TC concentrations of 10nM and higher. IFN-γ production induced by TC reached a plateau at 250 nM, whereas a similar concentration of CMVpp65

Figure 2. CMVpp65 peptide and TC trigger calcium release in CMV specific CTL. CMV-specific CTL were loaded with fluo-4 AM (2μM) and subsequently incubated with plate-adhered EBV transformed B cells that were treated with either CMVpp65 peptide or TC (peptide concentration 250 nM). Over time, changes in free calcium signal of the total CTL population were measured with via fluorometry (A488nm/ E535nm). Non-CMV treated target cells (-) served as a control. A representative calcium release experiment. The results are presented as percentage of maximal experimental release (see materials and methods).

B Quantification of the changes in free calcium signal per minute (Δ % of maximal release min⁻¹) within the CMV specific CTL population during the first 10 minutes after incubation with target cells (average of five independent experiments; error bars indicate SEM).
peptide induced more IFN-γ production (6.55 ± 1.53 vs 16.36 ± 3.42 % (p=0.02) of CD8+ T cells, respectively; figure 1C). IFN-γ production induced by CMVpp65 peptide only reached a plateau at concentrations higher than 10 μM (data not shown). Notably, upregulation of early activation marker CD69 on CD8+ T cells was comparable after stimulation with either CMVpp65 peptide or TC coated CLL cells (figure 1D). Besides small inter-donor variance, no difference was observed between HLA-A2/CMV and HLA-B7/CMV induced CTL activity (data not shown).

**TC induce calcium release in CMV-specific CTL**

The release of calcium from intracellular storage compartments into the cytoplasm is one of the earliest signaling events upon TCR triggering (25). To investigate the potency of TC to mobilize Ca++, cumulative changes in cytoplasmatic calcium levels in populations of either CMVpp65 peptide or TC triggered CTL were measured (see materials and methods). Both CMVpp65 peptide and TC treated target cells induced a marked increase in free calcium levels in CMV-specific CTL populations compared to CTL populations that were exposed to non-CMV treated target cells (figure 2A). Notably, there was no quantitative difference observed between CMVpp65 peptide and TC induced calcium release (measured as Δ % of maximal release min⁻¹; figure 2B).

**CD2 is not fully triggered in TC stimulated CTL**

Next, a more detailed analysis of synapse formation around autologous MHC class I presenting CMVpp65 peptide or TC was performed. To this end, the lytic capacity of CMV-specific CTL was tested after pre-incubation with antibodies against CD2, CD80 and LFA1. Lysis of both CMVpp65 peptide and TC-coated CLL could only be inhibited by antibodies against LFA1 (figure 3A). Notably, in agreement with published data on influenza specific T cell clones (36), antibodies against CD2 or CD80 did not affect lysis by CMV-specific CTL.

Next, the influence of antibodies against CD2 and CD80 on IFN-γ production by CMV-specific CTL was tested. CLL cells were coated with CMVpp65 peptide or TC (peptide concentration 250 nM) and subsequently used as stimulus for CMV-specific CTL that were pre-incubated with blocking antibodies. Since CMV-specific CTL lack expression of CD28, antibodies against CD80, as expected, did not affect IFN-γ production (figure 3B). Furthermore, block-
Figure 3. The effect of antibodies against synapse molecules on CMVpp65 peptide or TC induced lysis (A) and IFN-γ production (B) by CMV specific CTL.

CLL cells were coated with either CMVpp65 peptide or TC (peptide concentration 250 nM) and incubated with CMV-specific CTL (E:T ratio = 1:1) that were pre-treated with antibodies against CD2 (CLB-CD2.1 and CLB-T11/1), CD80 or LFA1. The effect of these antibodies on A lysis (4h) and B IFN-γ production (4h) is reflected as relative response ([response in presence of antibody]/[response without antibody]). In the absence of antibodies, TC coated targets induced IFN-γ production in 3.00% of CD8+ T cells compared to 9.87% of CD8+ T cells triggered with CMVpp65 loaded targets. Specific lysis observed in the absence of blocking antibodies was 22% for CMVpp65 peptide- and 14% for TC-coated CLL cells. The bars in both graphs represent the average of three independent experiments ± SEM.

C FACS plots of IFN-γ production by CMV-specific CTL (gated on CD3+CD8+ cells). Plots are representative pictures for CTL triggered with non-coated CLL cells (no CMV), CMVpp65 peptide coated CLL cells (pp65) or TC coated CLL (TC), respectively. The lower plots show the effect of pre-incubation of the effector cells with an antibody against CD2 (CLB-CD2.1). The percentage of IFN-γ+/CD69+ cells is indicated in the upper right corner of each plot.
ing CD2 with CLB-T11/1 monoclonal antibody seemed to have an (nonsignificant) inhibitory effect on CMVpp65 peptide induced IFN-γ production by CMV-specific CTL. Surprisingly, both CD2 specific antibodies CLB-CD2.1 and CLB-T11/1 induced increased production of IFN-γ by TC stimulated CMV-specific CTL (from 3.00% to 7.01% CD8+/IFN-γ+ cells), approaching the level of CMVpp65 induced IFN-γ production (9.87% CD8+/IFN-γ+ cells). Not only the percentage of IFN-γ positive CD8+ T cells increased in the presence of anti-CD2 antibody (for example from 2.89% to 15.33 % in the experiment depicted in figure 3C), but the presence of CD2 antibodies also increased the amount of IFN-γ produced per cell. In contrast, when CMV-specific CTL were pre-incubated with F(ab′)2 fragments of CD2 antibodies CLB-T11/1 and CLB-CD2.1, IFN-γ production by both CMVpp65 peptide and TC stimulated CTL was strongly inhibited, suggesting that the stimulatory effect of the CD2 antibodies was Fc-mediated.

Transmission electron microscopy imaging of the immunological synapse
Previously, Choudhuri et al. demonstrated that by introducing MHC class I molecules with an elongated ectodomain on antigen presenting cells, subsequent T cell activation (measured by cytokine production) was diminished due to an increased intermembrane separation distance within the immunological synapse(9). Since the size of the TC exceed that of natural occurring MHC-I molecules (~90 kDa vs ~45 kDa), we questioned whether this would affect the dimensions of the immunological synapses induced by these constructs. To evaluate this, sections of embedded conjugates between CTL and either CMVpp65 peptide or TC coated EBV transformed B cell targets were made and the dimensions of the formed synapses were examined using transmission electron microscopy. In two independent experiments, the intermembrane separation distance within the tight junctions formed between target and effector cells and the length of the tight junctions were determined. By measuring intermembrane separation distances in tight junctions formed between either CMVpp65 peptide or TC coated targets and effector cells, some variance in intermembrane separation distance within both CMVpp65 peptide and TC induced synapses was observed. Nevertheless, there was no difference observed in mean intermembrane separation distance between CMVpp65 peptide (mean ± SEM: 8.91 ± 1.85 nm) and TC (8.71 ± 1.85 nm) induced synapses (two-tailed non-parametric T-test: p= 0.48, figure 4B). In a subsequent experiment, the length of tight junctions in conjugates between
Figure 4. Transmission electron microscopy imaging of CMVpp65 peptide and TC induced synapses. Conjugates between CMV-specific CTL and either CMVpp65 peptide or TC coated EBV transformed B cells were fixed and embedded in paraffin (see materials and methods). Subsequently, sections of the embedded conjugates were stained with uranyl acetate and lead citrate and analyzed using transmission electron microscopy.

**A** images of conjugates between CMV-specific CTL and CMVpp65 peptide (upper images) or TC (lower images) coated targets. On the left hand overview images are displayed (scale bar represents 2 μm), on the right hand zoomed images are shown (scale bar represents 0.5 μm). The tight junctions are indicated by the black arrows.

**B** intermembrane separation distance within tight junctions. Results of a single experiment are shown. Bars represent mean of all measurements, the error bars indicate SEM.

**C** Percentage of tight junctions with a length shorter or longer than 300nm. For both CMVpp65 peptide and TC, 16 conjugates were analyzed and within these conjugates, the length of individual tight junctions was measured (total number of tight junctions measured: 40 CMVpp65 peptide- and 45 TC-induced tight junctions).
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target and effector cells was measured. We observed a broad variance in length of tight junctions and also in the number of tight junctions per conjugate. It appeared as if synapses between EBV cells loaded with CMVpp65 peptide and CMV-specific CTL were formed by less but longer tight junctions than synapses between TC coated EBV cells and CMV-specific CTL. Thus, TC induced synapses had a “patchy” appearance compared to CMVpp65 peptide induced counterparts (figure 4A and C). However, despite visible qualitative differences in length between TC and CMVpp65 peptide induced tight junctions, this difference was not quantifiable in a statistically significant manner (two-tailed non-parametric T-test: p=0.24).

CD2 is not recruited to TC induced synapses

The results of the CTL activation experiments in the presence of blocking antibodies suggested incomplete CD2 triggering in TC induced T cell activation. Taken together with the observed tight junction morphology of TC induced synapses this raised the question if and where CD2 is located in TC-induced synapses compared to MHC-I/peptide induced synapses. To investigate this, conjugate formation between CMV-specific CTL and either CMVpp65 peptide or TC-coated target cells was allowed and subsequently the position of CD2 in synapses was visualized through confocal fluorescence microscopy. Conjugate formation between T cells and target cells was antigen specific, since no conjugate formation was observed when CMV-specific T cells were incubated with non-coated target cells (data not shown). Furthermore, in about 50% of all CMVpp65 peptide and TC-induced conjugates, CD3 was concentrated in the synapse between target and effector cell (figure 5). CD2 was also concentrated in CMVpp65 peptide induced synapses, albeit to a lower extent (29.7 ± 0.4 % of all conjugates). In contrast, in TC-induced synapses CD2 remained randomly distributed on the surface of CMV-specific CTL. This could be manipulated by incubating CMV-specific CTL with an antibody against CD2 (CLB-CD2.1) prior to conjugate formation. As a result, CD2 was now recruited towards TC-induced synapses (21.0 ± 8.7%), while CD2 remained randomly distributed on CTL forming conjugates with CMVpp65 peptide loaded targets. This recruitment appeared to be Fc-mediated since pre-incubation of CMV-specific CTL with F(ab’)2 fragments of CLB-CD2.1 resulted in an even distribution of CD2 on CTL forming conjugates with TC coated targets (data not shown).
Figure 5 Confocal fluorescence microscopy of conjugates between CMVpp65 peptide or TC coated targets and CMV-specific CTL. EBV transformed B cells were coated with either CMVpp65 peptide or TC and loaded with CMTMR Orange to discriminate them from effector cells.

A Representative pictures of conjugates between target and effector cells demonstrate the position of CD3 (upper panels) and CD2 (middle panels) in the immunological synapse (IS; magnification 40x). The bottom pictures were taken from conjugates of CMVpp65 peptide or TC coated targets and CMV-specific CTL that were pre-incubated with an antibody against CD2 (CLB-CD2.1). The positioning of CD2 in these conjugates was subsequently visualized by a FITC labeled goat anti-mouse antibody.

(B) Percentage of conjugates in which either CD3 or CD2 is located in the IS (bars represent the mean of three experiments). In each experiment, at least 30 conjugates were evaluated per condition; error bars indicate SEM.

(Cells that do not stain for CD3, CD2 or CMTMR Orange are CLL cells that remain within the effector cell population after in vitro expansion of CMV-specific CTL)
Discussion

In previous studies, we demonstrated that CMV-specific CTL can be redirected towards CLL cells that are targeted with anti-CD20 scFv-MHC-I/CMVpp65 complexes (TC) (23). In the present study, we explored the mechanism behind TC induced CTL activation. We show that TC can efficiently trigger CTL mediated lysis. However, compared to CMVpp65 activated CTL, IFN-γ production by TC activated CMV-specific CTL is less efficient. We provide evidence that the reduced capacity to induce IFN-γ production by TC can be explained by incomplete CD2 triggering because: 1) CD2 did not translocate towards TC induced synapses and 2) CD2 antibodies restored TC-induced IFN-γ production to the level of CMVpp65 peptide stimulated CTL and induced the redistribution of CD2 towards the immunological synapse. The latter suggests that not only CD2 ligation, but importantly, clustering of CD2 in the immunological synapse is mandatory to give full CTL activation.

Various studies have addressed the role of CD2 in CTL activation (8;15;30;31), but the exact role of CD2 in human effector T cell activation is still not completely understood. A recent publication by Espagnolle et al. suggests that in helper T cells CD2 ligation is essential for sustained calcium signaling and subsequent helper cell activation (11). Our results suggest that in CTL the induction of both lytic activity and calcium mobilization appear to be relatively independent from CD2 ligation. These findings are in line with studies from other groups demonstrating that interfering with CD2-CD58 interaction does not affect TCR polarization towards the APC (38) nor the lysis of target cells (19). The latter shows that TCR signaling still occurs in the absence of CD2 ligation. Therefore, it seems that CD2 has a role in modulating certain aspects of TCR signaling in CTL rather than being essential for the initiation of proximal signaling events.

The TC used in our studies has a higher molecular weight than autologous MHC-I (± 90 kDa vs ± 45 kDa). Since the dimensions of CD2-CD58 conjugates seem to be similar to the dimensions of a (normal) TCR-MHC-I conjugate (35), the most likely position for CD2-CD58 interactions is in the centre of the immunological synapse (close to TCR-MHC-I). Therefore, it can be speculated that the size of TC changes the dimensions of the immunological synapse and thereby lead to inefficient CD2 recruitment. Choudhuri et al. have demonstrated that altering the dimensions of MHC-I indeed affects the dimensions of the immunological synapse resulting in impaired T cell activa-
In our current study, transmission electron microscopy analysis of CTL-target conjugates indicated that the synapse formation with TC coated B-cells is different from immunological synapse formation with CMVpp65 coated targets. The apparent predominance of short tight junctions in TC-induced conjugates compared to CMVpp65 peptide-induced conjugates (where there is a tendency towards long tight junctions) may reflect deviations from the normal immunological synapse structure in TC-induced synapses. If correct, this would explain both the inability of CD2 to fully redistribute towards the synapse and could also account for CD2 antibodies entering TC-induced synapses through the “gaps” between these tight junctions and engage with Fc receptors on the target cell, inducing an artificial clustering of CD2 and thereby full activation of the CTL (21). This would then also explain the effect of CD2 antibodies on CMVpp65 peptide-induced T cell activation, because according to the above-proposed model the predominant long synapses between CMVpp65 peptide-loaded target cells and CTL will not allow antibody-CD2 complexes to cluster in the vicinity of TCR-MHC-I complexes, a process which apparently seems to be essential for full CTL activation. Alternatively, it can be argued that the reduced IFN-γ production by CMV-specific CTL when triggered with TC is caused by a suboptimal amount of peptide present on the targeted CLL cell (12) as a result of the low surface expression of CD20 (3). However, since surface staining of CTL for the early activation marker CD69 showed a similar concentration-dependent upregulation for pp65 peptide and TC, whereas at the same time the curves for IFN-γ production diverge, this scenario seems unlikely. Thus, we favor the explanation that the observed structural differences between CMVpp65 peptide and TC-induced synapses account for the difference in IFN-γ production by CMV-specific CTL.

In the presence of an antibody against CD80, CMV-specific CTL exhibit both lytic activity and IFN-γ production. This was expected, as CMV-specific CTL have an effector-memory phenotype which allows them to become activated without receiving costimulatory signals (5). It is this capacity that makes them ideal effectors for a T cell based immunotherapy for CLL, since CLL is known to express only low levels of costimulatory ligands on its surface (14). Furthermore, the effector-memory phenotype suggests that only minute amounts of peptide presented on the CLL cells by means of TC may be sufficient to trigger CMV-specific CTL to lyse the targeted cancer cells. Indeed, our experiments indicate that lysis of target cells is achieved at much lower peptide concentrations than required for the induction of cytokine production by CMV-specific
CTL, thereby confirming the observations of Faroudi et al. (12). Finally, lysis of peptide loaded targets could only be blocked by antibodies against LFA1, demonstrating that CMV-specific CTL require only minimal stimulation to exert powerful lytic activity. Recently, it has been described that CLL cells induce impaired actin polymerization in T cells, resulting in defective immunological synapse formation with subsequent impairment of T cells function (28). Apparently this is not relevant in our model of redirected virus specific T cells, because these CTL induce efficient lysis of CLL target cells. Our study raises the question whether the reduced induction of cytokine production by TC as compared to the directly CMV peptide loaded CLL target cells, is good or bad news for the in vivo application of TC for anti-tumor therapy. Since it has been suggested that the immune system in CLL patients is skewed towards Th2 (13), the induction of Th1 cytokines may be crucial to support a proper anti-tumor response. On the other hand, it has also been described that one of the most important Th1 cytokines, IFN-γ, may serve as a survival factor for CLL cells (6). More importantly, full activation of the total pool of CMVpp65-reactive CTL at once might even result in a cytokine storm as a side-effect (17;33). In this respect, the relative inability of TC to induce the production and release of cytokines may in fact be favorable and broaden their clinical applicability. Whether the latter holds true is still to be tested in upcoming in vivo studies, where the TC will be injected into transgenic mice that express human CD20 on all B cells. These studies should point out to what degree activation of autologous virus-specific cells via these B cell-directed TC indeed induces lysis of the targeted B cells and release of cytokines in vivo. Nevertheless, we expect that redirecting viral immune responses in vivo through TC will be a feasible and highly tumor cell-specific immunotherapy.

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Reference List


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