Antigen receptor triggering and apoptotic pathways in neoplastic B cells
Mackus, W.J.M.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
CHAPTER 6

Expansion of CMV-specific CD8⁺CD45RA⁺CD27⁻ T cells in B-CLL


Wendelina J.M. Mackus¹,²
Florine N. Frakking¹,²
Annette Grummels¹,²
Laila E. Gamadia²
Godelieve J. de Bree²
Dörte Hamann²
René A.W. van Lier²
Marinus H.J. van Oers¹

¹Dept. of Hematology and
²Laboratory for Experimental Immunology,
Academic Medical Center, University of
Amsterdam, Amsterdam, The Netherlands.
ABSTRACT

In B-CLL patients the absolute number of T cells is increased. Although it has been suggested that these T cells might be tumor-specific, concrete evidence for this hypothesis is lacking. We performed a detailed immunophenotypical analysis of the T cell compartment in the peripheral blood of 28 B-CLL patients (Rai 0 n=12, Rai I-II n=10, Rai III-IV n=6) and 12 healthy age-matched controls and measured the ability of these patients to mount specific immune responses. In all Rai stages a significant increase in the absolute numbers of CD3+ cells was observed. Whereas the number of CD4+ cells was not different from controls, B-CLL patients showed significantly increased relative and absolute numbers of CD8+ cells which exhibited a CD45RA+CD27+ cytotoxic phenotype. Analysis of specific immune responses with tetrameric CMV-peptide complexes showed that B-CLL patients had significantly increased numbers of tetramer-binding CMV-specific CD8+ T cells. The rise in the total number of CD8+ cytotoxic T cells was only evident in CMV-seropositive B-CLL patients. Thus, our data suggest that in B-CLL patients the composition of T cells is shifted towards a CD8+ cytotoxic cell type in an effort to control infections with persistent viruses such as CMV. Moreover, they offer an explanation for the high incidence of CMV reactivation in CLL patients treated with T cell depleting agents, such as MabCampath (α-CD52 mAb). Furthermore, since in CMV-seronegative patients no increase in cytotoxic CD8+ T cells is found, our studies do not support the hypothesis that tumor-specific T cells account for T cell expansion in B-CLL.

INTRODUCTION

B cell chronic lymphocytic leukemia (B-CLL) is the most common adult leukemia in the Western world, and consists of a clonal expansion of CD5+CD19+CD23+ B cells. Patients with B-CLL suffer from recurrent infections and have an increased incidence of autoimmune diseases, suggesting T cell dysfunction. Previous studies have shown that in B-CLL the absolute numbers of both CD4+ cells and (more pronounced) CD8+ T cells are elevated. Phenotypical analysis of these cells shows expression of CD45RA and CD57, whereas CD28 is poorly expressed.

It has been suggested that these elevated numbers of T cells are related to tumor-antigen-directed immune responses. First, examination of T cell receptor V gene usage and determination of the CDR3 length revealed both clonal and oligoclonal expansions in CD8+ cells and (more frequently) in CD4+ cells. Second, it has been demonstrated that patient-derived CD4+ T cells and, to a lesser extent CD8+ T cells, can proliferate upon priming with autologous DC either loaded with autologous VH-CDR3-specific peptides or pulsed with apoptotic bodies of B-CLL cells. In addition, T cells can proliferate after stimulation with CD40-activated leukemic B cells. However, proliferation of patient-derived CD8+ or CD4+ T cells has not been detected upon stimulation with freshly isolated leukemic B cells. Third, whereas cytotoxic immune responses against stimulated and unstimulated CLL cells could be generated in allogeneic immune settings, autologous cytotoxic immune responses against CD40-activated or freshly isolated B-CLL cells have rarely been detected. Thus, since convincing evidence regarding tumor-specificity of the expanded pool of CD8+ cells in CLL patients expressing the cytotoxic phenotype is
still lacking, alternative explanations should be considered such as adaptive responses of the immune system to microbial antigens.

Encounter of viral antigen induces naïve CD4+ and CD8+ T cells to clonally expand and differentiate into helper T cells or effector T cells respectively. CD8+ effector cells can counteract an acute viral infection via both the production of cytotoxic effector molecules such as perforin/granzyme B and the Fas/Fas ligand pathway. After viral clearance, a pool of antigen-specific CD8+ cytotoxic CD45RA+CD27- cells as well as non-cytotoxic CD45RA-CD27+ cells persists, which is capable to generate a more efficient response when individuals are being rechallenged with the same antigen. Recent data suggest that in humans different persistent viral infections induce CD8+ T cells with different phenotypes corresponding to distinct stages of differentiation. In healthy individuals latently infected with Cytomegalovirus (CMV) virus specific CD8+ cells exhibit either the non-cytotoxic CD45RA-CD27+ phenotype or the cytotoxic CD45RA-CD27- phenotype. The latter phenotype is found at even higher frequencies in immunocompromised individuals. These CMV-specific cytotoxic T cells also express CD57 and lack CCR7 and L-selectin.

To elucidate the basis for the CD8+ T cell expansion in B-CLL we investigated the composition of the T cell compartment in B-CLL patients via extensive immunophenotypical analysis. In addition, we analysed antigen-specificity of the expanded CD8+ T cell pool using tetrameric CMV-peptide complexes. Our data show that only in CMV-seropositive B-CLL patients the composition of T cells is shifted toward a CD8+ cytotoxic T cell type which might have a key role in maintaining viral latency.

PATIENTS, MATERIAL AND METHODS

Patients
Peripheral blood samples were obtained from 28 B-CLL patients from the outpatient clinic of the department of Hematology of the Academic Medical Center. All individuals gave written informed consent and the study was approved by the Ethical Review Board. Patients were classified according to the criteria of Rai et al.; the group with good prognosis consisted of 12 patients with Rai stage 0 (mean age in years 68 ± 10), the group with intermediate prognosis consisted of 10 patients with Rai stage I or II (mean age in years 70 ± 14) and the group with poor prognosis consisted of 6 patients with Rai stage III-IV (mean age in years 66 ± 9). Two patients received therapy consisting of chlorambucil when included in the study: one patient (Rai stage IV) at time of bloodwithdrawal and the other patient (Rai stage I) 2 months before inclusion. The other patients had not received treatment at least 6 months before inclusion into the study. 79 % (22/28) of the patients never received any prior treatment. The control group consisted of 12 healthy age-matched volunteers (mean age in years 60 ± 9) which had not received any kind of treatment or medication.

Monoclonal antibodies
For analysis of cell surface markers conjugated antibodies were used for two-colour, three-colour or four-colour analysis of PBMCs. Different combinations of antibodies were used. CD4-fluorescin isothiocyanate (FITC), CD4-peridinin chlorophyll protein (PercP), CD8-FITC, CD8-phycocerythin (PE), CD8-PercP, CD8-allophycocyanin (APC) and CD19-PE were all purchased from Becton Dickinson ((BD), San Jose). CD3-FITC, CD27-FITC and CD45RA-PE were obtained from Sanquin (Amsterdam, The Netherlands). FITC-labeled CD45RO was obtained from (Dako A/S, Glostrup, Denmark). To detect Natural Killer cell receptors (NKR's)
biotinylated CD45RA purchased from Pharmingen was used in conjunction with streptavidin-APC (BD), combined with either NKB-1-PE (BD), CD94-PE (Pharmingen, San Diego, CA), CD158a-PE and CD158b-PE (both Immunotech, Marseille, France). APC-conjugated tetramers were produced as described below.

Isolation of peripheral blood mononuclear cells (PBMCs), immunofluorescent staining and flowcytometric analyses

Diluted EDTA-blood was layered on Lymphoprep (Nycomed, Pharma, Oslo, Norway) for standard density gradient centrifugation and PBMCs were harvested from the interphase and washed twice in Iscove's modified Dulbecco's medium (IMDM; Gibco Life Technology, Paisley, Scotland) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS; ICN Biomedicals GmbH, Meckenheim, Germany), 100 U/ml penicillin, 100 μg/ml streptomycin and L-glutamin (Gibco). Cells were used immediately or frozen and stored in liquid nitrogen at −196°C until the day of analysis.

Freshly isolated or thawed PBMCs were resuspended in medium and subsequently washed in phosphate-buffered saline (PBS) containing 0.5% (w/v) bovine serum albumin (PBA). 500,000 PBMCs were incubated with fluorescent-labeled conjugated monoclonal antibodies (mAbs; concentrations according to manufacturer's instructions) against cell surface markers in combination with APC-labeled tetrameric complexes at an appropriate concentration. Depending on the combination of subsets analysed two-, three- or four-colour analyses were performed. Antibody staining was performed in a volume of 50 μL for 30 minutes at 4°C protected from light.

For detection of the NKR's in combination with CD8+ T cell subsets a two-step staining protocol was performed. PBMCs were incubated with the anti-CD45RA-biotinylated mAb for 30 minutes at 4°C protected from light. Cells were washed twice with PBA after which they were incubated with anti-streptavidin-APC in combination with directly conjugated anti-CD27-FITC and anti-CD8-PercP mAbs for 30 minutes at 4°C protected from light.

After incubation cells were washed twice in PBA and expression of cell surface markers was analysed using a FACS Calibur flow cytometry and Cellquest Software (BD).

Peptides

The HLA-A2 binding CMV pp65 derived peptide NLVPMVATV and the HLA-B7 binding CMV pp65 derived peptide TPRVTGGAM were purchased from the IHB-LUMC peptide synthesis library facility (Leiden, The Netherlands).

Generation of CMV tetrameric complexes

Tetrameric complexes were generated essentially as described previously. In brief, purified HLA-A2.1 or HLA-B7 heavy chain and β2-microglobulin were synthesized using a prokaryotic expression system (PET; Novagen, Milwaukie, WI). The heavy chain was modified by deletion of the transmembrane/cytosolic tail and C-terminal addition of a sequence containing the BirA enzymatic biotinylation site. The HLA-A2.1-binding CMV pp65-derived peptide NLVPTMATV or the HLA-B7 CMV pp65-derived peptide TPRVTGGGA were used for refolding. Monomeric complexes were concentrated, biotinylated by BirA (expressed using the pET expression system and purified using Clontech cobalt beads, Palo Alto, CA) in the presence of biotin (Sigma Chemical, St. Louis, MO), adenosine triphosphate (ATP; Sigma Chemical) and MgCl2. The biotinylated product was separated from free biotin by FPLC using a Superdex 200 HR16 / 60 column (Amersham Pharmacia, Little Chalfont, UK). Streptavidin-APC conjugate (Molecular Probes, Europe BV, Leiden, The Netherlands) was added in a 1:4 molar ratio and subsequently tetramers were FPLC purified using the same column.
CMV-specific cytotoxic T cells in B-CLL

**Determination of CMV-PCR and anti-CMV IgG serology**
Quantitative PCR was performed in EDTA whole blood samples as described for plasma or serum\(^{281}\). Anti-CMV IgM and IgG were determined in serum using the AxSYM micro particle enzyme immunoassay according to the manufacturer’s instructions. Measurements were calibrated relative to standard serum. Results are expressed as a ratio of the measurements to a standard serum (IgM).

**Statistical analysis**
Two-sided Mann-Whitney test was used for analysis of differences between groups. For correlations Spearman non-parametric correlation test was used. P-values <0.05 were considered statistically significant.

**RESULTS**

**Phenotypical analysis of T cell subsets in B-CLL**
Relative and absolute numbers of CD19\(^+\) cells, CD3\(^+\), CD4\(^+\) and CD8\(^+\) cells were assessed via FACS analysis in 28 patients with B-CLL. All three prognostic groups showed increased relative and absolute numbers of CD19\(^+\) B cells (Table 1). Although the relative numbers of CD3\(^+\) T cells were diminished, a significant rise in the absolute numbers of CD3\(^+\) T cells was observed in all B-CLL patients. This was accounted for by the CD8\(^+\) cell population since absolute numbers of CD8\(^+\) cells were significantly elevated whereas no significant changes in the CD4\(^+\) cell population were observed (Table 1).

<table>
<thead>
<tr>
<th>Control, n = 12</th>
<th>CD19</th>
<th>CD3</th>
<th>CD3 CD4</th>
<th>CD3 CD8</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>13 ± 5</td>
<td>63 ± 12</td>
<td>55 ± 17</td>
<td>19 ± 10</td>
</tr>
<tr>
<td>No.</td>
<td>311 ± 152</td>
<td>1461 ± 481</td>
<td>805 ± 365</td>
<td>285 ± 171</td>
</tr>
<tr>
<td>Rai 0, n = 12</td>
<td>%</td>
<td>78 ± 13(^*)</td>
<td>15 ± 11(^*)</td>
<td>55 ± 87</td>
</tr>
<tr>
<td></td>
<td>No.</td>
<td>19280 ± 20090(^*)</td>
<td>2124 ± 814(^†)</td>
<td>1101 ± 521</td>
</tr>
<tr>
<td>Rai I-II, n = 10</td>
<td>%</td>
<td>81 ± 9</td>
<td>13 ± 6(^*)</td>
<td>44 ± 23</td>
</tr>
<tr>
<td></td>
<td>No.</td>
<td>20800 ± 15770(^*)</td>
<td>2794 ± 1414(^†)</td>
<td>1316 ± 983</td>
</tr>
<tr>
<td>Rai III-IV, n = 6</td>
<td>%</td>
<td>90 ± 8</td>
<td>5 ± 5(^*)</td>
<td>39 ± 22</td>
</tr>
<tr>
<td></td>
<td>No.</td>
<td>68000 ± 46220(^*)</td>
<td>2568 ± 1179(^†)</td>
<td>1183 ± 1065</td>
</tr>
</tbody>
</table>

**Table 1. Relative and absolute T cell numbers in B-CLL**
Distribution of B cells and subsets of T cells in controls and B-CLL patients of different Rai stages. Mean ± SD of relative (%) and absolute (no. of cells/μL) numbers are presented. Numbers of CD4\(^+\) and CD8\(^+\) cells were detected within the CD3\(^+\) gate. The relative number of CD3\(^+\) T cells was increased due to elevated numbers of CD8\(^+\) T cells in all Rai stages of B-CLL. \(^*\) P<0.001, \(^†\) P<0.01, \(^‡\) P<0.05).
We have previously demonstrated that, based on both expression of the cell surface markers CD45RA and CD27 and functional properties, a discrimination can be made within the CD8⁺ subset between CD45RA⁺CD27⁺ naïve cells, and two primed subsets, i.e. CD45RA⁺CD27⁻ cytotoxic cells and CD45RA⁺CD27⁺ non-cytotoxic cells.[271] To study whether the rise in CD8⁺ cells was accompanied by changes in subset distribution further phenotypical analyses of peripheral CD8⁺ T cells were carried out. An example of a staining of CD8⁺ cells from a healthy individual and a Rai stage 0 B-CLL patient is shown in Fig. 1. B-CLL patients display a shift towards the CD8⁺CD45RA⁺CD27⁻ cytotoxic compartment. In all B-CLL Rai stages the absolute numbers of CD8⁺CD45RA⁺CD27⁻ cytotoxic cells significantly exceeded those of healthy controls (Fig. 2), whereas absolute numbers of naïve T cells were not different between B-CLL and controls (Fig. 2), nor were numbers of CD45RA⁺CD27⁺ non-cytotoxic T cells (data not shown). Thus, the rise in absolute numbers of CD3⁺ T cells is largely caused by increased numbers of CD8⁺CD45RA⁺CD27⁻ cytotoxic T cells.

**Figure 1. CD8⁺ T cell subsets in controls and B-CLL**

Representative dotplot of a three colour immunostaining of CD8⁺ T cell subsets in a healthy control (upper panel) and a Rai stage 0 B-CLL patient (lower panel). Cells were gated on lymphogate via FSC/SSC and subsequently on CD8-PerCP after which the following subsets can be distinguished based on CD45RA-PE and CD27-FITC: CD45RA⁺CD27⁻ cytotoxic effector cells; CD45RA⁺CD27⁺ naïve cells; and CD45RA⁺CD27⁺ non-cytotoxic cells.

**Figure 2. Increased absolute number of CD8⁺ CD45RA⁺CD27⁻ cytotoxic T cells in B-CLL**

Distribution of different T cell subsets within CD8⁺ cells. Absolute numbers of CD8⁺ cytotoxic cells (open symbols) are significantly increased in all Rai stages of B-CLL (squares) when compared to healthy controls (circles) (Rai 0 P= 0.0011 (*); Rai I-II P= 0.0027 (**); Rai III-IV P= 0.0076 (***) whereas numbers of CD8⁺ naïve cells (filled symbols) were similar. Numbers represent mean ± SD of cells analysed by three colour immunostaining gated as described in Fig. 1.
Expansion of CD8$^+$ cytotoxic T cells in B-CLL correlates with CMV seropositivity

Increased numbers of CD8$^+$CD45RA$^+$CD27$^+$ cytotoxic T cells have been observed during chronic latent infection with CMV in both healthy individuals and even more pronounced in immunocompromised individuals$^{277-279}$. Thus, we investigated whether the rise in CD8$^+$CD45RA$^+$CD27$^+$ cytotoxic cells in B-CLL patients correlated with chronic latent CMV infection.

CMV-specific CD8$^+$ cells were visualised by using tetrameric HLA-A2.1/NLVPVMVATV or HLA-B7/TPRVTGGA complexes (Fig. 3A). We used CMV-specific tetramer complexes containing HLA-A2 and -B7 to analyze patients with the appropriate HLA type. Using a combination of four-colour staining with monoclonal antibodies against CD45RA and CD27 a characterization of the CMV-specific CD8$^+$ cells was performed. Almost all CD8$^+$CMV-tetramer$^+$ cells detected expressed the CD45RA$^+$CD27$^+$ cytotoxic phenotype (Fig. 3B). In this regard, no significant differences were observed between B-CLL patients and controls (CD8$^+$CMV-specific CD45RA$^+$CD27$^+$ cells in B-CLL patients: 49 ± 6 % mean ± SEM, in controls: 42 ± 11 % mean ± SEM). B-CLL patients showed both a relative and absolute increase in the number of CD8$^+$ T cells binding CMV-tetrameric complexes (B-CLL patients (n=10): 5.6 ± 1.5 % mean ± SEM, range 0.21 - 14.4 %, and 37 ± 10 cells/µL mean ± SEM, range 2 – 116 cells/µL; and controls (n=6): 2.5 % ± 0.5 mean ± SEM, range 0.8 - 3.8 %, P=0.22, and 13 ± 3 cells/µL, mean ± SEM, range 2 - 28 cells/µL, P=0.0225) (Fig. 3C&D).

Figure 3. CMV-specific CD8$^+$ T cells have a cytotoxic phenotype and are elevated in CMV-seropositive B-CLL

(A) A representative dotplot of CMV-specific CD8$^+$ T cells of a healthy control (upper panel) and a Rai stage 1 B-CLL patient (lower panel) using CD8-PercP (y-axis) versus HLA-A2 or HLA-B7 CMV-tetramer-APC (x-axis) gated on lymphocyte via FSC and SSC.

(B) Composition of the different T cell subsets of CD8$^+$ CMV-specific T cells was determined based on four-colour immunostaining of CD27-FITC, CD45RA-PE, CD8-PercP and CMV-tetramer-APC. Cells were gated on lymphocytes via FSC/SSC and CMV-specific CD8$^+$ T cells were additioanally gated on both CD8- and CMV-tetramer positivity as represented by the square box in upper right quadrant of the dotplot under A. CMV-specific CD8$^+$ T cells consist of the cytotoxic cell phenotype and show an increase in both the percentage (C) and absolute number (P=0.0225) (D) of CMV-specific CD8$^+$ T cells in patients with B-CLL (squares, n= 10) compared with controls (circles, n= 6).
Having established that CMV-specific T cells expand in B-CLL, we next investigated whether the increase in CD8^+CD45RA^+CD27^− cytotoxic T cells was related to CMV serology status. Indeed, CMV-seropositive CLL patients had significantly higher relative and absolute numbers of CD8^+CD45RA^+CD27^− cytotoxic T cells than CMV-seronegative individuals (CMV-seropositive B-CLL patients: 48 ± 4 %, mean ± SEM, and 461 ± 105 cells/μL, mean ± SEM; CMV-seronegative B-CLL patients: 14 ± 2 %, mean ± SEM, P=0.0002, and 124 ± 32 cells/μL, mean ± SEM, P=0.0041) (Fig. 4A&B). In healthy individuals a similar trend was observed (CMV-seropositive controls: 23 ± 5 %, mean ± SEM, and 73 ± 28 cells/μL, mean ± SEM; CMV-seronegative controls: 11 ± 1 %, mean ± SEM, and 30 ± 2 cells/μL, mean ± SEM). CMV-seropositive B-CLL patients showed a more pronounced increase in both relative (P=0.0022) and absolute (P=0.0003) numbers of CD8^+CD45RA^+CD27^− cells as compared to CMV-seropositive healthy individuals. Noteworthy, however, no significant difference was observed when comparing B-CLL patients and controls which both were CMV-seronegative. In all the controls and all but one CLL patient CMV load was undetectable as assessed by CMV-specific PCR indicating adequate viral control (data not shown). Furthermore, the percentage of CD8^+CD45RA^+CD27^− cytotoxic cells showed a positive correlation with the absolute number of CD8^+ cells in 27 CMV-seropositive individuals analysed (both patients (n=18) and controls (n=9), R= 0.624, P= 0.005), whereas no correlation was found in CMV-seronegative individuals (n=10, patients n=7 and controls n=3 R= -0.2364, P=0.5135) (data not shown). Thus, the increase in the numbers of cytotoxic T cells in the total CD8^+ cell population correlates with chronic latent CMV infection.
CMV-specific cytotoxic T cells in B-CLL

<table>
<thead>
<tr>
<th>NKB-1</th>
<th>CD94</th>
<th>CD158a</th>
<th>CD158b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, n = 9</td>
<td>3 ± 1</td>
<td>11 ± 3</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>CD8+</td>
<td>13 ± 3</td>
<td>29 ± 8</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>CD8+CD45RA+CD27-</td>
<td>3 ± 0.6</td>
<td>8 ± 2</td>
<td>2 ± 0.3</td>
</tr>
<tr>
<td>B-CLL, n = 18</td>
<td>4 ± 1*</td>
<td>14 ± 4†</td>
<td>5 ± 1†</td>
</tr>
</tbody>
</table>

Table 2. Decreased expression of NKR's on CD8+CD45RA+CD27- cytotoxic T cells in B-CLL

Expression of the NKR's NKB-1, CD94, CD158a and CD158b was analysed on different CD8+ T cell subsets in a four-colour immunostaining combining CD27-FITC, CD8-PercP, CD45RA-bio-Strep-APC, and PE-labeled α-NKR antibodies. Mean ± SEM of the percentage of cells is indicated (*P<0.01, †P<0.05).

Expression of Natural Killer cell Receptors (NKR's) on cytotoxic T cells of B-CLL patients

Specificity of NK cell-mediated killing is provided by inhibitory signals transduced by receptors for MHC class I, so called Natural Killer cell Receptors (NKR's) (review in 35). These receptors, both of the C-type lectin and Ig superfamily classes, have also been found on cytotoxic T cells282-285. CMV-specific T cells, irrespective of their CD27 phenotype, invariably express low amounts of NKR's compared to total CD8+ T cells277.

In view of our findings of increases in cytotoxic T cells in B-CLL patients, we investigated the expression of the NKR's on subsets of CD8+ T cells via immunostaining with mAbs against NKB-1, CD94, CD158a or CD158b in combination with mAbs against CD45RA and CD27. In agreement with the literature in healthy individuals highest expression of all four NKR's was detected on CD8+CD45RA+CD27- cells (Table 2). B-CLL patients, however, showed a significantly reduced expression of all NKR's (except CD94) on cytotoxic CD8+ T cells when compared to controls (Table 2). We next analysed whether the observed decrease in NKR expression on CD8+ cytotoxic T cells in B-CLL patients was correlated to latent CMV infection. Whereas on CD8+CD45RA+CD27- cytotoxic cells of CMV-seropositive controls the expression of CD158a was comparable to CMV-seronegative controls and B-CLL patients, the expression of this particular receptor was significantly diminished in CMV-seropositive B-CLL patients (CMV-seropositive: 3 ± 0.9% mean ± SEM, n=12; CMV-seronegative 11 ± 2.4%, n=4; P=0.0092) (Fig. 5). A similar tendency of diminished expression of both NKB-1 and CD158b on CD8+CD45RA+CD27 cytotoxic T cells in CMV-seropositive B-CLL patients was observed.

DISCUSSION

In this study we have shown that in B-CLL absolute numbers of CD3+ T cells are increased due to expansion of the CD8+ T cell population with a CD45RA+CD27 cytotoxic phenotype and that expansion of these cells is related to CMV infection.
Together, our data suggest that in B-CLL patients adequate immune responses are induced to control chronic viral infections such as CMV. Although expansion of CD4<sup>+</sup> T cells has been described in B-CLL<sup>253,256</sup> no significant changes in either relative or absolute numbers of CD4<sup>+</sup> T cells were detected in our study. On the other hand, the rise in the amount of CD8<sup>+</sup> T cells was prominent and resulted in an inverse CD4<sup>+</sup>/CD8<sup>+</sup> ratio (data not shown) confirming results from previous reports on T cell subsets in B-CLL<sup>251,253-255</sup>.

Our observation that B-CLL patients showed higher frequencies of CMV-specific CD8<sup>+</sup> T cells (even up to 14 % of total CD8<sup>+</sup> cells) when compared to healthy controls (Fig. 3C&D) indicates that these patients are capable of generating a cellular immune response to control persistent viral CMV infection. Indeed, in all controls and all but one CLL patient CMV load was undetectable as assessed by CMV-specific PCR (data not shown). Considering the fact that in our tetramer studies only two combinations of immunodominant peptides and HLA subtype have been used to detect CMV-specific CD8<sup>+</sup> responses it might be possible that our data largely underestimate the magnitude of responses against CMV. On the other hand, these remaining CD8<sup>+</sup> cytotoxic cells might represent CD8<sup>+</sup> cells specific for antigens of other persistent viruses e.g. EBV. We analysed the presence of EBV-specific CD8<sup>+</sup> cells by using tetrameric EBV-peptide complexes. Unfortunately due to limited availability of patient material we could only study 6 patients and 3 controls. In this group (which interestingly contained one patient with a recent primary EBV infection) we did not observe an increase in the percentage of CD8<sup>+</sup>EBV<sup>+</sup> cells when compared to controls. This is in agreement with recent data showing that memory CD8<sup>+</sup> T cells vary in differentiation phenotype in different persistent virus infections<sup>276</sup> with CMV inducing T cells of the CD45RA<sup>+</sup>CD27<sup>+</sup> phenotype. Indeed, we found in a cohort of 200 children that the frequencies of circulating CD45RA<sup>+</sup>CD27<sup>+</sup> CD8<sup>+</sup> T cells depend on infection with CMV but not on infection with other herpes viruses such as EBV and VZV<sup>286</sup>.

Our data are in line with those observed in other immuno-compromised patients such as renal transplant patients who showed significant increased frequencies of virus-specific CD8<sup>+</sup> cells<sup>277</sup>. It is striking that expansion of CD8<sup>+</sup>CD45RA<sup>+</sup>CD27<sup>+</sup>....

**Figure 5. Decreased expression of CD158a on CD8<sup>+</sup> cytotoxic T cell is related to CMV-seropositivity in B-CLL patients**

CMV-seronegative controls (open rounds, n=3), CMV-seropositive controls (filled rounds, n=5) and CMV-seronegative B-CLL patients (open squares, n=4) exhibit an elevated percentage of CD158a<sup>+</sup> cells within the CD8<sup>+</sup>CD45RA<sup>+</sup>CD27<sup>+</sup> subset as compared to the respective total CD8<sup>+</sup> cells, whereas the percentage of CD158a<sup>+</sup> within the CD8<sup>+</sup>CD45RA<sup>+</sup>CD27<sup>+</sup> subset of CMV-seropositive (filled squares, n = 12) B-CLL patients is similar for the total CD8<sup>+</sup> cells and significantly lower compared to CMV-seronegative B-CLL patients (P= 0.0092).
CMV-specific cytotoxic T cells in B-CLL

cytotoxic cells was not observed in CMV-seronegative B-CLL patients. This observation makes it highly unlikely that the expanded T cell pool in B-CLL is tumor-specific (Fig. 4A&B). Theoretically, it could be that the expansion of T cells is driven by cytokines produced by the leukemic cells\textsuperscript{287}. CLL cells have been shown to produce a number of cytokines such as TNF-\(\alpha\), TGF-\(\beta\), IL-1, IL-6, IL-8, and IL-10\textsuperscript{288}. However, none of these cytokines have been shown to induce expansion of CMV-specific memory effector cells\textsuperscript{287}, making this possibility highly unlikely.

The detection of oligoclonal T cell populations within B-CLL has been regarded as an indication for expansion of tumor-specific CD8\(^+\) T cells\textsuperscript{253,254,260}. However, proliferative and cytotoxic capacity of CD8\(^+\) cells in B-CLL has been detected only upon stimulation of T cells with either autologous leukemic B cells previously activated with CD40\textsuperscript{264-266}, or, with leukemic B cells engineered to improve antigen-presenting function\textsuperscript{289}, respectively. This might indicate that these oligoclonal T cell expansions are not related to the tumor at all. Increases in both CD4\(^+\) and CD8\(^+\) T cells with a skewed TCR V\(\beta\) repertoire have also been shown in other B cell malignancies such as hairy cell leukemia and multiple myeloma\textsuperscript{256,290,291}. Similarly, no proliferative or cytotoxic responses of autologous CD8\(^+\) T cells directed against these freshly isolated tumor cells have been detected\textsuperscript{256,292}.

CMV can be a considerable burden to the immune system. Periodic reactivation of CMV drives expansion and subsequent contraction of the CMV-specific T cell pool in healthy individuals\textsuperscript{277,278,293}. In this way virus and immune system keep a balance and clinical latency is maintained. Since viral replication might be less adequately repressed in immunocompromised individuals this delicate balance is presumably shifted towards increased numbers of T cells due to repeated rounds of subclinical virus reactivation. In case of B-CLL it can be envisaged that the increase in leukemic B cells hampers immunocompetence. Our data also offer an explanation for the frequent reactivation of CMV in patients treated with potent T cell depleting agents such as Alemtuzumab\textsuperscript{294} both when used in first line\textsuperscript{295} and in relapsed patients\textsuperscript{296}.

Another strong indication that changes in T cell subsets in B-CLL patients is related to chronic viral infection is derived from our analysis on the expression of the NKR's on CD8\(^+\) T cells. In line with earlier reports on T cells\textsuperscript{283,284} we show that CD8\(^+\) T cells differentiating towards the CD27 negative phenotype express NKR's in both controls and CMV-seronegative B-CLL patients. However, CMV-seropositive B-CLL patients show low expression of NKB-1, CD158a and CD158b especially on CD8\(^+\)CD45RA\(^-\)CD27\(^-\) cytotoxic T cells (Table 2 + Fig. 5). This is in agreement with earlier findings on NKR expression of CMV-tetramer positive CD8\(^+\) cells of renal transplant patients which was invariably low\textsuperscript{277}. The fact that in B-CLL patients expression of NKR's is low on cytotoxic T cells again suggests that, in comparison to healthy individuals, a large proportion of these cells are involved in containment of - persistent - viral infection.

In summary, our data suggest that repeated antigenic stimulation in vivo as induced by chronic viral infections such as CMV is responsible for the disturbed balance in the composition of T cells in B-CLL patients. In addition, our data indicate that B-CLL patients are capable of mounting adequate CD8\(^+\) cytotoxic T cell responses. Importantly, since CMV-negative B-CLL patients do not show a rise in cytotoxic CD8\(^+\) T cells our studies are a very strong argument against the hypothesis that B-CLL-specific T cells expand in B-CLL.
Chapter 6

Acknowledgements

The authors would like to thank the patients and volunteers for their blood donations, Mette Hazenberg for technical assistance, Jan Weel and technicians from the department of Clinical Virology for performing CMV-PCR’s and detection of CMV-serology, technicians from the department of Hematology for technical assistance with patient material and Debbie van Baarle for reagents.