The number of cytomegalovirus-specific CD4+ T cells is markedly expanded in patients with B-cell chronic lymphocytic leukaemia and determines the total CD4+ T cell repertoire

Short title: CMV-specific CD4+ T cells are increased in CLL

Category: Immunobiology

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Abstract
B-cell chronic lymphocytic leukaemia is associated with immune suppression and an altered T cell repertoire with expansion of memory cells. Cytomegalovirus is a common herpes virus that elicits a strong virus-specific T cell immune response after infection. We studied the CMV-specific CD4+ T cell response in 45 patients and 35 control subjects and demonstrate it to be markedly expanded in the patient group, averaging 11% of the CD4+ pool compared to 4.7% in controls. The magnitude of the CMV-specific CD4+ immune response increased with disease stage and was particularly high in patients who received chemotherapy. Within this group the CMV-specific response comprised over 46% of the CD4+ T cell repertoire in some patients. Serial analysis revealed that CMV-specific immunity increased during treatment with chemotherapy and remained stable thereafter. CMV seropositive patients exhibited a markedly altered CD4+ T cell repertoire with increased numbers of CD45R0+ T cells and a reduction in CD27, CD28 and CCR7 expression. Overall survival was reduced by nearly 4 years in CMV seropositive patients although this did not reach statistical significance. CLL patients therefore demonstrate an expansion of the CD4+ CMV-specific immune response which is likely to contribute to the immunological and clinical features of this disease.
Introduction

Infectious disease is a major cause of morbidity and mortality in patients with B cell chronic lymphocytic leukaemia. Hypogammaglobulinemia and impaired function of the cellular immune response can occur early in the course of disease and contribute to the state of immune suppression. In addition, treatments used to control the disease can themselves render patients susceptible to infection. Many studies of T cell phenotype and function have been undertaken in patients with B-CLL and have shown abnormalities in the phenotype of CD4 and CD8 T cells including inversion of the normal CD4:8 ratio and the accumulation of terminally differentiated effector T cells with relative absence of naïve precursors. These abnormalities have been suggested to contribute to the immunosuppression of B-CLL and have also been suggested to indicate the presence of a tumour-specific CD4+ T cell response.

Cytomegalovirus is a human herpes virus that infects the majority of the human population during their lifetime. Infection is associated with the development of a vigorous CMV-specific immune response which typically comprises over 2% of the CD8+ T cell repertoire. In states of immunosuppression, such as following allogeneic transplantation or during HIV infection, this value is often increased and is felt to represent cellular response to subclinical viral reactivation occurring during immunosuppression. It has been shown that CMV-specific CD8+ T cell response is similarly increased in patients with B-CLL and the phenotype of CMV-specific cells was that of late differentiated effector cells. Much less is known concerning the activity of the CD4+ CMV-specific T cell response in either
healthy donors or in patient groups. The development of intracellular staining for cytokine production has led to the opportunity to study CMV-specific CD4+ T cell immunity in more detail.

CMV sero-positivity is associated with the accumulation of memory T cells and has been shown as a confounding factor in the analysis of T cell phenotype in patients with rheumatoid arthritis and in normal ageing 13-15. Here, we have analysed CD4+ CMV-specific T cell responses in patients with B-CLL and have compared these to an age matched control group. We find that the CMV-specific CD4+ T cell response is markedly increased in patients with B-CLL and show that this is associated with chemotherapy. These changes are so marked as to determine the total CD4+ T cell repertoire of the patient.
Material / Methods

Patient population

73 patients with B-cell chronic lymphocytic leukaemia were recruited from the University Hospitals NHS Trust, Birmingham, UK. Ethical permission was obtained from the South Birmingham Ethics Committee and written informed consent was provided in all cases in accordance with the Declaration of Helsinki. Patients were studied at all stages of disease. A control group of 49 healthy donors was recruited from a local cohort of volunteers with a median age of 66 years (range 62-81) of which 35 donors were CMV seropositive. CMV seropositivity was determined by serological testing and 45 patients were found to be CMV seropositive (Table 1). There were no differences between the CMV seropositive and seronegative groups with respect to age, stage of disease or treatment history. 10ml of blood was obtained at each timepoint and analysis used either whole blood samples or peripheral blood mononuclear cells (PBMC) obtained by density centrifugation and maintained in RPMI with 10% FCS. The absolute lymphocyte count on each sample was measured by automated cell analysis on a Coulter counter.

Measurement of the absolute CD3+, CD4+ and CD8+ T cell counts

These were determined by use of FACS analysis in combination with the combined lymphocyte and monocyte counts obtained from FBC analysis. The
antibodies anti-CD3-FITC, CD4-PE and CD8-Tricolor were used to stain whole blood samples and the percentage of each population was determined by FACS analysis. This percentage was then used to determine the absolute number of each T cell subset.

**Detection of CMV-specific CD4+ T cells**

CMV-specific CD4+ T cells were determined according to the previously described method \(^{16}\). Briefly, whole blood in sodium heparin was aliquoted into 15 ml propylene tubes and the co-stimulatory mAbs anti CD28 and anti CD49d were added to the samples at the 1µg/ml final concentration. CMV lysate or SEB (positive control) was added to the tubes and a negative control without antigen was used in all cases. Tubes were incubated at 37°C for 6 hours with the presence of 10µg/ml of the cytokine secretion inhibitor, brefeldin A, for the last 4 hours. EDTA was added at final concentration of 2mM for 15 minutes at room temperature and then the red cells lysed and leukocytes fixed by incubation of cultures with FACS lysing solution (Becton-Dickinson, USA) for 10 min. Cells were washed in PBS containing 0.5% bovine serum albumin, 0.1% sodium azide and permeabilized by incubation in FACS permeabilization buffer (Becton-Dickinson, USA) for 10 min at room temperature. The cells then were washed in PBS with BSA and sodium azide prior to staining.
Immunofluorescent staining and flowcytometric analysis.

The following monoclonal antibodies were used in this study: Anti IFNγ (FITC, PE), anti-IL2 (FITC, PE), anti-TNFα (FITC, PE), mouse IgG2a (FITC, PE), mouse IgG1 (FITC, PE), CD49d (pure), CD28 (pure) were obtained from Becton-Dickinson Immunocytometry Systems. CD4 (ECD, PC5), CD8 (FITC), CD3 (PE), CD69 (PC5), CD57 (FITC, PE), CD28 (FITC), CD45RO (FITC, PE), CD45RA (FITC), CD27 (FITC), CD38 (FITC), HLA-DR (FITC) and mouse IgG1 (ECD, TC) were obtained from Coulter Immunology. CD28 (TC) and CD8 (TC) were from Caltag; CCR7 (FITC) was obtained from R&D. Cells were incubated with directly conjugated mAbs for 30 min in room temperature at dark. After staining, cells were washed, fixed in 1% paraformaldehyde in PBS and then kept at 4°C until flow cytometric analysis. Four colour flowcytometric analysis was performed on a flow cytometer. Between 2x10⁴ to 5x10⁴ CD4+ cells were typically collected. Cells gated on SSC versus FSC to collect lymphocytes and then on CD4+ cells versus SSC. Data were analysed using WinMDI software and displayed as dot plots of cytokine versus CD69 Fluorescence.

CMV Ag, strain AD169, was purchased from Microbix Biosystems Inc. SEB and Brefeldin A were purchased from Sigma; and FACS lysing and Permeabilization solution from Becton Dickinson.
Statistical analysis

Kaplan-Meier analysis was used to determine the influence of CMV seropositivity on survival in patients with chronic lymphocytic leukaemia. A log-rank test was used to test the difference between the two survival curves.
Results

The T cell count is increased in patients with B-CLL and CMV seropositive patients exhibit an increment in the CD3+ and CD8+ T cell counts

The number of CD3+, CD3+CD4+ and CD3+CD8+ lymphocytes was initially determined in peripheral blood from patients with B-CLL and an age-matched control group. The CD3+ T cell count was increased by over two fold in B-CLL patients compared to controls (Table 2). Furthermore, CMV seropositivity was associated with an increased CD3+ cell count in both the patients (CMV seropositive 2648/ml (175-9936) vs CMV seronegative 2225/ml (113-8996), p=0.042) and the control group (CMV seropositive 1100/ml (290-2670) vs CMV seronegative 792/ml (192-976), p=0.02). The CD4+ T cell count was also increased in B-CLL patients in comparison to the control group but was not influenced by CMV serostatus. Indeed, the CD4+ T cell count was marginally decreased in CMV seropositive individuals in both the patient (1139/ml vs 1262) and control groups (467/ml vs 566) but this did not reach statistical significance.

CMV seropositivity had a particularly marked effect on the CD8+ T cell count in both the patient and control groups. Within the patient group the mean CD8+ T cell count in CMV seropositive donors was 1460/ml compared to 1070 in the CMV seronegative group (p=0.019). In the control group the corresponding values were 541/ml and 175/ml respectively (p<0.0007) and this observation is
likely to reflect the reported increase in the number of CMV-specific CD8+ T cells that has been observed in CLL patients (12).

Of interest, patients with B-CLL exhibited increased T cell counts compared to the control group irrespective of CMV serostatus. The reasons for this are unknown but could reflect an increased immune component against other infectious agents or potentially a tumour-specific immune response.

**The number of CMV-specific CD4+ T cells is increased in patients with B-CLL**

Blood samples were obtained from CMV seropositive B-CLL patients and age matched controls and the number of CMV-specific CD4+ T cells was determined by cytokine flow cytometry (CFC). CMV lysate was used as a source of antigenic stimulation prior to analysis of IFNγ, TNFα or IL-2 expression within the CD3+CD4+ T cell subset (Figure 1).

The CMV-specific CD4+ T cell response was expressed as a percentage of the total CD4+ T cell repertoire and was found to be significantly increased in B-CLL patients (Figure 1). This was the case when CFC was determined by expression of either IFNγ, TNFα or IL-2 (mean frequency 11% compared to 4.3% for IFNγ; 11% vs 4.6% for TNFα; 3.6% vs 1.3% for IL2). A lower frequency IL-2 response to CMV stimulation was seen in both the patient and control groups. The absolute number of CMV-specific CD4+ T cells was more markedly increased (3.5 fold) in CLL patients compared to the control group (83,900/ml vs 24100 for IFN-γ, 79,300/ml vs 26,900 for TNFα; 23,200/ml vs 7850 for IL-2).
CMV-specific CD4+ T cells are most markedly increased in CLL patients with advanced stages of disease

In order to determine the parameters that might influence the increase in CMV-specific CD4+ T cells in B-CLL patients, the frequency of CMV-specific CD4+ T cells was compared in patients with different stages of disease. A significant increase in the frequency of CMV-specific CD4+ T cells was seen in patients at Binet stages B and C compared to patients at stage A disease (Figure 2). The mean value in patients with advanced disease was 15.2% (range 1.7-43%) of the CD4+ pool compared to 8.2% (1.4-34%) in patients at stage A disease (p=0.03).

An increase in CMV-specific CD4+ T cells is associated with treatment for CLL

The increase in the CMV-specific CD4+ T cell response that was observed with advanced disease might reflect a response to treatment and so patients were subdivided according to whether or not they had been treated for their disease. A significant increase in the CMV-specific CD4+ T cell response was observed in association with a history of treatment (Figure 3A,B). The mean percentage of CMV-specific CD4+ T cells was 17%, 16% and 5.8% for IFNγ, TNFα and IL2 CFC respectively in patients with a history of treatment compared to 8.06%, 7.08% and 1.41% in untreated patients (p=0.017, 0.015 and 0.019). As treatment can be associated with a decrease in the lymphocyte count it was felt important to identify the absolute CMV-specific T cell count in these groups. Despite the
potentially immune suppressive effects of chemotherapy the mean number of CMV-specific CD4+ T cells was increased in treated patients at 138,000, 116,000 and 39,200 cells/ml for IFNγ, TNFα and IL2 responses respectively compared to 55,900, 42,400 and 6,270/ml in untreated patients (p=0.047, 0.045, and 0.015 respectively).

These data raised the possibility that the increment in the CMV-specific CD4+ T cell response was influenced solely by treatment for CLL rather than the underlying disease itself. However, a comparison between the untreated patient group and controls revealed that a significant increase in both the frequency and number of CMV-specific CD4+ T cells between untreated patients and the control group (mean 8.06% of CD4+ T cells vs 4.3%, p=0.03 and 55900/ml compared to 24100/ml, p=0.04) (Figure 3 C).

**The CMV-specific CD4+ T cell immune response increases during episodes of treatment with chemotherapy**

In order to understand the observation of an increased CMV-specific immune response in patients who had undergone treatment, we then elected to perform serial analysis of the immune response during treatment episodes. Two patients receiving monthly courses of fludarabine (40mg/m² orally for 5 days) were studied at monthly intervals as well as an additional patient receiving chlorambucil at a dose of 4mg daily.

The magnitude of the CMV-specific immune response expressed either as a percentage of the CD4+ T cell pool or as an absolute number of cells, increased
markedly during treatment with chemotherapy, peaking at approximately 4 months following fludarabine treatment compared to a slower rise in those receiving chlorambucil (Figure 4A). In contrast, serial analysis on several patients who received chemotherapy more than 1 year ago showed stable or slowly falling numbers of CMV-specific T cells (Figure 4B). Patients who had not been treated also demonstrated stable T cell numbers.

The expanded CMV-specific CD4+ T cell pool determines the CD4+ T cell repertoire in CMV seropositive B-CLL patients

CMV-specific CD4+ populations exhibit a characteristic effector memory phenotype with a pattern of CD45RO+CD27-CD28-CD57+ expression. This profile was also seen in the analysis of CMV-specific responses in the CLL patient cohort (data not shown). Several abnormalities of the CD4+ T cell repertoire have been reported in B-CLL patients and it was felt to be important to determine if CMV infection contributed to these features. CD28- and CD57+ T cells have previously been correlated with specific clinical features in CLL such as neutropenia and clonal T cell expansions. Use of multicolour cytometry allowed the study of co-expression of CD28 with CD57 and we observed that 9.5% of CD4+ T cells showed a CD28-CD57+ phenotype in the CMV seropositive patients compared to less than 1% in the uninfected group (Figure 5A; p=0.00002).

The CD4+ T cell repertoire in healthy CMV seropositive elderly donors shows a unique profile in relation to uninfected individuals and we therefore went on to
compare the CD4+ pool in CMV seropositive subjects in both the CLL patient and control groups. CLL patients showed an altered pattern of expression of all phenotypic markers on the total CD4+ T cell repertoire when compared to the control group (Figure 5B). In particular there was reduced expression of the important co-stimulatory and survival molecules CD27 and CD28, as well as CD45RA and CCR7 which are observed on naïve and/or central memory cells respectively. In contrast the expression of CD57 and CD45RO, both of which are expressed on late differentiated effector memory cells, was increased in CMV seropositive patients. These findings show that, although CMV modulates the CD4+ T cell repertoire in healthy donors, these effects are much more marked in the CLL patient group which reflects the great increase in the absolute CMV-specific CD4+ T cell count.

More infectious episodes were seen in the CMV seropositive cohort but CMV seropositivity has no difference on overall patient survival.

Comparison of the clinical features of the CLL cohort in relation to CMV serostatus had shown that major infectious episodes were more commonly observed within the CMV seropositive cohort (Table 2). Clinical data were therefore obtained on the whole cohort in order to see if CMV seropositivity had any influence on patient survival. Information was available on 53 patients of which 23 were dead and 30 still alive at the time of analysis. For patients still alive the median follow-up time was 105 months (range 6 to 212). Reverse
Kaplan-Meier analysis of all patients gives median follow-up time for all patients of 117 months.

16 patients were CMV seronegative (5 dead; 11 censored) and 37 were CMV seropositive (18 dead; 19 censored). No differences were observed in the type of chemotherapy used in each group. Of these patients, those seronegative for CMV demonstrated an increase in survival compared to CMV positive patients, with median survival estimated as 157 months compared to 112 months. (Figure 6) However, although the hazard ratio is 0.67 (95% CI: 0.25 to 1.81), indicating an observed 33% reduction in the risk of death for CMV negative patients, this effect does not reach statistical significance due to the small cohort size (p=0.42).
Discussion

A wide range of abnormalities within the T lymphoid system have been observed in patients with B cell chronic lymphocytic leukaemia. A characteristic feature is an increase in the CD45RO+ memory T cell population and a comparable reduction in the CD45RA+ naive subset. Functional abnormalities and clonal expansions of T cells have also been observed and it has been suggested that this may represent a tumour-specific immune response, although no antigenic specificity has been demonstrated. High numbers of cytotoxic T cells which express perforin have also been reported in the CD4+ department and, importantly, changes in the T cell repertoire are associated with clinical complications. In particular, the development of neutropenia has also been specifically associated with increased numbers of CD57+ T lymphocytes.

In this report we have characterized the CD4+ T cell response to cytomegalovirus in patients with chronic lymphocytic leukaemia and have identified a number of important observations. The most striking finding is that the absolute magnitude of the CMV-specific CD4+ T cell response is increased by over three times in patients with CLL. Indeed, in two patients the proportion of the CD4+ T cell pool directed against this virus was measured at 44% and 46% respectively which are amongst the highest values ever reported for the CMV-specific CD4+ T cell response. As some CMV-specific CD4+ T cells may not have the capacity to produce IFN-γ, and so would not be detectable by our cytokine assay, the true value may be even higher.
Having observed this effect, it became important to address the potential factors that might be related to the expansion. Initially we looked at the effect of disease stage on the magnitude of CMV-specific immunity. This revealed that the CD4+ T cell response doubled as patients progressed from Rai stage A to stage C, and patients with the greatest absolute levels of CMV-specific immunity were almost always those with progressive disease. When patients were subdivided on the basis of treatment history the size of the CMV-specific immune response was shown to be far greater in patients who had undergone chemotherapy. The effect of treatment with either chlorambucil or fludarabine was to double the magnitude of the CMV-specific cellular response. However, although treatment further expands the CMV-specific immune repertoire, it should be noted that the CD4 T cell response is increased in all CLL patients, irrespective of treatment history.

A prospective analysis in patients undergoing chemotherapy allowed us to determine the kinetics of the CMV-specific immune response. The CMV-specific CD4+ T cell immune response increased by approximately 3 fold in patients during treatment with fludarabine or chlorambucil. Interestingly, the rate of this increase was dependent on the nature of the treatment, and the half life of T cell expansion was measured at 40 days during fludarabine treatment and 120 days in association with chlorambucil.

The observation that the CMV-specific CD4+ T cell immune response increases in patients with CLL is somewhat paradoxical given the immune suppression that is associated with this disease. However, the magnitude of the CMV-specific
immune response is strongly boosted by episodes of viral reactivation and we suspect that the immune suppression associated with CLL leads to subclinical viremia which in turn triggers an increase in the CMV-specific immune response. Strong evidence for this is seen in our prospective studies where the CMV-specific immune response increases markedly within the first three months following immunosuppressive chemotherapy. Interestingly we were not able to detect CMV reactivation by PCR within this cohort which indicates that viral replication is effectively controlled in CLL patients undergoing chemotherapy with fludarabine or chlorambucil. However, it is to be expected that more intensive chemotherapy would subvert the ability of the immune response to respond to viremia and this is likely to explain the high rates of CMV reactivation that are observed after treatment with alemtuzumab.

CMV-specific CD4+ T cells exhibit a characteristic phenotype with high levels of expression of CD57 and CD45RO, but with greatly reduced levels of CD28. When we compared the total CD4+ T cell pool in CLL patients in comparison to healthy donors it was seen that CMV seropositivity markedly influenced the global T cell repertoire. This reinforces the understanding that chronic CMV infection is one of the most profound modulators of the lymphoid pool and should be taken into account in studies of immune profile in relation to disease.

As features such as an increase in memory or CD57+ T cells have been associated with clinical complications of CLL, it became important for us to determine the influence of CMV seropositivity on the clinical characteristics of the patients within this cohort. Serious infections such as pneumonia and shingles
were seen only in the CMV seropositive patients and as the relative usage of fludarabine was comparable in both groups this might suggest that CMV infection increases the degree of immune suppression within patients. Survival data were only available on 53 patients but importantly overall survival was reduced by nearly 4 years in the CMV seropositive group. This observation was not statistically significant with this sample size but demonstrates an urgent need to use larger cohorts to determine the importance of CMV serostatus on the clinical course of patients with CLL. There is increasing interest in the potential contribution of CMV infection to immune senescence and the potential mechanisms by which CMV carriage could impair immune function include reduction in the naïve T cell pool or secretion of soluble immunomodulatory mediators from the expanded memory population.

Chronic herpes virus infections are ubiquitous in all populations and relatively little attention has been given to their potential to modulate the natural history of human disease. Our studies suggest that CMV infection has a profound influence on the immune system in patients with CLL and that this effect increases with disease progression. Further studies are required to understand the clinical importance of this association and to investigate the potential therapeutic opportunities that may be revealed.

**Acknowledgements**

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Author Contributions

B.P. performed the experimental work and helped in experimental design. R.B. assisted in developing the experimental assays. H.P. assisted with data collection and study analysis. L.B. performed the statistical analysis for the paper. C.F. helped with the design of the study and P.M. led the design and writing of the project.

Conflict of Interest

The authors have no conflicts of interest to declare
References


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Table 1. Clinical characteristics of the patients studied
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Table 2 Absolute T cell count in B-CLL patients and control group
Figure Legends

**Figure 1. Higher frequencies of CMV-specific CD4+ T cells are present in patients with B-CLL.** CMV-specific CD4+ responses were assessed using cytokine flow cytometry following stimulation of whole blood using CMV lysate. CMV-specific T cells were determined by production of intracellular (A) IFNγ, (B) TNFα or (C) IL-2. A higher frequency and absolute number of CMV-specific CD4+ T cells were present in CMV-seropositive patients relative to the age-matched CMV-seropositive control group (n=35).

**Figure 2. The frequency of CMV-specific CD4+ T cells in B-CLL patients is increased in advanced disease.** The frequency of CMV-specific CD4+ T cells was determined by IFNg production and grouped according to the Binet stage of disease. CMV-specific CD4+ T cells represented 8.2% of the CD4+ T cell pool in patients with stage A disease (n=29) compared to 15.2% in patients at Binet stages B and C (n=16).

**Figure 3. The frequency of CMV-specific CD4+ T cells is increased in patients with a history of treatment.** CMV-specific CD4+ T cells were measured in CMV-seropositive patients and expressed (A) as a percentage of the CD4+ T cell pool or (B) as an absolute number. The mean percentage of CMV-specific CD4+ T cells was 17%, 16% and 5.8% for IFNγ, TNFα and IL2 responses respectively in patients with a history of treatment compared to 8.06%,


7.08% and 1.41% respectively in untreated patients (p=0.017, 0.015 and 0.019). The mean number of CMV-specific CD4+ T cells in CMV-seropositive treated patients was 138300, 115800 and 39200 for IFNγ, TNFα and IL2 responses respectively compared to 55900, 42400 and 6270 in untreated patients (p=0.047, 0.045, and 0.015 respectively). (C) A significant difference was observed in the frequency and number of IFNγ positive CMV-specific CD4+ T cells between untreated CMV-seropositive patients and the CMV-seropositive control group (mean 8.06% of CD4+ T cells compared to 4.3%,p=0.03 and 55900/ml compared to 24100/ml, p=0.04; n=35 ).

Figure 4  The frequency of CMV-specific CD4+ T cells increases markedly during treatment with chemotherapy  (A) The temporal kinetics of the CMV-specific CD4+ T cell response was assessed in relation to treatment history. The frequency and absolute number of IFN producing CD4+ T cells increases sharply during treatment periods with chlorambucil or fludarabine.  (B) Examples of the kinetics of the CMV-specific CD4+ T cell immune response in three patients who were studied serially at 3 monthly intervals. Patient 1 showed a stable immune response prior to commencing treatment with fludarabine after which the CMV-specific CD4+ T cell response peaked at 37.7% of the CD4+ T cell pool after 2 months and then declined. Patient 2 received treatment with chlorambucil and also exhibited an increase in the CMV-specific CD 4+ T cell immune response which increased up to 5 months after treatment. Patient 3 was untreated and showed a stable CMV-specific immune response. FACS analysis was gated on
CD4+ T cells and IFNγ expression is shown on the x-axis and CD69 on the y-axis. The absolute CD4 T cell counts (cells/microlitre) were measured at (A) 671, 540, 530 and 642 at 0, 4, 8 and 12 months; (B) 1104, 829, 800 and 983 at 0, 4, 8 and 14 months and (C) 389, 377, 417 and 97 at 6, 10, 12 and 16 months respectively.

**Figure 5** CMV seropositive patients have an altered CD4+ T cell repertoire. (A) CD28-CD57+ CD4+ T cells are present almost exclusively in CMV seropositive patients. PBMC were stained with monoclonal antibodies against CD8, CD28 and CD57 and the relative expression of CD28+ and CD57+ was determined on the CD8+ T cell populations of CMV seropositive and seronegative patients. Significant differences in the number of CD57+, CD28- and CD57+CD28- populations were observed between the two groups. (B) The phenotype of the CD4+ T cell repertoire is markedly altered in CMV seropositive patients (n=12) compared to an age-matched CMV seropositive control group (n=10). PBMC were stained with monoclonal antibodies against CD4, CD27, CD28, CD57, CD45RA, CD45RO and CCR7 and the relative expression of each marker was determined on the CD4+ T cell population. Significant differences were observed between the two groups.

**Figure 6:** CMV seronegative CLL patients show a trend towards increased overall survival compared to the CMV seropositive group. Kaplan-Meier analysis was used to determine the influence of CMV seropositivity on survival in
patients with chronic lymphocytic leukaemia. A log-rank test was used to test the difference between the two survival curves. The median survival of the CMV seronegative group was estimated at 157 months compared to 112 months within the CMV seropositive patients (p=0.42)
Figure 1

A. IFNγ

- Left: Patients
- Right: Control

<table>
<thead>
<tr>
<th>% CMV-specific CD4+ T cells</th>
<th>Number of CMV-specific CD4+ T-cells/ml</th>
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<tbody>
<tr>
<td>Frequency</td>
<td>Absolute Number</td>
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<tr>
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P = 0.0004  P = 0.0005

B. TNFα

- Left: Patients
- Right: Control

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<th>Number of CMV-specific CD4+ T-cells/ml</th>
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P = 0.003  P = 0.02

C. IL2

- Left: Patients
- Right: Control

<table>
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P = 0.04  P = 0.05
Figure 3

A

CMV-Specific CD4+ T Cells as a percentage of CD4+ T cells

IFNγ  TNFα  IL2

P = 0.017  P = 0.015  P = 0.019

Treated Patients  Untreated Patients

B

Number of CMV-Specific CD4+ T cells/ml

IFNγ  TNFα  IL2

P = 0.047  P = 0.045  P = 0.015

Treated Patients  Untreated Patients

C

Percentage of CMV-Specific CD4 T cells

Number of CMV-Specific CD4+ T cells/ml

P = 0.03  P = 0.04

Frequency  Absolute Number

Untreated Patients  Control

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Figure 4A
B

3 months
Pre-treatment

1 month
pre-treatment

2 months
post-treatment

5 months
post-treatment

Patient 1

7.42%

6.69%

37.70%

15.6%

Patient 2

4.3%

3.88%

13.57%

14.07%

Patient 3, untreated

8.51%

8.38%

7.4%

7.21%

Figure 4b
Figure 5

A

% CD3+CD4+ T cells

Phenotyping marker

CD57+  \( P=0.00001 \)

CD28-  \( P=0.002 \)

CD57+CD28-  \( P=0.00002 \)

Left: CMV seropositive

Right: CMV seronegative

B

% CD4+ T cells expressing specific marker

Phenotyping marker

CD27  ***

CD28  *

CD57  ***

CD45RA  ***

CD45RO  ***

CCR7  **

Left: Elderly donors

Right: CLL patients

* \( P<0.05 \)

** \( P<0.01 \)

*** \( P<0.001 \)
Figure 6
Levels of cytomegalovirus-specific CD4+ T cells are markedly expanded in patients with B-cell chronic lymphocytic leukaemia and determine the total CD4+ T cell repertoire

Batoul Pourgheysari, Rachel Bruton, Helen Parry, Lucinda Billingham, Chris Fegan, Jim Murray and Paul Moss